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Original Article

Argonaute 2 promotes angiogenesis via the PTEN/VEGF signaling pathway in human hepatocellular carcinoma

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Aim: Argonaute2 (AGO2) protein is the active part of RNA-induced silencing complex, cleaving the target mRNA strand complementary to their bound siRNA. An increasing number of miRNAs has been identified as essential to angiogenesis of hepatocellular carcinoma (HCC). In this study we investigated how AGO2 affected HCC angiogenesis.
Methods: Human HCC cell lines HepG2, Hep3B, Huh7, SMMC-7721, Bel-7404, MHCC97-H and LM-3, and human umbilical vein endothelial cells (HUVEC) were tested. The expression of AGO2 in HCC cells was knocked down with siRNA and restored using recombinant adenovirus expressing Ago2. The levels of relevant mRNAs and proteins were examined using RT-PCR, Western blot and EILSA. Nude mice were implanted with Huh7 or SMMC-7721 cells, and tumor volumes were measured. After the mice were euthanized, the xenograft tumors were used for immunohistological analysis.
Results: In 6 HCC cell lines, AGO2 protein expression was significantly correlated with VEGF expression (r=+0.79), and with VEGF secretion (r=+0.852). Knockdown of Ago2 in Huh7 cells and SMMC-7721 cells substantially decreased VEGF expression, whereas the restoration of AGO2 reversed both VEGF expression and secretion. Furthermore, knockdown of Ago2 significantly up-regulated the expression of PTEN (a tumor suppressor involved in the inhibition of HCC angiogenesis), and vice versa. Moreover, the specific PTEN inhibitor bisperoxovanadate (7, 14, 28 nmol/L) dose-dependently restored the expression of VEGF and the capacity of HCC cells to induce HUVECs to form capillary tubule structures. In the xenograft nude mice, knockdown of Ago2 markedly suppressed the tumor growth and decreased PTEN expression and CD31-positive microvascular in the xenograft tumors.
Conclusion: A direct relationship exists between the miRNA processing machinery AGO2 and HCC angiogenesis that is mediated by the AGO2/PTEN/VEGF signaling pathway. The results suggest the high value of Ago2 knockdown in anti-angiogenesis therapy for HCC.

Keywords: hepatocellular carcinoma; angiogenesis; Argonaute 2; microRNAs; VEGF; PTEN; bisperoxovanadate

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Introduction

Hepatocellular carcinoma (HCC), the third most common cause for cancer mortality worldwide[1, 2], is characterized by neovascularization, which is closely associated with its metastasis, progression and therapy resistance[3, 4]. Like other hypervascular solid tumors, angiogenesis of HCC is intricately regulated by vascular endothelial growth factor (VEGF), basic fibroblast growth factor(bFGF), platelet-derived growth factor (PDGF), and other related factors[5, 6]. However, the mechanisms for HCC angiogenesis have still not been fully elucidated.

AGO2 is one of the four members of Argonaute protein family, which is characterized by typical PAZ (Piwi-Argonaute-Zwille) and PIWI domains[7]. However, in humans, AGO2 is the only member with slicer activity, essential to microRNA (miRNA) maturation as well as the gene-silencing process guided by small RNAs[8]. Recent studies have revealed that AGO2 was frequently up-regulated in cancers and was linked to carcinogenesis, including head and neck squamous cell carcinoma[9], colon cancer[10], colorectal carcinomas[11], ovarian carcinoma[12], gastric carcinoma[13], and urothelial carcinoma of the bladder[14]. In our previous study, we found that AGO2 was over-expressed in HCC cells and that Ago2-knockdown significantly impaired HCC growth *in vivo*, but barely affected HCC cell propagation *in vitro*[15]. This led us to speculate that

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AGO2 might promote HCC tumorigenesis through angiogenesis.

In this study, we investigated the involvement of AGO2 in HCC angiogenesis. Bi-directional manipulation of the Ago2 expression via RNAi-based knockdown and recombinant adenovirus-mediated expression restoration revealed that the expression of AGO2 protein was intensely associated with VEGF expression and secretion. Moreover, we found that PTEN was a mediator of AGO2 and VEGF interaction. These results provided new insight on the role of AGO2 in HCC angiogenesis via the PTEN/VEGF signaling pathway.

Materials and methods

Cell culture

The human HCC cell lines HepG2, Hep3B, Huh7, and Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from the ATCC (Manassas, VA, USA), and human HCC cell lines SMMC-7721, Bel-7404, MHCC97-H, and LM-3 were obtained from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured according to the instructions of the providers, that is, HepG2, Hep3B, Huh7, Bel7404, MHCC97-H, LM-3, HUVEC cells were cultured in high glucose DMEM with 10% FBS, and SMMC-7721 cells were cultured in RPMI-1640 medium with 10% FBS. All cells were incubated under 37 °C, 5% CO_2 conditions.

