

## ORIGINAL ARTICLE

# RNA-binding protein SAMD4A inhibits breast tumor angiogenesis by modulating the balance of angiogenesis program

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## Abstract

Tumor-induced angiogenesis is important for further progression of solid tumors. The initiation of tumor angiogenesis is dictated by a shift in the balance between proangiogenic and antiangiogenic gene expression programs. However, the potential mechanism controlling the expression of angiogenesis-related genes in the tumor cells, especially the process mediated by RNA-binding protein (RBP) remains unclear. SAMD4A is a conserved RBP across fly to mammals, and is believed to play an important role in controlling gene translation and stability. In this study, we identified the potential role of SAMD4A in modulating angiogenesis-related gene expression and tumor progression in breast cancer. SAMD4A expression was repressed in breast cancer tissues and cells and low SAMD4A expression in human breast tumor samples was strongly associated with poor survival of patients. Overexpression of SAMD4A inhibited breast tumor angiogenesis and cancer progression, whereas knockdown of SAMD4A demonstrated a reversed effect. Mechanistically, SAMD4A was found to specifically destabilize the proangiogenic gene transcripts, including *C-X-C motif chemokine ligand 5 (CXCL5)*, *endoglin (ENG)*, *interleukin 1 $\beta$  (IL1 $\beta$ )*, and *angiopoietin 1 (ANGPT1)*, by directly interacting with the stem-loop structure in the 3' untranslated region (3' UTR) of these mRNAs through its sterile alpha motif (SAM) domain, resulting in the imbalance of angiogenic genes expression. Collectively, our results suggest that SAMD4A is a novel breast tumor suppressor that inhibits tumor angiogenesis by specifically downregulating the expression of proangiogenic genes, which might be a potential antiangiogenic target for breast cancer therapy.

## KEYWORDS

metastasis, mRNA stability, RNA-binding protein, SAMD4A, tumor angiogenesis

**Abbreviations:** 3'UTR, 3' untranslated region; ActD, Actinomycin D; ADGRB1, adhesion G protein-coupled receptor B1; ANGPT1, angiopoietin 1; ARE, AU-rich element; CXCL5, C-X-C motif chemokine ligand 5; DRB, 5, 6-dichlorobenzimidazole riboside; ENG, endoglin; IL1 $\beta$ , interleukin 1 $\beta$ ; PHAT, pseudo heat analogous topology; RIP-ChIP, RNA immunoprecipitation-chromatin immunoprecipitation; SAMD4A, sterile alpha motif domain-containing protein 4A; SERPINF1, serpin family F member 1; THBS1, thrombospondin 1; VEGFC, vascular endothelial growth factor C.

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## 1 | INTRODUCTION

Tumor angiogenesis plays an important role in facilitating the tumor growth and progression.<sup>1</sup> Vascular endothelial cells could be activated by a series of angiogenesis-related factors secreted and released by different tumor cells,<sup>2,3</sup> which can form a rich vascular network in the tumor tissues.<sup>4</sup> The new-formed vascular network in turn could provide nutrition to the tumor cells and facilitate tumor metastasis to distant organs through circulation.<sup>5</sup> Therefore, the strategies to control the gene expression of angiogenesis-related factors in the tumor cells would help to control the tumor angiogenesis. Angiogenesis-related factors include proangiogenic factors, such as angiopoietin 1 (ANGPT1),<sup>6</sup> C-X-C motif chemokine ligand 5 (CXCL5),<sup>7</sup> endoglin (ENG),<sup>8</sup> interleukin 1 $\beta$  (IL1 $\beta$ ),<sup>9</sup> and vascular endothelial growth factors (VEGFs)<sup>10</sup>; and antiangiogenic factors, such as tissue inhibitor of metalloproteinases 1 (TIMP1),<sup>11</sup> adhesion G protein-coupled receptor B1 (ADGRB1),<sup>12</sup> thrombospondin 1 (THBS1),<sup>13</sup> and serpin family F member 1 (SERPINF1).<sup>14</sup> In general, tumor angiogenesis can be turned on by either increasing the gene expression of proangiogenic factors or decreasing the expression of antiangiogenic factors. Although significant advances have expanded our understanding of the mechanisms controlling angiogenesis-related gene expression, however how these genes are regulated in the tumor cells at post-transcriptional level, especially by RNA-binding protein (RBP), remains unclear.

Human SAMD4A, also known as Smaug1 in *Drosophila*, is a conserved RBP encoded by the *SAMD4A* gene.<sup>15</sup> Initially, studies on *Drosophila* showed that Smaug1 could play an important role in suppressing mRNA translation<sup>16-19</sup> and destabilizing mRNA.<sup>20,21</sup> Smaug1 can directly interact with Ago1 protein to inhibit gene translation in a miRNA-independent manner.<sup>22</sup> Further studies have shown that Smaug1 could also degrade mRNA by binding directly to mRNA via Smaug Recognition Region Elements (SREs)<sup>23</sup> or recruiting the CCR4/POP2/NOT deadenylase complex.<sup>24</sup> Crystal structure analysis also showed that Smaug1 could bind RNA through its sterile alpha motif (SAM) domain.<sup>25-27</sup> Moreover, Smaug1 has also been reported to be essential for miRNA biogenesis.<sup>28</sup> In mammalian cells, human SAMD4A can form a kind of mRNA-silencing foci in the cytoplasm,<sup>29</sup> acting differently from processing body and stress granules.<sup>30,31</sup> Recently, it was reported that human SAMD4A can protect the host from viral infection by degrading viral RNA.<sup>32</sup> Overall, these studies suggested that SAMD4A can potentially play an important role in post-transcriptional regulation of gene expression. However, it remains unknown how SAMD4A affects the expression of various genes in the human tumor cells, especially that of tumor angiogenesis-related genes.

In this study, we identified that human SAMD4A could act as a novel breast tumor angiogenesis inhibitor by selectively downregulating proangiogenic genes expression at the post-transcriptional level. The expression of SAMD4A was reduced in the human breast tumor tissues. Ectopic expression of SAMD4A in human breast tumor cells significantly inhibited tumor-induced

angiogenesis and tumor progression, whereas knockdown of SAMD4A expression by shRNAs demonstrated a reverse effect. Furthermore, induction of SAMD4A in vivo by adenovirus could significantly inhibit breast tumor angiogenesis and progression. Notably, low SAMD4A expression in breast tumors was strongly associated with the poor survival of breast cancer patients. These results demonstrated that SAMD4A could function as a potential breast tumor suppressor and was involved in the regulation of tumor angiogenesis by specifically inhibiting proangiogenic gene expression.

## 2 | MATERIAL AND METHODS

### 2.1 | Cell lines

Human breast cancer cell lines (MDA-MB-231, MDA-MB-468, MCF7 and T47D), human normal mammary epithelial cell lines (MCF-10A, MCF-12A) were obtained from the ATCC and cultured in DMEM or PRM1640 medium with 10% FBS plus 1% penicillin/streptomycin, respectively. Immortalized HUVEC, HEK293T, and HEK293 cells were obtained from the National Infrastructure of Cell Line Resource (Beijing, China).

### 2.2 | Quantitative real-time polymerase chain reaction (qRT-PCR) and PCR array

Total RNA was extracted from tissues or cultured cells using TRIzol (Invitrogen) and reverse transcribed to cDNA for qPCR using SYBR Green Fast Master Mix (Roche). The expression level of each gene was normalized to the mRNA level of GAPDH based on the  $\Delta\Delta C_t$  method. A QIAGEN Human Angiogenesis PCRArray Kit was used to analyze angiogenesis-related gene expression in accordance with the manufacturer's instructions. All the PCR array data are listed in Table S2.

