lncRNA CASC2 SUPPRESSES THE GROWTH OF HEMANGIOMA CELLS BY REGULATING miR-18a-5p/FBXL3 AXIS

Y-X. DAI, M-K. QIU, S-Q. WANG, C. PAN, Y. WANG and J-M. OU

Department of General Surgery, Xinhua Hospital, Shanghai Jiao Tong University, School of Medicine, Shanghai, China

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Dysregulation of lncRNA cancer susceptibility candidate 2 (CASC2) is involved in the pathogenesis of multiple malignancies. However, the underlying mechanisms by which lncRNA CASC2 regulates the proliferation of hemangiomas (HAs) remain undocumented. Herein, the expression levels of lncRNA CASC2 and VEGF in proliferating or involuting phase HAs were assessed by qRT-PCR analysis, and the effects of lncRNA CASC2 on HAs cell growth were evaluated by MTT, colony formation assays and Western blot analysis. lncRNA CASC2 specific binding with miR-18a-5p was confirmed by luciferase report assay. Consequently, we found that the expression of lncRNA CASC2 was reduced in proliferating phase HAs as compared with the involuting phase HAs or normal tissues, and possessed a negative correlation with VEGF expression in proliferating phase HAs. Restored expression of lncRNA CASC2 repressed cell viability and colony formation and downregulated VEGF expression, while silencing lncRNA CASC2 showed the opposite effects. Moreover, lncRNA CASC2 was confirmed to bind with miR-18a-5p, which could reverse lncRNA CASC2-induced anti-proliferative effects by targeting FBXL3 in HAs cells. Altogether, our findings demonstrated that lncRNA CASC2 suppressed the growth of HAs cells by regulating miR-18a-5p/FBXL3 axis.

Hemangiomas (HAs) as one of the most frequently recurring vascular benign tumors, are linked to the proliferation of vascular endothelial cells, and classified into proliferating phase HAs (PPH) and involuting phase HAs (IPH) (1). The mutations in CCM2 and von Hippel-Lindau contribute to cerebral cavernous malformation and hemangioblasts (2, 3). Our previous studies revealed that VEGF/VEGFR signaling is implicated in the proliferation and apoptosis escape of HAs, and blockage of this signaling produces a therapeutic effect (4, 5).

Long non-coding RNAs (lncRNAs) exceeding 200 nucleotides in length is implicated in cell growth, invasion and metastasis in a variety of tumors including HAs (6-8). lncRNA cancer susceptibility candidate 2 (CASC2) is downregulated in multiple malignancies, such as thyroid carcinoma (9, 10), osteosarcoma (OS) (11), hepatocellular carcinoma (HCC) (12), and gastric cancer (13), and decreased expression of lncRNA CASC2 is associated with advanced TNM stage and tumor metastasis, predicting a poor prognosis in tumor patients (10-12). lncRNA CASC2 represses cell growth and induces cell apoptosis in thyroid carcinoma by inactivating AKT/ERK1/2 signaling (9), and retards epithelial-mesenchymal transition in HCC by regulating miR-367/FBXW7 axis (12), whereas

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Corresponding Author:
Dr. Jingmin Ou,
Department of General Surgery, Xinhua Hospital,
Shanghai Jiao Tong University, School of Medicine,
No.1665 Kongjiang Road, 200092, Shanghai, China
Tel.: +86 021 25077875 - Fax: +86 021 65795173
e-mail: oujingmin17@163.com

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downregulation of lncRNA CASC2 promotes the tumorigenesis, cell invasion and cisplatin resistance by sponging miR-181a/-19a (10, 11, 13). These studies indicated that lncRNA CASC2 can act as a potential biomarker in cancer.

Dysregulation of lncRNAs is associated with the tumorigenesis of HAs (8). The expression of lncRNA UCA1, Linc00152, SNHG16 and lncRNA CASC9 is increased in PPH samples, and facilitates the proliferation and invasion of HAs cells (14-17). However, the role and underlying mechanisms of lncRNA CASC2 in HAs remain unknown. Herein, we uncovered the mechanisms by which lncRNA CASC2 inhibited HAs cell growth by regulating miR-18a-5p/FBXL3 axis.

MATERIALS AND METHODS

HDEC and CRL-2586 EOMA cell lines used were stored at our laboratory. lncRNA CASC2 plasmids or control vector pEX-3, lncRNA CASC2 siRNA (si-CASC2) or control vector (si-NC), and miR-18a-5p mimic or inhibitor were purchased from GenePharma (Shanghai, China). The primer sequences of CASC2, miR-18a-5p and FBXL3 were synthesized by Sangon Biotech (Shanghai, China).