Virus construction

To conduct RNAi-based knockdown, two previously identified oligonucleotides encoding the Ago2-specific siRNA sequence and a loop sequence separating the complementary domains were synthesized and inserted into the lentiviral transfer vector pGC-LV (Genechem, Shanghai, China)[15]. A non-silencing-siRNA was used as a control. Then, the transfer vector with Ago2-specific shRNA was obtained and co-transfected with the lentiviral help plasmids pHelper 1.0 and pHelper 2.0 into HEK-293T cells using Lipofectamine 2000 reagent (Invitrogen) to package lentiviral particles. To transiently restore the expression of AGO2, a replication-defective recombinant adenovirus carrying the expression cassette of Ago2, Ad-Ago2, was constructed according to a previously described procedure[16]. The adenovirus was used to infect Ago2-knockdown HCC cells at five multiplicity of infection (MOI), and the adenovirus without transgene (*ie*, Ad-blank) served as the control.

Quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Life Technology, USA) when cells covered approximately 80% of the bottom of 6-well plates. The reverse transcriptase reactions used the Quantscript RT Kit (TIANGEN, Beijing, China) and 1.0 μg RNA of each sample in a 20 μL reaction. Then, total cDNA was diluted to a volume of 100 μL, of which, 2 μL was used for target mRNA expression measurements by real-time PCR with SYBR Green Realtime PCR Master Mix (TOYOBO, Japan) according to the manufacturer's protocol and normalized to GAPDH. All reactions were performed in triplicate.

Table 1. Sequences of primers for RT-PCR.

Target	Sequence (5′–3′)
AGO2	F: CGTGCCTGCTGGAATGTTTC
	R: CCATCCGTGAGGCCTGTATC
PTEN	F: CGGCAGCATCAAATGTTTCAG
	R: AACTGGCAGGTAGAAGGCAACTC
VEGF	F: CTACCTCCACCATGCCAAGT
	R: AGCTGCGCTGATAGACATCC
FGF1	F: GTCAGTGCTGCCTGAATGCT
	R: CTCCCGAAGGATTAAACGACG
FGF2	F: GGAGAAGAGCGACCCTCAC
	R: AGCCAGGTAACGGTTAGCAC
TGF-β	F: CCCTGGACACCAACTATTGC
	R: AAGTTGGCATGGTAGCCCTT
PDGFA	F: GGCACTTGACACTGCTCGT
	R: GCAAGACCAGGACGGTCATTT
PDGFB	F: GCGCTCTTCCTGTCTCTCTG
	R: TCGAGTGGTCACTCAGCATC
GAPDH	F: GGAGGAGTGGGTGTCGCTGT
	R: GTGGACCTGACCTGCCGTCT

However, for semi-quantitative reverse transcription and polymerase chain reaction, a volume of 5 μL was used, and analyzed by nucleic acid gel electrophoresis. The relative expression values of different samples were determined using Huh7 or SMMC-7721 cells as reference samples. The sequences of the primers used in these experiments are listed in Table 1.

Western blot

Protein lysates from cell lines were prepared in lysis buffer and centrifuged at 12 000×*g* at 4 °C. Western blotting was performed according to a previously described procedure[15]. The primary antibodies used were rabbit anti-AGO2 (CST, USA), rabbit anti-PTEN (CST, USA), mouse anti-VEGF (Beyotime, China), mouse anti-GAPDH (Beyotime, China). The secondary antibodies were HRP-labeled goat anti-rabbit or anti-mouse IgG (H+L) (Beyotime, China). The expression of each band was quantitatively analyzed using the Image Lab™ Software (Bio-Rad, USA) and normalized to the expression of GAPDH in the same lane.

Enzyme-linked immunosorbent assay

Suspensions from Huh7, SMMC-7721, Ago2-knockdown Huh7 (*ie* Huh7-siAgo2), Ago2-knockdown SMMC-7721 (*ie*, 7721-siAgo2) cells were collected and diluted five-fold for VEGF secretion tests by Enzyme-linked Immunosorbent Assay using the Human VEGF Quantikine ELISA Kit (R&D, USA). Assays were carried out according to the manufacturer's instructions.

Capillary tube assay

The ability of Huh7, SMMC-7721, Huh7-siAgo2, and 7721-siAgo2 cells to induce endothelial cells to proliferate and organize into capillary-like sprouts was examined using HUVECs as previously described[15] with a slight modification. In sum-

mary, 6.0×10^4 HUVEC cells were collected, re-suspended in conditioned medium, which was the suspension from Huh7, SMMC-7721, Huh7-siAgo2, 7721-siAgo2 cell culture for 48 h, and seeded in 24-well plates, each of which was coated with 50 μL growth factor-reduced matrigel (BD, USA). Photos were taken 24 h later.

