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Role of microRNA-27a in down-regulation of angiogenic factor AGGF1 under hypoxia associated with high-grade bladder urothelial carcinoma

Yan Xu [a], Ming Zhou [b], Jingjing Wang [a], Yuanyuan Zhao [a], Sisi Li [a], Bisheng Zhou [a], Zhenhong Su [a], Chengqi Xu [a], Yue Xia [d], Huijun Qian [d], Xin Tu [a], Wei Xiao [e], Xiaoping Chen [e], Qiuyun Chen [c], Qing K. Wang [a,c,*]

[a] The Key Laboratory of Molecular Biophysics of the Ministry of Education, Department of Genetics and Developmental Biology, College of Life Science and Technology, Center for Human Genome Research, Cardio-X Institute, Huazhong University of Science and Technology, Wuhan, PR China
[b] Department of Anatomic Pathology, Cleveland Clinic, Cleveland, OH, USA
[c] Center for Cardiovascular Genetics, Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA
[d] Department of Urology, Renmin Hospital of Wuhan University, Wuhan 430060, PR China
[e] Hepatic Surgery Centre, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, PR China

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ABSTRACT

Hypoxia stimulates angiogenesis under a variety of pathological conditions, including malignant tumors by inducing expression of angiogenic factors such as VEGFA. Surprisingly, here we report significant association between down-regulation of a new angiogenic factor AGGF1 and high-grade urothelial carcinoma. The proportion of strong AGGF1 expression cases was significantly lower in the high-grade urothelial carcinoma group than that in the low-grade urothelial carcinoma group ($P = 1.40 \times 10^{-5}$) or than that in the normal urothelium tissue group ($P = 2.11 \times 10^{-4}$). We hypothesized that tumor hypoxia was responsible for differential expression of the AGGF1 protein in low- and high-grade urothelial carcinomas, and therefore investigated the molecular regulatory mechanism for AGGF1 expression under hypoxia. Under hypoxic conditions, AGGF1 protein levels declined without any change in mRNA levels and protein stability. Hypoxia-induced down-regulation of AGGF1 was mediated by miR-27a. Overexpression of miR-27a suppressed AGGF1 expression through translational inhibition, but not by RNA degradation. Moreover, the hypoxia-induced decrease of AGGF1 expression disappeared after miR-27a expression was inhibited. Furthermore, down-regulation of AGGF1 reduced hypoxia-induced apoptosis in cancer cells. Taken together, the results of this study indicate that (1) hypoxia down-regulates expression of the AGGF1 protein, but not *AGGF1* mRNA, by inducing expression of miR-27a; (2) Down-regulation of AGGF1 had an apparent protective role for cancer cells under hypoxia; (3) Down-regulation of the AGGF1 protein confers a significant risk of high-grade human urothelial bladder carcinoma.

1. Introduction

Hypoxia occurs during a range of diverse physiological and pathophysiological states, including development, cancer, and chronic inflammation [1]. Most solid tumors contain substantial hypoxic regions, and the extent and severity of the region correlate with poor prognosis in patients [2]. Moreover, hypoxic cancer cells usually show resistance to chemotherapy and radiotherapy, which are also associated with poor clinical prognosis [3]. Hypoxic conditions trigger coordinated regulation of many genes, including those involved in increased angiogenesis, up-regulation of glycolytic enzymes and genetic instability [4]. Studies of the mechanisms underlying the regulation of such genes have emphasized the dominant role of the hypoxia-inducible factor (HIF) transcriptional system [5]. Recently, another mechanism of gene regulation mediated by microRNAs (miRNAs) in hypoxia was identified [5–7]. MiRNAs are small non-coding RNAs that repress target gene expression through RNA degradation and/or translational inhibition [8–10]. MiRNAs have been shown to affect fundamental cellular processes, including cell cycle regulation, proliferation, and apoptosis [11,12].

AGGF1 is a newly identified angiogenic factor which is highly expressed in endothelial cells and promotes angiogenesis as potently as VEGF in a chick embryo angiogenesis assay [13]. Increased *AGGF1* expression is associated with vascular disease Klippel–Trenaunay syndrome [13,14]. Our group has demonstrated that *AGGF1* overexpression in zebrafish embryos activates the AKT signaling pathway to induce the specification of vein identity and angiogenesis [15]. More recently, we have found that *AGGF1* is the master regulatory gene that drives differentiation of the mesodermal cells to multipotential hemangioblasts [16]. Increased *AGGF1* expression increases angiogenesis and blood flow and improves limb functions in a mouse hindlimb ischemia model of peripheral artery disease [17]. Tumor growth and expansion require angiogenesis, which may involve hypoxia-induced expression of angiogenic factors.

* Corresponding author at: Center for Human Genome Research, Huazhong University of Science and Technology, Wuhan, Hubei 430074, PR China.
E-mail addresses: qkwang@mail.hust.edu.cn, wangq2@ccf.org (Q.K. Wang).

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The association and potential contribution of AGGF1 in cancer progression and metastasis, however, remains unknown.

Bladder carcinoma is the fourth most common malignancy in men in the United States. Urothelial carcinoma is the most common histological subtype of bladder cancer and accounts for 90%–95% of cases, while squamous cell carcinoma represents only 5% of cases [18]. In 2013, it was estimated that 72,570 new patients would be diagnosed with bladder cancer, and 15,210 were projected to die from the disease [19]. Most cases of urothelial cancer are papillary tumors that have been classified into low-grade and high-grade papillary urothelial carcinomas. The latter is biologically more aggressive and carries a high probability of progression into muscle-invasive carcinoma and cancer-specific mortality [20]. Early detection and a deeper molecular understanding of urothelial bladder cancer are needed to improve diagnosis and treatment [21]. Many markers were implicated in identification of patients who may have an aggressive form of urothelial carcinoma and also in affecting outcome after treatment. But until now none of the current prognostic molecular markers are sufficiently validated [22].

In the present study, we showed that high-grade bladder carcinoma was associated significantly with down-regulation of the AGGF1 protein as compared to normal bladder tissues and low-grade bladder carcinoma. To investigate the molecular mechanisms of down-regulation of AGGF1 expression in high-grade bladder carcinoma, we hypothesized that hypoxia regulates AGGF1 expression. Our study showed that hypoxia induced a dramatic decrease in the expression of AGGF1 in different human cancer cells, whereas expression of control proteins HIF1α and VEGF-A (markers of hypoxia) increased as expected. The hypoxia-induced decrease in AGGF1 expression only occurred at the protein level instead of transcriptional suppression, and was mediated by miR-27a.

2. Materials and methods

2.1. Evaluation of AGGF1 expression by immunohistochemistry in human bladder tissues

Urothelial carcinoma and normal urothelial tissue samples were collected from 59 patients undergoing surgery at the Cleveland Clinic. The World Health Organization classification of bladder tumors was used to assess the histologic grading of urothelial carcinomas. The grading of tumors was evaluated by at least two pathologists and surgeons. Thirty tumors were classified as low-grade urothelial carcinomas (LGUCC) and 22 were high-grade urothelial carcinomas (HGUCC). Seven normal non-neoplastic urothelial tissue samples were obtained from patients with non-neoplastic bladder diseases. The thirty low grade and 22 high grade urothelial carcinomas were used to construct the tissue microarray (TMA). The thirty low grade tumors were all transurethral resection specimens. Twenty nine of them were pTa and one case was pT1. For 22 high grade tumors, 15 were radical cystectomy specimens and 7 were transurethral resections. The pT stage was Ta in 3, T1 in 1, T2 in 6 and T3 in 12. The pN stage was Nx in 7, N0 in 11, N1 in 3 and N2 in 4.

All 59 cases were retrieved from the surgical pathology files and re-reviewed to confirm the original diagnoses by independent pathologists. One or more tissue representative blocks were used to construct a TMA. For each case, three 1.5-mm cores were taken to construct the TMA. This TMA was then sectioned into 4-μm sections. Hematoxylin-eosin (H&E) staining was performed to verify the histological diagnoses. The sections were used for immunostaining analysis using an anti-AGGF1 antibody as described [13]. Briefly, 4-μm tissue sections were antigen-retrieved in 0.1 M citrate buffer, pH 6.0, in a pressure steamer for 15 min. The slides were then incubated sequentially with the primary antibody, a biotinylated secondary antibody, avidin-peroxidase complex (Ventana, Tucson, AZ) and chromogenic substrate diaminobenzidine. The immunostaining was performed on a Ventana Benchmark automatic stainer (Ventana, Tucson, AZ).

In the follow-up confirmation study, urothelial carcinoma tissue samples along with normal adjacent urothelial tissue samples were collected from 4 patients undergoing surgery at Huazhong University of Science and Technology (B35, B76, B84 and B85). In B35, the tumor invaded the full thickness of the bladder and was staged as pT3N0M0. In B76, the tumor invaded the inner muscularis propria with left iliac lymph node metastasis. B84 represented an inverted papilloma. In B85, the tumor was low grade and was staged as T2N2M0. However, it invaded lamina propria with right iliac lymph node metastasis. It should be noted that it is rare for a low grade bladder cancer to metastasize, thus a caution should be taken with interpretation of the stage of the B85 tumor.

Immunohistochemistry analysis was used for assessing the expression level of the AGGF1 protein in TMAs with a polyclonal anti-AGGF1 antibody reported previously [13] and in the confirmation study with another polyclonal anti-AGGF1 antibody (Proteintech, Wuhan, Hubei, China). Normal urothelial cells strongly expressed AGGF1, but stromal cells did not (Fig. 1A). Therefore, urothelial carcinomas with an AGGF1 expression level similar to normal urothelial cells were graded as “strongly positive”, while those with an expression level intermediate between normal urothelial cells and stromal cells were graded as “weakly positive” (Fig. 1A).