Frozen HAs tissue samples, consisting of PPH (n=15), IPH (n=15) and normal tissues (n=15), were stored at our laboratory. This study was approved by the Ethics Committee of Xinhua Hospital.

Cell culture

HDEC and CRL-2586 EOMA cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Sigma, Aldrich, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, Sigma, Aldrich, USA), 100U/ml of penicillin and 100μg/ml of streptomycin (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time PCR (qRT-PCR)

To assess the expression of lncRNA CASC2, miR-18a-5p and FBXL3 in HAs tissues and cells, qRT-PCR analysis was performed according to previous reports (4, 5). PCR primer sequences were listed as follows: lncRNA CASC2 forward: 5'-TACAGGACAGTCAGTGGT GGTA-3' and reverse:5'-ACATCTAGCTTAGGA ATG TGGC-3'; FBXL3 forward: 5'- GCAGCTTG TGATATACTATCGCA-3' and reverse: 5'-TGGTCGAGCAGTTGAAATAAGTC-3'; miR-18a-5p forward: 5'-GTCCGGAC TCAGATCTCGAGCTT-G A A T C T A C T G C A G T G A A G G C A C T T G - 3 ' , reverse: 5'-TATCTAGATCCGGTGGATCCG-TGCAACTATGCAAAACTAACAG-3'; GAPDH forward: 5'-CAACGAATTTGG C TACAGCA-3' and reverse: 5'-AGGGGTCTACAT GGCAACTG-3'. This experiment was repeated three times.

Western blot analysis

To evaluate the protein levels of VEGF in HAs cells, Western blot analysis was performed according to previous reports (4, 5). The primary antibody against VEGF (1:1000, ab46154, Abcam, Cambridge, MA, USA) was diluted and incubated overnight at 4°C. Horseradish peroxidase-linked secondary antibodies (1:1000) were added, and incubated at room temperature for 2 h. The membranes were washed with PBS and the bands were visualized using ECL-PLUS/Kit according to the kit's instruction.

MTT and colony formation assays were conducted as previously reported (4, 5).

Luciferase reporter assay

The fragments of lncRNA CASC2 or FBXL3 3′ UTR were amplified by PCR and inserted into a pMIR-Report luciferase reporter vector (Ambion, Austin, TX, USA). After 24-h incubation, they were co-transfected with 60 μM miR-18a-5p mimic or inhibitor. A luciferase reporter assay system was used to analyze the luciferase activities.

Statistical analysis

Statistical analyses were carried out by SPSS 20.0 (IBM, SPSS, Chicago, IL, USA) and GraphPad Prism. Student's *t*-test was used to analyze the statistical significance for comparisons between two groups. Pearson correlation analysis was used to analyze the correlations. $P < 0.05$ was considered statistically significant.

RESULTS

lncRNA CASC2 had a negative correlation with VEGF expression in PPH

Our previous study indicated that VEGF can be used to distinguish PPH and IPH (4, 5). Thus, we assessed the expression of lncRNA CASC2 and VEGF in PPH and IPH by qRT-PCR analysis

and found that lncRNA CASC2 expression was substantially decreased ($P < 0.0001$; $P = 0.0292$), while VEGF expression was increased in PPH ($P = 0.0253$; $P < 0.0001$) as compared with IPH or normal tissues (Fig. 1A). Pearson correlation analysis uncovered that lncRNA CASC2 had a negative correlation with VEGF expression in PPH (r=-0.6676, $P = 0.0055$; Fig.1B) rather than in IPH (r=-0.3809, $P = 0.1613$; Fig.1C) and normal tissues (r=-0.0577, $P = 0.8383$).

lncRNA CASC2 reduced the proliferation and colony formation of HAs cells

MTT and colony formation assays were conducted to assess the role of lncRNA CASC2 in HAs cells. After lncRNA CASC2 plasmids had been transfected into HDEC and CRL-2586 EOMA cells for 48 h, their overexpression efficiencies were determined by qRT-PCR analysis ($P < 0.01$; Fig. 2A). Ectopic expression of lncRNA CASC2 repressed the cell viability (Fig. 2B) and colony formation ability (Fig. 2C) in these two cell lines as compared with the pEX-3 group ($P < 0.01$). Then, Western blot analysis showed that VEGF protein levels were significantly reduced by lncRNA CASC2 as compared with the pEX-3 group in these two cell lines (Fig. 2D).

