

## RESEARCH ARTICLE

# Long noncoding RNA TUG1 upregulates VEGFA to enhance malignant behaviors in stomach adenocarcinoma by sponging miR-29c-3p

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**Abstract**

**Background:** Long noncoding RNA (lncRNA) TUG1 has been reported to display a pivotal role in the tumorigenesis and malignant progression of various types of cancers, including stomach adenocarcinoma (STAD). However, the contribution of aberrant expression of TUG1 and the mechanism by which it serves as a competing endogenous RNA (ceRNA) in STAD remains largely obscure.

**Methods:** The human STAD cell lines (MGC-803 and AGS), human normal gastric epithelial cell line (GES-1), human umbilical vein endothelial cells (HUVECs), and human embryonic kidney cells (HEK293T) were purchased and cultured to investigate the roles of TUG1 in STAD. Twenty BALB/c nude mice were purchased to establish a xenograft model to explore the roles of TUG1 in vivo.

**Results:** Bioinformatics analysis revealed that TUG1 was upregulated in STAD, of which expression was negatively and positively correlated with miR-29c-3p and VEGFA, respectively. Functional analyses indicated that TUG1 functioned as an oncogene to promote malignant behaviors (proliferation, migration, and angiogenesis) of STAD cells; whereas miR-29c-3p exerted the opposite role. Mechanistically, the interaction between miR-29c-3p with TUG1 and VEGFA was demonstrated. It was observed that miR-29c-3p could reverse the TUG1-induced promotion effect on cell proliferation, migration, and angiogenesis in STAD. Furthermore, TUG1 overexpression promoted STAD cell proliferation, metastasis, and angiogenesis, whereas VEGFA silence restored these effects, both in vitro and in vivo.

**Conclusion:** This finding confirmed that lncRNA TUG1 acts as a ceRNA for miR-29c-3p to promote tumor progression and angiogenesis by upregulating VEGFA, indicating TUG1 as a therapeutic target in STAD management.

**KEYWORDS**

angiogenesis, miR-29c-3p, stomach adenocarcinoma, TUG1, VEGFA

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## 1 | INTRODUCTION

Stomach adenocarcinoma (STAD), also known as gastric cancer, is the most common gastrointestinal malignancy with more than 1 million new cases worldwide in 2018.<sup>1</sup> Since STAD is diagnosed at the advanced stage in more than 80% of patients, its mortality ranks third among all cancers, accounting for about 8.2% of the total cancer deaths in 2018.<sup>1</sup> Although substantial improvements in the therapeutic effects have been witnessed in STAD in the last decades, the outcome of patients with STAD is still unsatisfactory. In order to develop novel effective strategies for prolonging STAD patient survival, expanding the knowledge of molecular mechanisms behind STAD progression remains imperative.

Protein-coding RNA (mRNAs), constituting only approximately 2% of the RNAs made from the human genome, is essential for gene expression, whose aberrance driving the most common subtypes of numerous cancers.<sup>2</sup> In the past, noncoding RNA (ncRNA) that is not involved in the production of proteins was considered as simply non-functional “junk.”<sup>3</sup> However, in the past 20 years, a growing number of studies demonstrated that multiple types of ncRNA, such as microRNA (miRNA) and long ncRNA (lncRNA), are involved in regulating cellular processes and pathways in physiology and the development of cancer.<sup>4,5</sup> Increasing studies implicated the important role of lncRNA in driving cancer progression.<sup>6</sup> It has been widely proposed that the lncRNA can “sponge” miRNA to modulate the activity of miRNAs on target genes, thereby regulating the mRNA splicing, transcription, and expression of target genes.<sup>7</sup> In recent years, taurine-upregulated gene 1 (TUG1) has been reported to serve as a potential oncogenic lncRNA in various types of cancer, such as colorectal cancer,<sup>8</sup> pancreatic cancer,<sup>9</sup> and STAD.<sup>10</sup> Zhang et al<sup>10</sup> revealed that TUG1 may be a regulator of GC proliferation *in vitro* and *in vivo*. Besides, Ren et al<sup>11</sup> indicated that TUG1 played a promotion role in the tumorigenesis and progression of STAD by acting as competing endogenous RNA (ceRNA) for miR-145-5p both *in vitro* and *in vivo*. Nevertheless, the regulatory network of TUG1 is still largely unknown.

It is well established that tumor angiogenesis plays a crucial role in the progression of solid tumors,<sup>12</sup> including STAD.<sup>13</sup> Folkman<sup>14</sup> proposed that both tumor growth and metastasis rely on the angiogenesis within a tumor. Therefore, inhibiting angiogenesis has become one of the effective strategies in cancer therapy.<sup>15</sup> Numerous signaling molecules, such as VEGFA and TGF- $\beta$ , have been proven to modulate angiogenesis during cancer progression over the past decades.<sup>16,17</sup> Many studies reported that TUG1 is involved in angiogenesis during the progression of various diseases.<sup>18,19</sup> One miRNA in particular, miR-29c-3p, could be directly targeted by TUG1 to stimulate angiogenesis in endothelial progenitor cells and diabetic mouse ischemic limb.<sup>20</sup> Besides, previous studies showed that TUG1 exerts oncogenic functions by interacting with miR-29c-3p in several cancers.<sup>9,21</sup> Additionally, it has been reported that miR-29c-3p could suppress cell proliferation and induce apoptosis in lung cancer by targeting angiogenic factor VEGFA.<sup>22</sup> Inspired by the above literature, we hypothesized that TUG1 functions as an oncogenic lncRNA

to upregulate VEGFA expression via sponging miR-29c-3p, contributing to the malignant behaviors in STAD.

Here, we studied the pro-tumorigenic effects of TUG1 in STAD *in vivo* and *in vitro*. Moreover, we demonstrated for the first time TUG1 as an important driver of cell proliferation, metastasis, and angiogenesis in STAD, through upregulation of VEGFA by functioning as a miR-29c-3p sponge.

## 2 | MATERIALS AND METHODS

### 2.1 | Integrative analysis based on public data

RNA-seq data downloaded from the Genotype Tissue Expression project (GTEx) (<https://gtexportal.org/>) and The Cancer Genome Atlas (TCGA) (<http://cancergenome.nih.gov/>) was used to perform differential expression analysis for TUG1 and miR-29c-3p.

Starbase (<http://starbase.sysu.edu.cn/panCancer.php>)<sup>23</sup> was applied to perform the coexpression analysis for TUG1/miR-29c-3p, VEGFA/miR-29c-3p, and VEGFA/TUG1.

### 2.2 | Cell lines and culture

The human STAD cell lines (MGC-803 and AGS), human normal gastric epithelial cell line (GES-1), human umbilical vein endothelial cells (HUVECs), and human embryonic kidney cells (HEK293T) obtained from the ATCC (VA, USA) were cultured in RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin in a humidified environment of 5% CO<sub>2</sub> at 37°C.

### 2.3 | RT-qPCR

Total RNA extracted from cells or tissues using TRIzol (Invitrogen) was subjected to synthesizing the cDNA of TUG1, miR-29c-3p, and VEGFA. Then, RT-qPCR was conducted to quantify the relative mRNA levels with iTaq™ universal SYBR Green I Kit (Bio-Rad). GAPDH and U6 served as endogenous controls to normalize against the expression of studied RNAs (TUG1, miR-29c-3p, and VEGFA) using the 2<sup>- $\Delta\Delta$ Ct</sup> method.<sup>24</sup> The primers used in this study are listed in Table S1.

