



ORIGINAL ARTICLE

Extracellular ATP promotes angiogenesis and adhesion of TNBC cells to endothelial cells via upregulation of CTGF

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Abstract

Our previous works have indicated that extracellular ATP is an important prometastasis factor. However, the molecular mechanism involved needs to be further studied. We demonstrated that extracellular ATP treatment could upregulate the expression of connective tissue growth factor (CTGF) in both triple-negative breast cancer (TNBC) cells and endothelial cells (ECs). Extracellular ATP stimulated the migration of TNBC cells and ECs, and angiogenesis of ECs via the P2Y₂--YAP-CTGF axis. Furthermore, we demonstrated that adenosine triphosphate (ATP) stimulated TNBC cell adhesion to ECs and transmigration through the EC layer via CTGF by upregulation of integrin β 1 on TNBC cells and VCAM-1 on ECs. Both apyrase (ATP-diphosphohydrolase) and CTGF shRNA treatments could inhibit the metastasis of inoculated tumors to lung and liver in a mouse model, and these treated tumors had fewer blood vessels. Collectively, our data indicated that extracellular ATP promotes tumor angiogenesis and the interactions between TNBC cells and ECs through upregulation of CTGF, thereby stimulating TNBC metastasis. The pleiotropic effects of ATP in angiogenesis and cell adhesion suggest that extracellular ATP or CTGF could be an effective target for TNBC therapy.

KEYWORDS

angiogenesis, cell adhesion, CTGF, extracellular ATP, integrin β 1

1 | INTRODUCTION

Triple-negative breast cancer is a subtype of highly malignant breast cancer, which is prone to distant metastasis and has a poor prognosis.¹ Tumor metastasis involves multiple steps including tumor cell detachment from the primary tumor, invasion through the extracellular matrix, penetration of blood vessels, interaction with circulating

tumor cells with vascular endothelium, and extravasation into and invasion of the target organ.² In addition, tumor angiogenesis not only plays an important role in tumor growth, but is an essential step for tumor metastasis. A high degree of tumor vascularization increases the chance for cancer cells to reach the bloodstream, and newly formed blood vessels are more permeable to cancer cells, which also promotes tumor metastasis.³

Abbreviations: ATP, adenosine triphosphate; Ca-CM, cancer cell conditioned medium; CCN2, cellular communication network factor 2; CTGF, connective tissue growth factor; ECs, endothelial cells; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; NC, Negative Control; OS, overall survival; TNBC, triple-negative breast cancer.

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The tumor microenvironment is characterized by an unusually high concentration of adenosine triphosphate (ATP).⁴ When tumor tissue, especially fast growing tumor tissue, is in a hypoxic and ischemic condition, ATP is released from cells undergoing necrosis, apoptosis, and autophagy, and extracellular ATP could reach several hundred micromoles.⁵ Extracellular ATP mediates a wide range of biological functions in tumors, such as stimulating tumor cell proliferation, migration, and invasion into the tumor microenvironment.^{6,7} Extracellular ATP can also modulate EC migration and angiogenesis,⁸ and increase the adhesion of polymorphonuclear granulocytes to ECs.⁹ Previous research has demonstrated that hypoxia can induce angiogenesis and cancer cell adhesion to ECs.^{10–12} Whether extracellular ATP could stimulate cancer cell adhesion to ECs remained to be investigated.

Connective tissue growth factor (CTGF), also known as cellular communication network factor 2 (CCN2), is one of the most widely studied members of the CCN family. CTGF is highly expressed in many cancer types, including breast cancer, pancreatic cancer, glioma, etc.,¹³ and plays an important role in tumor cell proliferation, migration, invasion, and metastasis.^{14,15} CTGF could promote cell proliferation by regulating cell cycle-related proteins in TNBC cells, and promote bone metastasis of breast cancer through the integrin $\alpha_v\beta_3$ -ERK1/2 signaling pathway.¹⁶ CTGF could also promote the epithelial-mesenchymal transition (EMT) of tumor cells^{17,18} and chemotherapeutic resistance.^{19,20} In addition to its direct effect on tumor cells, CTGF causes the fibrosis and inflammatory microenvironment of tumors, and induces angiogenesis through paracrine or autocrine pathways, which further supports its promotion of tumor progression.²¹ For example, CTGF could increase the expression of vascular endothelial growth factor (VEGF) and ANGPT2 in tumor tissue.^{22,23}

To reveal the molecular mechanism behind extracellular ATP stimulation of tumor invasion and metastasis, we used cDNA microarray and RNA-seq to identify differentially expressed genes in breast cancer cells MCF-7 and fibroblast cells HFF1, before and after 100 μ M ATP treatment. We found that CTGF mRNA was upregulated in HFF1 cells after ATP treatment. qRT-PCR confirmed this finding in TNBC cells, fibroblast cells HFF1, and human ECs HUVECs. Through functional tests, we found that ATP could promote angiogenesis and TNBC cell adhesion to ECs. We hypothesized that CTGF exerted a stimulating effect on ATP-driven angiogenesis and TNBC cell adhesion to the endothelium.