### 2.3 | RNA-sequencing analysis

RNA sequencing was performed as described previously,<sup>33</sup> and completed by Allwegene Technology Inc in Beijing. The RNA-sequencing data are listed in Table S1 and have been deposited in NCBI SRA (<https://www.ncbi.nlm.nih.gov/sra>) (accession number PRJNA699986).

### 2.4 | RNA immunoprecipitation (RIP)

RIP experiment was conducted as previously described.<sup>33</sup> The protein-RNA complexes were immunoprecipitated by protein A/G beads, and total RNA extracted with TRIzol, followed by detection with RT-PCR.

## 2.5 | mRNA stability

For the mRNA stability test, after the de novo RNA synthesis was blocked by actinomycin D and 5,6-dichlorobenzimidazole riboside, mRNA abundance in SAMD4A stable breast tumor cells was analyzed by qPCR.

## 2.6 | Luciferase reporter assays

SAMD4A/GFP, mutants, or GFP-control constructs and pGL3 luciferase reporter constructs containing full-length or mutant of 3'UTR of different genes were cotransfected into HEK293 cells using Lipofectamine 3000 reagent, respectively. After 48 h, the cells were lysed with 1× reporter lysis buffer and luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega, USA). All experiments were conducted in triplicate and repeated at least 3 times.

## 2.7 | RNA-EMSA and Supershift

RNA-EMSA and Supershift were performed as described in Doc S1. The probes used are listed in Table S3.

## 2.8 | Animal study

Animal experiments were performed as described in Doc S1.

## 2.9 | Statistical analysis

Statistical tests were performed with GraphPad Prism 6 software. Data in bar graphs represent mean  $\pm$  SEM of at least 3 biological repeats. Student *t* test was used to compare treatment vs vehicle control or otherwise as indicated. All tests were two-tailed, and a *P*-value < .05 was considered to be statistically significant.

# 3 | RESULTS

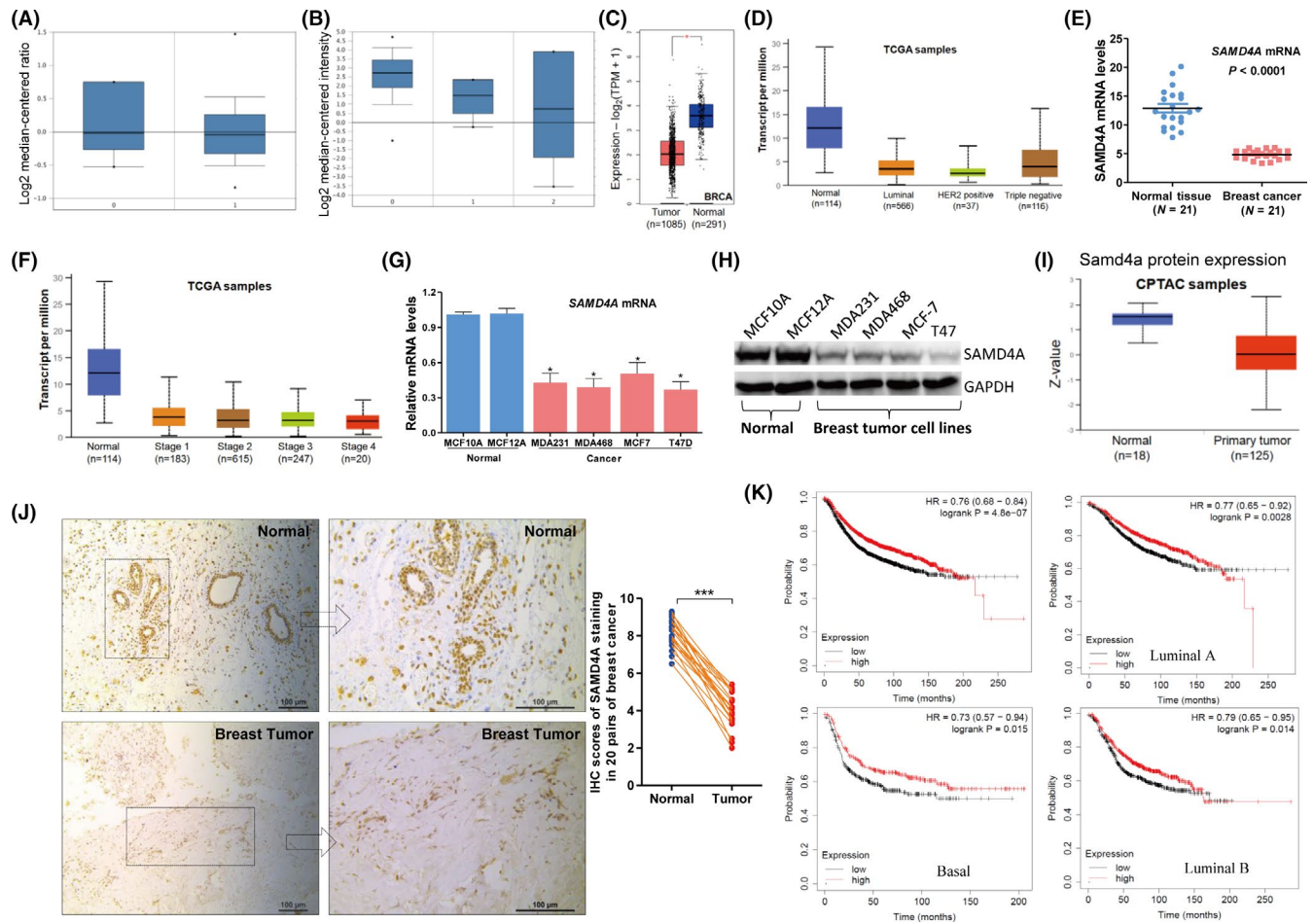
## 3.1 | SAMD4A expression was reduced in breast tumor tissues and associated with poor survival

To detect the expression level of SAMD4A in breast tumors, we compared the expression of SAMD4A in breast cancer tissues and normal mammary gland tissues by analyzing TCGA database. SAMD4A mRNA expression levels in invasive breast carcinoma (Figure 1A), invasive ductal breast carcinoma, invasive lobular breast carcinoma (Figure 1B), and breast tumor tissues (Figure 1C) were reduced compared with normal breast tissues. SAMD4A

was also expressed at relatively low levels in different subtypes of breast cancers (Figures 1D and S1A) and a breast cancer cohort (Figure 1E). Furthermore, a correlation between SAMD4A expression and the pathological stages of breast cancers was also observed (Figure 1F). Additionally, SAMD4A mRNA (Figure 1G) and protein (Figure 1H) were downregulated in several human breast tumor cells. The data from the CPTAC dataset showed lower expression of SAMD4A protein in the primary breast tumors (Figure 1I). Our immunohistochemistry (IHC) staining with SAMD4A from an independent breast cancer cohort further confirmed these results (Figure 1J). These findings clearly demonstrated that SAMD4A expression was impaired in breast tumors and cell lines. In addition, Kaplan-Meier plots showed that the breast cancer patients with higher tumor SAMD4A mRNA levels were more likely to survive compared with patients whose tumors expressed lower levels of SAMD4A (Figure 1K, S1B-D).<sup>34</sup> Although no significant correlation was observed between SAMD4A expression and patient survival in HER2<sup>+</sup> subsets, a similar trend suggested that the tumors with lower SAMD4A expression exhibited poorer survival (Figure S1E). Overall, these results demonstrated that SAMD4A expression was inhibited in breast cancer and was strongly associated with patient survival.