Immunofluorescence assay

Cells were plated onto a confocal dish and fixed by paraformaldehyde for 20 min and solubilized with Triton X-100 for 10 min. The primary antibody rabbit anti-PTEN was used at a concentration of 1:100. Alexa Fluor 555-labeled donkey anti-rabbit IgG (H+L) (Beyotime, China) secondary antibody was used for the immunofluorescence assay. After immunolabeling, cells were washed, stained with DAPI, and then scanned with a confocal laser scanning microscope (Zeiss LSM700, Germany).

In vivo assay

All animal experiments were undertaken in accordance with the National Institute of Health guidelines for the care and use of laboratory animals, with the approval of the Scientific Investigation Board at the Second Military Medical University, Shanghai. Huh7, SMMC-7721 and Huh7-siAgo2, 7721-siAgo2 cells were injected subcutaneously into nude mice (1×10^7/mouse, 6 per group). Twelve days post injection, tumor volumes were measured every 4 d thereafter [volume=($W^2\times L$)/2; W, width; L, length, in cubic millimeters]. The mice were euthanized four weeks post transplantation to weight the xenograft tumors, and the tumor tissues cells were separated for immunohistological analysis using the avidin biotin-peroxidase complex (ABC) method. The used primary antibodies were AGO2 (1:250, CST, USA), PTEN (1:250, CST, USA), and CD31 (1:200, Abcam, USA).

Statistical analysis

All data are presented as the mean±standard deviation. Independent Student's *t*-test was used to analyze the variation of two selected groups, and Pearson's chi-square test was used to analyze the correlation of two parameters. A $P<0.05$ was considered statistically significant and $P<0.01$ was considered highly statistically significant. All statistical analyses were performed with SPSS (Statistical Package for the Social Sciences) version 18.0 software.

Results

The expression and secretion of VEGF was significantly correlated with AGO2 protein in HCC cells

In our previous study, we had surprisingly noticed that Ago2-knockdown impaired the tumorigenesis of Huh7 cells *in vivo*, but barely affected the growth of Huh7 cells *in vitro*. These results indicate that AGO2 may be involved in HCC angiogenesis. To determine the validity of this hypothesis, we first examined the expression of the angiogenesis-related factors VEGF, TGF-β, PDGFA, PDGFB, FGF1, FGF2 in the Ago2-knockdown HCC cells. The results showed that VEGF expression was remarkably decreased ($P=0.0068$), while the expression of the other factors was almost unchanged under the condition when AGO2 was specifically reduced (Figure 1A, 1B). Thus, the correlation between AGO2 and VEGF was investigated by examining the expression of AGO2 and VEGF in a list of HCC cell lines (Figure 1C). From the results of