2 | MATERIALS AND METHODS

2.1 | Cell lines and culture

MDA-MB-231, SK-BR-3, and MCF10A cells were purchased from ATCC. MCF-7, HS578T, and EA.hy926 cells were bought from the Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. HUVEC and HMEC-1 cells were bought from Zhong Qiao Xin Zhou Biotechnology. HUVEC and HMEC-1 cells were cultured in endothelial culture medium (ECM) bought from Zhong Qiao Xin Zhou Biotechnology. MCF-7, SK-BR-3, MDA-MB-231, HS578T, and EA.hy926 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (GIBCO).

2.2 | Statistical analysis

All the experiments were performed in triplicate. All the data were analyzed using Prism 6 (GraphPad Software). Student's *t* test was used to determine the difference between each two groups. Error bars in the experiments indicated standard deviation (SD). Any values of *p* < 0.05 were considered statistically significant.

Details of additional methodologies can be found in Appendix S1.

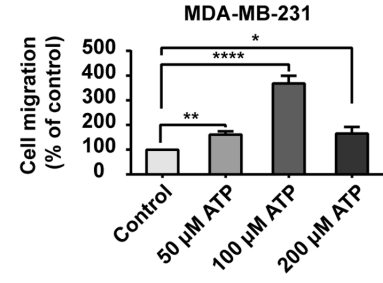
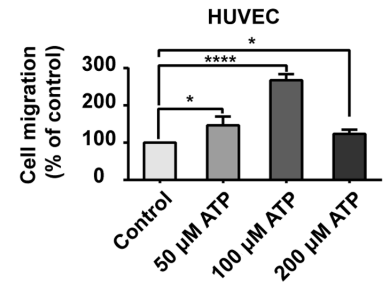
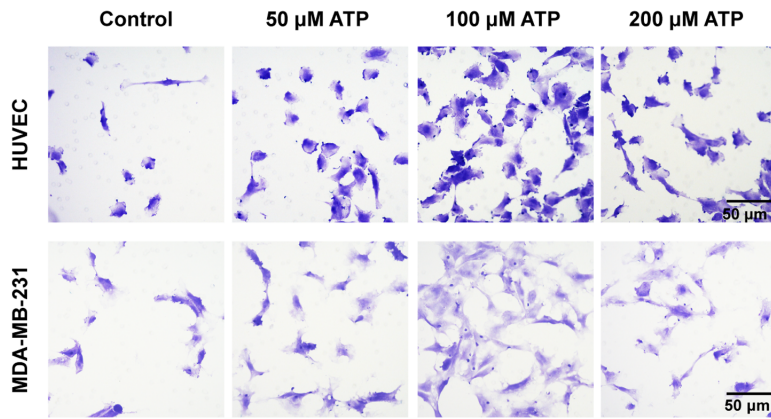
3 | RESULTS

3.1 | ATP induces ECs migration, angiogenesis, and cancer cell adhesion to ECs in vitro