This study was approved by Institutional Review Boards on Human Subject Research at Cleveland Clinic and Huazhong University of Science and Technology. The informed written consent was obtained from each patient.

2.2. Cell culture

The human bladder cancer cell line (J82), human colon cancer cell line (HCT116), human cervical cancer cell line (HeLa), human non-small cell lung carcinoma cell line (H1299) and human embryonic kidney cell line (HEK293) were from the American Type Culture Collection (ATCC, Rockville, MD, USA). J82 cells were maintained in the Minimal Essential Medium with Earle's (MEM/EBSS, Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone). Other cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% FBS (Hyclone). The cells were cultured at 37 °C in a humidified incubator with 5% CO_2.

2.3. Cell transfection

For plasmid DNA, cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

For studies of miR-27a, cells were transiently transfected with 100 nM of a miR-27a mimics or an inhibitor (Ribobio, Guangzhou, Guangdong, China) with Lipofectamine 2000 according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested and lysed for preparation of protein extracts.

For RNAi, cells were transfected with 100 nM siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4. Hypoxic exposure conditions

The standard hypoxic exposure condition was 3% O_2 (or 1% O_2 as indicated in the text), 5% CO_2, and 92% N_2 (or 94% N_2) in a Hypoxia Chamber (HF100 Incubator, Heal Force Bio-Meditech Holdings Limited, Shanghai, China). At the start of each experiment, the culture media were replaced with pre-equilibrated normoxic or hypoxic media. Cells were cultured in 6-well plates and exposed to hypoxia for a period of 2, 6, 24 or 48 h. Hypoxic conditions were also mimicked using a hypoxia-mimetic agent, cobalt (II) chloride ($CoCl_2$, Sigma-Aldrich, St. Louis, MO, USA) [23–25].

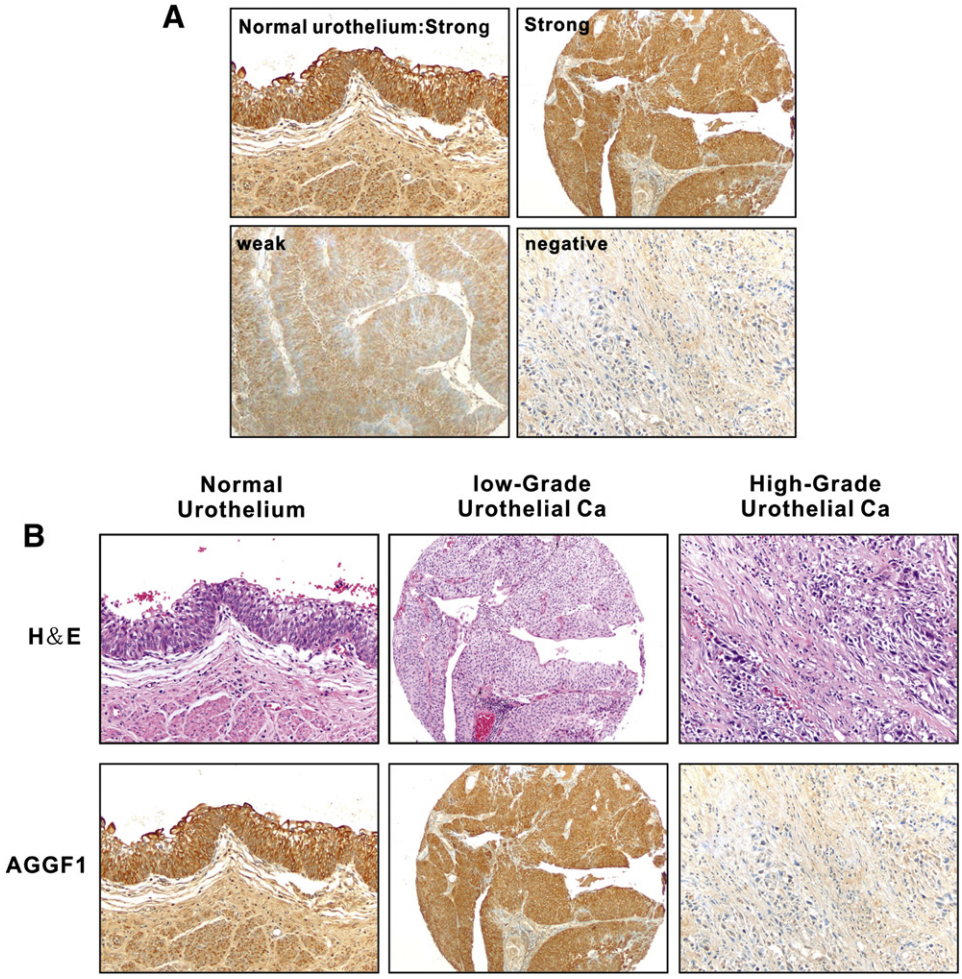

Fig. 1. Differential expression of AGGF1 in normal urothelium and different histological grades of urothelial carcinomas. (A) Immunostaining analysis of AGGF1 expression in representative samples: no AGGF1 expression (negative −), weak AGGF1 expression (weak +), and strong AGGF1 expression (strong +). (B) Normal urothelium and a low-grade urothelial carcinoma exhibited strong immunostaining for AGGF1 protein. In contrast, negative or weak AGGF1 immunoreactivity was detected in a high-grade urothelial carcinoma.

2.5. Bioinformatic analysis

We used three programs and related databases, TargetScan 5.2 [26], PicTar [27] and MiRanda [28], to predict potential binding sites of miRNAs to the 3′-untranslated region (3′-UTR) of *AGGF1*.

2.6. Plasmids and siRNAs

The 3′-UTR of *AGGF1* with a putative miR-27a binding site was amplified by a polymerase chain reaction (PCR) using human genomic DNA as the template. The PCR product was digested with *Spe I* and *Hind III*, and sub-cloned into the pMIR-REPORT luciferase plasmid (Applied Biosystems, Foster City, CA, USA), resulting in pMIR-AGGF1-3′-UTR-wt. Primers used for constructing pMIR-AGGF1-3′-UTR-wt were 5′-GGACTAGTTTGCGAAAACATGGGTAGTG-3′ (forward) and 5′-CCCAAGCTTTGCCTGCAATGGTCTTTATC-3′ (reverse).

The miR-27a binding site was mutated in pMIR-AGGF1-3′-UTR-wt using PCR-based site-directed mutagenesis and the construct was referred to as pMIR-AGGF1-3′-UTR-mut. The seed region of the predicted miR-27a site (CUGUGAA) was mutated to CCUAUAA.

Two siRNAs targeting the *AGGF1* mRNA sequence were chemically synthesized by RiboBio. Silencer scrambled negative control siRNA (siNC) having no significant homology to any known gene sequence from humans was also purchased from RiboBio. The sequences of siRNA duplexes were as follows: 5′- GUCGGAAGAUGUUGGAGAAdTdT-3′ (sense) for siAGGF1-B and 5′- GCUGUAAACCCUGCUACAAdTdT-3′(sense) for siAGGF1-C.

2.7. Luciferase assays

For luciferase assays, HEK 293 cells were plated in a 96-well plate for 24 h before transfection. Cells were co-transfected with 50 ng of either the pMIR-AGGF1-3′-UTR-wt or pMIR-AGGF1-3′-UTR-mut and 100 nM of either a miRNA mimics negative control or a miR-27a mimics (RiboBio). Forty-eight hours after transfection, cells were lysed in passive lysis buffer (Promega, Madison, WI, USA) and activities of firefly and renilla luciferase were measured with the dual-luciferase assay system (Promega) with a GloMax luminometer (Promega).

2.8. Quantitative reverse-transcription PCR (qRT-PCR) analysis

Total RNA was isolated from cells using TRIzol reagent (TaKaRa Biotech, Dalian, Liaoning, China) and quantified. Two micrograms of RNA from each sample was treated with DNase I (Promega, Madison, WI, USA) to remove contaminating genomic DNA and used for synthesis of cDNA with M-MLV reverse transcriptase (Promega). The cDNA was then analyzed by real-time quantitative PCR with FastStart SYBR Green Master (Roche Applied Science, Mannheim, Germany). The primer sequences used for qRT-PCR were as follows: *AGGF1*, 5′-AATCCCTGAA GTTGGTGTCA-3′ and 5′-GTTTTCGGCTGAAGAATCTG-3′; *β-actin*, 5′-CATCGTCCACCGCAAATG-3′ and 5′-CACCTTCACCGTTCCAGTT-3′.

For the measurement of the expression level of mature hsa-miR-27a, Bulge-loop™ miRNA qRT-PCR Primer Sets (RiboBio, Guangzhou, Guangdong, China) were used. The U6 small RNA was used as an internal control.

Table 1
Immunohistochemical analysis of AGGF1 expression in urothelial carcinomas.

	Negative −	Weak +	Strong +
Normal urothelium	0	0	7/7 (100%)[a]
Low-grade urothelial carcinoma	1/30 (3.3%)	5/30 (16.7%)	24/30 (80%)[b]
High-grade urothelial carcinoma	7/22 (31.8%)	11/22 (50%)	4/22 (18.2%)

[a] HGUCC versus normal urothelium, $P = 2.11 \times 10^{-4}$ by a Fisher's exact test; Odds ratio or OR could not be estimated because one of the variables is 0.
[b] HGUCC versus LGUCC, $P = 1.40 \times 10^{-5}$, OR (95% confidence interval) = 0.056 (0.014–0.226) by a Pearson's Chi-square test.