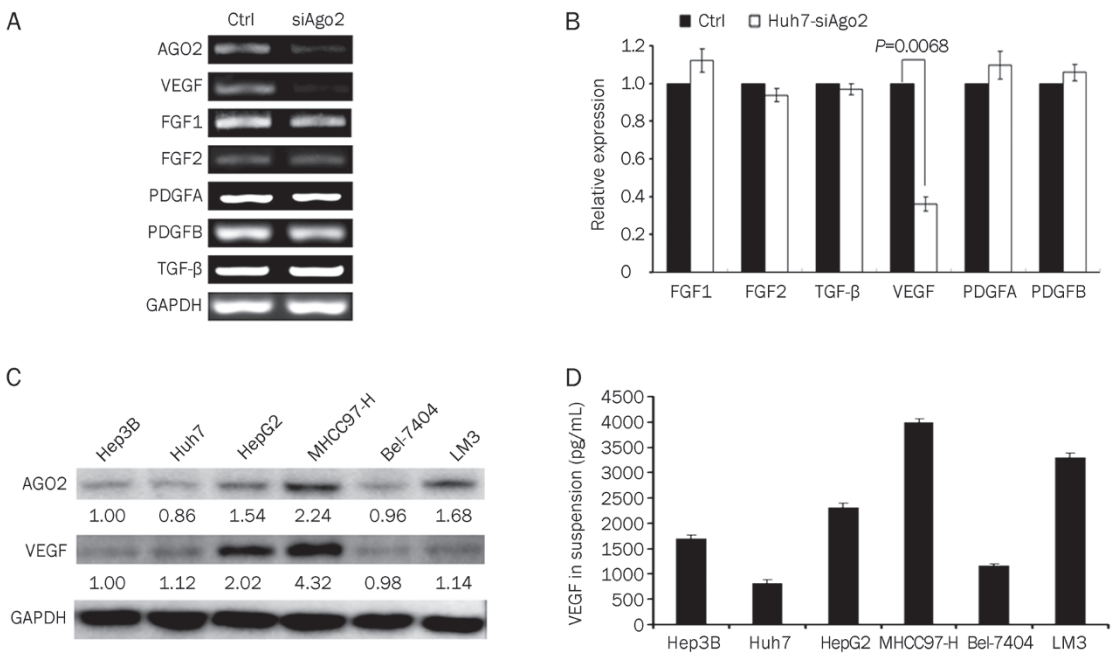

Figure 1. Expression of AGO2 and VEGF was significantly correlated in HCC cell lines. (A and B) RT-PCR was performed to detect the expressions of angiogenesis related factors in Huh7-ctrl and Huh7-siAgo2 cells; (C) Western blot was performed to detect VEGF and AGO2 expressions in HCC cell lines. (D) ELISA was performed to detect VEGF secretion in HCC cell lines suspensions.

quantitative Western blot analysis, we found that the expression of AGO2 and VEGF was significantly correlated at the protein level (r=+0.79, P=0.031). Furthermore, a more significant correlation was revealed when we analyzed AGO2 intracellular expression and VEGF secretion in HCC cell culture suspensions (r=+0.852, P=0.001).