## 3.2 | SAMD4A regulates the expression of angiogenesis-related genes in breast tumor cells and inhibits tumor-induced angiogenesis

To determine the genes regulated by SAMD4A at the whole transcriptome level, RNA sequencing was performed in SAMD4A-overexpressing MDA-MB-468 and MDA-MB-231 cells (Figure S2A). Overall, 3976 genes were commonly downregulated and 4677 genes were commonly upregulated in these 2 breast tumor cell lines (Figure S2B). KEGG pathway analysis on these commonly regulated genes showed that the 'Metabolic pathways, MAPK signaling pathway, Ribosome, and Lysosome' pathways were significantly enriched (Figure S2C). Gene ontology analysis further revealed that the regulated genes were strongly enriched for the transcripts associated with poly(A) RNA-binding (Figure S2D), cell-cell adhesion (Figure S2E), cytosol (Figure S2F), and angiogenesis (Figure S2G,H). These computational analyses suggested that SAMD4A may be involved in controlling angiogenesis pathway in human breast tumor cells. We found that a set of proangiogenic genes was downregulated by SAMD4A, including *CXCL5*, *ENG*, *IL1 $\beta$* , *ANGPT1*, and *VEGFC*. In addition, the antiangiogenic genes were upregulated, including *TIMP1*, *THBS1*, *ADGRB1*, and *SERPINF1* (Figure 2A). A clear trend was further displayed that the expression of proangiogenic genes was downregulated and that of antiangiogenic genes was upregulated (Figure 2B). Indeed, the levels of proangiogenic mRNAs were decreased in a time-dependent manner in SAMD4A-expressing cells (Figures 2C and S2I,K). In contrast, an increase in the levels of 4 antiangiogenic genes did not exhibit a time-dependent response



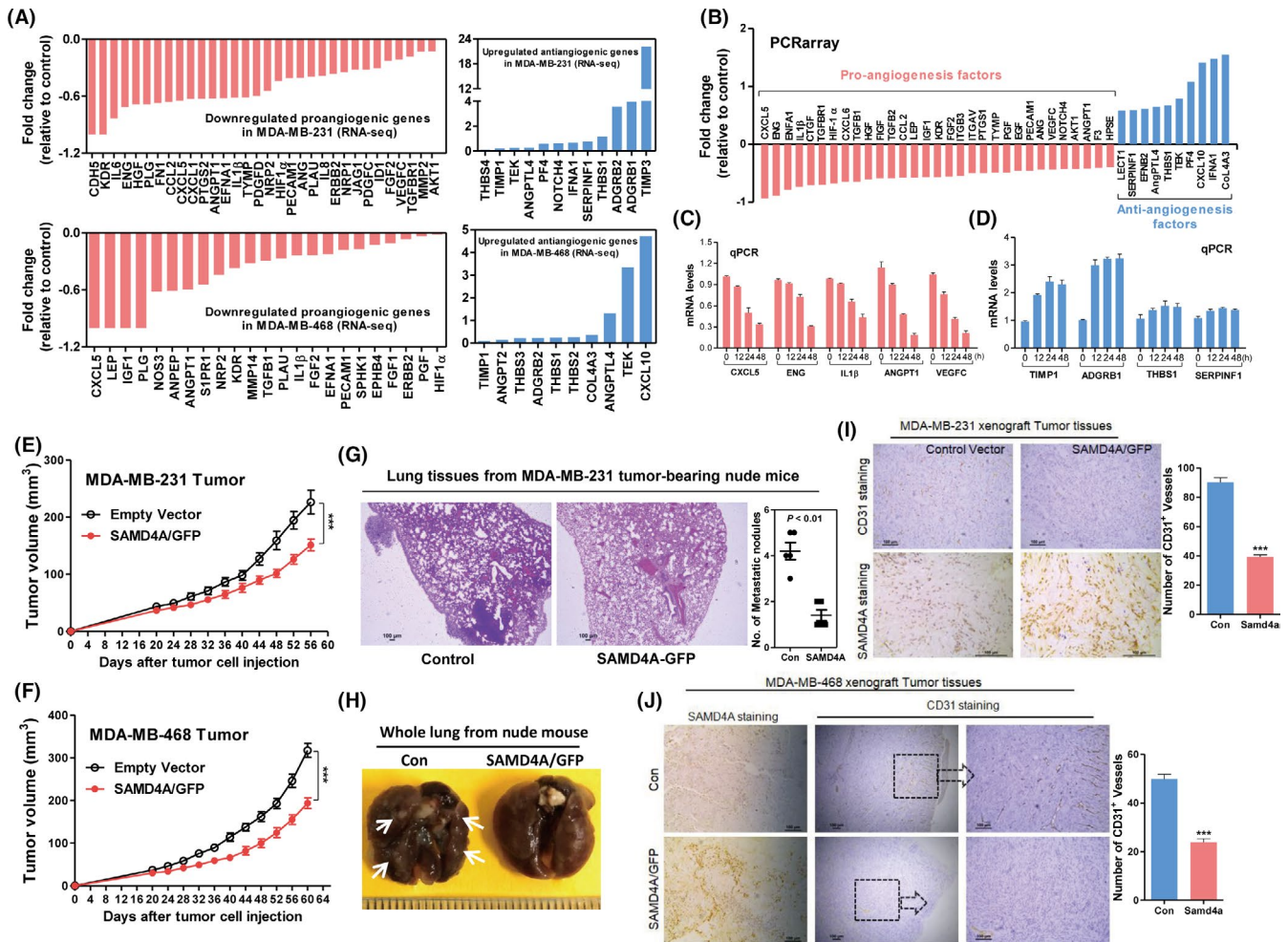
**FIGURE 1** SAMD4A expression is reduced in breast tumor tissues and associated with poor survival. A, Comparison of SAMD4A expression between normal (0) ( $n = 4$ ) and invasive breast carcinoma (1) ( $n = 154$ ). B, SAMD4A mRNA expression among normal (0) ( $n = 20$ ), invasive ductal breast carcinoma (1) ( $n = 5$ ), and invasive lobular breast carcinoma (2) ( $n = 5$ ). C, Expression level of SAMD4A between normal tissue and primary breast tumor tissue was analyzed (<http://gepia2.cancer-pku.cn/#analysis>). D, Expression level of SAMD4A between different subtypes of breast cancers was analyzed (<http://ualcan.path.uab.edu/analysis-prot.html>). E, SAMD4A expression was measured by qPCR in human breast tumors and surrounding "normal" tissues ( $n = 21$  pairs). F, Expression levels of SAMD4A were analyzed by the main pathological stages of breast cancers (<http://ualcan.path.uab.edu/analysis-prot.html>). G, H, SAMD4A mRNA and protein levels were measured, respectively, by qPCR (G) and western blot (H) in human normal mammary gland epithelial cell lines and breast tumor cell lines. I, The SAMD4A protein levels between normal tissue and primary breast tumor tissue were analyzed (<http://ualcan.path.uab.edu/analysis-prot.html>). J, Left: Representative images of SAMD4A IHC staining in normal mammary gland tissues and breast tumor tissues were shown. Scale bars, 100  $\mu\text{m}$ . Right: IHC scores of matched breast tumor and normal tissues ( $n = 20$ ) were evaluated based on SAMD4A staining. K, Kaplan-Meier relapse free survival curves of breast cancer patient, basal subtype, luminal A subtype, luminal B subtype breast cancer patients with low and high tumor SAMD4A transcripts. \*  $P < .05$ ; \*\*\* $P < .0001$

(Figures 2D and S2J). We did not find that SAMD4A could significantly regulate the expression of VEGFs (Figure S2K). These data led us reasonably to speculate that SAMD4A may play a role in regulating tumor-induced angiogenesis.