2.9. Western blot analysis

Cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, proteinase inhibitor cocktail), and total protein extracts were used for Western blot analysis as previously described [13]. The primary antibodies used include a rabbit polyclonal anti-AGGF1 antibody (1:2000, Proteintech, Wuhan, Hubei, China), a rabbit polyclonal anti-HIF-1α antibody (1:2000, Proteintech) and a mouse monoclonal anti-alpha tubulin (1:5000, Merck Millipore, Darmstadt, Germany). The secondary antibodies were either a goat anti-rabbit or a goat anti-mouse HRP-conjugated secondary antibody (1:20,000, Sigma-Aldrich, Castle Hill, NSW, Australia). The images were developed with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL, USA) using a ChemiDoc XRS (Bio-Rad Laboratories, Richmond, CA, USA), and analyzed with Gel-Pro analyzer 4. The data were presented as the fold change in the ratio of the intensity of the AGGF1 band to that of the corresponding tubulin band.

2.10. Enzyme-linked immunosorbent assays (ELISA)

AGGF1 and VEGF-A levels in culture supernatants were analyzed using a human AGGF1 ELISA kit (USCN Life Science, Wuhan, Hubei, China) and a human VEGF-A ELISA kit (R&D Systems, Minneapolis, MN, USA) according to manufacturers' instructions. Standard curves were created by a series of concentrations of purified AGGF1 or VEGF-A using the CurveExpert 1.3 software program.

2.11. Apoptosis assays

Transfected cells were treated with a hypoxia-mimetic, $CoCl_2$, harvested and used for apoptosis assays using FC-500 flow cytometry (Beckman Coulter, Miami, FL, USA). Briefly, cells were treated with annexin V-FITC and propidium iodide (PI) from an Apoptosis Detection Kit (KeyGEN, Nanjing, Jiangsu, China). Annexin V-FITC-positive cells were counted as early apoptotic cells. Cells that were positive for both annexin V-FITC and PI were counted as late apoptotic cells. Viable cells were negative for both annexin V-FITC and PI [29].

2.12. Statistical analysis

The association analysis between the expression levels of AGGF1 and different grades of urothelial carcinomas was performed using either a

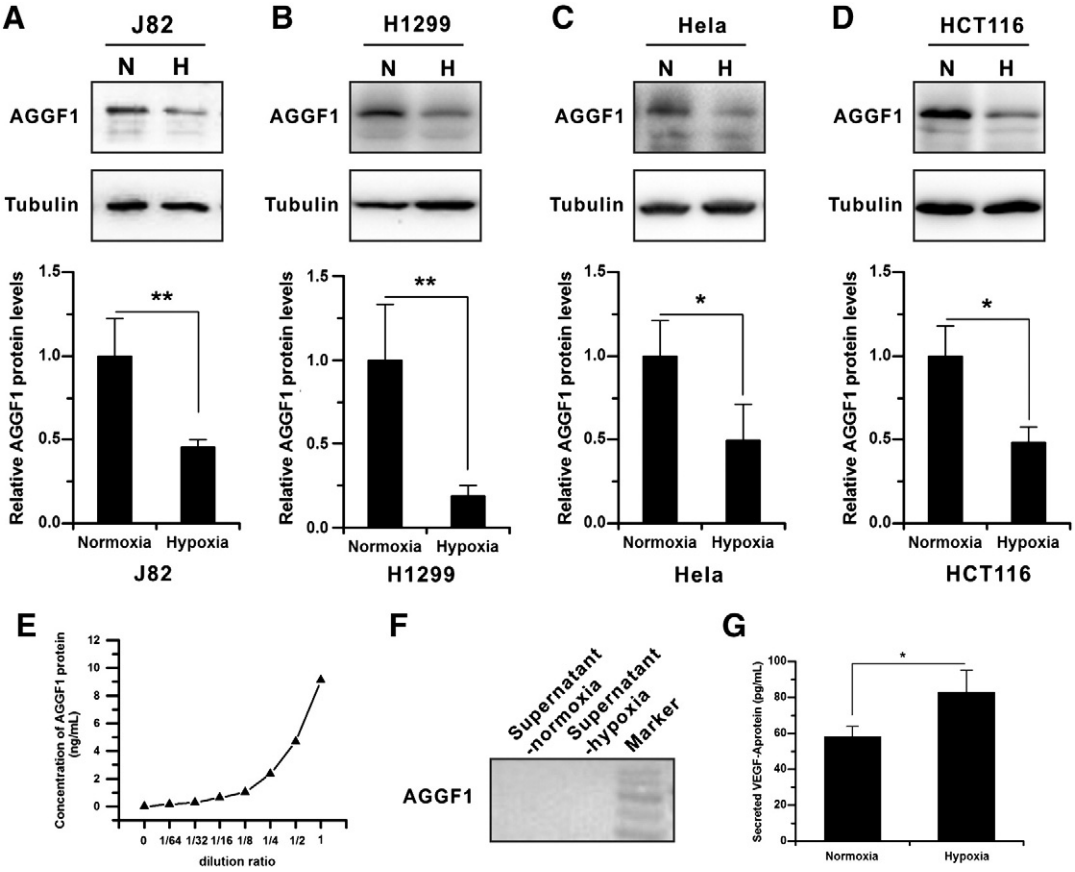

Fig. 2. AGGF1 protein levels are down-regulated under hypoxic conditions. Four different human cancer cell lines, J82, H1299, HeLa and HCT116, were cultured under either normoxic (21% O_2) or hypoxic conditions (3% O_2) for 48 h and then used for measuring the protein levels of AGGF1 by Western blot analysis. β-Actin was used as an equal sample loading control. (A, B, C, D) Representative Western blot images. N, normoxia; H, hypoxia. Protein expression was quantified by densitometric analysis. The ratio of AGGF1/β-actin is shown as the mean fold change. *, $P < 0.05$, **, $P < 0.01$. (E) Test of validity of the ELISA kit. The ELISA kit successfully detected purified AGGF1 protein in a concentration-dependent manner. (F) Western blot analysis for secreted AGGF1 in culture supernatants. J82 cells were cultured under either normoxic or hypoxic conditions for 48 h and then culture media were collected for measuring the secreted AGGF1 protein. (G) Hypoxia increased the secreted VEGFA protein (positive control). *, $P < 0.05$.

Pearson's Chi-square test or a Fisher's exact test with SPSS version 16.0 software.

Other data were expressed as mean ± standard deviation (SD). The results were from at least three independent experiments. Statistical analysis for comparison of means between two groups was carried out with Student's t-tests using SPSS version 16.0 software.

A *P*-value of 0.05 or less was considered to be statistically significant.

3. Results

3.1. Significant association of low expression of the AGGF1 protein with high grade urothelial carcinoma

Angiogenesis-related factors have important clinical applications as predictive markers of tumors [30–32]. As a newly-identified angiogenic factor, the expression status of AGGF1 (GeneID: 55109) protein in