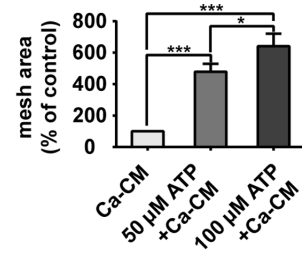
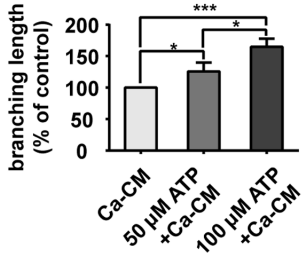
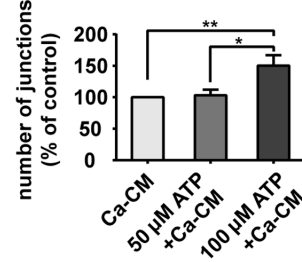
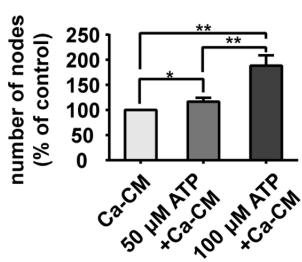
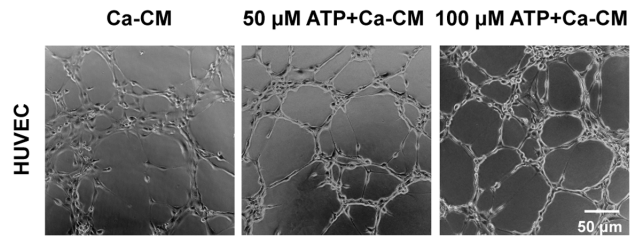
Our previous research works demonstrated that ATP promotes BC cell invasion and metastasis.^{24,25} We wondered whether extracellular ATP could affect EC migration, angiogenesis, and cancer cell adhesion to ECs. As shown in Figure S1, ATP dramatically promoted the migration of ECs EA.hy926 and HMEC-1 in wound healing assays. Transwell migration assays demonstrated that 100 μ M ATP was most effective in enhancing migration (Figures 1A and S1B) of ECs (HUVECs, HMEC-1) and TNBC cells (MDA-MB-231, HS578T). Tube formation assay was performed to detect angiogenesis. Compared with cancer cell conditioned medium (Ca-CM), the addition of ATP (ATP + Ca-CM) obviously promoted angiogenesis (Figure 1B). In addition, we found that 100 μ M ATP-treated TNBC cells were more

FIGURE 1 ATP induces cell migration, angiogenesis, and cancer cell adhesion to endothelial cells (ECs) in vitro. (A) Representative images and quantification of MDA-MB-231 and HUVEC cell migration in a transwell migration assay. (B) Tube formation of HUVEC cells after the indicated treatment. Statistical analysis was undertaken from four aspects: number of nodes, number of junctions, branching length, and mesh area using Image J software. Ca-CM, conditioned medium derived from breast cancer cells. (C) Representative images and quantification of adhesion assay. After the indicated treatment, FITC tracer-labeled MDA-MB-231 cells were added to HUVEC cells. After 30 min of incubation, the adhesive MDA-MB-231 cells were quantified. (D) Immunohistochemistry (IHC) staining of CTGF expression in human breast cancer tissues. Bar graphs represent means \pm SD from triplicates. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001

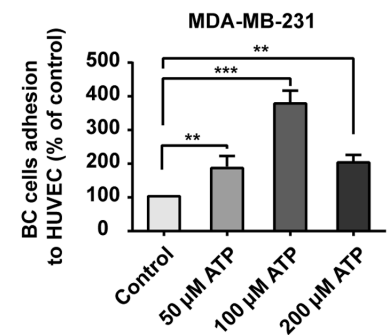
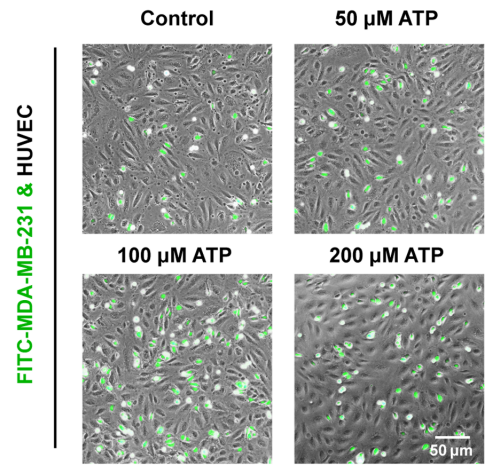
(A)



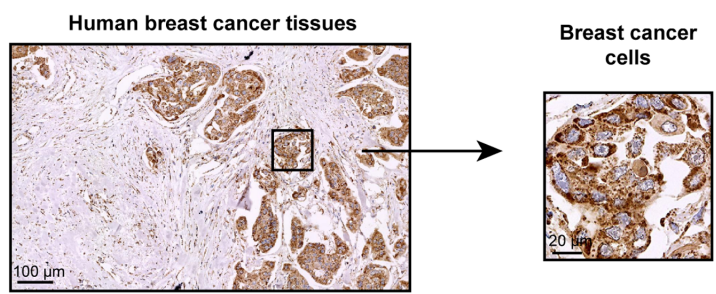
(B)



(C)



(D)



likely to adhere to ECs compared with non-ATP-treated TNBC cells (Figures 1C and S1C). These data suggested that ATP stimulated ECs migration, angiogenesis, and cancer cells' adhesion to ECs.