For verification, we first examined the effect of conditioned medium (CM) from SAMD4A-overexpressing breast tumor cells on HUVEC migration and tube formation in vitro. The CMs harvested from tumor cells overexpressing SAMD4A could significantly inhibit endothelial cell migration (Figure S3A) and tube formation (Figure S3B). The proliferation ability of HUVECs was not affected by the tumor CMs (Figure S3C). In addition, overexpression of SAMD4A could significantly inhibit the proliferation of

MDA-MB-231 and MCF7 cells (Figure S3D), but had no significant effect on the migration of breast tumor cells (Figure S3E). The in vivo study also showed that the growth of tumors overexpressing SAMD4A was significantly inhibited (Figure 2E,F), and the number of metastatic foci (Figure 2G) and metastatic white nodules (Figures 2H and S3F) in the lungs of nude mice bearing tumors overexpressing SAMD4A was significantly less than that of the control group. Notably, the number of CD31-positive vessels in SAMD4A overexpressing tumor tissues was significantly reduced in both breast tumor models (Figure 2I,J). These results confirmed that SAMD4A could inhibit breast tumor-induced angiogenesis in vitro and in vivo.





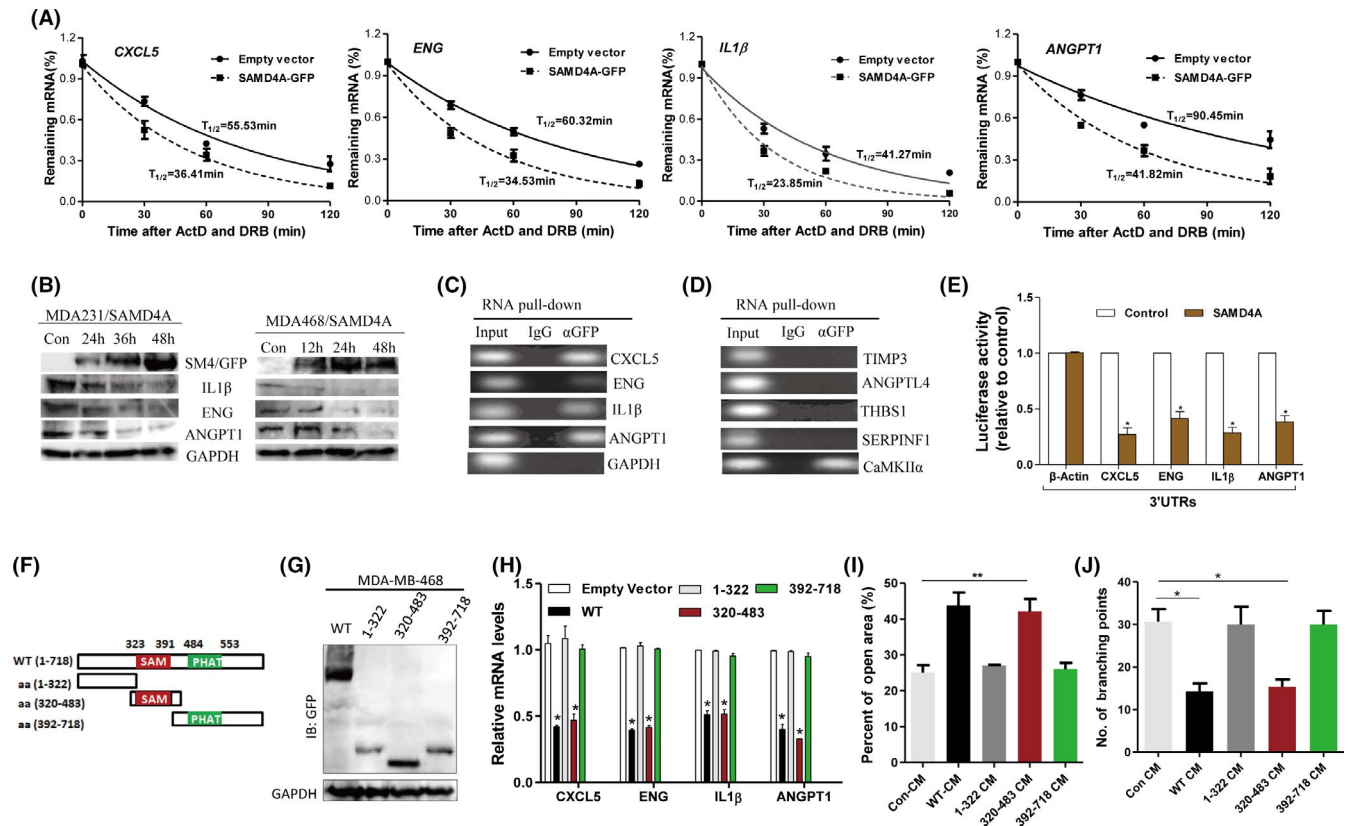
**FIGURE 2** SAMD4A regulates the expression of angiogenesis-related genes in breast tumor cells and inhibits tumor-induced angiogenesis. A, RNA-seq data showing that the proangiogenic mRNAs were downregulated, and the antiangiogenic mRNAs were upregulated by SAMD4A in breast tumor cells. B, PCR array data showing that the angiogenesis-related genes were regulated by SAMD4A in MDA-MB-468 cells. C, D, qPCR confirming the downregulation of indicated proangiogenic mRNAs (C) and upregulation of indicated antiangiogenic mRNAs (D) after SAMD4A overexpression in MDA-MB-231 cells. E, F, Tumor growth curves in nude mice received MDA-MB-231/SAMD4A-GFP, MDA-MB-468/SAMD4A-GFP and their control cells, respectively. G, H&E staining of lung tissue sections from nude mice bearing MDA-MB-231/GFP or MDA-MB-231/SAMD4A-GFP tumors. Scale bars, 100  $\mu$ m. Quantification of metastatic nodules is shown in the right panel. H, Whole lungs from nude mouse bearing MDA-MB-468/GFP or MDA-MB-468/SAMD4A-GFP tumors were compared. White arrows indicate the metastatic foci. I, J, Representative histological images from MDA-MB-231 (I) and MDA-MB-468 xenografts (J) stained with anti-CD31 and anti-SAMD4A antibodies. Scale bars, 50  $\mu$ m. \*\*\* $P < .001$

### 3.3 | SAMD4A selectively destabilizes proangiogenic mRNAs by SAM domain

Next, we examined if SAMD4A could affect the mRNA stability of proangiogenic mRNAs.<sup>20</sup> The half-lives of proangiogenic mRNAs, including *CXCL5*, *ENG*, *IL1 $\beta$* , and *ANGPT1*, were significantly shortened by approximately 2-fold in SAMD4A-overexpressing tumor cells (Figure 3A). However, overexpression of SAMD4A had little effect on the half-lives of antiangiogenic transcripts (Figure S4A-C), indicating that SAMD4A selectively decreases the stability of proangiogenic mRNAs rather than antiangiogenic mRNAs. The protein expression of *IL1 $\beta$* , *EBG*, and *ANGPT1* was reduced by SAMD4A in a time-dependent manner (Figure 3B). ELISA results also indicated that

SAMD4A could reduce the content of *IL1 $\beta$*  and *CXCL5* proteins in the tumor CMs (Figure S4D).