The knockdown efficiencies of si-CASC2 were defined in HDEC and CRL-2586 EOMA cells ($P < 0.01$; Fig. 3A). Reduced expression of lncRNA CASC2 facilitated cell viability (Fig.3B) and colony formation (Fig.3C) in these two cell lines as compared with the si-NC group ($P < 0.05$; $P < 0.01$). Western blot analysis indicated that VEGF protein levels were elevated by silencing lncRNA CASC2 as compared with the si-NC group in these two cell lines (Fig. 3D).

miR-18a-5o was identified to bind with lncRNA CASC2 in HAs cells

Online starBasev2.0 (http://starbase.sysu.edu.cn/starbase2/index.php) was used to identify lncRNA CASC2-specific binding with miR-18a-5p in tumors. qRT-PCR analysis showed that miR-18a-

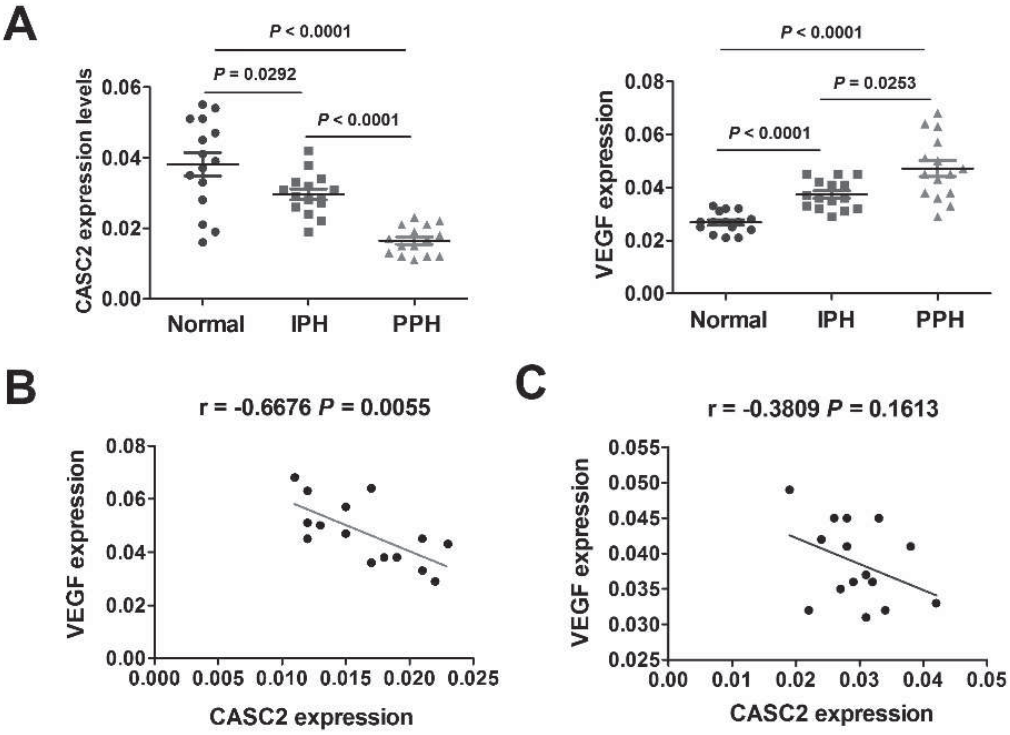

Fig. 1. *The expression levels of lncRNA CASC2 and VEGF in HAs tissue samples.* ***A****) qRT-PCR analysis of the expression levels of CASC2 and VEGF in PPH, IPH and normal tissues.* ***B****) Pearson correlation analysis of the correlation of CASC2 with VEGF expression in PPH.* ***C****) Pearson correlation analysis of the correlation of CASC2 with VEGF expression in IPH.*