### 2.4 | Cell transfection

Short hairpin RNAs (shRNA) for TUG1 (sh-TUG1-1 and sh-TUG1-2) and VEGFA (sh-VEGFA), and the corresponding negative control (NC), TUG1-overexpressing vector (TUG1), and negative control pcDNA3.1 (Vector) were all supplied by RiboBio (Table S2: <https://portals.broadinstitute.org/gpp/public/gene/search>). The mimic and inhibitor for miR-29c-3p (miR-29c-3p mimic and miR-29c-3p inhibitor) and their corresponding negative control (NC mimic and

NC inhibitor) were obtained from GenePharma. Lipofectamine<sup>®</sup> 2000 reagent (Invitrogen) was used for the transfection of the above-indicated plasmids. 48 h later, cells were harvested for further analysis, whose transfection efficiency was confirmed by RT-qPCR.

## 2.5 | CCK-8 assay

Cells were seeded into 96-well plates for 24 h, 48 h, and 72 h incubation. Then, CCK-8 reagent (10%, v/v) was added to each well at designed time points. After an additional 1.5 h incubation, the absorbance at 450 nm was measured under a Microplate reader (Bio-Rad).

## 2.6 | Clonogenic assay

The procedures were performed as Franken et al<sup>25</sup> described previously. Staining colonies were photographed and counted with a BX51 microscope (Olympus).

## 2.7 | Transwell assay

In brief, 700  $\mu$ l medium with 10% FBS was added to the bottom chamber. Meanwhile, 200  $\mu$ l cells suspension (serum-free) was added to the top chamber with 8 mm pores of filter membrane, followed by 24 h incubation. The cells migrated to the bottom chamber were fixed and stained, followed by photographed and counted under a BX51 microscope.

## 2.8 | Western blot

Total protein extracted from cells using RIPA buffer (Abcam) was quantified with a BCA Kit (Beyotime). Western blot analysis was carried out as previously reported.<sup>26</sup> All antibodies used in this study were purchased from Abcam: Anti-BMP4 antibody (ab235114), Anti-ICAM1 antibody (ab171123), Anti-VCAM1 antibody (ab134047), Anti-VEGFA (ab46154), Anti-Ki67 (ab16667), Anti-CD31 (ab9498), Anti-GAPDH antibody (ab8245), and Rabbit Anti-Human IgG H&L (HRP) (ab6759).

## 2.9 | Tube formation assay

Tube formation assay was conducted according to a previous study.<sup>27</sup> HUVECs were seeded into Matrigel-coated plates and incubated with a conditioned cell culture medium (CM). Finally, the formation of capillary tubes was observed and photographed under a BX51 microscope and the number of branches was counted with ImageJ software.

## 2.10 | Dual-luciferase assay

The binding sites of TUG1 and VEGFA with miR-29c-3p were predicted by TargetScan (<http://www.targetscan.org/>).<sup>28</sup> The sequences of wild-type (WT) or mutant (MUT) 3'-UTR for TUG1 or VEGFA were synthesized and cloned into pmirGLO Vector (Promega). HEK293T and AGS cells were co-transfected with miR-29c-3p mimic or NC mimic, together with each vector. Using the Dual-Luciferase<sup>®</sup> Reporter Assay System (Promega), the luciferase activity of each group was examined.

## 2.11 | Ago2-RNA immunoprecipitation (RIP) assay

Ago2-RIP assay was performed by using a Magna RIP Kit (Millipore). Briefly, cell extract was immunoprecipitated with magnetic beads conjugated with antibodies against Ago2 (ab32381, Abcam). IgG was served as the negative control. Finally, immunoprecipitated TUG1 or VEGFA was analyzed by RT-qPCR.

## 2.12 | Biotin-coupled RNA pull-down

The biotinylated RNAs (miR-29c-3p, TUG1, and VEGFA) were acquired by using T7 RNA polymerase (Promega) and Biotin RNA Labeling Mix (Roche), followed by purified with RNeasy Mini Kit (Qiagen). Pull-downs were performed with biotinylated RNAs and cell extracts. Biotin-coupled RNA complex was isolated by streptavidin-coated magnetic beads (Invitrogen) and subjected to detect the enrichment of miR-29c-3p or TUG1 or VEGFA by RT-qPCR.

## 2.13 | Enzyme-linked immunosorbent assay (ELISA)

The production of VEGF proteins was measured using a human VEGF-A ELISA Kit (Sigma-Aldrich).

## 2.14 | In vivo study

Twenty BALB/c nude mice (4–5 weeks, male) purchased from Guangdong Medical Laboratory Center were randomized into four groups ( $N = 5$  per group). To establish the xenograft model, MGC-803 cells ( $1 \times 10^6/0.1$  ml PBS) with diverse transfections were subcutaneously injected into the right armpit of mice in different groups as followed: vector group (cells transfected with empty vector), TUG1 group (cells overexpressing TUG1), TUG1 + sh-NC (overexpressed TUG1-cells transfected with scrambled RNA), TUG1 + sh-VEGFA (overexpressed TUG1-cells transfected with sh-VEGFA). Two weeks later, the mice were sacrificed to collect tumor samples. Tumor weight and volume were immediately recorded after tumor resection and subsequently stored in  $-80^{\circ}\text{C}$  for further analysis. Tumor volume was calculated following the equation:  $\text{length} \times \text{width}^2 \times 1/2$ .

All procedures performed were approved by the Animal Ethics Committee of the Second Affiliated Hospital of Nanchang University.

## 2.15 | Immunohistochemistry (IHC)

Paraffin-embedded tumor tissues were sliced into 4  $\mu\text{m}$  thick sections for immunohistochemical staining to examine the expression of Ki-67, BMP4, ICAM1, VCAM1, and CD31 as Mattioli et al<sup>29</sup> previously described.

## 2.16 | Statistical analysis

All experiments were performed thrice at least. Data are expressed as mean  $\pm$  SD. Prism 8.0.1 was applied to conduct a statistical analysis of all data. The comparisons among groups were performed using student's *t* test or one-way ANOVA followed by Bonferroni's post hoc test. It is considered to be statistically significant when  $p < 0.05$ .

# 3 | RESULTS

## 3.1 | The aberrant expressions of TUG1 and miR-29c-3p were observed in STAD tissues and cells

Integrative analysis of GTEx and TCGA revealed that TUG1 is highly expressed in STAD tissues when compared with normal tissues (Figure 1A). Further analysis of the paired tumor-normal tissue samples from STAD available in TCGA confirmed this finding (Figure 1B). The same analyses performed for miR-29c-3p showed an opposite result to TUG1, which showed that miR-29c-3p was significantly downregulated in STAD tissues compared with normal tissues (Figure 1C,D). We further validated their expression levels in human STAD cell lines and normal gastric epithelium cell line GES-1 by RT-qPCR. In comparison to the GES-1 cells, TUG1 was upregulated while miR-29c-3p was downregulated in MGC-803 and AGS cells, (Figure 1E,F). These results hinted that TUG1 upregulation and miR-29c-3p downregulation might be involved in the progression of STAD.