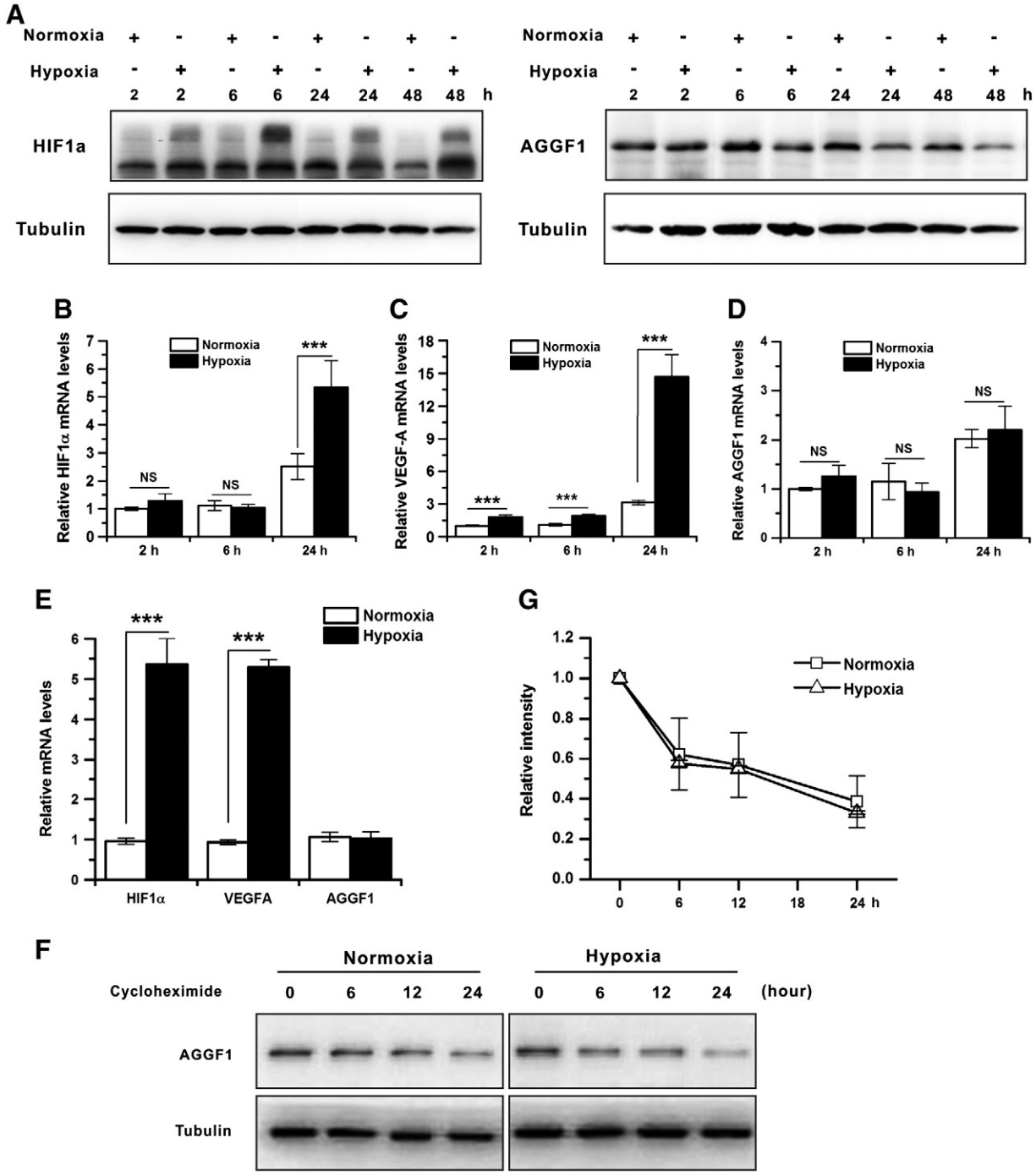

Fig. 3. Hypoxia-induced down-regulation of AGGF1 was independent of transcriptional inhibition. (A) Protein levels of HIF-1α, AGGF1 and α-tubulin were analyzed by Western blot analysis under normoxic or hypoxic conditions. (B, C, D) The mRNA levels of *HIF-1α*, *VEGF-A* and *AGGF1* were determined by real-time RT-PCR analysis under normoxic or hypoxic conditions. The ratios of *HIF-1α*/*β-actin*, *VEGF-A*/*β-actin* and *AGGF1*/*β-actin* were shown as the fold change. (E) Real-time RT-PCR analysis for *HIF1α*, *VEGFA* and *AGGF1* in J82 cells cultured under normoxia or hypoxia. ***, $P < 0.001$. (F) AGGF1 protein stability assays. The degradation of the AGGF1 protein was assessed after inhibition of de novo protein synthesis with cycloheximide treatment. J82 cells were cultured under the indicated conditions for 20 h, treated with cycloheximide for 6 to 24 h, and used for Western blot analysis of AGGF1. (G) Quantification of AGGF1 levels at different time points from Western blot images in (F). The ratio of AGGF1/tubulin was plotted against time points. The ratio at time point 0 was set at 1.0 and other ratios were converted as relative values to that at time point 0. All experiments were performed in triplicate (n = 3).

neoplastic tissues has not been investigated. Here, normal urothelium and neoplastic tissues from urothelial carcinomas of different histological grades were used for evaluation of the expression level of the AGGF1 protein using immunohistochemistry analysis. The expression levels of the AGGF1 protein were classified into three tiers: no expression (negative), weak expression (weak +) and strong expression (strong +) as shown in Fig. 1A.

Normal urothelium showed strong expression of AGGF1 proteins with strong circumferential membranous staining in all 7 samples (100%; Fig. 1B, Table 1). Urothelial cancers of different grades exhibited differential AGGF1 expression. Strong AGGF1 expression was detected in 80% (24/30) of LGUCC and 18.2% (4/22) of HGUCC, respectively (Fig. 1B, Table 1). Weak AGGF1 expression was detected in 0/7 normal urothelium, 16.7% (5/30) LGUCC and 50% (11/22) HGUCC. No AGGF1 was detected in 0% (0/7) normal urothelium, 3.3% (1/30) LGUCC and 31.8% (7/22) HGUCC. The proportion of strong AGGF1 expression cases was significantly lower in the high-grade urothelial carcinoma group than those in the low-grade urothelial carcinoma group ($P = 1.40 \times 10^{-5}$, OR = 0.056) or than those in the normal urothelium tissue group ($P = 2.11 \times 10^{-4}$). There was no difference between normal urothelium and LGUCC in terms of AGGF1 protein expression ($P = 0.57$). Taken together, these data suggested that down-regulation of AGGF1 protein expression was significantly associated with a risk of HGUCC.

3.2. Hypoxia down-regulates expression of AGGF1

Tumor hypoxia is more common in cases of high-grade superficial urothelial carcinoma because of increased proliferative activity of tumor cells [33]. Thus, hypoxia may be responsible for the finding that AGGF1 protein expression was high in normal urothelium and low-grade urothelial carcinomas, but low in high-grade urothelial bladder carcinomas. The key to test this hypothesis is to demonstrate that AGGF1 is down-regulated in hypoxic conditions. Human bladder cancer cell line, J82, was exposed to hypoxia (3% O_2, 5% CO_2, and 92% N_2) for 48 h and then used for measuring the expression level of AGGF1. Western blot analysis showed that AGGF1 expression was decreased by >2-fold in response to hypoxia as compared to normoxia (Fig. 2A).

To verify whether the decrease of AGGF1 expression in response to hypoxia is cell-specific, we analyzed three other human cancer cell lines, including human non-small cell lung carcinoma cell line (H1299), human cervical cancer cell line (HeLa) and human colon carcinoma cell line (HCT116). Hypoxia-induced down-regulation of AGGF1 was also observed in all three cell lines (Fig. 2B–D). All the results indicated that AGGF1 expression was significantly decreased in human cancer cells after exposure to hypoxia. It is interesting to note that hypoxia-induced down-regulation of AGGF1 expression was more pronounced in H1299 cells (>5-fold) than that in other cancer cells (Fig. 2B).

Since AGGF1 is known to be a secreted angiogenic growth factor in human vascular endothelial cells, we next examined the levels of AGGF1 secreted in the culture supernatant from cancer cells using ELISA. To prevent detection of exogenous AGGF1 in FBS, cells were cultured in DMEM without FBS. As shown in Fig. 2E, the ELISA kit can detect the purified AGGF1 protein in a concentration-dependent manner. However, ELISA failed to detect any AGGF1 protein in the culture supernatant from J82 cells. Consistent with the data, Western blot analysis also failed to detect expression of AGGF1 in the culture supernatant under normoxia or hypoxic conditions (Fig. 2F). In contrast, an increased level of VEGFA (positive control) was detected (Fig. 2G).

3.3. Down-regulation of AGGF1 protein under hypoxic conditions was independent of transcriptional inhibition

To further assess AGGF1 expression levels under hypoxic conditions, we analyzed AGGF1 expression at different time-points during hypoxia. We exposed HCT116 cells to hypoxia (3% O_2, 5% CO_2, and

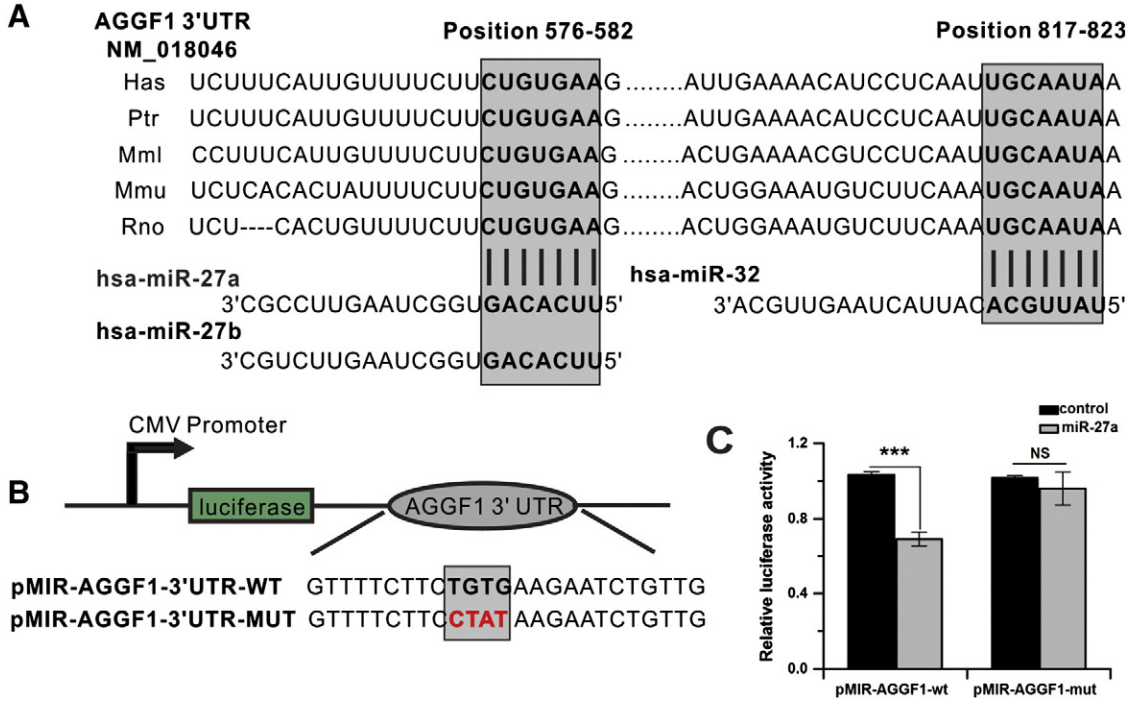

Fig. 4. MiR-27a regulates AGGF1 expression by binding to a putative binding site in the 3′-UTR of *AGGF1*. (A) A schematic diagram shows the predicted binding sites of miR-27a/b and miR-32 in the 3′-UTR of *AGGF1* mRNA, and the conserved sites in the human, chimpanzee, rhesus, mouse and rat. (B) A schematic diagram shows luciferase reporters containing the potential miR-27a binding site or the related mutated site. (C) Data from luciferase assays in HEK293 cells. Reporters containing the wild-type or 4-bp mutant 3′-UTR of AGGF1 were co-transfected with a miR-27a mimics or a negative control mimics into HEK293 cells. Cells were harvested 48 h after transfection and luciferase activities were measured and normalized to the renilla activities. Three independent experiments were performed. Error bars represent standard deviation (SD). ***, $P < 0.001$.

92% N_2) for 2, 6, 24, or 48 h or normoxia (21% O_2) as a control. The expression of HIF-1α, a marker for hypoxia, was induced at 2 h after hypoxia. The magnitude of HIF-1α induction peaked at 6 h, and then moderately decreased (Fig. 3A). By contrast, decreased AGGF1 protein levels were observed at time points of 6 h, 24 h, and 48 h under hypoxic conditions (Fig. 3A). These results suggest that expression of AGGF1 is independent of HIF-1α in HCT116 cells under hypoxia.