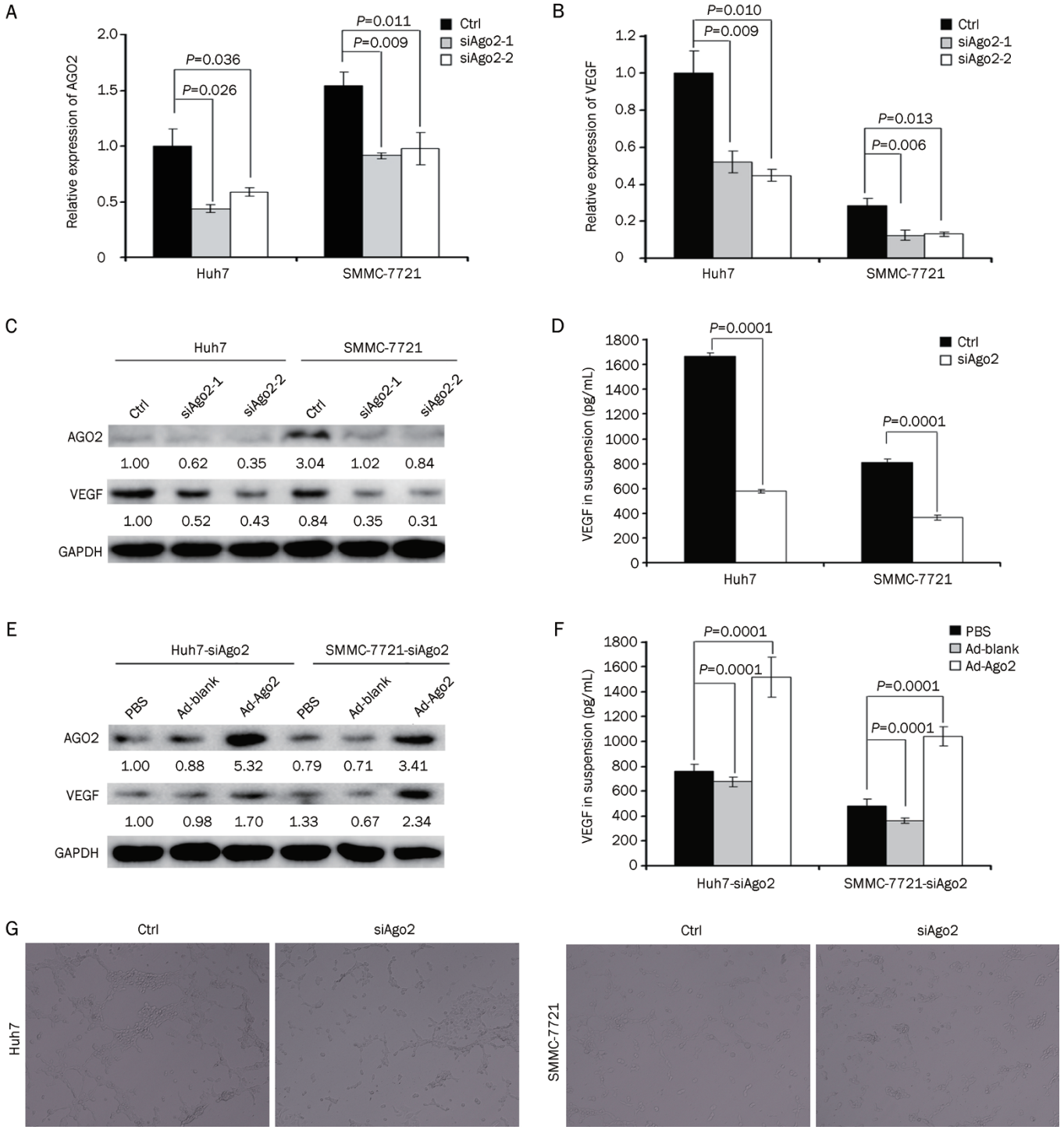

Figure 2. Ago2-knockdown impaired HCC angiogenesis through reduction of VEGF. (A–D) Quantitative real-time RT-PCR, Western blot, and ELISA were performed to test the effect of Ago2-knockdown on VEGF expression. (E and F) Western blot and ELISA were performed to detect the effect of Ago2 restoration on VEGF expression. (G) 3-D matrigel system was used to test the ability of Ago2-knockdown HCC cells to induce capillary tubules formation from HUVECs (200×). Ad-Ago2 and Ad-blank indicated the recombinant adenovirus expressing Ago2 and blank adenovirus without transgene, respectively.

Ago2-knockdown impaired angiogenesis by reducing VEGF expression *in vitro*

To confirm the correlation between AGO2 and VEGF, and due to their respective minimum and maximum up-regulation of AGO2 expression compared to normal liver cells, Huh7 and SMMC-7721 cells were chosen to conduct Ago2 knocking down with two individual siRNAs targeting different locations of the Ago2 gene (Figure 2A). The data showed that the expression of VEGF was decreased at the RNA level in all Ago2-knockdown HCC cells (*ie*, Huh7-siAgo2-1/Huh7-siAgo2-2 and 7721-siAgo2-1/7721-siAgo2-2) relative to the corresponding control cells expressing a non-silencing-siRNA ($P<0.05$, Figure 2B). These results were consistent with those from the Western blot and EILSA analyses, indicating that the expression of AGO2 and VEGF was synchronously down-regulated ($P<0.001$, Figure 2C and 2D). Interestingly, both the expression and secretion of VEGF were increased when we restored the expression of AGO2 by infection with the recombinant adenovirus expressing AGO2 ($P<0.001$, Figure 2E and 2F). These results suggested that AGO2 could promote HCC angiogenesis by the up-regulation of VEGF.

In vitro co-culture of cell culture suspensions with HUVECs in a three dimensional matrigel system has been ubiquitously applied for angiogenesis analysis[6, 17]. Thus, we next co-cultured HCC cell culture suspensions with HUVECs in matrigel-coated 6-well plates to examine the influence of Ago2-knockdown on the capacity of the HCC cells to induce HUVECs to form capillary tubules. These results showed that the ability of HCC cells to induce HUVEC capillary tubule formation was greatly attenuated due to Ago2-knockdown in Huh7 and SMMC-7721 cells relative to the control (Figure 2G). These results indicated that AGO2 could promote HCC angiogenesis by up-regulating the expression of VEGF, illustrating that AGO2 could function in HCC angiogenesis through regulating the expression of VEGF.

Ago2-knockdown impaired HCC angiogenesis *in vivo*

To examine the function of AGO2 on HCC angiogenesis *in vivo*, Huh7-siAgo2/Huh7-ctrl and 7721-siAgo2/7721-ctrl cells were subcutaneously transplanted into nude mice. Twelve days post transplantation, the solid tumors could be detected in all mice. However, the tumor growth of the test group (*ie*, Huh7-siAgo2 and 7721-siAgo2) was significantly slower than those of the control group (*ie*, Huh7-ctrl and 7721-ctrl) during the following half month ($P<0.05$, Figure 3A and 3B). On the day of euthanasia, the sizes of xenograft from Huh7-siAgo2 and 7721-siAgo2 group were remarkably smaller than those from Huh7-ctrl and 7721-ctrl, respectively (Figure 3C and 3D). The xenograft tumor weights between the test group and control group also exhibited a significant difference ($P<0.0001$, Figure 3E and 3F).

Immunohistochemistry analysis was then performed to examine the variance of microvascular richness between the xenografts from the test and control groups. As a result, we found that the CD31-postive microvascular within the xenograft tissues was greatly decreased with the knockdown of Ago2 (Figure 3G) in two HCC transplantation models. These results indicated that Ago2-knockdown could effectively impair HCC angiogenesis and thereby inhibit tumor growth *in vivo*, suggesting a potent utilization of Ago2-knockdown in antiangiogenic therapy for HCC.