## 3.2 | The effect of TUG1 and miR-29c-3p on cell proliferation, migration, and angiogenesis in STAD

In order to clarify the biological functions of TUG1 and miR-29c-3p in STAD, different vectors were respectively transfected into MGC-803 and AGS cells. RT-qPCR analysis confirmed that each vector was successfully transfected into cells (Figure 2A–D). The suppression efficiency for TUG1 expression of sh-TUG1-1 was slightly higher than that of sh-TUG1-2 (Figure 2B); hence sh-TUG1-1 was chosen to perform the following experiments. CCK-8 assay showed that overexpressing TUG1 significantly enhanced the proliferation of STAD,

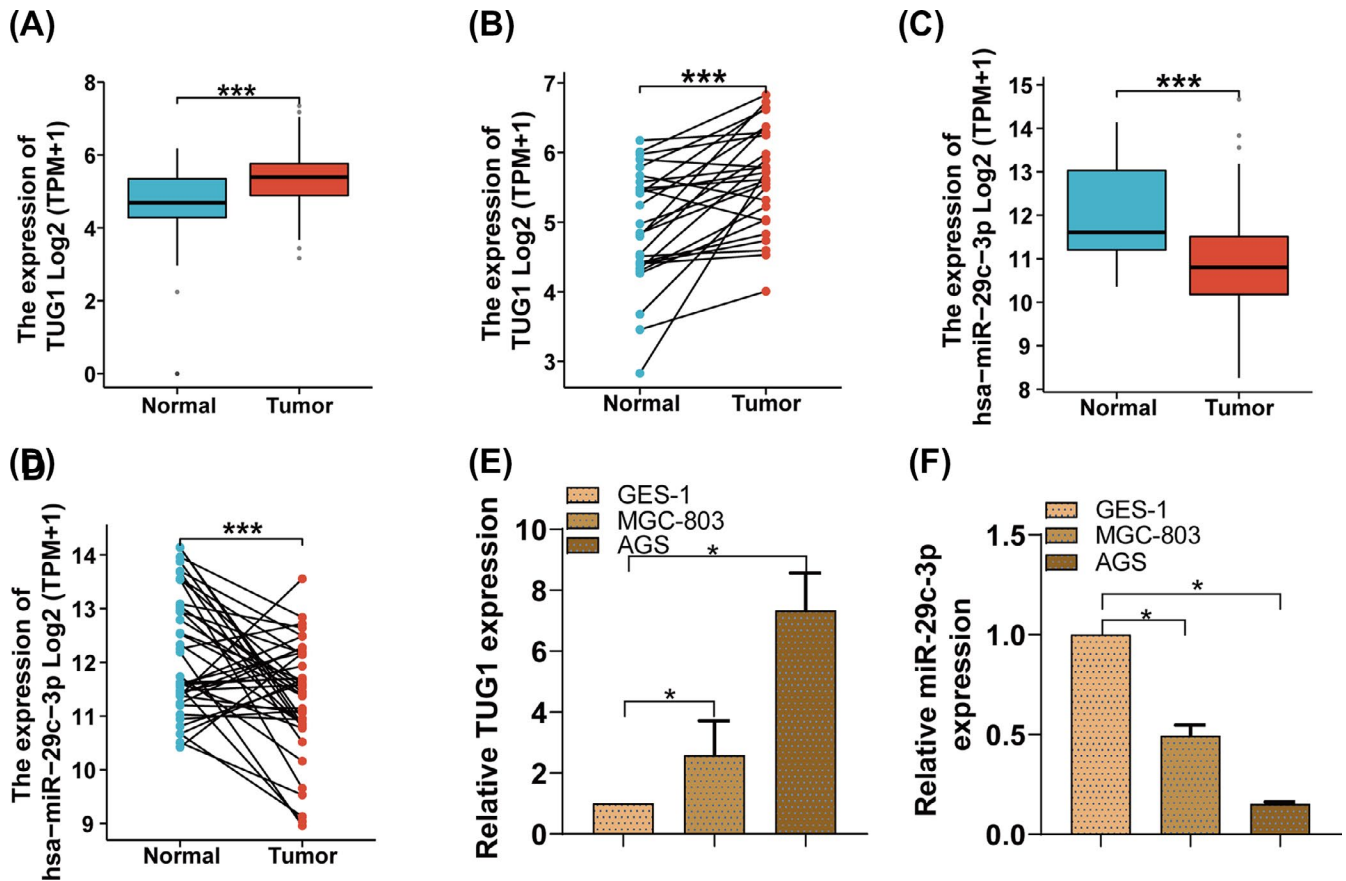
whereas silencing TUG1 exerted the opposite effects (Figure 2E). Simultaneously, clonogenic assay showed that clone forming capacity of STAD cells was strengthened by overexpressing TUG1, but impaired by knocking down TUG1 (Figure 2F). The above experiments collectively confirmed the promotion role of TUG1 on cell proliferation in STAD. In addition, CCK-8 and clonogenic assays also revealed that the cell proliferation capacity of STAD was repressed by miR-29c-3p mimic, whereas enhanced by miR-29c-3p inhibitor (Figure 2G,H), suggesting the inhibitory effect of miR-29c-3p on cell proliferation in STAD.

To evaluate the effect of TUG1 and miR-29c-3p on STAD cell migration, transwell assay and western blot detecting BMP4, ICAM1, and VCAM1 expression were subsequently performed in STAD cells. The overexpression of TUG1 enhanced not only the migratory characteristic but also the expression of BMP4, ICAM1, and VCAM1 of STAD cells (Figure 3A,B). The knockdown of TUG1 had the opposite effects (Figure 3A,B). That means TUG1 plays a promotion role in the migration of STAD. For miR-29c-3p, it is observed that STAD cells migration and the expression of BMP4, ICAM1, and VCAM1 were negatively correlated with miR-29c-3p expression (Figure 3D,E), revealing the repression role of miR-29c-3p in STAD migration.

Since angiogenesis is the prerequisite to tumor growth and metastasis, a tube formation assay was subsequently performed to investigate the role of TUG1 and miR-29c-3p in regulating the pro-angiogenic ability of STAD cells. The culture medium (CM) of STAD cells with different transfections was harvested to treat HUVECs. After treatment, we found that the CM from STAD cells with TUG1 overexpression or miR-29c-3p silencing induced HUVECs to form more capillary tubes than the control groups (vector or NC inhibitor) (Figure 3C). In contrast, when HUVECs were treated using CM from STAD cells with TUG1 depletion or miR-29c-3p overexpressing, HUVECs sprouted fewer capillary tubes compared with negative controls (NC or NC mimic) (Figure 3F). These results demonstrated that the expression of both TUG1 and miR-29c-3p in STAD cells has a great impact on the tube formation of HUVECs, suggesting that TUG1 upregulation and miR-29c-3p downregulation are involved in the angiogenesis of endothelial cells during STAD progression.