To identify the possible mechanisms involved in the hypoxia-induced change in AGGF1 expression, we examined mRNA expression of *AGGF1* using real-time RT-PCR analysis. After cultured in normoxic or hypoxic conditions, HCT116 cells were harvested to

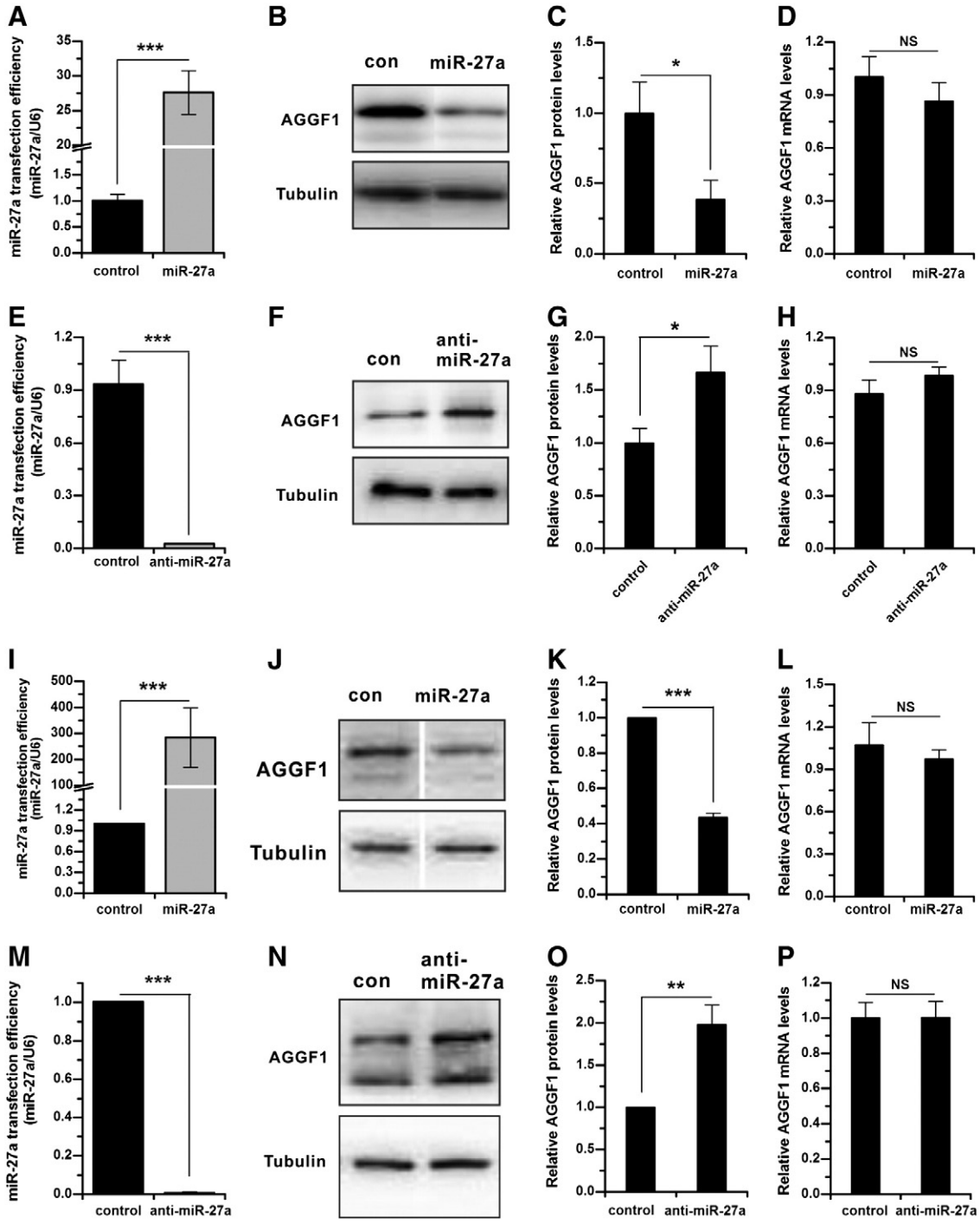

Fig. 5. MiR-27a inhibits expression of AGGF1 at the protein level. (A, E, I, M) The levels of miR-27a were validated by semiquantitative end-point PCR analysis in HCT116 and J82 cells transfected with 100 nM of miR-27a mimics or miR-27a inhibitor. U6 small nuclear RNA was used as an internal control. (B, C, J, K) Overexpression of miR-27a significantly decreased the levels of the AGGF1 protein. Representative Western blot images are shown. α-Tubulin serves as a loading control. (D, L) Quantitative RT-PCR analysis was used to measure the *AGGF1* mRNA levels after transfection with a miR-27a mimics or a negative control mimics. The results were normalized to β-actin. (F, G, N, O) Inhibition of miR-27a increased AGGF1 protein expression. Representative Western blot images are shown. α-Tubulin serves as a loading control. (H, P) Quantitative RT-PCR analysis was used to measure the *AGGF1* mRNA levels after transfection with a miR-27a inhibitor or a negative control inhibitor. The results were normalized to β-actin. Three independent experiments were performed. Error bars represent standard deviation (SD). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

detect the mRNA expression of *HIF-1α*, *VEGF-A*, *AGGF1* and *β-actin* at different treatment times. The results showed that hypoxia induced increased mRNA expression of *HIF-1α* and *VEGF-A* in a time-dependent manner. Increased expression of *HIF-1α* mRNA was detected starting at the 24-h time point (Fig. 3B), while increased HIF-1α protein was observed at 2 h (Fig. 3A) because of increased protein stability [34]. Transcript levels of *VEGF-A*, a target of HIF1α, increased at 2 h and reached a very high level at 24 h (Fig. 3C). However, *AGGF1* mRNA levels did not change after exposure to hypoxic conditions compared to normoxic conditions at all three time points (Fig. 3D).

Similar findings were made in J82 bladder cancer cells (Fig. 3E). Compared to normoxia, hypoxia induced significantly increased expression of *HIF1α* mRNA and *VEGFA* mRNA, but not *AGGF1* mRNA. Together, these data show that hypoxia does not affect the expression level of *AGGF1* mRNA, instead, regulates AGGF1 protein expression, suggesting that down-regulation of AGGF1 protein by hypoxia was independent of transcriptional inhibition.

Down-regulation of AGGF1 protein by hypoxia may also be caused by changes in AGGF1 protein stability. To test this possibility, we exposed J82 cells to hypoxia (1% O_2) and then assessed degradation of the AGGF1 protein using Western blot analysis after inhibition of de novo protein synthesis with cycloheximide treatment. Time-dependent degradation of AGGF1 was observed both in normoxia and hypoxia. No significant difference of the AGGF1 half-life was detected between normoxia and hypoxia (Fig. 3F and G). These results suggest that changes in AGGF1 stability are not the major cause of down-regulation of AGGF1 by hypoxia.

3.4. MicroRNA-27a down-regulates AGGF1 expression via translational inhibition but not RNA degradation

Under hypoxic conditions, AGGF1 protein levels dropped without changes in mRNA levels. Therefore, we hypothesized that miRNAs were involved in hypoxia-induced down-regulation of AGGF1. The downstream target genes of miRNAs can be predicted using multiple algorithms [35]. We screened for miRNA binding sites in the 3′-UTR of *AGGF1* using three prediction algorithms: TargetScan 5.2, PicTar, and miRanda. MiRNAs predicted to bind to the 3′-UTR of *AGGF1* by all three algorithms included miR-27a, miR-27b, and miR-32. The mature sequences of the three miRNAs are shown in Fig. 4A. Interestingly, previous microarray-based expression profiling found that miR-27a was induced in response to hypoxia [36], and hence we selected only miR-27a for further study.

To verify that miR-27a regulates AGGF1 expression via the potential binding site at the 3′-UTR of *AGGF1*, we constructed two luciferase reporters: pMIR-AGGF1-3′-UTR-wt and pMIR-AGGF1-3′-UTR-mut with a 4-bp mutation in the seed region of the putative binding site. The wild-type *AGGF1* 3′-UTR or mutant 3′-UTR (Fig. 4B) was inserted downstream of the *firefly luciferase* gene and transfected in HEK293 cells. For the wild-type reporter, overexpression of miR-27a mimics (100 nM) repressed the luciferase activity significantly ($P < 0.001$) compared to negative control miRNA mimics (Fig. 4C). In contrast, a decrease in luciferase activity was not observed when miR-27a mimics was co-transfected with the mutant reporter in HEK293 cells (Fig. 4C). The results suggest that the predicted miR-27a binding site is responsible for repressing the expression of AGGF1.