Ago2-knockdown impaired HCC angiogenesis through the up-regulation of PTEN

We further investigated the underlying mechanism through which AGO2 regulates HCC angiogenesis. In our previous study[15], Ago2-knockdown was revealed to elicit an up-regulation of PTEN, a tumor suppressor which has been identified as a typical inhibitor of angiogenesis and VEGF expression[18–22]. Therefore, it was reasonable that PTEN might serve as a mediator of the association between AGO2 and VEGF. In accordance with our previous finding, the expression of PTEN was significantly increased in either Huh7 or SMMC-7721 cells when Ago2 was knocked down both *in vitro* and *in vivo* ($P<0.05$, Figure 4A, 4B, and 3G). The results were further confirmed by immunofluorescence analysis, in which PTEN signal was stronger in the HCC cells expressing Ago2-specific siRNA (eGFP was co-expressed with the siRNA) relative to the control (Figure 4C). Conversely, the expression of PTEN could be reduced by the restoration of AGO2 (Figure 4D).

To further investigate the role of PTEN in mediating the capacity of AGO2 to regulate VEGF, the function of PTEN was blocked with the PTEN inhibitor bisperoxovanadate (bpv). In agreement with our expectations, we found that VEGF was increased when bpv was utilized at four different concentrations (*ie*, 0, 7, 14, and 28 nmol/L), while the expression of AGO2 was barely affected (Figure 4E). Subsequently, capillary tube assays were also conducted with HUVECs. The results showed that the capacity of Huh7-siAgo2 and 7721-siAgo2 cell culture suspensions to induce capillary tubule formation with HUVECs could be restored when the function of PTEN was inhibited (Figure 4F). Taken together, these results indicated that AGO2 might regulate HCC angiogenesis through the deregulation of PTEN and the consequent up-regulation of VEGF.

Discussion

In the previous study, we had demonstrated that up-regulation of AGO2, an executor of miRNA-guided gene silencing, could regulate HCC growth by specifically enhancing the capacity of oncogenic miRNAs (*eg*, miR-21a) to repress their target tumor suppressor genes (*eg*, PTEN), as expression profiling showed that oncogenic miRNAs such as miR-21a[15] and miR-221[23] are ectopically over-expressed while tumor suppressive miRNAs such as miR-199a[24] and let-7a[25] are dysfunctionally restricted or even lose expression. In this study, we have further documented the role of AGO2 and its role in the regulation of HCC angiogenesis. It was revealed that the expression and secretion of VEGF are significantly correlated with the expression of AGO2 protein among several HCC cell lines, whereas Ago2-knockdown can induce down-regulation of VEGF and consequently lead into the suppression of HCC