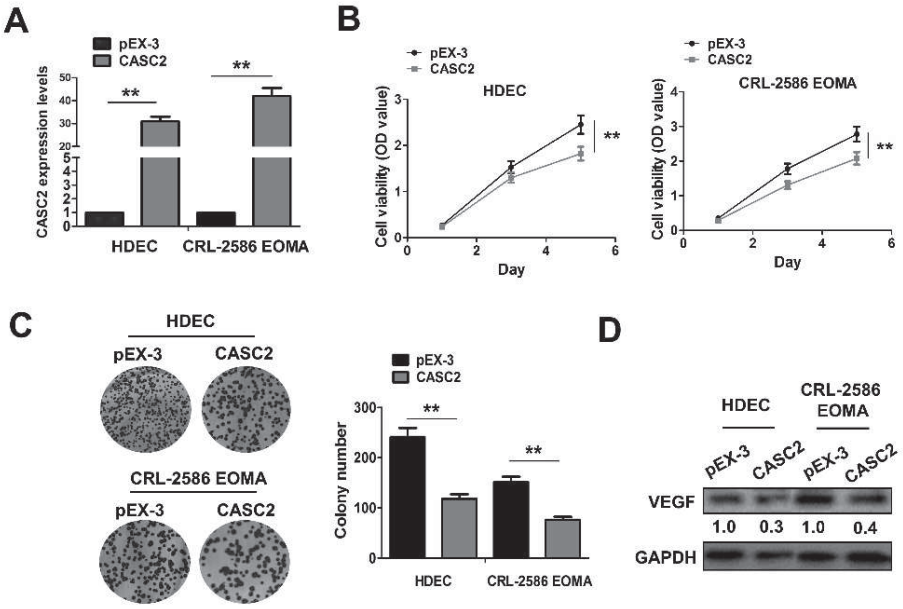

Fig. 2. *CASC2 overexpression inhibited HAs cell proliferation and colony formation. **A)** qRT-PCR analysis of the transfection efficiency of CASC2 plasmids in HDEC and CRL-2586 EOMA cells. **B)** MTT analysis of the cell proliferative viability after transfection with CASC2 plasmids in HAs cells.(**C**) Colony formation analysis of the cell colony formation number after transfection with CASC2 plasmids in HAs cells. **D)** Western blot analysis of the expression levels of VEGF after transfection with CASC2 plasmids in HAs cells. Data are the means ± SEM of three experiments. **P<0.01.*

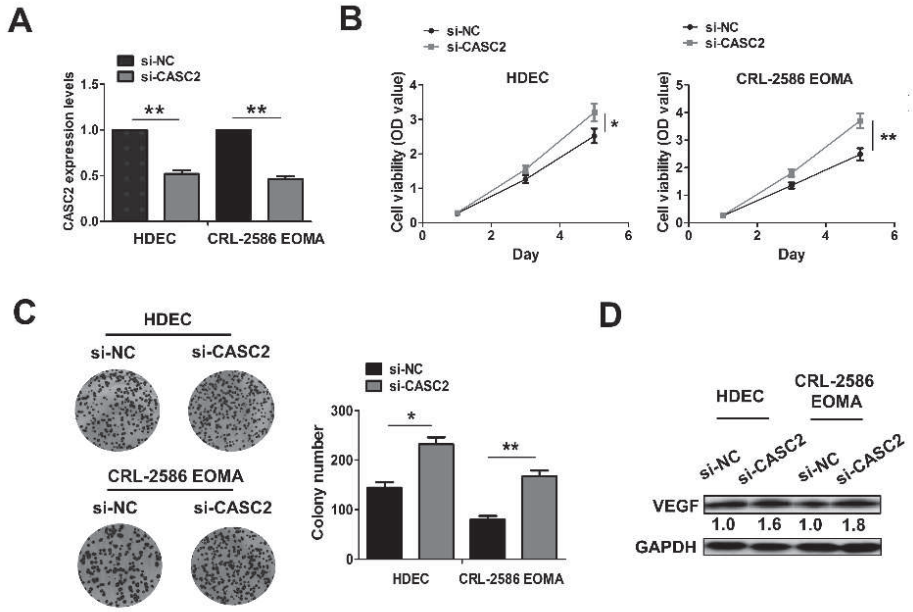

Fig. 3. *CASC2 knockdown promoted HAs cell proliferation and colony formation. **A)** qRT-PCR analysis of the transfection efficiency of si-CASC2 plasmids in HDEC and CRL-2586 EOMA cells. **B)** MTT analysis of the cell proliferative viability after transfection with si-CASC2 plasmids in HAs cells. **C)** Colony formation analysis of the colony formation number after transfection with si-CASC2 plasmids in HAs cells. **D)** Western blot analysis of the expression levels of VEGF after transfection with si-CASC2 plasmids in HAs cells. Data are the means ± SEM of three experiments. *P<0.05; **P<0.01.*