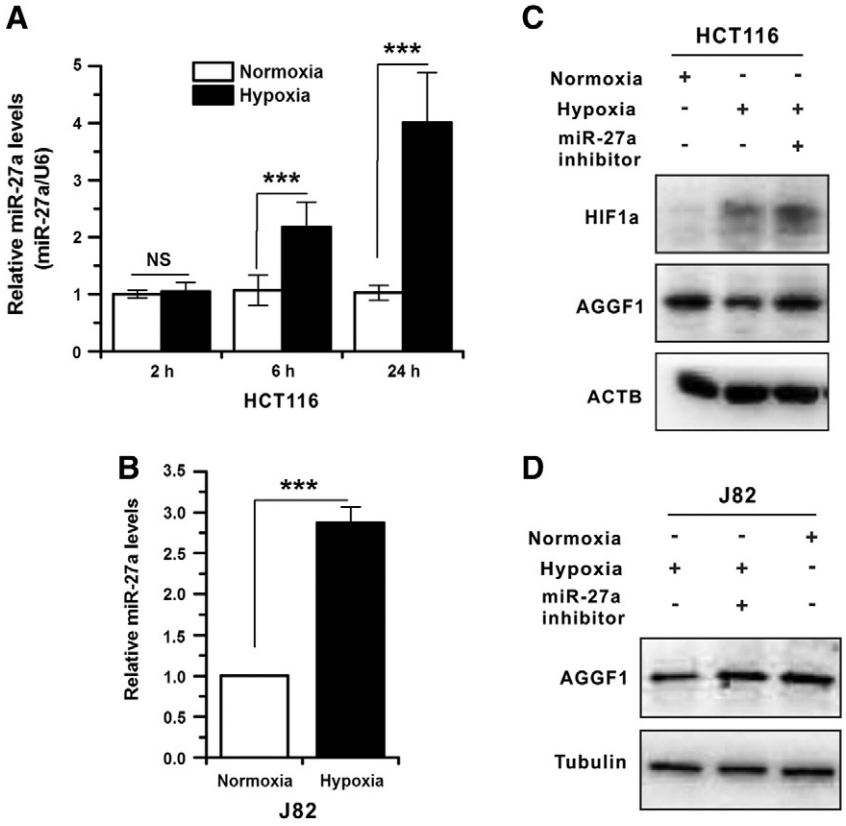

Fig. 6. MiR-27a was up-regulated under hypoxic conditions and inhibition of miR-27a can rescue hypoxia-induced down-regulation of AGGF1 in HCT116 and J82 cells. (A, B) The levels of the mature MiR-27a were assessed in HCT116 and J82 cells after exposure to hypoxia. U6 small nuclear RNA was used as an internal control. ***, $P < 0.001$. (C, D) HCT116 and J82 cells were transfected with a miR-27a inhibitor and then exposed to hypoxic conditions. Western blot analysis was used to measure the expression level of the AGGF1 protein. β-Actin was used as the loading control.

To further validate that miR-27a regulates expression of AGGF1, and to understand the precise mechanism, we transfected HCT116 with a miR-27a mimics (100 nM) or a miR-27a inhibitor (100 nM) for 72 h and then examined *AGGF1* mRNA and protein levels. Semiquantitative end-point PCR analysis confirmed the efficacy of transfection by revealing markedly increased expression of mature miR-27a after transfection with the miR-27a mimics (Fig. 5A). Compared to the control mimics, the miR-27a mimics significantly reduced AGGF1 protein expression by 2.5-fold (Fig. 5B and C). Moreover, knocking down of miR-27a (Fig. 5E) resulted in a consistent increase in AGGF1 protein expression by about 1.6 fold as quantitated by densitometric analyses (Fig. 5F and G). However, there was no change in mRNA levels of *AGGF1* regardless of overexpression or knockdown of miR-27a (Fig. 5D and H). Similar results were obtained with J82 bladder cancer cells (Fig. 5I–P). Taken together, these results indicate that miR-27a down-regulates AGGF1 expression through translational inhibition but not RNA degradation.

3.5. Up-regulation of miR-27a correlates with the down-regulation of AGGF1 protein levels under hypoxic conditions and in urothelial carcinomas

The status of miR-27a expression was tested in HCT116 cells after exposure to hypoxia. The expression levels of mature miR-27a were assessed using miR-27a specific stem-loop reverse transcription primers and PCR amplification primers. Semiquantitative end-point

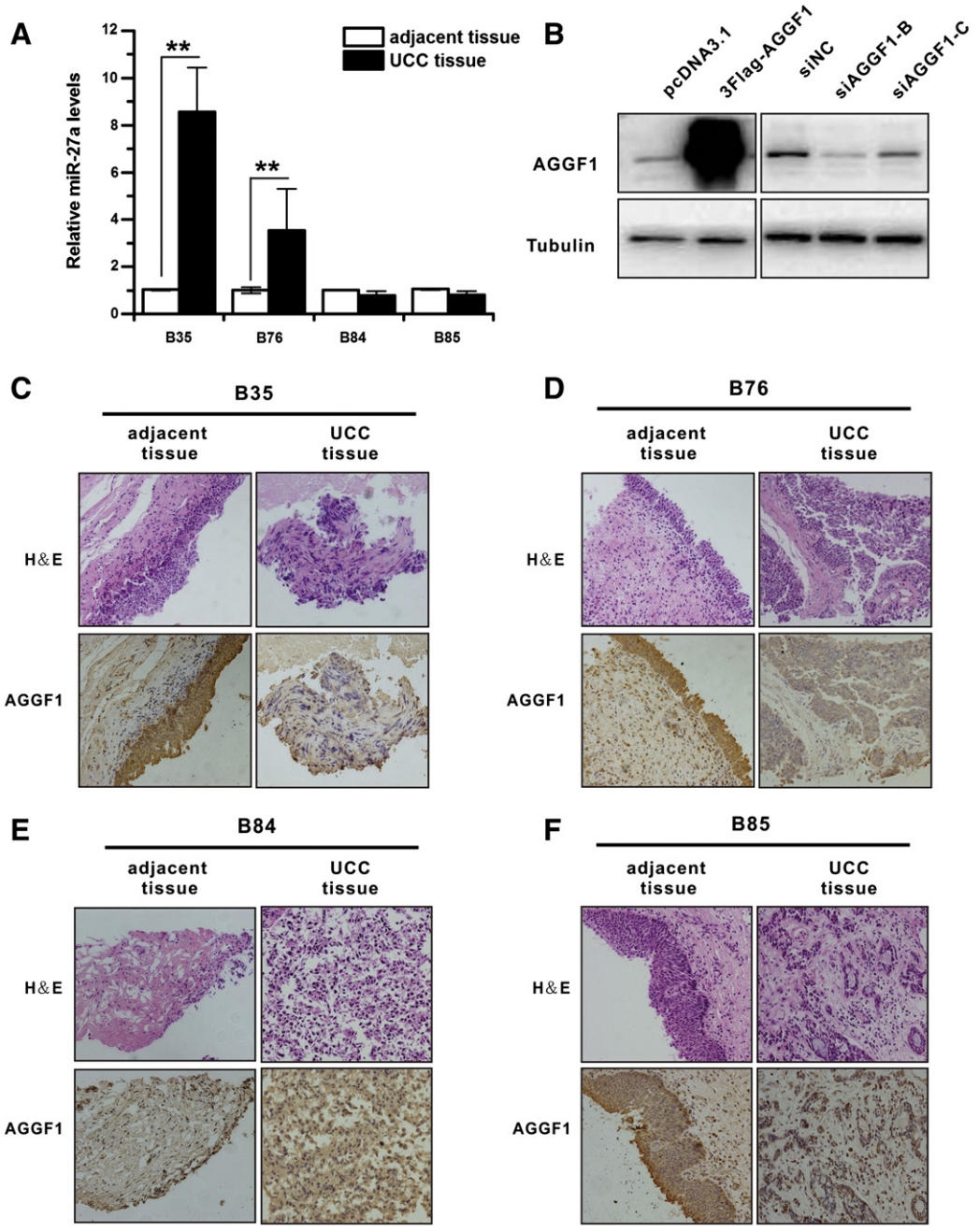

Fig. 7. Correlation between up-regulation of miR-27a in high grade urothelial carcinomas and down-regulation of AGGF1. (A) The relative expression levels of miR-27a in four pairs of urothelial carcinomas (UCC) and matched adjacent normal tissues were measured by qPCR analysis. B35 and B76 were classified as high grade UCC, whereas B84 and B85 were low grade tumors. **, $P < 0.01$. (B) The specificity of the polyclonal anti-AGGF1 antibody was verified by Western blot analysis with protein extracts from cells with or without overexpression of AGGF1 and from cells with knockdown of *AGGF1* (siAGGF1-B, siAGGF1-C) or control siRNA (siNC). (C-F) The levels of the AGGF1 protein in UCC tumor tissues and matched adjacent tissues were characterized using immunostaining analysis.

PCR analysis showed an approximately 2-fold increase in miR-27a expression at the time-point of 6 h under hypoxic conditions (Fig. 6A, $P = 6.9 \times 10^{-6}$). At 24 h, miR-27a expression was increased by up to 4-fold under hypoxic conditions compared to normoxic conditions (Fig. 6A, $P = 2.2 \times 10^{-8}$). Similar results were obtained in J82 bladder cancer cells (Fig. 6B, $P = 7.2 \times 10^{-5}$).

To correlate the miR-27a expression levels with the hypoxia-induced decrease in AGGF1 expression, cells were transfected with a miR-27a inhibitor or a control inhibitor and exposed to hypoxic conditions. The hypoxia-induced decrease in AGGF1 expression disappeared after transfection with the miR-27a inhibitor in HCT116 cells (Fig. 6C). Similar results were obtained in J82 bladder cancer cells (Fig. 6D). Taken together, these data suggested that miR-27a was induced by hypoxia and that the up-regulation of miR-27a expression correlates with the down-regulation of AGGF1 protein level under hypoxic conditions.

To further correlate the expression of miR-27a to that of AGGF1, we have collected urothelial carcinoma tissue samples along with normal adjacent urothelial tissue samples from 4 patients (B35, B76, B84 and B85). B35 and B76 were classified as high grade urothelial carcinomas, whereas B84 and B85 were low grade urothelial carcinomas. Semiquantitative end-point RT-PCR analysis showed that for B35 and B76, the expression levels of miR-27a in tumors were significantly higher than that in adjacent normal tissue samples, whereas for B84 and B85, there was no significant difference between the expression levels of miR-27a in tumors and in adjacent normal tissue samples (Fig. 7A). These data are consistent with the relative expression levels of the AGGF1 protein (Fig. 7B–F). Immunohistochemical analysis with a specific anti-AGGF1 antibody showed that the expression levels of the AGGF1 protein appeared to be higher in B35 and B76 than those in B84 and B85 (Fig. 7B–F). These results are consistent with the findings from the TMA studies described above (Table 1).