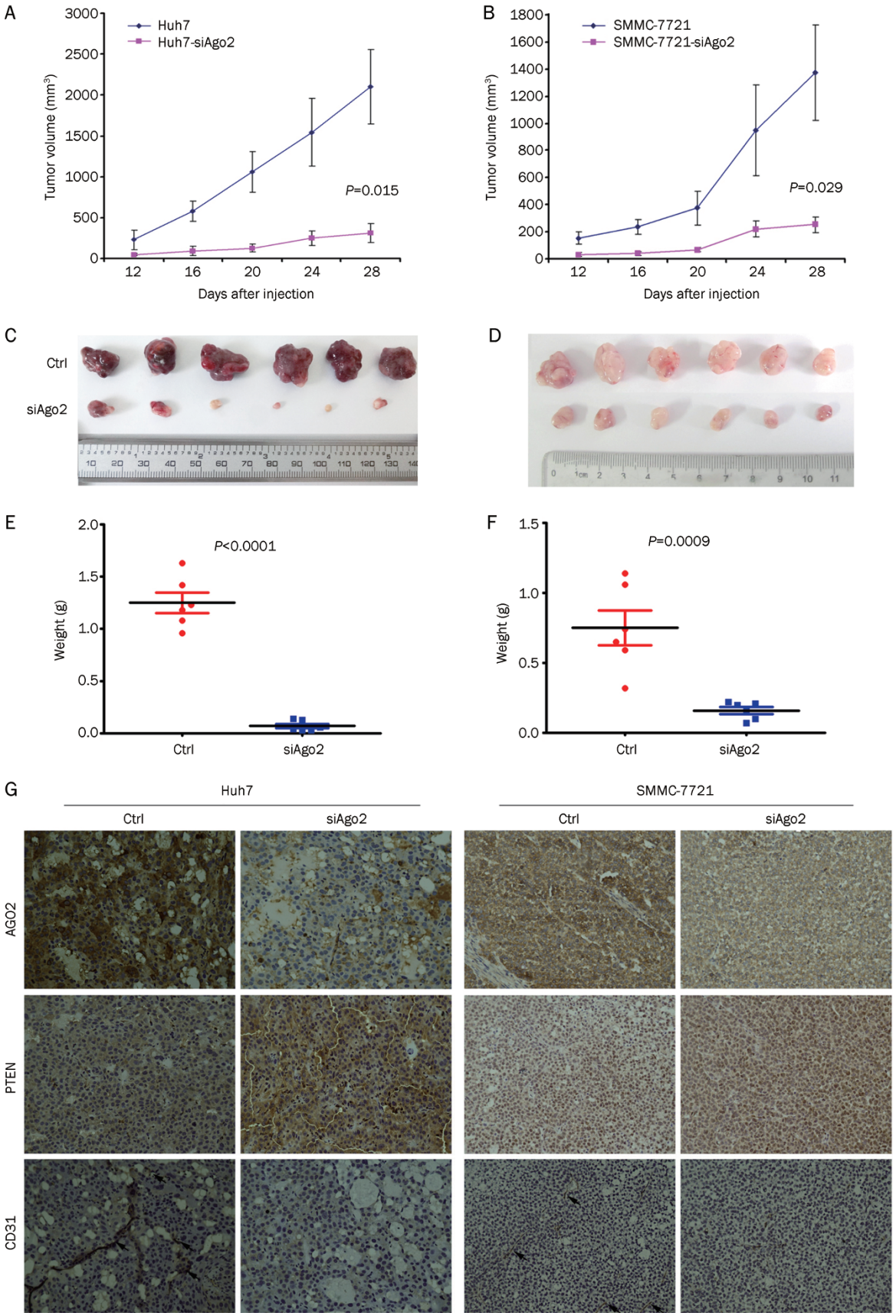

Figure 3. Ago2-knockdown impaired HCC angiogenesis and tumor growth *in vivo*. (A–G) The growth curve, morphology, and weight of xenograft were measured. (H) Immunochemistry was conducted by using antibody for AGO2, PTEN, and CD31 (200×).