## 3.3 | TUG1 functions as a ceRNA for miR-29c-3p in STAD

Based on our above results and a previous finding that there existed a direct interaction between TUG1 and miR-29c-3p in pancreatic cancer,<sup>9</sup> we wondered whether TUG1 modulates malignant behaviors of STAD cells via sponging miR-29b. The correlation analysis from the starbase platform revealed an inverse correlation between the expression of TUG1 and miR-29c-3p in STAD ( $r = -0.244$ ,  $p < 0.001$ ; Figure 4A). The level of miR-29c-3p expression was decreased when TUG1 was overexpressed but elevated when TUG1 was knocked down (Figure 4B,C). Then, dual-luciferase assay was performed and determined that the level of miR-29c-3p is directly regulated by TUG1 (Figure 4D). The result indicated that miR-29c-3p



**FIGURE 1** The expression profile of TUG1 and miR-29c-3p in STAD. (A) Integrative analysis based on GTEx and TCGA databases for the expression of TUG1 in STAD. (B) The differential expression analysis of TUG1 between STAD tumor and adjacent normal tissues based on TCGA database. (C) Integrative analysis based on GTEx and TCGA databases and for the expression of miR-29c-3p in STAD. (D) The differential expression analysis of miR-29c-3p between STAD tumor and adjacent normal tissues based on TCGA database. The expression of (E) TUG1 and (F) miR-29c-3p in a normal gastric epithelial cell line (GES-1) and STAD cell lines (AGS and MGC-803) detected by RT-qPCR

mimic dramatically reduced the luciferase activity of TUG1-WT in HEK293T and AGS cells (Figure 4D,E). Ago2-RIP assay was conducted and validated the physical interaction between miR-29c-3p and its potential binding lncRNA TUG1 in AGS cells (Figure 4F). Consistently, biotin-coupled RNA pull-down further confirmed that miR-29c-3p directly interacted with TUG1 in AGS and MGC-803 cells (Figure 4G,H). The abovementioned data demonstrated that TUG1 directly binds to miR-29c-3p to serve as its molecular sponge, thereby playing a negative regulatory role for miR-29c-3p expression in STAD cells.

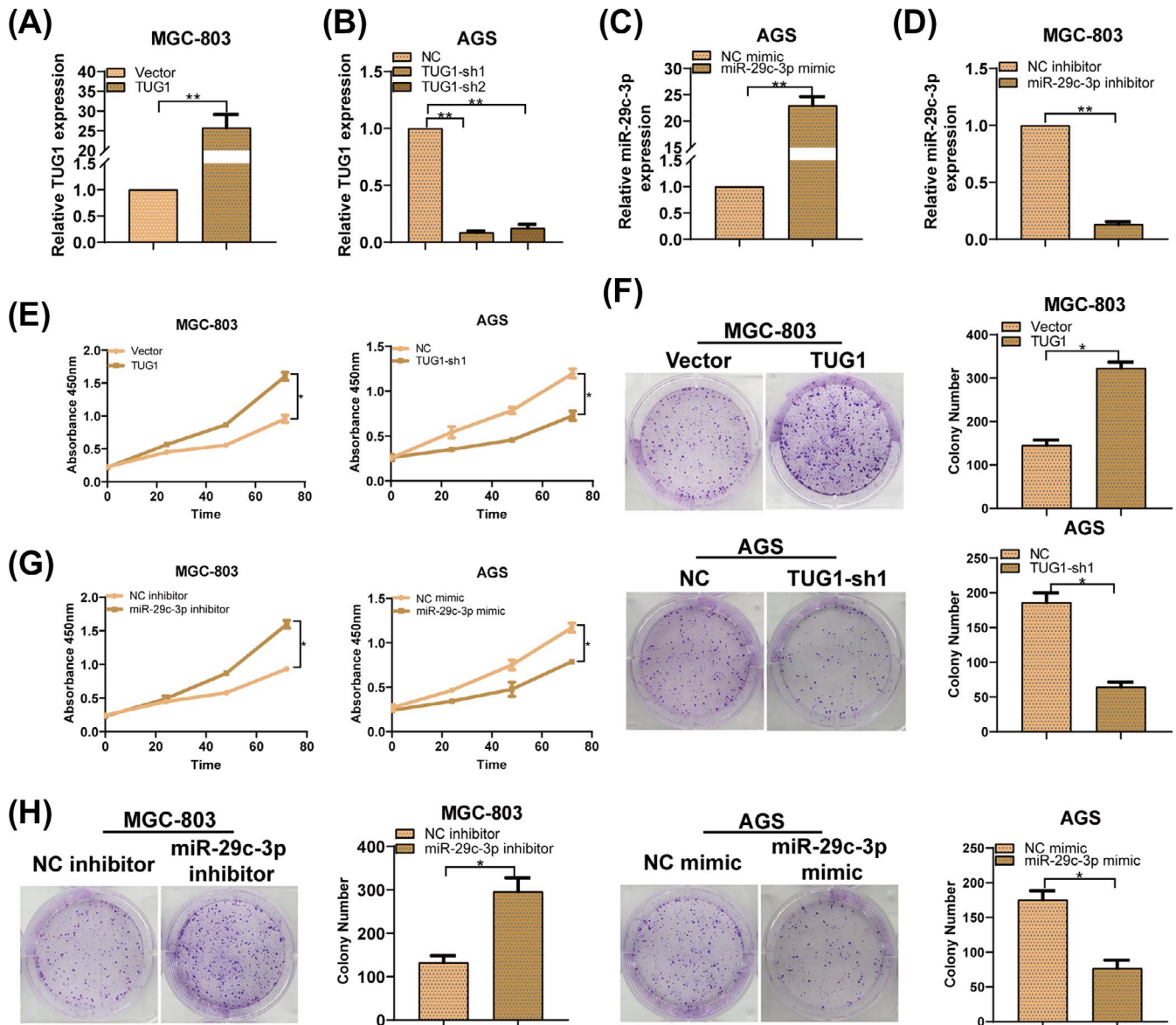
### 3.4 | miR-29c-3p is a mediator of the effects induced by TUG1 on STAD cells

Next, the functional relevance of the TUG1/miR-29c-3p interaction was further investigated in STAD cells (AGS and MGC-803). CCK-8 and clonogenic assays revealed that miR-29c-3p is capable of reversing the effects of TUG1 on the cell proliferation of STAD (Figure 5A-D). In the meantime, transwell assay revealed that the role of TUG1 overexpression and depletion in STAD migration

was respectively antagonized by miR-29c-3p mimic and inhibitor (Figure 5E,F). This finding was confirmed by the detection of BMP4, ICAM1, and VCAM1 expressions (Figure 5G,H). Moreover, miR-29c-3p was also implicated in regulating TUG1-induced biological effects of HUVEC. When MGC-803 cells overexpressing TUG1, its CM could facilitate the tube formation of HUVECs; but when TUG1 overexpressed MGC-803 cells were simultaneously upregulating miR-29c-3p, the promotion role of its CM on tube formation of HUVECs was attenuated (Figure 5I). This finding was further confirmed by AGS cells that were transfected with sh-TUG1 alone or combined with miR-29c-3p inhibitor (Figure 5J). Taken together, the effect of TUG1 on cell proliferation, migration, and angiogenesis in STAD was predominantly mediated through the miR-29c-3p.

### 3.5 | VEGFA is directly targeted by miR-29c-3p and regulated by TUG1

As the most extensively studied angiogenic factor, VEGFA has been reported to be regulated by miR-29c-3p to suppress malignant