5p expression was markedly increased in PPH as compared with IPH or normal tissues ($P = 0.0079$, $P = 0.0048$; Fig. 4A), and had a negative correlation with VEGF expression in PPH (r=-0.5931, $P = 0.0198$; Fig. 4B). The binding sites of miR-18a-5p with wild type (WT) or mutant (Mut) lncRNA CASC2 3'UTR are indicated in Fig. 4C. The transfection efficiency of miR-18a-5p mimic or inhibitor in HDEC and CRL-2586 EOMA cell lines was confirmed by qRT-PCR analysis (Fig. 4D). Then, the luciferase activities of WT lncRNA CASC2 3'UTR were lowered by miR-18a-5p mimic, but raised by its inhibitor, and those of Mut lncRNA CASC2 3'UTR were unaffected by miR-18a-5p in HAs cells (Fig. 4E). Moreover, miR-18a-5p expression was markedly depressed by lncRNA CASC2, but elevated by silencing lncRNA CASC2 (Fig. 4F), but lncRNA CASC2 expression was unaffected by miR-18a-5p in HAs cells (Fig. 4G).

miR-18a-5p reversed lncRNA CASC2-induced anti-proliferative effects by targeting FBXL3

After si-CASC2 plasmids were co-transfected with miR-18a-5p inhibitor into HDEC and CRL-2586 EOMA cells, we found that miR-18a-5p inhibitor repressed cell viability and reversed si-CASC2-induced cell proliferation in these two cell lines ($P < 0.05$, $P < 0.01$; Fig. 5A). Online TargetScanHuman7.1 (http://www. targetscan.org/