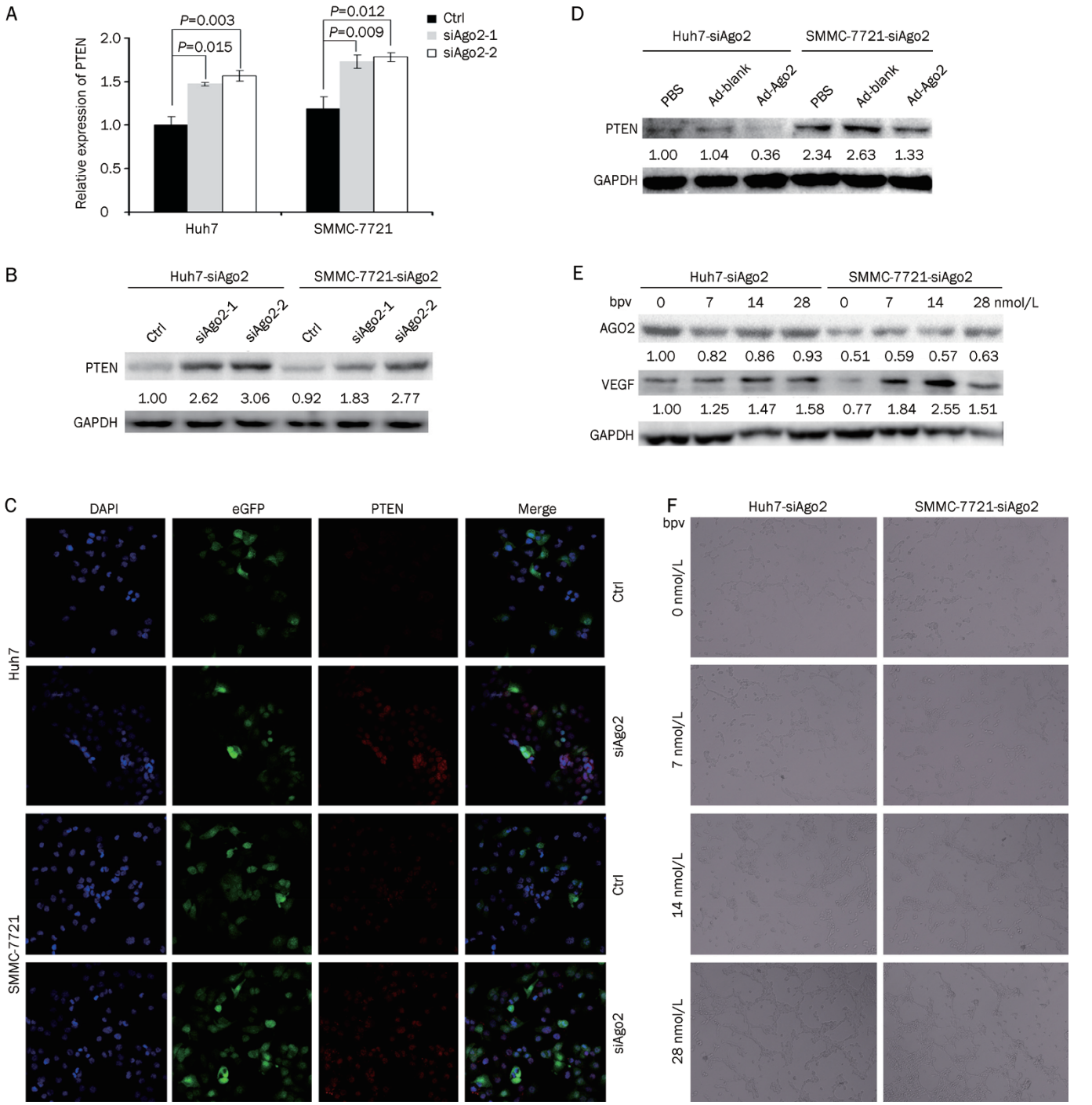

Figure 4. Ago2-Knockdown impaired HCC angiogenesis by up-regulation of PTEN. (A–C) Quantitative real-time RT-PCR, Western blot, and immunofluorescence assay (200×) was used to test the effect of Ago2-knockdown on PTEN expression, respectively. (D) Western blot was performed to test the effect of Ago2 restoration on PTEN expression. (E) Western blot was performed to test the impact of PTEN inhibitor bisperoxovanadate (bpv) at four different concentrations on AGO2 and VEGF expression. (F) 3-D matrigel system was used to test Ago2-knockdown HCC cells' ability to induce capillary tubules formation from HUVECs after utilizing PTEN inhibitor bpv (200×).

angiogenesis *in vitro* and *in vivo*. Interestingly, this suppression can be reversed through either restoration of AGO2 or functional blockade of PTEN, which is frequently mutated or down-regulated in HCC cells (Supplementary Figure 1A). Thus, the results presented here have confirmed the regulatory relationship between AGO2 and PTEN, and then further extended it to VEGF. The results have broadened our understanding of HCC angiogenesis by providing the novel signaling pathway of AGO2/PTEN/VEGF and offering a reasonable explanation for the irrelevant impact of Ago2-knockdown *in*

vitro and *in vivo*.

It has been well documented that specific miRNAs play important roles in the regulation of cancer angiogenesis[26]. In HCC, miRNAs such as miR-21a and miR-195, among others, have been identified to regulate HCC angiogenesis[27]. Recently, Dicer, a miRNA processing machinery that has universal function in miRNA maturation, has been demonstrated to regulate cancer angiogenesis[28, 29]. Data reported by Tomohiro *et al* have also indicated that AGO2 participated in controlling the growth of endothelial cells, thereby directly regulating angiogenesis[30]. In this study, another member of miRNA processing machinery-Ago2, has been revealed to indirectly regulate HCC angiogenesis through its regulatory effect on VEGF but not HIF1α (Supplementary Figure 1B). It might be of great interest to investigate the role of AGO2 in angiogenesis in other types of malignancy because its ectopic expression is present in various cancers[9–14].

As an indispensable mediator of miRNA-related regulatory pathways, AGO2 also has been shown to directly interact with common cancer associated genes, such as EGFR[31], FAK[32], AKT[33], MAPK[34], P4H[35], *etc*. Among them, several genes have already been proven to be involved in regulating angiogenic-related factors (*ie*, VEGF). It is noted that EGFR and P4H are effectors of hypoxia, which is a major factor in angiogenesis promotion. Under hypoxic conditions, EGFR can phosphorylate AGO2 and inhibit its capacity to regulate the maturation of tumor suppressive miRNAs[36, 37], and P4H can also regulate the stability of HIF1α and AGO2[38]. Therefore, it is possible that AGO2 may exert its regulation of angiogenesis through different signaling pathways besides AGO2/PTEN/VEGF. Nevertheless, the data showed here that the expression of VEGF is significantly correlated with AGO2 and Ago2-knockdown in two different HCC cells (*ie*, Huh7 and SMMC-7721) can remarkably impair HCC angiogenesis *in vitro* and *in vivo*. This finding clearly supports the regulatory role of AGO2 in HCC angiogenesis and suggests that AGO2 might serve as a potential target for anti-angiogenic therapy against malignant tumors.

Acknowledgements

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Author contribution

Qi-jun QIAN, Hua-jun JIN and Zhen-long YE designed research and revised the manuscript; Zhen-long YE and Yao HUANG performed research and wrote the manuscript; Linfang LI contributed new reagents; Hai-li ZHU assited with animal experiment; Hai-xia GAO assisted with Immunofluorescence experiment ; Hui LIU, Jing-lei ZHANG and Sai-qun LV assisted with ELISA, Zeng-hui XU, Luo-ning ZHENG and Tao LIU assisted with the confocal laser scanning microscope operation.

Supplementary information

Supplementary figures are available at the Acta Pharmacologica Sinica website.

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