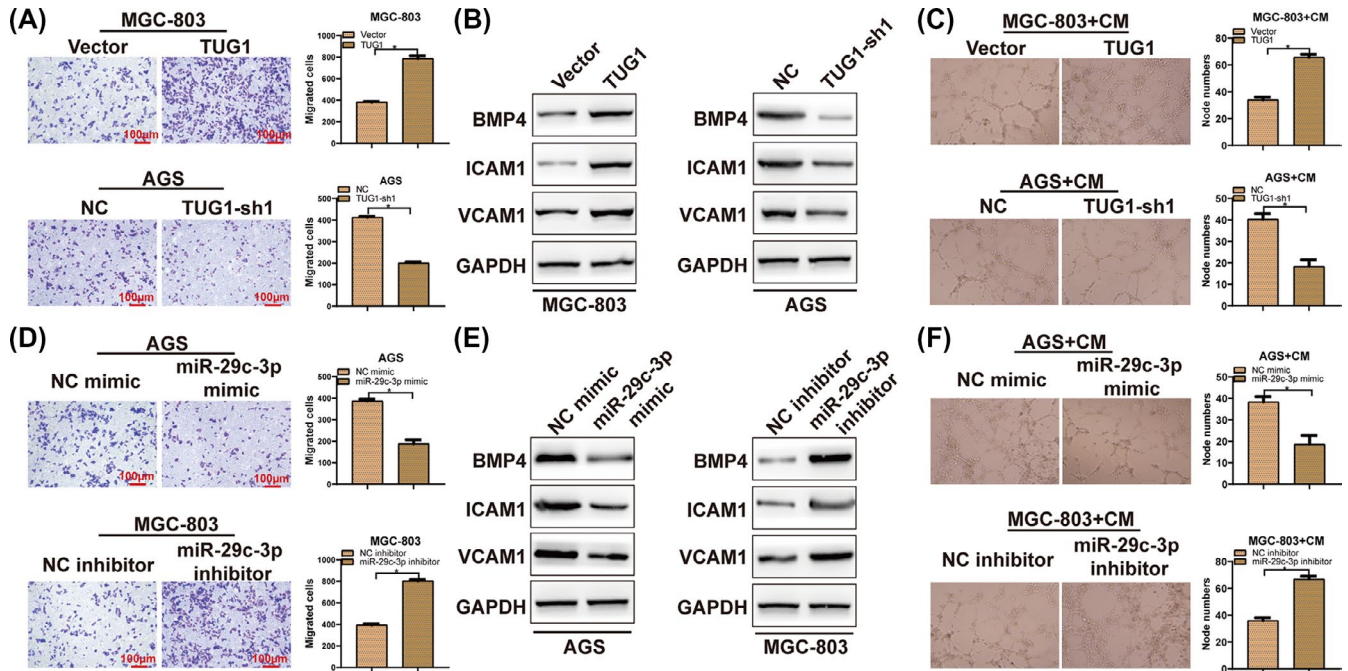


**FIGURE 2** The effect of TUG1 and miR-29c-3p on the proliferation of STAD cells. MGC-803 cells were transfected with TUG1 overexpression vector or miR-29c-3p inhibitor; AGS cells were knocked down TUG1 or transfected with miR-29c-3p mimic. RT-qPCR assay examined the expression of TUG1 in (A) MGC-803 and (B) AGS cells, as well as the expression of miR-29c-3p in (C) AGS and (D) MGC-803 cells. The effect of TUG1 on cell proliferation detected by (E) CCK-8 and (F) clonogenic assays. The effect of miR-29c-3p on cell proliferation detected by (G) CCK-8 and (H) clonogenic assays

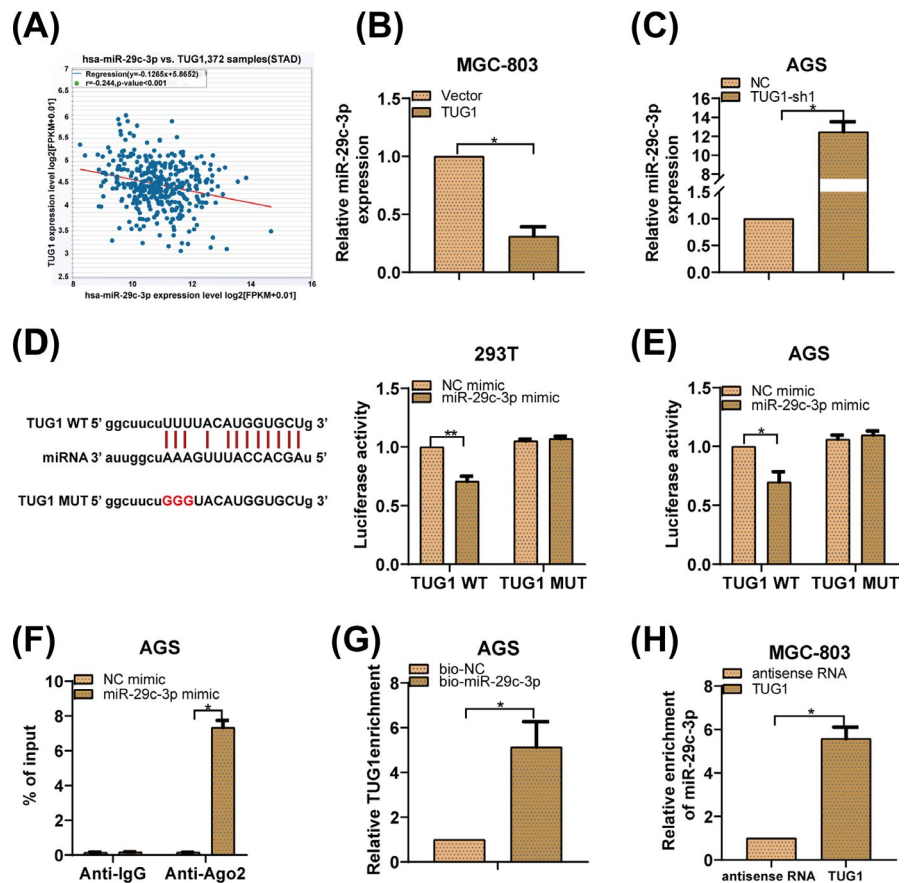
phenotypes of lung cancer cells.<sup>22</sup> Hence, we speculated that TUG1/miR-29c-3p modulated the malignant behaviors of STAD via VEGFA. Based on starbase, we found that VEGFA expression in STAD is negatively related to miR-29c-3p ( $r = -0.161$ ,  $p < 0.001$ ) but positive correlated with TUG1 ( $r = 0.237$ ,  $p < 0.001$ ) (Figure 6A). RT-qPCR and ELISA results demonstrated that TUG1 overexpression and miR-29c-3p inhibitor significantly elevated VEGFA levels in MGC-803 cells, while the TUG1 depletion and miR-29c-3p mimic obviously decreased the expression of VEGFA in AGS cells (Figure 6B–E). Dual-luciferase assay demonstrated that miR-29c-3p is directly bound to VEGFA (Figure 6F,G). Consistently, Ago2-RIP and RNA pull-down assays further confirmed miR-29c-3p directly interacts with VEGFA (Figure 6H–I).