Previous studies have shown that SAMD4A can directly bind to mRNA.<sup>20,23,32</sup> Therefore, an RNA pull-down assay was performed to determine whether SAMD4A could bind to these proangiogenic transcripts. Four proangiogenic mRNAs were found to be amplified by PCR (Figure 3C), but not the antiangiogenic mRNAs (Figure 3D). *CaMKII  $\alpha$*  (calcium/calmodulin-dependent kinase II $\alpha$ ) was used as the positive control.<sup>30</sup> We next investigated whether SAMD4A could also target the 3'UTRs of proangiogenic mRNAs.<sup>16</sup> A series of reporter vectors containing proangiogenic mRNAs 3'UTRs was constructed by cloning the 3'UTRs of *CXCL5*, *ENG*, *IL1 $\beta$* , and *ANGPT1* downstream of the luciferase gene (Figure S4E). As shown



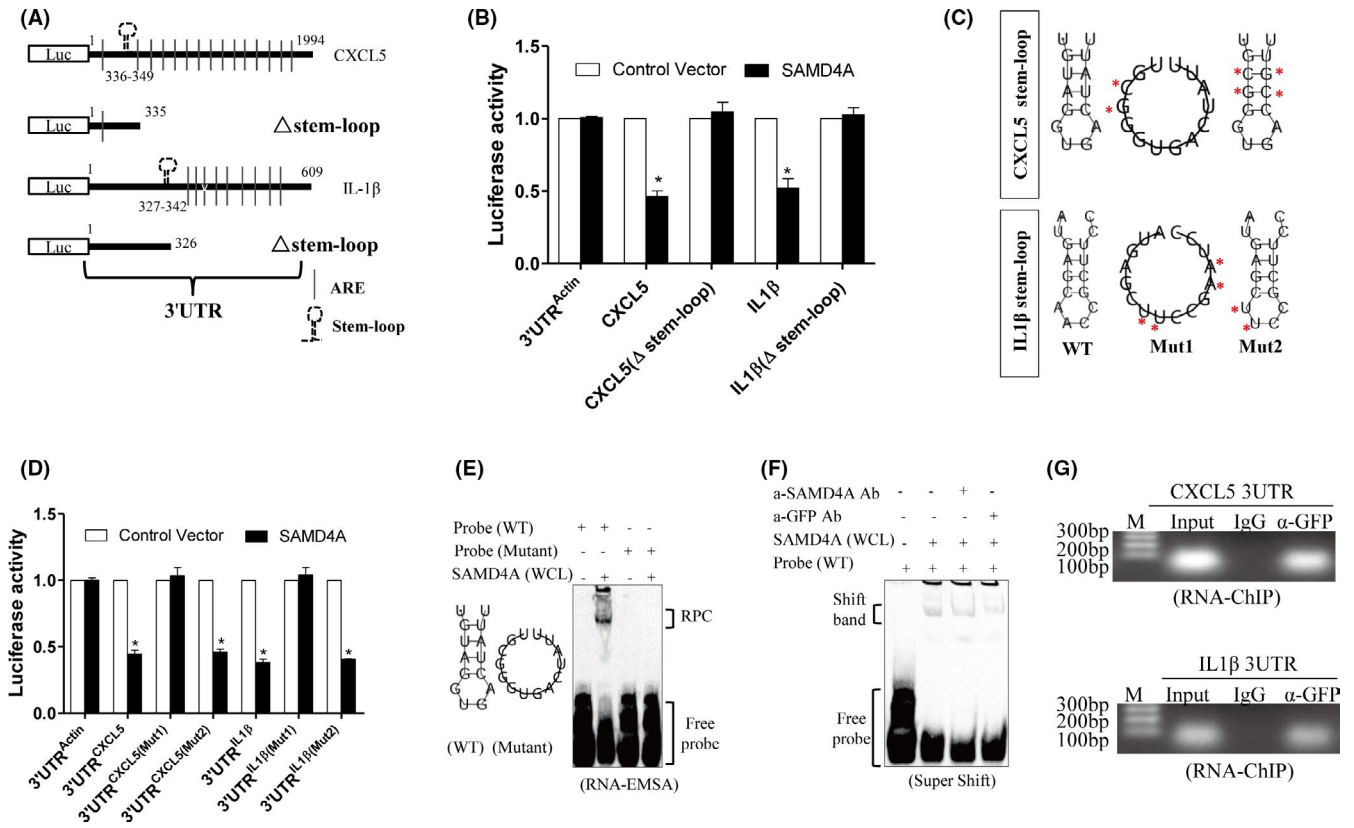
**FIGURE 3** SAMD4A selectively destabilizes proangiogenic mRNAs and inhibits tumor angiogenesis by SAM domain. A, Half-lives of indicated proangiogenic mRNAs were shortened by SAMD4A in MDA-MB-231 cells. B, SAMD4A/GFP, IL1 $\beta$ , ENG, ANGPT1, and GAPDH protein expression were analyzed by western blotting in MDA-MB-231 and MDA-MB-468 cells. C, D, RNA-IP was performed using anti-GFP antibody or IgG in extraction of MDA-MB-231/SAMD4A-GFP cells. Proangiogenic (C) but antiangiogenic factors transcripts (D) were enriched by SAMD4A. E, Measurement of luciferase activities of reporters containing the 3'UTRs of indicated proangiogenic genes. F, Schematic representation of the domains in SAMD4A and the truncation mutants. G, Expression of SAMD4A truncation mutants was confirmed by immunoblotting with anti-GFP antibody. H, Expression of indicated proangiogenic mRNAs was measured by qPCR after overexpressed SAMD4A and its mutants in MDA-MB-231 cells. I, Quantification of open area of HUVECs treated with indicated tumor CMs in the wound-healing assay. J, Quantification of the number of branching points of HUVECs treated with indicated tumor CMs in the tube formation assay. \* $P < .05$ , \*\* $P < .01$

in Figure 3E, SAMD4A suppressed luciferase activities of all the 4 reporters containing different proangiogenic factor genes 3'UTRs, suggesting that SAMD4A targeted the 3'UTRs of proangiogenic mRNAs and downregulated their mRNAs expression.

To identify the domain of SAMD4A required for downregulation of proangiogenic mRNAs, 3 different truncated mutants of SAMD4A were prepared: amino acid (aa) 1-322 (N-terminal), aa 320-483 (containing SAM domain), aa 392-718 (containing PHAT domain and C-terminal) (Figure 3F), and their expression was confirmed by western blot (Figure 3G). Wild-type (wt) and mutant aa 320-483 could effectively inhibit the luciferase activities of 4 3'UTR reporters (Figure S4F) and their mRNA expression (Figure 3H), whereas aa 1-322 and aa 392-718 mutants completely lost their inhibitory functions. Moreover, the CMs from MDA-MB-231 cells expressing WT and aa 320-483 mutant could inhibit HUVEC migration (Figures 3I and S4G) and tube formation (Figure 3J). Overall, these results demonstrated that SAMD4A could downregulate proangiogenic genes expression by destabilizing their mRNAs, and its SAM domain was necessary for the inhibition of tumor angiogenesis.