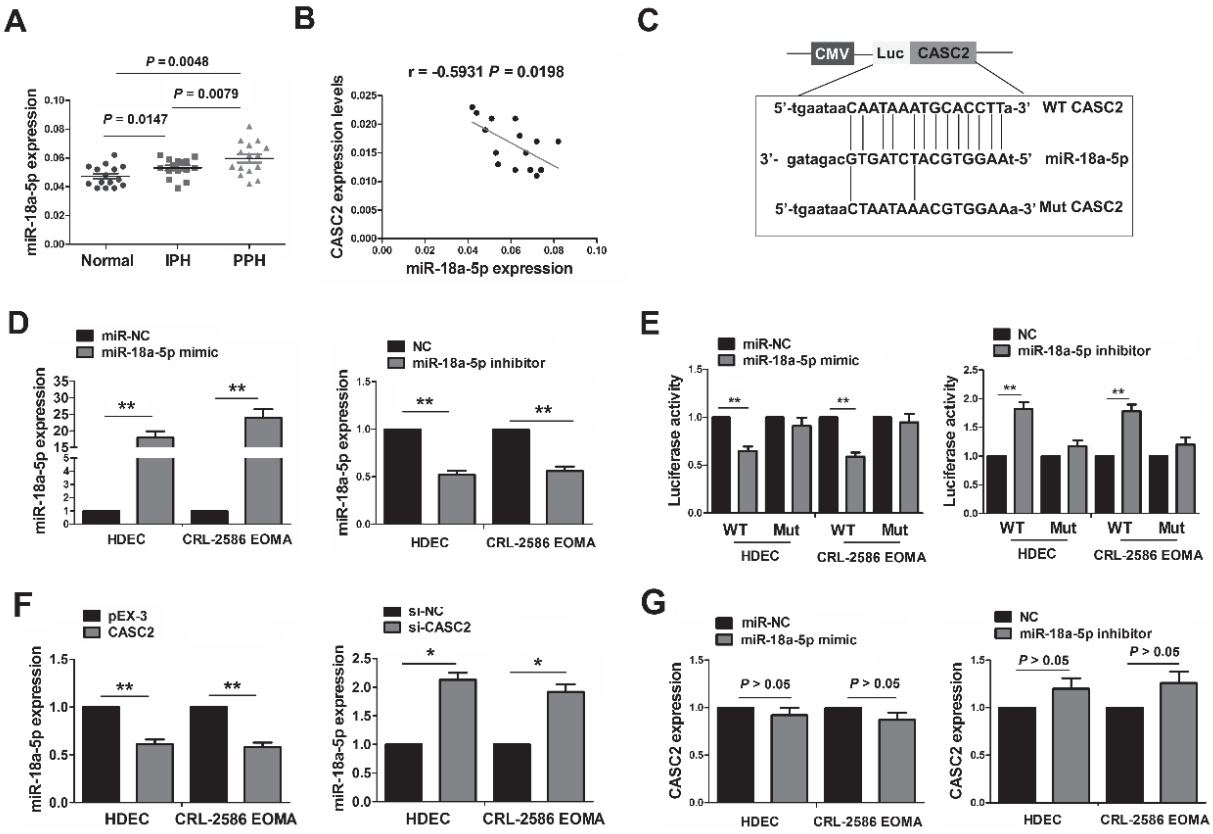

Fig. 4. *LncRNA CASC2 could bind with miR-18a-5p in HAs cells.* ***A****) qRT-PCR analysis of the expression levels of miR-18a-5p in PPH, IPH and normal tissues.* ***B****) Pearson correlation analysis of the correlation of CASC2 with miR-18a-5p expression in PPH.* ***C****) Schematic representation of the binding sites of WT or Mut CASC2 with miR-18a-5p.* ***D****) qRT-PCR analysis of the transfection efficiency of miR-18a-5p mimic or inhibitor in HDEC and CRL-2586 EOMA cells.* ***E****) Luciferase activities of WT or Mut CASC2 after transfection with miR-18a-5p mimic or inhibitor in HDEC and CRL-2586 EOMA cells.* ***F****) qRT-PCR analysis of the expression levels of miR-18a-5p after transfection with CASC2 or si-MEG3 plasmids in these two cell lines.* ***G****) qRT-PCR analysis of the effects of miR-18a-5p mimic or inhibitor on CASC2 expression levels in HDEC and CRL-2586 EOMA cells. Data are the means ± SEM of three experiments. *P<0.05; **P<0.01.*

vert_71/) was used to identify F-box and leucine-rich repeat protein 3 (FBXL3) as a direct target of miR-18a-5p (Cumulative weighted context score = - 0.80). FBXL3 expression levels were substantially decreased in PPH as compared with IPH or normal tissues ($P < 0.0001$, $P < 0.0001$) and displayed a negative correlation with miR-18a-5p expression in PPH (r= - 0.7175, $P = 0.0026$; Fig. 5B). The binding sites of miR-18a-5p with WT or Mut FBXL3 3'UTR are indicated in Fig. 5C. Then, the luciferase activities of WT FBXL3 3'UTR were lowered by miR-18a-5p mimic, but those of Mut FBXL3 3'UTR were unaffected by miR-18a-5p in HAs cells (Fig. 5D). qRT-PCR and Western blot analysis indicated that miR-18a-5p mimic significantly reduced the expression levels of FBXL3 as compared with miR-NC group in HDEC and CRL-2586 EOMA cells.

DISCUSSION

Accumulating data have demonstrated that the expression levels of lncRNA CASC2 are reduced in a variety of malignancies, and downregulation of lncRNA CASC2 can be used to predict a poor survival in cancer (9-13), but the expression of lncRNA CASC2 is elevated in HAs and favors the proliferation of HAs cells (17). Our previous study indicated that VEGF can act as a proliferating marker in PPH (4). Herein, lncRNA CASC2 expression levels were diminished as compared with IPH and normal tissues, and displayed a negative correlation