3.2 | Extracellular ATP upregulates CTGF expression in TNBC cells and ECs

We used cDNA microarray to identify differentially expressed genes in MCF-7 cells before and after 100 μ M ATP treatment (accession number: GSE113757), furthermore, we applied RNA-seq to identify differentially expressed genes in co-cultured MCF-7 and fibroblast cells HFF1 before and after 100 μ M ATP treatment. By GO analysis, we found that ATP significantly increased the expression of some genes related to migration and angiogenesis (Figure S2A). Among these genes, CTGF was related to both migration and angiogenesis and, as a secreted protein, CTGF was more able to explain the role of ATP from the angle of tumor microenvironment and cell-cell interaction. It is also worth noting that CTGF is an important protein in breast cancer. It is related to large tumor size, lymph node metastasis, and HER-2 status.¹³ Secreted CTGF could mediate the proliferation and migration of TNBC cells.²⁶ This is why we chose CTGF for further study.

By immunohistochemical assay, we found that CTGF was expressed in tumor cells, fibroblast cells, and ECs in human breast cancer tissues (Figure 1D). The expression of CTGF in breast cancer tissues was higher than in paired adjacent breast tissues (Figure S2B). To verify the cDNA microarray and RNA-seq data and further study the molecular mechanism, we performed qRT-PCR in BC cells, fibroblast cells, and ECs. We found that CTGF was upregulated upon ATP treatment in TNBC cells MDA-MB-231 and HS578T, as well as in ECs EA.hy926, HMEC-1, and HUVECs (Figure 2A and Table S2). However, CTGF was not upregulated upon ATP treatment in normal breast epithelial cells MCF10A and non-TNBC cells SK-BR-3 and MCF-7 (Figure 2A and Table S2). Western blotting and ELISA assays verified the upregulation of CTGF protein upon ATP treatment in TNBC cells and ECs, and the upregulation was time dependent (Figure 2B,C).

3.3 | CTGF is involved in ATP-driven migration and formation of pseudopodia in TNBC cells

To investigate the role of CTGF in ATP-driven migration, we inhibited its expression in TNBC cells and ECs using shRNAs against CTGF

(Figure 2D and Figure S3A). Wound healing assays and transwell migration assays showed that ATP-enhanced migration could be attenuated by CTGF shRNAs in TNBC cells, and that this phenomenon could be rescued by rCTGF (Figure 2E and Figure S3B). Pseudopodia are actin-rich cellular protrusions facilitating the migration and metastasis of tumor cells. Immunofluorescence showed that TNBC cells formed more and longer pseudopodia upon ATP treatment, and CTGF shRNAs decreased the ATP-promoted formation of pseudopodia (Figure 2F).

We then examined the phosphorylation levels of focal adhesion kinase (FAK), which is closely related to BC cell migration and angiogenesis.²⁷ Western blotting verified that ATP significantly increased pFAK (Y397) levels in TNBC cells, and that this phenomenon disappeared after knockdown of CTGF (Figure 2G). These results indicated that CTGF plays an important role in ATP-driven migration of TNBC cells.