### 3.4 | SAMD4A binds to the conserved stem-loop structure in the 3'UTR of proangiogenic transcripts for mRNA destabilization

Previous studies have shown that Smaug could bind conserved hairpin secondary structured SRE in 3'UTR of mRNA.<sup>16</sup> We compared the 3'UTR sequence of the 4 proangiogenic transcripts among the different species and identified a conserved sequence that could form a similar stem-loop structure (Figure S5A-D). To confirm the role of the stem-loop structure in SAMD4A-mediated destabilization of proangiogenic mRNAs, CXCL5 and IL1 $\beta$  3'UTR stem-loop deletion ( $\Delta$ stem-loop) reporters were generated by deleting the 3'UTR sequence containing the stem-loop structure (Figure 4A). SAMD4A could significantly inhibit the luciferase activities of reporters containing CXCL5 and IL1 $\beta$  3'UTR but had no effect on the luciferase activities of stem-loop structure deletion reporters (Figure 4B). These results suggested that the stem-loop structure rather than ARE could be important for RNA destabilization mediated by SAMD4A, because an ARE existed in the CXCL5



**FIGURE 4** SAMD4A binds to the conserved stem-loop structure in the 3'UTR of proangiogenic transcripts for mRNA destabilization. A, Diagram of the luciferase reporter constructs of CXCL5 and IL1β containing truncated 3'UTRs without the stem-loop structure. B, Relative luciferase activities of the indicated reporters were determined by the luciferase reporter assay. C, The predicted stem-loop structures of CXCL5 (upper) and IL1β (lower) in their 3'UTRs and mutation strategy (asterisks indicate base substitution). Mutant1 becomes unable to form stem-loop structure (middle) and Mutant2 still forms a stem-loop structure (right). D, Relative luciferase activities of the indicated reporters were determined by the luciferase reporter assay. E, RNA-EMSA was performed with biotin-labeled probes corresponding to the CXCL5 3'UTR in the presence of whole-cell lysates (WCL) extracted from MDA-MB-231/SAMD4A cells. F, Supershift assay was performed using anti-SAMD4A or anti-GFP antibodies. G, RNA-ChIP was conducted with genome fragments from MDA-MB-231/SAMD4A-GFP cells. \*P < .05

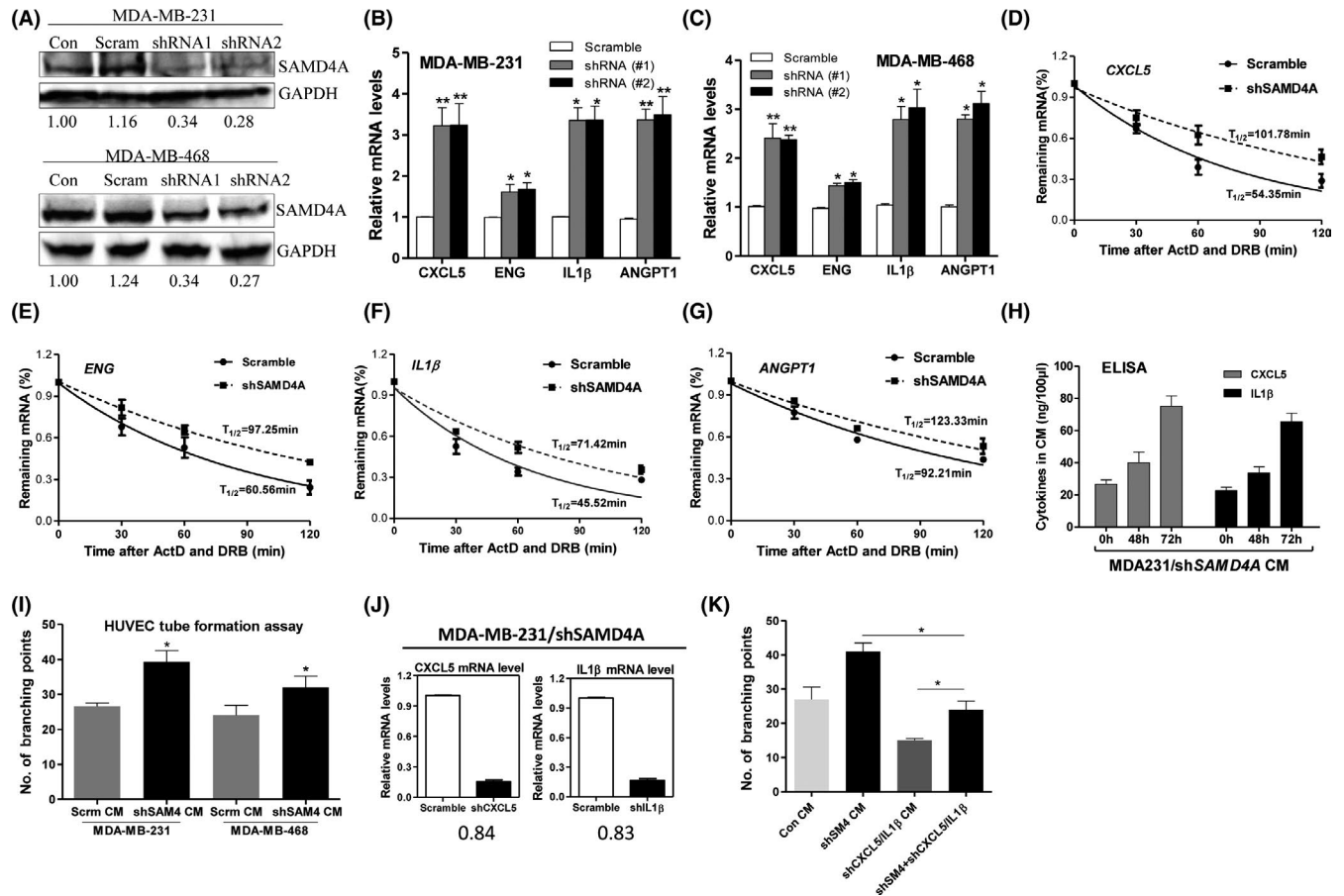
3'UTR truncated reporter. To determine whether the RNA secondary configuration was required for SAMD4A-mediated mRNA destabilization, 2 different 3'UTR mutant reporters of CXCL5 and IL1β were generated. The stem-loop structure of mutant1 was deleted through replacing 2 or 4 nucleotides, and the stem-loop structure of mutant2 remained intact although the substitution of nucleotides in the stem and loop region (Figure 4C). Luciferase assay results indicated that the mut1 completely resisted the inhibitory effect of SAMD4A, while mut2 was still sensitive to SAMD4A suppression (Figure 4D). RNA-EMSA results also showed that a clear RNA-protein binding complex band was formed only by the wild-type probe but not by the mutant probe of CXCL5 (Figure 4E). Supershift RNA-EMSA results showed that the binding density was substantially reduced after adding anti-SAMD4A and anti-GFP antibodies to the cytoplasmic proteins, suggesting that SAMD4A could physically bind to the stem-loop structure in 3'UTR of CXCL5 (Figure 4F). To confirm the binding of SAMD4A to the stem-loop structure in vivo, a modified RIP-ChIP was performed. The stem-loop sequences of CXCL5 and IL1β 3'UTRs were found to be amplified in the anti-GFP antibody pulled down groups, but not in isotype IgG groups (Figure 4G), indicating that

SAMD4A bound the stem-loops in the 3'UTRs inside the tumor cells. Taken together, these data demonstrated that SAMD4A primarily recognized the stem-loop structure in the 3'UTRs of proangiogenic gene mRNAs for promoting RNA destabilization.