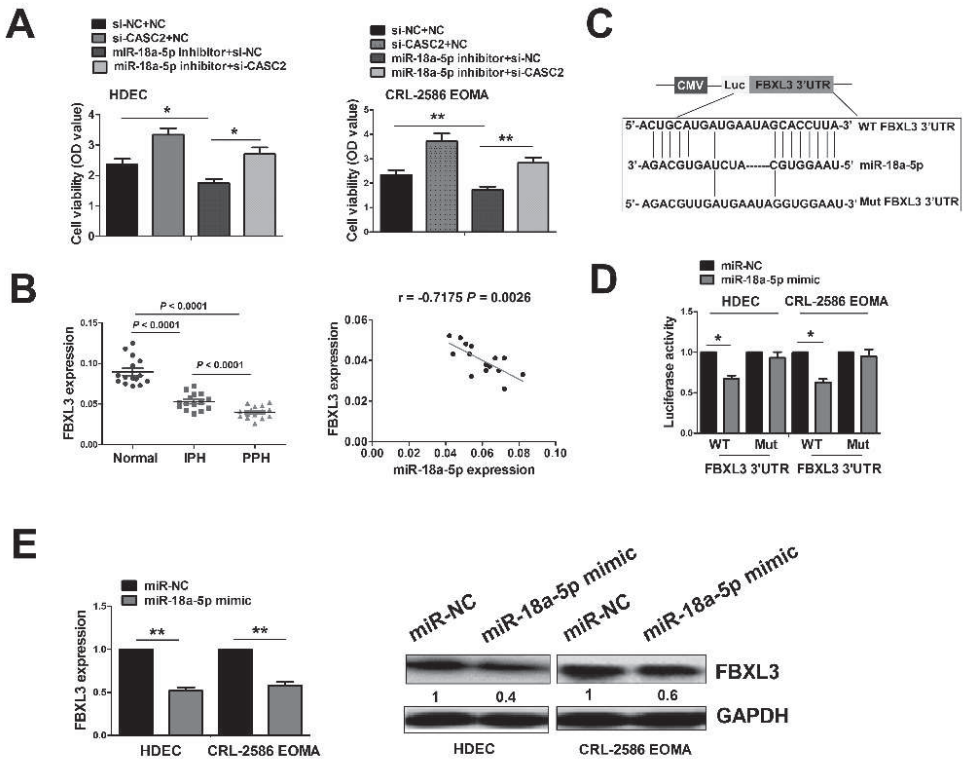

Fig. 5. *miR-18a-5p reversed CASC2-induced anti-proliferation effects by targeting FBXL3.* ***A****) MTT analysis of the cell proliferative viability after transfection with si-CASC2 and (or) miR-18a-5p inhibitor in HDEC and CRL-2586 EOMA cells.* ***B****) qRT-PCR analysis of the expression levels of FBXL3 in PPH, IPH and normal tissues, and Pearson correlation analysis of the correlation of FBXL3 with miR-18a-5p expression in PPH.* ***C****) Schematic representation of the binding sites of miR-18a-5p with WT or Mut FBXL 3'UTR.* ***D****) Luciferase activity of WT or Mut 3'UTR of FBXL3 after transfection with miR-18a-5p mimic in HDEC and CRL-2586 EOMA cells.* ***E****) qRT-PCR and Western blot analysis of the effects of miR-18a-5p mimic on FBXL3 expression levels in HAs cells. Data are the means ± SEM of three experiments. *P<0.05; **P<0.01.*

with VEGF expression in PPH rather than IPH, indicating that lncRNA CASC2 might be a negative factor in PPH.

lncRNA CASC2 functions as a tumor suppressive factor in multiple cancers (9-12). We found that lncRNA CASC2 suppressed HAs cell viability and colony formation, while downregulation of lncRNA CASC2 exerted an opposite effect. In terms of the association of VEGF with the proliferation of HAs (4), we found that, lncRNA CASC2 downregulated VEGF expression, thereby leading to the inhibition of HAs cell proliferation.

lncRNA CASC2 can act as the sponge of miRNAs to modulate cancer growth. For example, lncRNA CASC2 sponges miR-18a/-367 to retard tumor proliferation in OS and HCC (11, 12). Herein, we identified that miR-18a-5p had the potential to bind with lncRNA CASC2. It was upregulated in PPH as compared with IPH and normal tissues, and possessed a negative correlation with lncRNA CASC2 in PPH. lncRNA GAS5 also negatively regulates miR-18a-5p to inhibit glioma cell invasion (18). We also found that lncRNA CASC2 negatively regulated miR-18a-5p to inhibit HAs cell growth.

MiR-18a-5p is upregulated in renal cell carcinoma (RCC) and non-small cell lung cancer (NSCLC) and accelerates the carcinogenesis of RCC and NSCLC (19, 20). On the other hand, miR-18a-5p is downregulated in breast cancer and irradiated lung cancer and inhibits the migration and invasion of breast cancer and increases the radio-sensitivity of lung cancer by targeting HIF-1α (21, 22). Herein, miR-18a-5p was upregulated in PPH, and its inhibitor reduced HAs cell proliferation and reversed si-CASC2-induced cell proliferation. Furthermore, we identified FBXL3 as a direct target of miR-18a-5p in HAs cells and FBXL3 displayed a negative correlation with miR-18a-5p expression in PPH.

In conclusion, our findings demonstrated that lncRNA CASC2 suppressed HAs cell growth by regulating miR-18a-5p/FBXL3 axis and represents a potential therapeutic target for HAs.

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