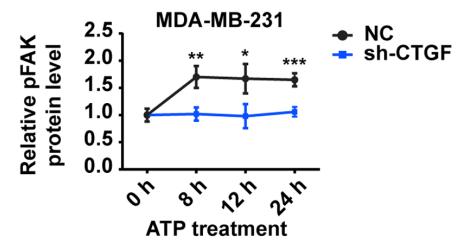
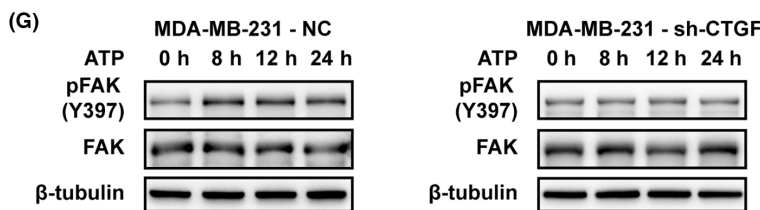
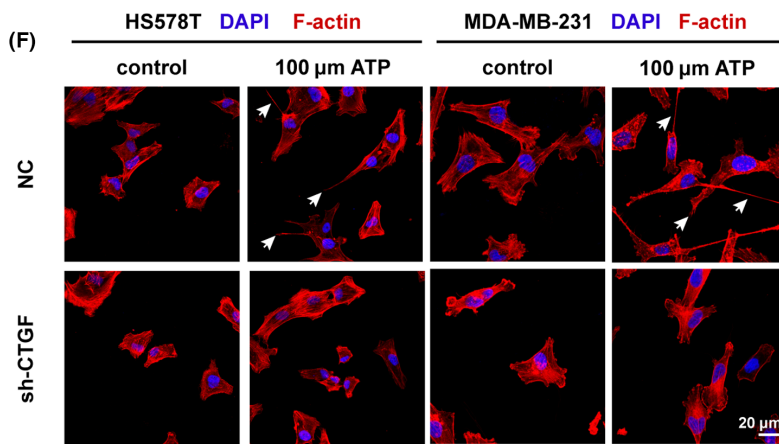
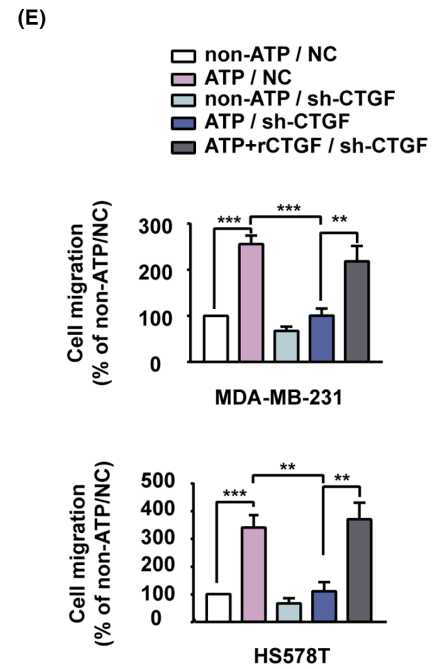
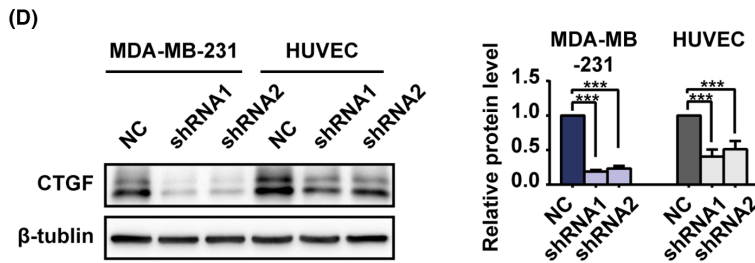
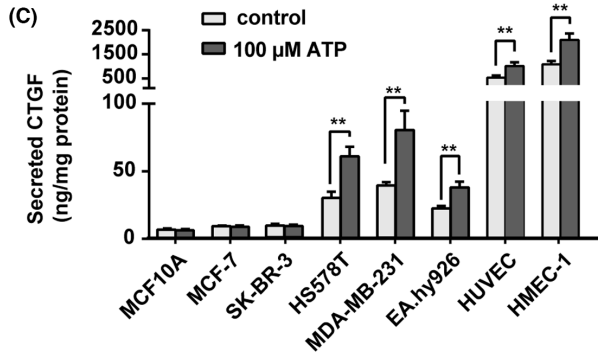
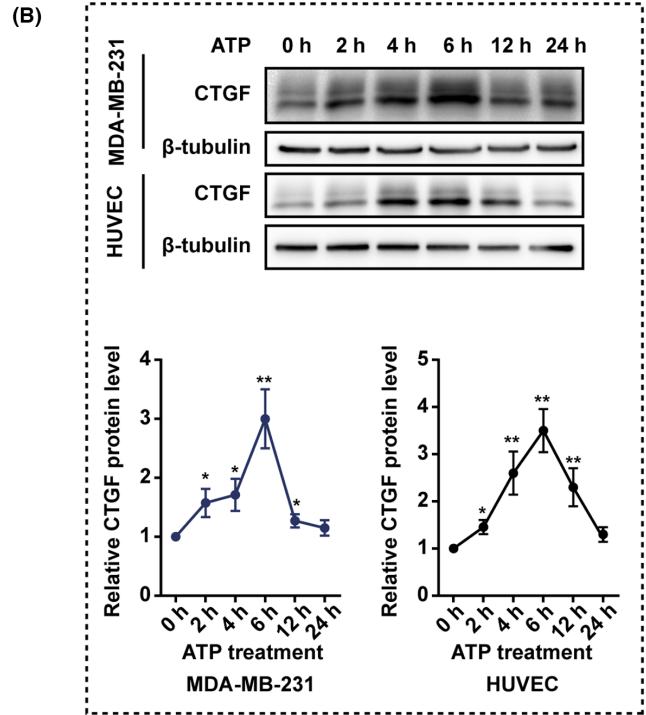
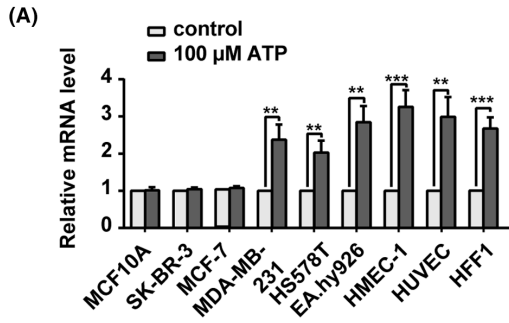
3.4 | CTGF is involved in ATP-driven migration of ECs and angiogenesis