### 3.5 | SAMD4A depletion increased proangiogenic mRNAs stability and enhanced tumor angiogenesis

To further confirm the inhibitory effects of SAMD4A on breast tumor angiogenesis, SAMD4A expression was silenced with 2 different shRNAs in MDA-MB-231 and MDA-MB-468 cells, respectively. The SAMD4A expression was found to be knocked down c. 66% and 72% by # 1 shRNA and # 2 shRNA, respectively (Figures 5A and S6A). Knockdown of SAMD4A significantly increased the mRNAs expression of proangiogenic genes in breast tumor cells (Figure 5B,C). In contrast, there was no significant change in mRNA levels of antiangiogenic genes after SAMD4A knockdown (Figure S6B). In addition, knockdown of SAMD4A significantly increased the half-lives of proangiogenic mRNAs in MDA-MB-231 cells (Figures 5D-G and S6C), but did not substantially affect the half-lives of antiangiogenic





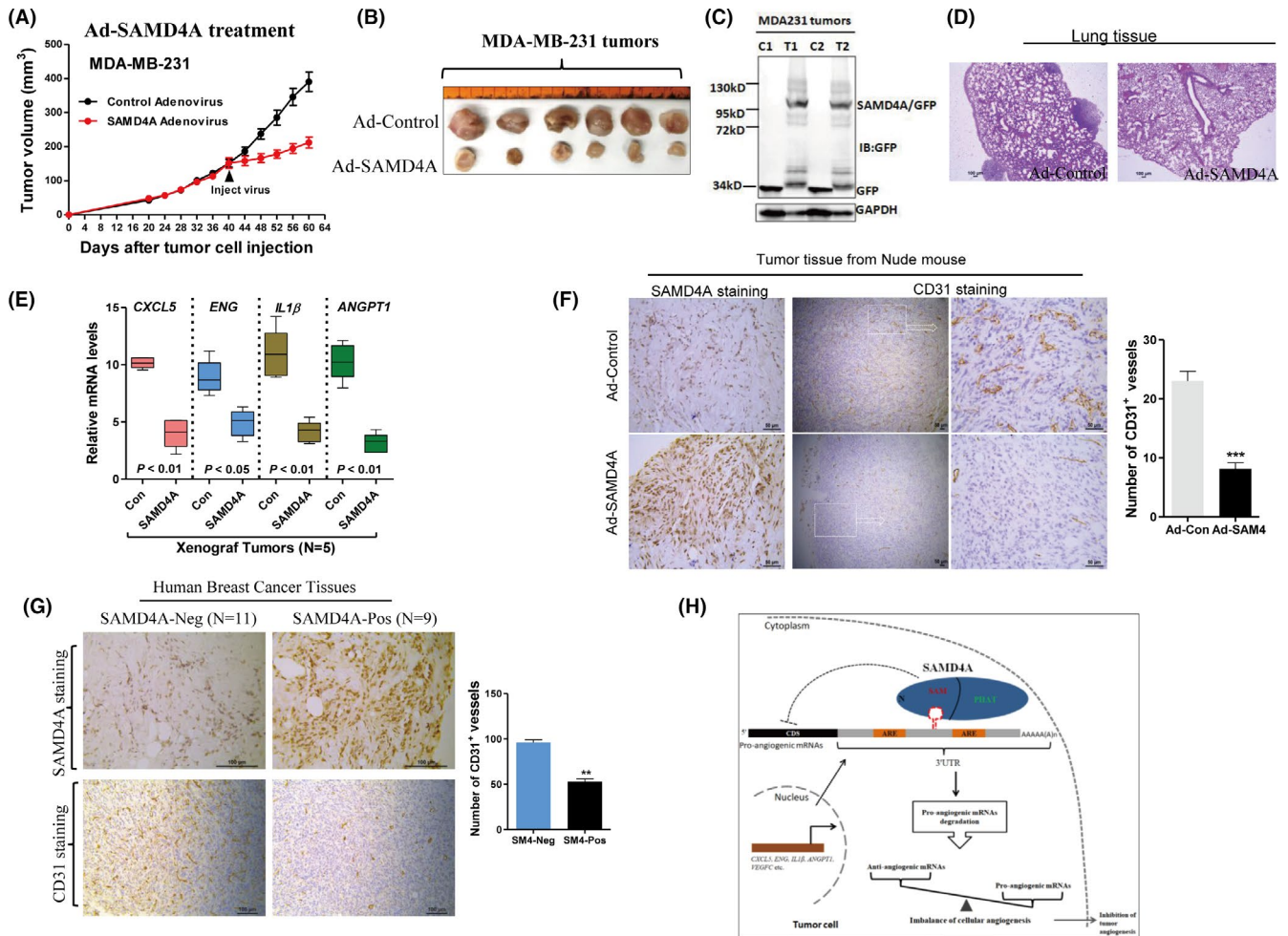
**FIGURE 5** SAMD4A depletion increased proangiogenic mRNAs stability and enhanced tumor angiogenesis. A, Stable knockdown of SAMD4A protein expression by infecting SAMD4A-shRNAs/lentivirus and scramble-shRNA/lentivirus in MDA-MB-231 and MDA-MB-468 cells, respectively. Western blot quantification was assessed by ImageJ software. B, C, Expression of proangiogenic mRNAs was measured by qPCR in the MDA-MB-231 (B) and MDA-MB-468 (C) cells, respectively, after knocking down SAMD4A expression. D–G, Half-lives of indicated proangiogenic mRNAs were increased after SAMD4A knockdown in MDA-MB-231 cells. H, ELISA quantification of CXCL5 and IL1 $\beta$  in serum-free culture medium of MDA-MB-231/shSAMD4A cells. I, Quantification of the number of branching points of HUVECs treated with indicated tumor CMs. J, MDA-MB-231/shSAMD4A cells were infected with scramble/lentivirus or shRNA-lentivirus targeting CXCL5 and IL1 $\beta$ , respectively. Total RNA extracted to measure mRNAs of CXCL5 and IL1 $\beta$ . K, Quantification of the number of branching points of HUVECs treated with indicated tumor CMs. \* $P < .05$ , \*\* $P < .01$

mRNAs (Figure S6D). ELISA results also showed that CXCL5 and IL1 $\beta$  protein levels were increased in the CMs of MDA-MB-231/shSAMD4A (Figures 5H and S6E) and MDA-MB-468/shSAMD4A cells (Figure S6F). Corresponding to the results obtained from over-expression experiments, the CMs from MDA-MB-231/shSAMD4A and MDA-MB-468/shSAMD4A cells could significantly promote HUVEC tube formation (Figure 5I). To confirm that the proangiogenic genes were involved in SAMD4A-mediated tumor angiogenesis, CXCL5 and IL1 $\beta$  genes were knocked down by shRNA lentiviruses in MDA-MB-231/shSAMD4A cells (Figure 5J). After co-knockdown of SAMD4A and its targets CXCL5 and IL1 $\beta$ , HUVEC tube formation was partially restored to the level of the control group (Figures 5K and S6G). We also found that the migration and tube formation of HUVECs were attenuated by adding neutralizing antibodies against CXCL5 and IL1 $\beta$  (Figure S6H,I), suggesting that proangiogenic genes indeed played an important role in SAMD4A-mediated tumor angiogenesis.