3.6. Down-regulation of AGGF1 protein reduced hypoxia-induced apoptosis in cancer cells

To assess the functional role of AGGF1 suppression under hypoxic conditions, the proportion of apoptotic cells was examined under various culture conditions. *AGGF1* expression was knocked down in HCT116 cells using two small interfering RNAs (siAGGF1-B and siAGGF1-C, Fig. 8C). Cells were then exposed to hypoxic mimetic $CoCl_2$ (1.5 mM) for 16 h and used for apoptosis assays. The results of flow cytometry analysis showed that the proportion of apoptotic cells increased after $CoCl_2$ treatment (Fig. 8A). We calculated the total apoptosis ratio, including early apoptotic cells and late apoptotic cells. Knock-down of *AGGF1* expression had little effect on apoptosis under normoxic conditions, but inhibited apoptosis by 30% under hypoxic conditions (Fig. 8A and B). These results suggested that down-regulation of AGGF1 protected cancer cells from apoptosis only under hypoxic conditions.

In addition to mimicking hypoxia using $CoCl_2$ treatment, we also analyzed apoptosis after exposing either HCT116 or J82 cells to 1% O_2. As shown in Fig. 9, under normoxia, knockdown of *AGGF1* expression by two different siRNAs did not have any effect on apoptosis of both

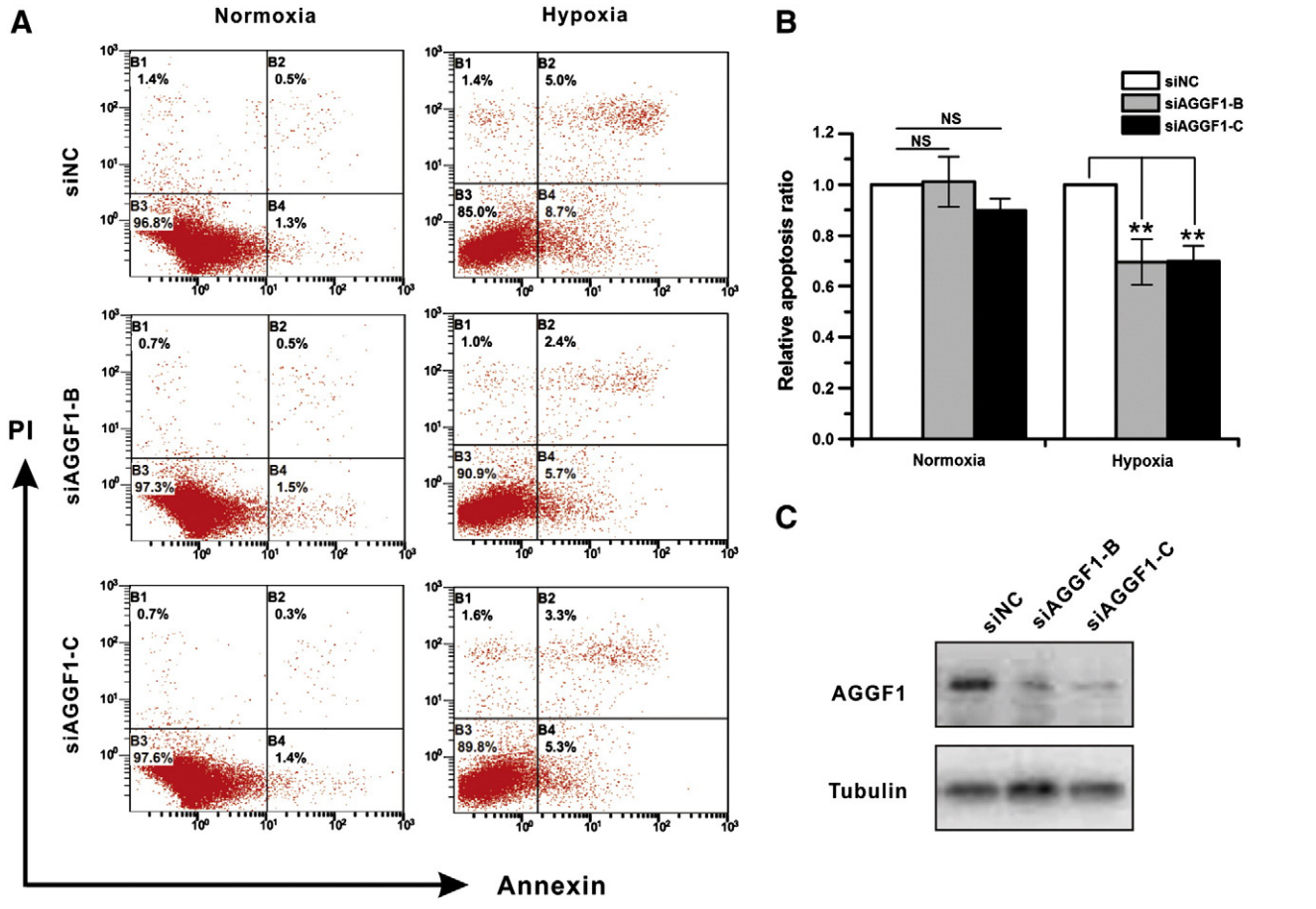

Fig. 8. Down-regulation of AGGF1 protein levels reduces hypoxia-induced apoptosis of HCT116 cells by treatment with $CoCl_2$. (A) Apoptosis was analyzed by flow cytometry analysis using antibodies against annexin-V and PI under various culture conditions. *AGGF1* expression was knocked down using two *AGGF1* specific siRNAs (siAGGF1-B and siAGGF1-C) or control siRNA (siNC), and exposed to hypoxia mimetic $CoCl_2$ (1.5 mM) for 16 h. (B) Changes in the proportion of apoptotic cells are shown as fold changes. (C) After flow cytometry analysis, the samples were used for Western blot analysis to ensure knockdown of AGGF1 expression. **, $P < 0.01$.

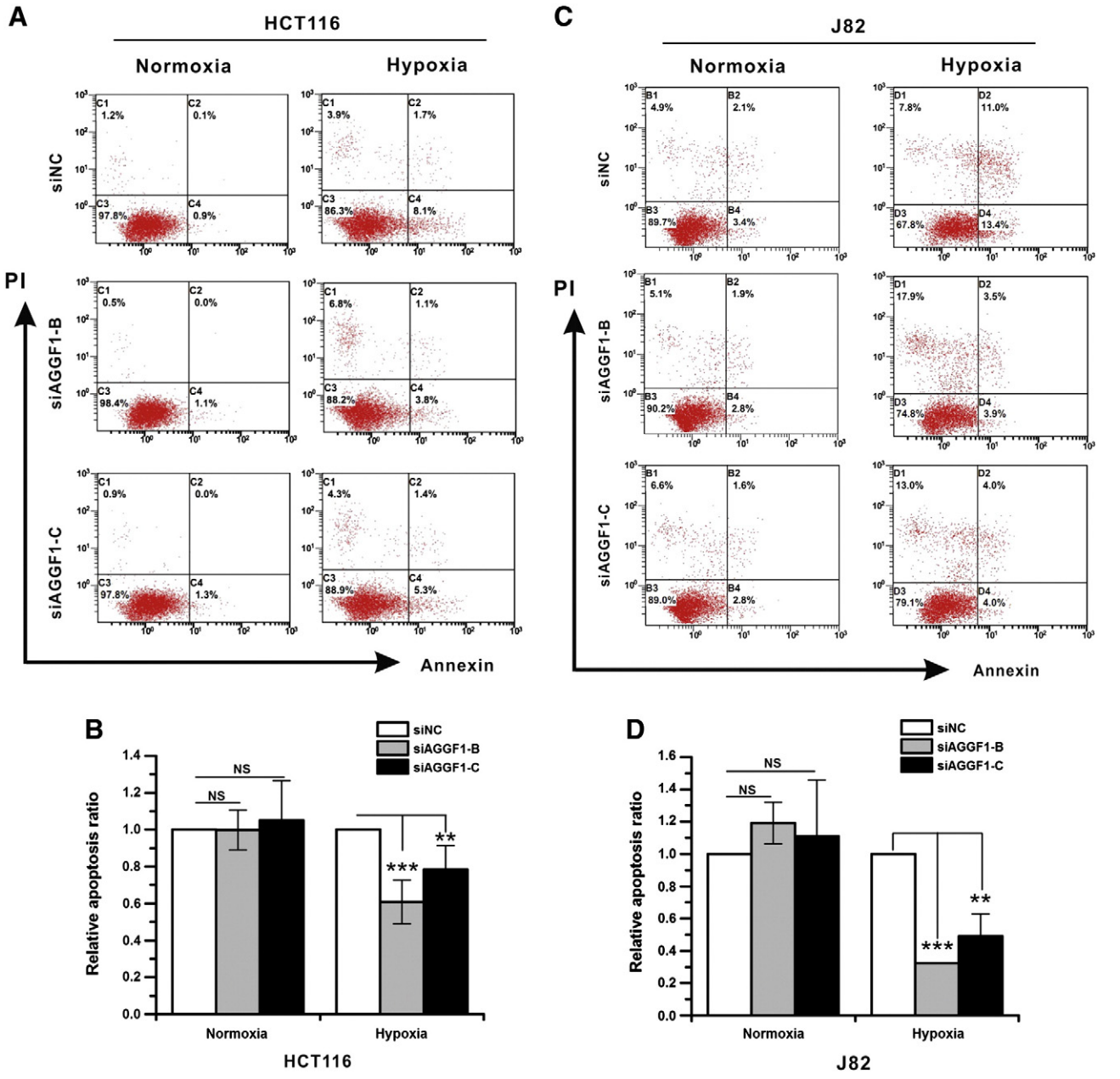

Fig. 9. Down-regulation of the AGGF1 protein reduces hypoxia-induced apoptosis of HCT116 and J82 cells by exposure to 1% O_2. (A, C) HCT116 and J82 cells were transfected with two *AGGF1* specific siRNAs (siAGGF1-B and siAGGF1-C) or control siRNA (siNC), and exposed to hypoxia (1% O_2). Apoptosis was analyzed by flow cytometry analysis. (B, D) Changes in the proportion of apoptotic cells are shown as the fold changes. Data are shown as mean ± SD (n = 3, **, $P < 0.01$. ***, $P < 0.001$).