Similarly, ATP-enhanced migration could be attenuated by CTGF shRNAs in ECs (Figures 3A and S3C), and this phenomenon could be rescued by recombinant CTGF protein (rCTGF). Spheroid sprouting assays and tube formation assays showed that ATP-enhanced sprouting (Figure 3B) and angiogenesis (Figure 3C) could be attenuated by CTGF shRNAs, and that this phenomenon could be rescued by rCTGF. ATP-induced phosphorylation of FAK (Y397) could also be attenuated by sh-CTGF (Figure 3D) in ECs. These results all indicated that CTGF plays an important role in ATP-driven EC migration and angiogenesis.

3.5 | P2Y2-YAP signaling activation is involved in ATP-promoted upregulation of CTGF

Connective tissue growth factor has been reported to be regulated by TGF β 1 signaling and YAP signaling.²⁸⁻³⁰ KEGG analysis showed that both pathways were activated in HFF1 cells after ATP treatment (Figure S4A). Therefore, we examined whether ATP could activate the two signaling pathways in TNBC cells and ECs. qRT-PCR demonstrated that several downstream genes of YAP signaling were significantly upregulated in MDA-MB-231 and HUVECs (fold change > 2)

FIGURE 2 Extracellular ATP upregulates connective tissue growth factor (CTGF) expression in triple-negative breast cancer (TNBC) cells and endothelial cells (ECs); CTGF is involved in ATP-driven migration and formation of pseudopodia in TNBC cells. (A) CTGF mRNA levels were detected by qRT-PCR in cells with/without ATP treatment. The levels of CTGF mRNA in each cells without ATP treatment were defined as 1. (B) MDA-MB-231 and HUVEC cells were treated with 100 μ M ATP for 0, 2, 4, 6, 12, and 24 h, and CTGF expression was measured by western blotting. (C) The secreted levels of CTGF with/without ATP treatment for 24 h in different cells were detected by ELISA. The concentrations of secreted protein of different samples were normalized to the corresponding total protein. (D) CTGF shRNAs (sh-CTGF) were transfected into MDA-MB-231 and HUVEC cells, and CTGF expression levels were measured by western blotting. (E) Quantification of breast cancer cell migration after the indicated treatment in a transwell migration assay. (F) The numbers of pseudopodia (white arrowheads) were increased after ATP treatment, and this effect was attenuated by sh-CTGF. (G) MDA-MB-231-NC and MDA-MB-231-sh-CTGF cells were treated with 100 μ M ATP; pFAK (Y397) and total FAK expression were measured by western blotting. Bar graphs represent means \pm SD from triplicates. * p < 0.05, ** p < 0.01, *** p < 0.001