### 3.6 | SAMD4A suppresses breast tumor angiogenesis and progression

To simulate the clinical treatment of breast cancer, adenovirus expressing the SAMD4A/GFP fusion gene and its control virus were generated to treat established MDA-MB-231 tumors in nude mice. After injection of SAMD4A/GFP-expressing adenovirus, tumor growth was observed to be inhibited, while the tumors treated with control adenovirus grew continuously (Figure 6A,B). Western blot was used to confirm the expression of SAMD4A/GFP fusion protein in the tumor tissues (Figure 6C). In addition, the number of metastatic lesions in lung tissue of nude mice bearing tumors treated with SAMD4A/GFP-adenovirus was significantly reduced (Figure 6D). These results clearly demonstrated that in vivo induction of SAMD4A could effectively suppress the growth and metastasis of breast tumor. The expression of CXCL5, ENG, IL1 $\beta$ , and ANGPT1 was decreased by SAMD4A in tumor xenografts (Figure 6E). In line with





**FIGURE 6** Adenoviral expressing SAMD4A in vivo suppresses tumor progression and angiogenesis of the established breast tumors. A, Tumor growth curves in nude mice after treatment with adenovirus. Black arrowhead indicated the time point of adenovirus injection. B, Comparison of MDA-MB-231 tumors treated with control adenovirus or SAMD4A-expressing adenovirus. C, SAMD4A-GFP fusion protein expression in xenograft tumors was confirmed by immunoblotting with an anti-GFP antibody. D, H&E staining of lung sections of tumor-bearing mice treated with control adenovirus or SAMD4A-expressing adenovirus, respectively. Scale bars, 100 μm. E, qRT-PCR was used to examine the mRNA expression level of *CXCL5*, *ENG*, *IL1β* and *ANGPT1* in xenograft tumors. F, Left: Representative histological sections from tumors treated with adenovirus, tumors stained with a specific anti-CD31 antibody. Scale bars, 50 μm. Right: Quantification of the number of CD31<sup>+</sup> vessels per section. G, Left: Representative images of IHC staining for SAMD4A (upper) and CD31 (lower) in human breast cancer tissues. Scale bars, 100 μm. Right: Quantification of the number of CD31<sup>+</sup> vessels per section. H, Working model of SAMD4A-mediated proangiogenic mRNAs degradation and inhibition of tumor angiogenesis in breast cancer. \*\**P* < .01

our previous in vivo results, the number of CD31-positive microvessels in the tumor tissues treated with SAMD4A/GFP-adenovirus was significantly less than that in the tumors treated with control adenovirus, as observed in the immunohistochemistry results (Figure 6F). Furthermore, in total, 20 human breast tumor samples were analyzed by IHC staining with anti-SAMD4A antibody, 11 samples were evaluated as higher SAMD4A expression (SAMD4A-positive) and 9 samples showed lower SAMD4A expression (SAMD4A-negative). Notably, more CD31-positive microvessels were observed in SAMD4A-negative tumors compared with in SAMD4A-positive tumors (Figure 6G), although no correlation between SAMD4A and proangiogenic gene expression was observed in human cancers (Figure S7). Finally, we proposed a model to elucidate the potential role of SAMD4A in regulating breast tumor angiogenesis (Figure 6H).

SAMD4A destabilized the proangiogenic mRNAs through recognizing and binding to the conserved stem-loop structure in the 3'UTR of these transcripts by its SAM domain, thereby resulting in the imbalance of angiogenesis-related gene expression programs in the tumor cells. Collectively, these results strongly demonstrated that SAMD4A may function as a novel tumor angiogenesis inhibitor, and inducing SAMD4A expression in the tumors might be a potentially useful antiangiogenic therapy for breast cancer treatment.

#### 4 | DISCUSSION

To date, the role of SAMD4A in human cancer progression remains unclear. In this study, we found, for the first time, that the expression

of SAMD4A was decreased in breast tumor tissues and cell lines. In addition, SAMD4A expression was strongly associated with the survival of patients with different breast cancer subsets, indicating that SAMD4A might function as a predictive biomarker in the breast cancer patients. Although no significant correlation was found between SAMD4A expression and patient survival in the HER2<sup>+</sup> subsets, the trend was consistent with other subsets. These results demonstrated that SAMD4A could be important in the prognosis of breast cancer patients.

Studies on the regulation of gene expression by SAMD4A in mammalian cells, especially in the human tumor cells, are still limited.<sup>35</sup> Here, we demonstrated the potential effect of SAMD4A on the whole transcriptome of breast tumor cells by performing RNA-seq. Depending on our research interests, we found that SAMD4A could modulate the balance of the angiogenesis program in human breast tumor cells. SAMD4A can potentially act as a "switch" for tumor angiogenesis. It has the ability to suppress the expression of a set of proangiogenic genes and therefore enhances antiangiogenic genes expression in tumor cells. We hypothesized that many antiangiogenic mRNAs that were upregulated might be a secondary effect of SAMD4A overexpression, because SAMD4A neither enriched the antiangiogenic mRNAs nor bound to their respective 3'UTR. Moreover, the knockdown of SAMD4A did not affect the expression of these antiangiogenic genes. These findings enhanced our understanding on the molecular functions of SAMD4A in regulating angiogenesis.

We found that human SAMD4A can also control proangiogenic gene expression at the post-transcriptional level. 3'UTR is believed to be important for the RBP-mediated post-transcriptional regulation of various genes.<sup>36</sup> Many RBPs, including Hu-antigen R (HuR),<sup>37</sup> Trans-activation response (TAR) RNA-binding protein 2 (TARBP2),<sup>38</sup> and monocyte chemotactic protein-induced protein 1 (MCP1P1),<sup>39</sup> can affect mRNA stability by targeting 3'UTR. We demonstrated that SAMD4A could inhibit luciferase activity through the 3'UTR of proangiogenic mRNAs, and reduce their mRNA stability. It has been reported that the SAM domain may be essential for SAMD4A-mediated RNA substrate recognition and binding.<sup>26</sup> In this study, we further demonstrate that the SAM domain of SAMD4A is crucial for the destabilization of proangiogenic genes and inhibition of tumor angiogenesis by truncating the domains of SAMD4A.

Smaug1 has been reported to bind its target transcripts by the conserved SRE in 3'UTR.<sup>15</sup> Many conserved elements responsible for mRNA binding are located in the 3'UTR, such as ARE,<sup>40</sup> GU-rich element (GRE),<sup>41</sup> stem-loop structure.<sup>42</sup> We found that the conserved stem-loop structures in the 3'UTR of proangiogenic transcripts were required for SAMD4A-mediated mRNA destabilization. However, no common stem-loop sequence has been identified among the proangiogenic gene 3'UTRs, further suggesting that SAMD4A recognizes the secondary stem-loop structure, which is consistent with previous study.<sup>43</sup> In summary, we demonstrate that SAMD4A can significantly suppress breast tumor-induced angiogenesis by disrupting the balance of angiogenesis-related genes in the

tumor cells, which might be a promising molecular target for breast cancer antiangiogenic therapy.

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## DISCLOSURE

The authors have no conflict of interest.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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