### 3.6 | TUG1 exerts an oncogenic role in STAD through regulating VEGFA both in vitro and in vivo

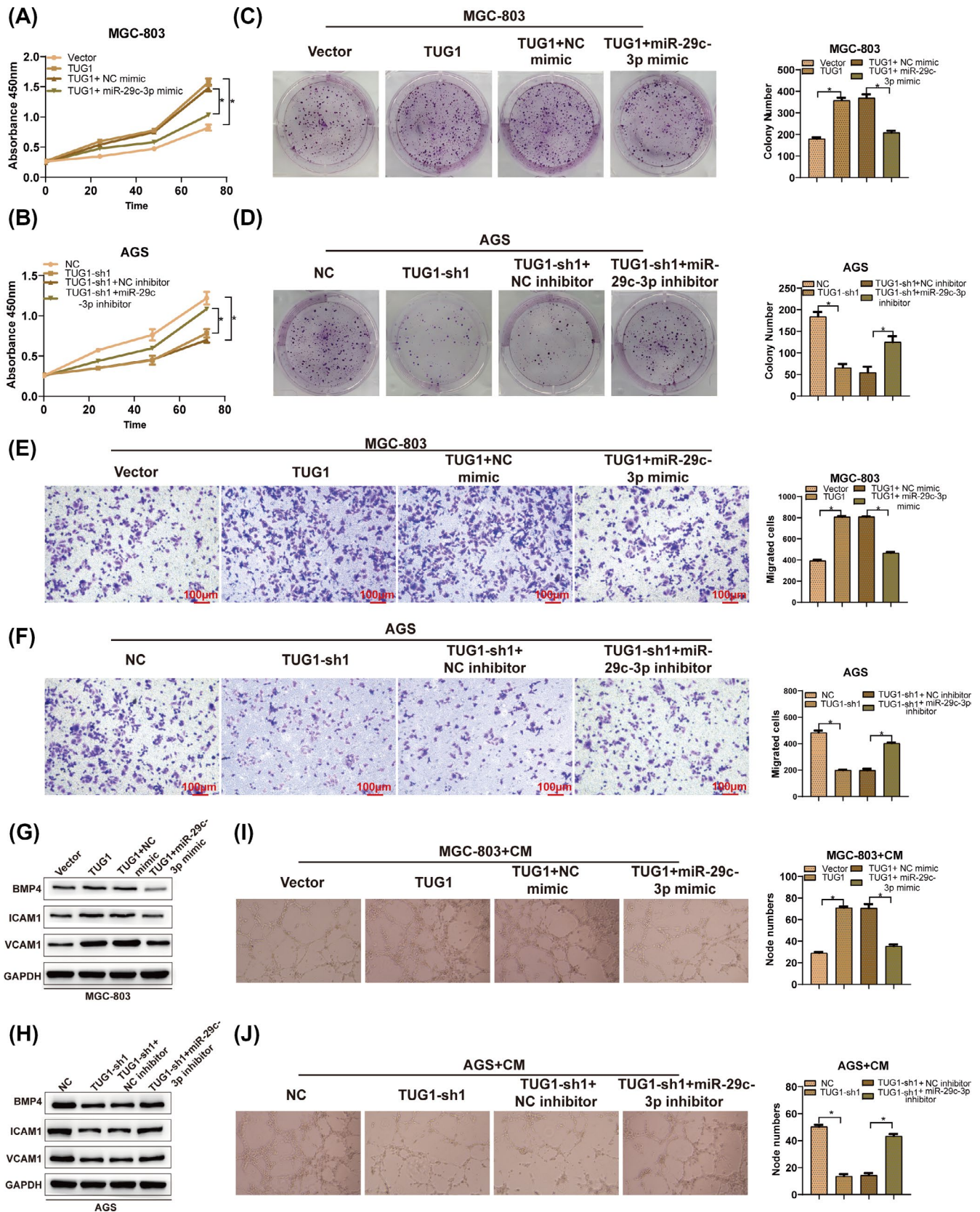
In an attempt to delineate whether TUG1-mediated oncogenic activity in STAD relies on VEGFA, TUG1 overexpression vector was transfected into MGC-803 cells alone or together with sh-VEGFA. The transfection efficiency of sh-VEGFA detected by RT-qPCR and WB was displayed in Figure 7A. In the CCK-8 assay, the increase of cell proliferation due to TUG1 overexpression was partially attenuated by silencing of VEGFA in MGC-803 (Figure 7B). Overexpressing TUG1 led to the elevation of BMP4, ICAM1, and VCAM1 expression, which was partly antagonized by the inhibition of VEGFA in MGC-803 (Figure 7C). Transwell assay showed



**FIGURE 3** The effect of TUG1 and miR-29c-3p on cell migration and angiogenesis in STAD. (A) The role of TUG1 in STAD cell migration investigated by transwell assay. (B) The expression of BMP4, ICAM1, and VCAM1 in STAD cells with TUG1 overexpression or depletion detected by western blot. (C) The effect of TUG1 on angiogenesis assessed by the tube formation of HUVECs. (D) The role of miR-29c-3p in STAD cell migration investigated by transwell assay. (E) The expression of BMP4, ICAM1, and VCAM1 in STAD cells with miR-29c-3p mimic or inhibitor detected by western blot. (F) The effect of miR-29c-3p on angiogenesis assessed by the tube formation of HUVECs