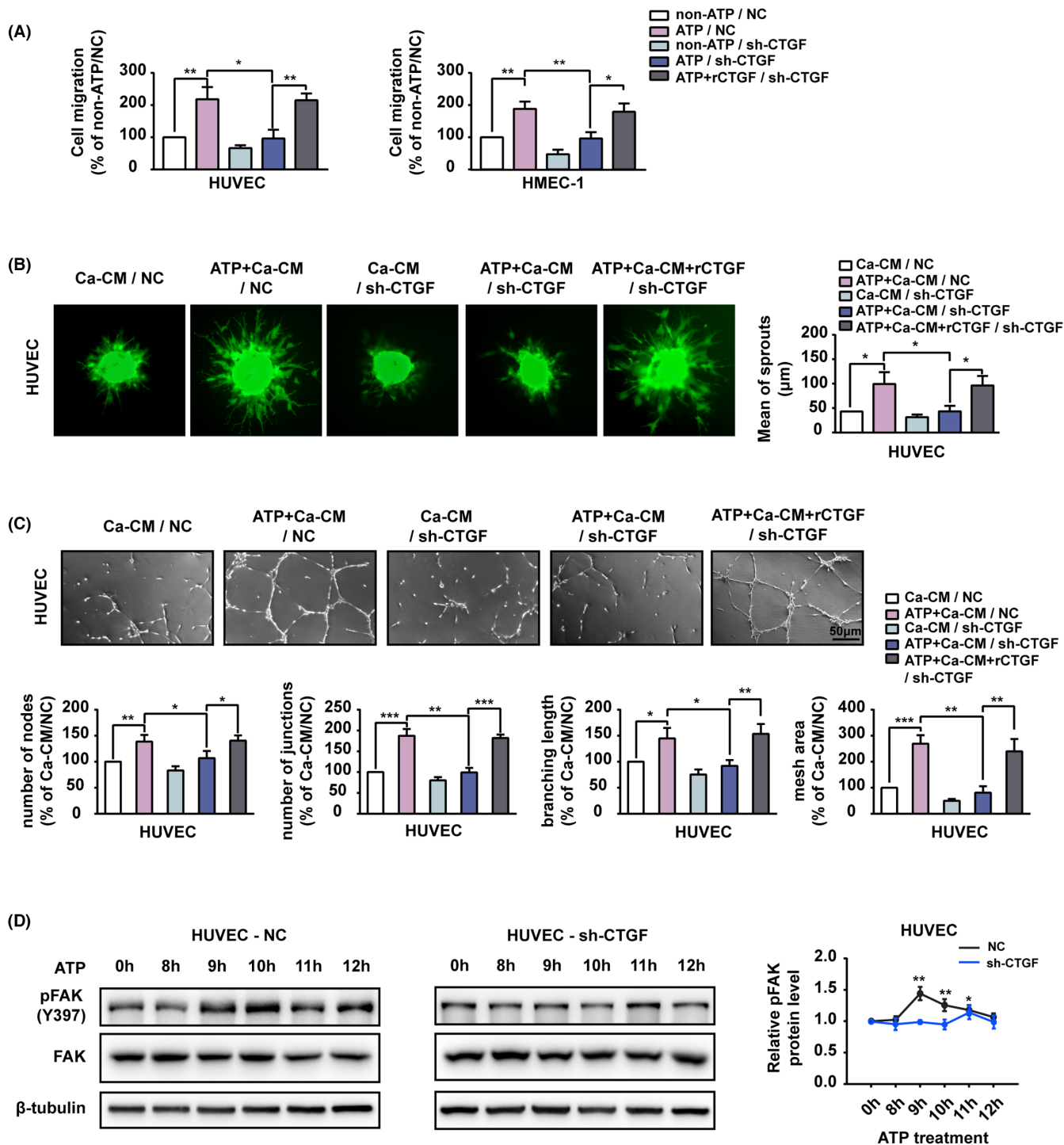


FIGURE 3 Connective tissue growth factor (CTGF) is involved in ATP-driven migration of endothelial cells (ECs) and angiogenesis. (A) Quantification of EC migration after the indicated treatment in a transwell migration assay. (B) Representative images and quantification of HUVEC-NC and HUVEC-sh-CTGF cells with the indicated treatment in a spheroid-based sprouting assay. The lengths of sprouts were measured. (C) Tube formation of HUVEC-NC and HUVEC-sh-CTGF cells after the indicated treatment. (D) HUVEC-NC and HUVEC-sh-CTGF cells were treated with 100 μ M ATP; pFAK (Y397) and total FAK expression levels were measured by western blotting. Bar graphs represent the means \pm SD from triplicates. * p < 0.05, ** p < 0.01, *** p < 0.001

after ATP treatment (Figure S4B). Western blotting showed that extracellular ATP reduced the phosphorylation of YAP (S127) in TNBC cells and ECs (Figures 4A and S4C). It is reported that the dephosphorylated YAP protein can translocate from the cytoplasm to the

nucleus and mediate transcription of downstream genes, including CTGF.³⁰ We demonstrated the translocation of YAP from cytoplasm to nucleus upon ATP treatment using immunofluorescence staining (Figures 4B and S4D). Moreover, we found Peptide 17 (YAP-TEAD