HCT116 and J82 cells. However, hypoxia induced apoptosis of HCT116 and J82 cells by about 10-fold and 5-fold, respectively, and knockdown of *AGGF1* expression inhibited hypoxia-induced apoptosis of HCT116 by 20%–40% and J82 cells by 50%–70% (Fig. 9). These results further indicate that down-regulation of *AGGF1* expression inhibits hypoxia-induced hypoxia in cancer cells.

We also assessed the effect of overexpression of *AGGF1* on apoptosis. We found that overexpression of *AGGF1* in HCT116 and J82 cells did not affect apoptosis under either normoxia or hypoxia (Fig. 10).

4. Discussion

Increased angiogenesis is involved in the growth, metastasis, and survival of various tumors, including urothelial carcinoma [37]. Angiogenic factors such as VEGFA or HIF-1α (regulating expression of VEGFA) were considered as potential biological markers for progression of bladder carcinomas to invasive tumors [38,39]. Therefore, in this study, we tested the hypothesis that an increased expression level of AGGF1, a newly identified angiogenic factor, might be observed in high-grade urothelial carcinomas compared to low-grade urothelial carcinomas or normal urothelial tissues. Surprisingly, opposite, unexpected results were obtained. Strong positive expression of AGGF1 was more frequently seen in normal urothelium samples and low-grade urothelial bladder carcinomas while no or weak expression of AGGF1 was dominant in high-grade urothelial bladder carcinomas (Table 1). Statistical analysis identified significant association between down-regulation of AGGF1 and development of high-grade urothelial bladder carcinomas (Table 1). Although the results were unexpected, they nevertheless indicate that AGGF1 expression levels are associated

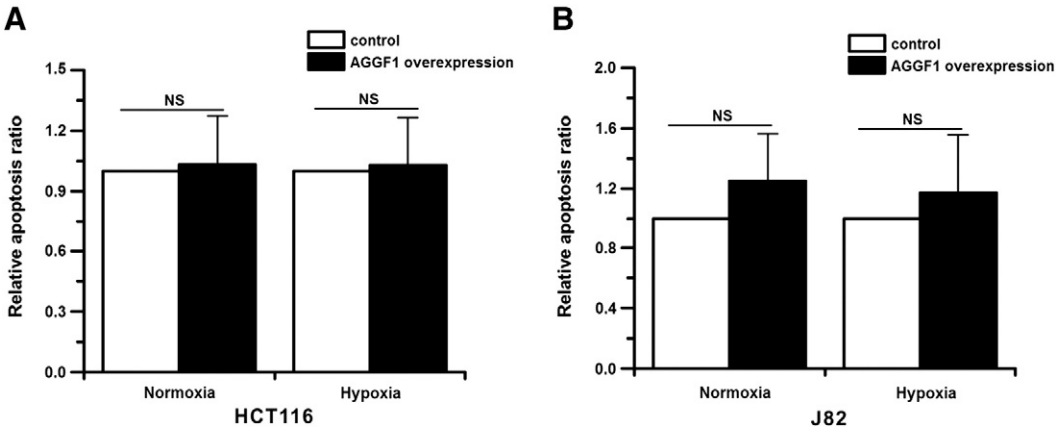

Fig. 10. Overexpression of AGGF1 protein had little effect on apopotosis under normoxia and hypoxia. HCT116 (A) and J82 (B) cells were transfected with an *AGGF1* expression plasmid or empty vector, cultured under the indicated conditions, and used for flow cytometry analysis. Changes in the proportion of apoptotic cells are shown as the fold changes. Data are shown as mean ± SD (n = 3, $P > 0.05$).

with risk of high-grade urothelial bladder carcinomas and may serve as a potentially useful marker of bladder cancer progression from low-grade to high-grade tumors.

One limitation of the above finding is that the identified association does not distinguish whether down-regulation of AGGF1 is the cause of high-grade urothelial bladder carcinomas or may be a mere consequence. The priori possibility is supported by the results that knockdown of AGGF1 expression significantly reduces apoptosis of tumor cells under hypoxia (Figs. 8 and 9). Tumor growth creates areas of hypoxia because of the limitation in oxygen diffusion provided by the host vasculature [31]. Moreover, hypoxia has been reported to be more common in high-grade urothelial carcinomas [33]. Western blot analysis showed that hypoxia induced a significant decrease in AGGF1 protein levels in different human cancer cell lines (Figs. 2, 3). Reduced expression of AGGF1 under hypoxia may help survival of tumor cells (Figs. 8 and 9), possibly resulting in development of high-grade urothelial bladder carcinomas. The second limitation is that the sample size for the human tumor study was small due to difficulties in collecting the matched tumors and adjacent normal tissue samples. Therefore, the conclusion derived from this analysis should be considered to be preliminary and future studies with larger sample sizes are needed to validate the finding. The third limitation is related to the data that down-regulation of AGGF1 reduced HCT116 and J82 apoptosis under hypoxia (Figs. 8 and 9), but overexpression of AGGF1 in the same cells did not affect apoptosis under hypoxia (Fig. 10). The reason underlying this discrepancy is unknown. It is possible that the endogenous level of AGGF1 was high and an additional increase of AGGF1 expression did not have further effect on apoptosis specifically under hypoxia.

The molecular mechanism by which hypoxia down-regulates AGGF1 expression was investigated in great detail. The finding that the mRNA level of *AGGF1* did not change under hypoxia suggested that the down-regulation was not at the transcriptional level and miRNA may be involved. Bioinformatic analysis and follow-up molecular and cellular studies demonstrated that the hypoxia-induced decrease of AGGF1 expression was dependent on miR-27a. First, a miR-27a mimics significantly decreased the expression level of AGGF1 and inhibition of miR-27a increased AGGF1 protein expression (Fig. 5). Second, miR-27a expression was up-regulated in high grade urothelial carcinomas, which was accompanied by down-regulation of AGGF1 (Fig. 7). Third, miR-27a showed up-regulation under hypoxic conditions (Fig. 6). Fourth, the hypoxia-induced decrease of AGGF1 expression disappeared with knockdown of miR-27a expression (Fig. 6). On the other hand, it is not clear whether miR-27a is the sole microRNA involved in regulation of AGGF1 under hypoxia. It is a generally accepted rule that each miRNA regulates many target genes and each gene (e.g. AGGF1) may be regulated by multiple miRNAs. As described earlier, bioinformatic analysis also identified two other miRNAs, miR-27b and miR-32, that may also be involved in regulation of AGGF1 expression. This possibility will be investigated in the future.

Our results suggest that down-regulation of AGGF1 expression by hypoxia-induced miR-27a expression represents a new signaling network for development of high-grade urothelial bladder carcinomas, however, it may not be the sole network. Besides miR-27a, other miRNAs have been found to be regulated by hypoxia [36]. Among them, miR-210 is the most predominantly up-regulated miRNA under hypoxia. MiR-210 is involved in tumor initiation, cell cycle regulation, mitochondrial metabolism and angiogenesis by targeting E2F3, MYC antagonist (MNT), ephrin-A3 (EFNA3) and iron-sulfur cluster scaffold protein (ISCU) [6,7,40–45]. MiR-21 induction by Akt2 during hypoxia promotes tumor resistance via its targets, PDCD4 and Spry1 [46]. Additionally, p53-mediated repression of miR-17-92 expression likely has an important function in hypoxia-induced apoptosis [47]. All of these data support the dominant role of miRNAs in development of more invasive, high-grade tumors.

AGGF1 has been shown to be an angiogenic factor critical to vasculogenesis (specification of venous fate) and angiogenesis [15,17]. On the other hand, it has been puzzling to us that AGGF1 contains putative functional domains of the FHA domain and G-patch motif that are more important for cellular functions in the nucleus [48,49]. The results in this study suggest that AGGF1 may be a multi-functional protein with roles in angiogenesis and other functions. This is supported by several pieces of evidence. First, AGGF1 does not appear to play a role as an angiogenic factor in high-grade urothelial bladder carcinomas because increased angiogenesis, meaning increased AGGF1 expression, may be expected in this type of tumors. Second, the expression pattern of AGGF1 was not consistent with that of HIF1-a (Fig. 3), which induces expression of angiogenic factors to increase angiogenesis under hypoxia. Third, little AGGF1 appeared to be secreted by cancer cells. Finally, down-regulation of AGGF1 played a protective role for tumor cells under hypoxia, although the detailed molecular mechanism needs future elucidation. Therefore, AGGF1 may turn out to be like FGF2 that has important functional roles both inside and outside the nucleus.

Our initial immunohistochemical analysis of AGGF1 protein expression focused on urothelial carcinomas simply because of the availability of tissue samples. Interestingly, we found that regulation of AGGF1 expression by hypoxia and by miR-27a appeared to be universal in several different cancer cell lines, e.g. the human bladder cancer cell line (J82), human colon cancer cell line (HCT116), human cervical cancer cell line (HeLa), human non-small cell lung carcinoma cell line (H1299) and human embryonic kidney cell line (HEK293). Thus, down-regulation of AGGF1 protein expression may be linked not only to high-grade urothelial carcinoma, but also to other high-grade tumors. Future studies are needed to verify this hypothesis.

In conclusion, the results in this study identify significant association between down-regulation of AGGF1 protein expression and risk of development of high-grade urothelial carcinomas. We also report that hypoxia-induced down-regulation of AGGF1 expression is dependent on hypoxia-induced up-regulation of miR-27a, and may promote survival of cancer cells.

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