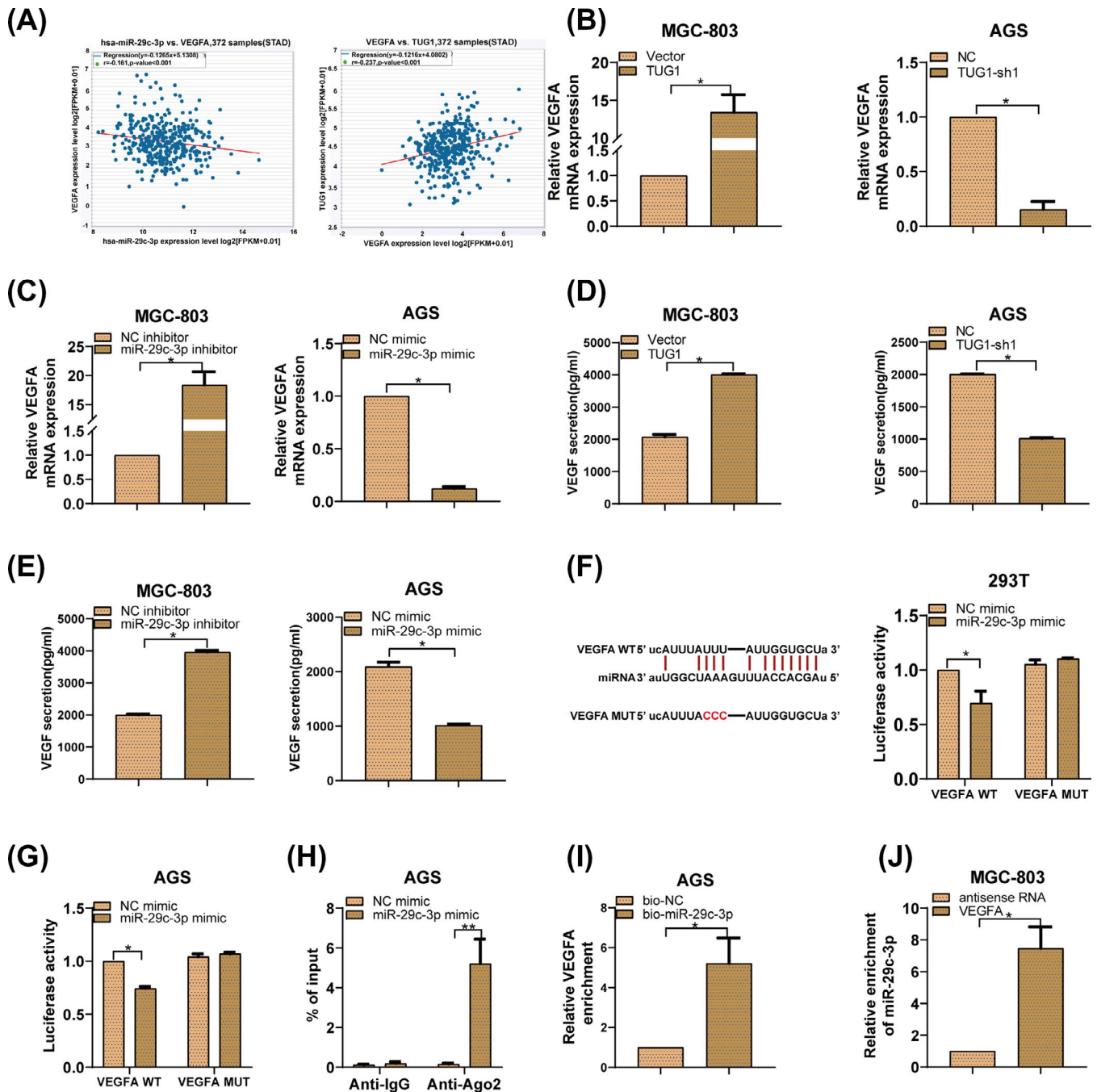


**FIGURE 4** TUG1 is a ceRNA for miR-29c-3p in STAD cells. (A) The correlation analysis between TUG1 and miR-29c-3p based on starbase platform. RT-qPCR detected the expression of miR-29c-3p in (B) TUG1 overexpressing MGC-803 cells and (C) TUG1 silencing AGS cells. (D) Dual-luciferase assay confirmed the interaction between TUG1 and miR-29c-3p (left: Binding sites of TUG1 in miR-29c-3p predicted using TargetScan; right: luciferase activity in HEK293T cells). (E) Dual-luciferase assay conducted in AGS cells. (F) Ago2-RIP assay evaluated whether TUG1 was enriched in Ago2 after overexpressing miR-29c-3p. RNA pull-down assay examined the binding between TUG1 and miR-29c-3p in (G) AGS and (H) MGC-803 cells



**FIGURE 5** The effects of TUG1 on STAD cells are mediated by miR-29c-3p. MGC-803 cells were transfected with TUG1 overexpression vector alone, or together with miR-29c-3p mimic or NC mimic; silencing AGS cells were transfected with sh-TUG1 alone, or together with miR-29c-3p inhibitor or NC inhibitor; STAD cells transfected with empty vector considered as the control group. (A-B) CCK-8 assay detected the cell viability. (C-D) Clonogenic assay showed the formation of colonies. (E-F) Transwell assay tested the STAD cell migration ability. (G-H) Western blot detected the expression of BMP4, ICAM1, and VCAM1. (I-J) The tube formation of HUVECs after treating with CM derived from STAD cells with different transfections

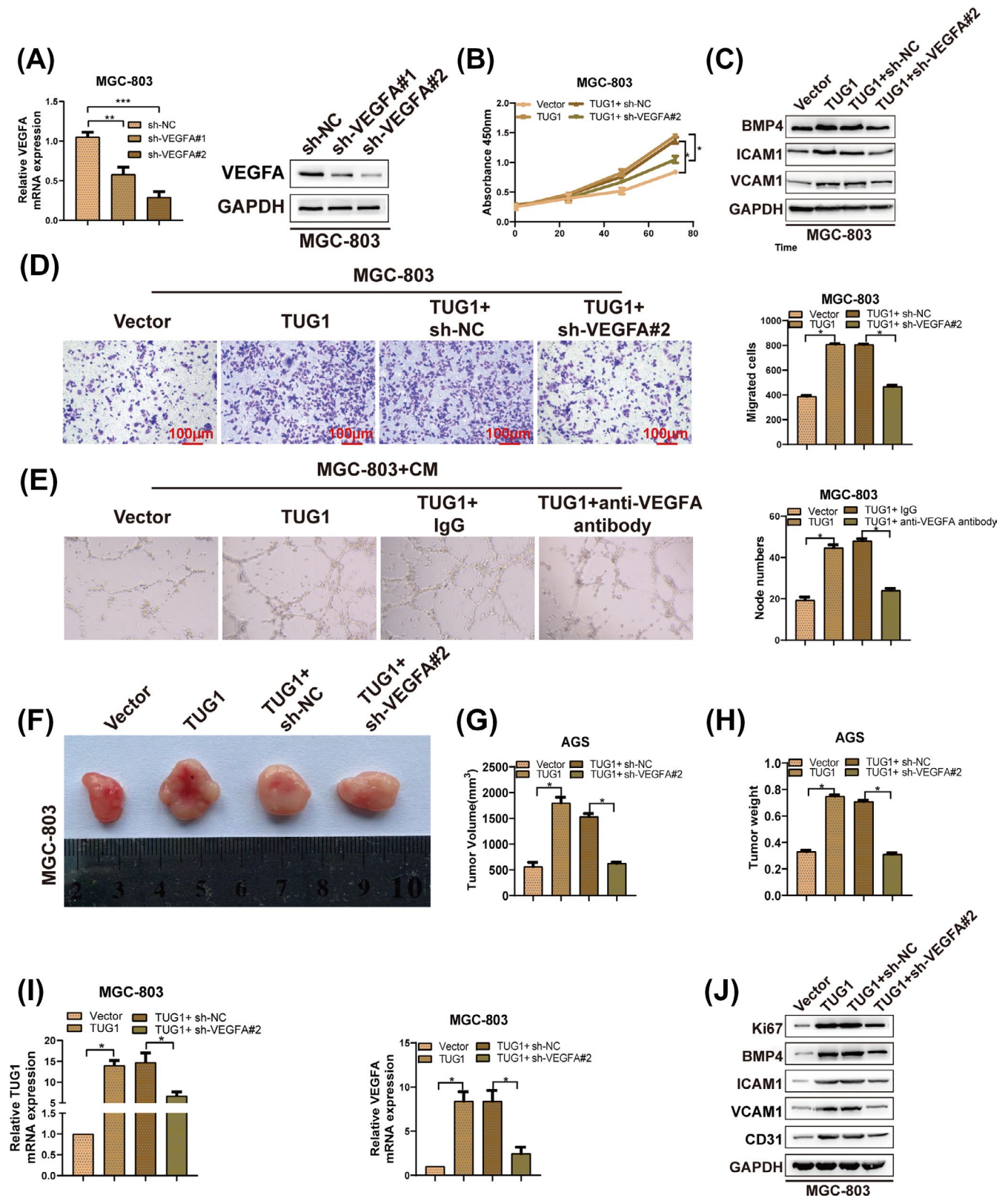




**FIGURE 6** TUG1 regulates VEGFA expression via sponging miR-29c-3p in STAD cells. (A) The correlation analysis of miR-29c-3p vs. VEGFA, and TUG1 vs. VEGFA in STAD based on starbase platform. The effect of (B) TUG1 and (C) miR-29c-3p on VEGFA mRNA expression in AGS cells by RT-qPCR. The effect of (D) TUG1 and (E) miR-29c-3p on VEGFA protein expression in AGS cells by ELISA. (F) Dual-luciferase assay confirmed the interaction between VEGFA and miR-29c-3p (left: Binding sites of VEGFA in miR-29c-3p predicted using TargetScan; right: luciferase activity in HEK293T cells). (G) Dual-luciferase assay conducted in AGS cells. (H) Ago2-RIP assay evaluated whether VEGFA was enriched in Ago2 after overexpressing miR-29c-3p. RNA pull-down assay examined the binding between VEGFA and miR-29c-3p in (I) AGS and (J) MGC-803 cells

that cell migration ability was significantly strengthened by TUG1 overexpression, and this enhancement was partly reversed by VEGFA depletion in MGC-803 (Figure 7D). Moreover, a similar trend was observed in tube formation assay (Figure 7E), suggesting that TUG1-induced promotion role in angiogenesis is dependent on VEGFA.

To further corroborate the above findings in vivo, MGC-803 cells transfected with TUG1 overexpression alone or together with vector and sh-VEGFA or sh-NC were used to establish the xenograft tumor model. MGC-803 cells transfected with empty vector considered as the control. As expected, overexpressing TUG1 significantly promoted tumor growth compared with the



**FIGURE 7** TUG1 promotes STAD progression by regulating VEGFA in vitro and in vivo. (A) RT-qPCR and WB analysis respectively examined the expression of VEGFA. TUG1 overexpression vector was transfected alone or combined with sh-VEGFA#2 or sh-NC into MGC-803 cells. (B) CCK-8 assay examined cell proliferation. (C) Western blot detected the expression of BMP4, ICAM1, and VCAM1. (E) Angiogenesis assessed by the tube formation of HUVECs. (D) Transwell assay evaluated cell migration ability. (F) The representative photograph, (G) volume, and (H) weight of xenograft tumors. (I) The expression of TUG1 and VEGFA in xenograft tumors by RT-qPCR. (J) WB analysis detected the protein levels of Ki-67, BMP4, ICAM1, VCAM1, and CD31 in xenograft tumors

control; however, this promotion could be partly weakened by VEGFA depletion (Figure 7F–H). RT-qPCR analysis on xenograft tumor tissues manifested that TUG1 overexpression caused a significant increase in both TUG1 and VEGFA expressions; while this increase was abrogated in part by silencing VEGFA (Figure 7I). Furthermore, WB analysis for Ki-67, BMP4, ICAM1, VCAM1, and CD31 was performed to verify that TUG1 drove STAD tumor growth, metastasis, and angiogenesis via VEGFA *in vivo*. The results showed that TUG1 overexpression led to an observably increased expression of Ki-67, BMP4, ICAM1, VCAM1, and CD31 in tumor tissues (Figure 7J). Notably, the expression of Ki-67, BMP4, ICAM1, VCAM1, and CD31 in the TUG1 + sh-VEGFA#2 group was obviously lower than those in the TUG1 and TUG1 + sh-NC groups, suggesting that VEGFA might be a mediator participating in the role of TUG1 in STAD tumor growth, metastasis, and angiogenesis.

## 4 | DISCUSSION

Increasing studies provide evidence that lncRNAs play considerable functional roles in tumor progression, which can be used as therapeutic targets and biomarkers for various types of cancers.<sup>30,31</sup> During the past decade, multiple lncRNAs have been reported as tumor suppressors or oncogenes in STAD,<sup>32</sup> such as MEG3,<sup>33</sup> HOTAIR,<sup>34</sup> and TUG1.<sup>11</sup> In many previous publications, TUG1 has been considered an oncogenic lncRNA, whose elevated expression was observed in diverse malignancies.<sup>35,36</sup> TUG1 is highly expressed in several hematologic malignancies, which has been indicated to be correlated with increased disease risk and stage in multiple myeloma and acute lymphocytic leukemia.<sup>37,38</sup> It has been previously reported that the elevated levels of TUG1 usually led to a poor prognosis in STAD patients.<sup>10</sup> Moreover, TUG1 has been implicated in the malignant behaviors of STAD cells *in vitro*, and proven to participate in tumorigenesis *in vivo*. However, studies on its biological functions and the underlying mechanisms in STAD progression are limited. Several studies documented that miR-29c-3p participates in STAD development and represses STAD cell proliferation and migration.<sup>39,40</sup> Given that TUG1 has been reported to “sponge” miR-29c-3p to promote tumor progression in several cancers, we speculated that there may also be a TUG1/miR-29c-3p axis in regulating STAD progression.

Initially, we performed integrative analysis for TCGA and GTEx data and found the upregulation of TUG1 and the downregulation of miR-29c-3p in STAD tissues, which is consistent with previous studies.<sup>41,42</sup> Baratieh et al<sup>42</sup> demonstrated that TUG1 contributes to the progression of STAD, which might serve as a diagnostic biomarker. However, a global expression profiling of lncRNAs in gastric cancer by Mo et al<sup>43</sup> identified TUG1 as the most downregulated lncRNAs in 104 pairs of gastric carcinoma and adjacent non-tumor tissues. This paradoxical result might be because of different sample size and the heterogeneity of cancerous tissues. Moreover, an array of functional analyses in our study, such as CCK-8, clonogenic, and

transwell assays, indicated that TUG1 exhibits a promotion role in the proliferation and migration of STAD cells. As known, the upregulation of ICAM1 and VCAM1 is closely related to the cancer metastasis.<sup>44</sup> Several previous studies indicated that BMP4 signaling exerts a crucial role in the metastasis of STAD.<sup>45,46</sup> Western blot further demonstrated that upregulating TUG1 could potentiate STAD cell migration ability, as indicated by an elevation in the expression of BMP4, ICAM1, and VCAM1. Our study also revealed that TUG1 contributes to tumor-related angiogenesis in STAD. In short, TUG1 may serve as an oncogenic lncRNA in STAD by promoting cell proliferation, migration, and angiogenesis. In the functional analysis of miR-29c-3p, miR-29c-3p displayed the totally opposite effect to TUG1 on the cell proliferation, migration, and angiogenesis in STAD, which led us to speculate there exists a regulatory relationship between miR-29c-3p and TUG1 in STAD progression. By dual-luciferase analysis, RIP assay, and RNA pull-down assay, the direct interaction between TUG1 and miR-29c-3p in STAD cells was demonstrated in this study. Additionally, in STAD cells, overexpressing TUG1 caused a significant decrease in the expression of miR-29c-3p, while silencing TUG1 led to an increase in miR-29c-3p expression. In addition to this, miR-29c-3p mimic abolished the TUG1 overexpression-induced promotion on the malignant phenotypes of STAD cells. Likewise, miR-29c-3p inhibitor could block the inhibitory effect mediated by TUG1 knockdown on STAD cells. These results corroborated that TUG1 served as a ceRNA for miR-29c-3p during STAD progression.

VEGFA signal transduction is critical to tumor-related angiogenesis during the progression of malignancies,<sup>47</sup> which has been proven of great significance in numerous clinical studies on STAD.<sup>48,49</sup> As demonstrated in a previous study, miR-29c-3p could serve as a tumor suppressor of lung cancer by directly regulating VEGFA.<sup>22</sup> Our study verified that VEGFA is a downstream effector of TUG1/miR-29c-3p in STAD. More importantly, knocking down VEGFA could impair the malignant behaviors of STAD cells and tumor growth induced by TUG1 overexpression to some extent *in vitro* and *in vivo*. These data support that the upregulation of TUG1 facilitated tumorigenesis via regulating the miR-29c-3p/VEGFA axis in STAD. IHC analysis on tumor tissues showed that the upregulation of TUG1 led to the increased levels of Ki-67, BMP4, ICAM1, VCAM1, and CD31, further indicating the promotion effect of TUG1 on STAD growth, metastasis, and angiogenesis *in vivo*. On the other hand, this effect could be partly counteracted by downregulating VEGFA. Collectively, our study highlighted the significance of TUG1 as a sponge for miR-29c-3p to upregulate VEGFA, thereby promoting tumor proliferation, metastasis, and angiogenesis in STAD. Certainly, the clinical significance of TUG1/miR-29c-3p/VEGFA during STAD progression is required to be investigated in further studies.

In conclusion, the study first confirmed that TUG1 facilitated cell proliferation, metastasis, and angiogenesis of STAD via the upregulation of VEGFA by sponging miR-29c-3p both *in vitro* and *in vivo*. The present study discloses a TUG1/miR-29c-3p/VEGFA regulatory axis in STAD pathogenesis, providing a theoretical foundation for employing TUG1 as a promising therapeutic target in the management of STAD.

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None.

## CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

## DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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#### SUPPORTING INFORMATION

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