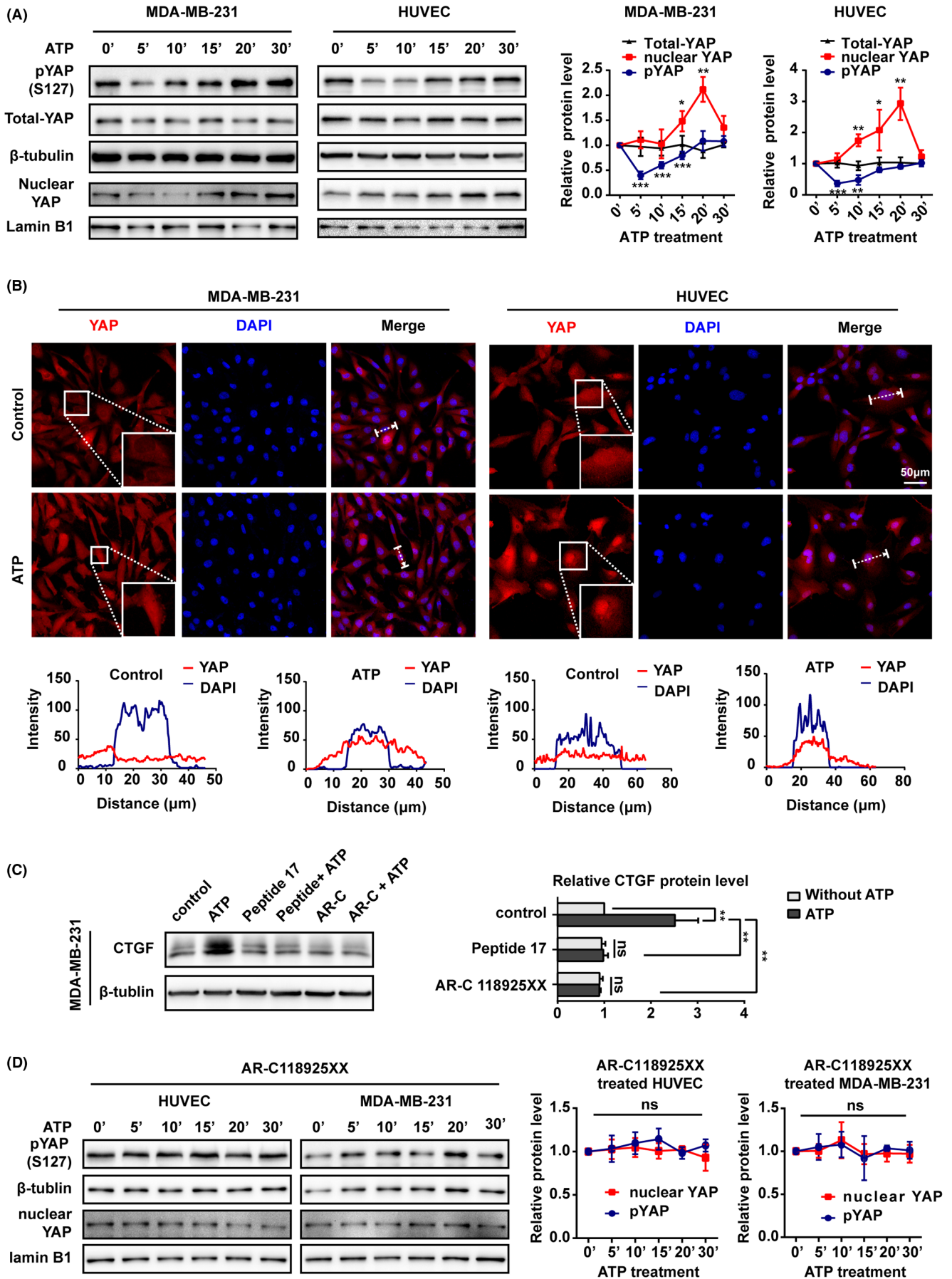


FIGURE 4 (Legend on next page)

FIGURE 4 P2Y2-YAP signaling activation is involved in ATP-promoted upregulation of connective tissue growth factor (CTGF). (A) MDA-MB-231 and HUVEC cells were treated with 100 μ M ATP; pYAP (S127), total YAP and nuclear YAP expression levels were measured by western blotting. (B) Immunofluorescence assay demonstrated the translocation of YAP from cytoplasm to the nucleus upon ATP treatment for 30 min. YAP is labeled in red and the cell nucleus is labeled in blue. The direction of the abscissa in the line graph is the same as the arrowhead of the dotted line in the graph. The abscissa of the line graph represents the distance between the dotted line and its origin. (C) CTGF expression in MDA-MB-231 cells after the indicated treatment measured by western blotting. AR-C, AR-C 118925XX (P2Y2 competitive receptor antagonist); Peptide 17, YAP-TEAD inhibitor 1. (D) After treatment with 10 μ M AR-C 118925XX, MDA-MB-231 and HUVEC cells were treated with 100 μ M ATP; pYAP (S127) and nuclear YAP expression levels were measured by western blotting. Bar graphs represent the means \pm SD from triplicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant

inhibitor 1) could block ATP-induced CTGF upregulation in TNBC cells and ECs (Figures 4C and S4E). These data showed that YAP signaling was indeed involved in ATP-driven upregulation of CTGF. We did not find activation of TGF β 1 signaling upon ATP treatment (data not shown).

As P2 receptors are the preferred receptors for ATP and involved in ATP-mediated migration in cancer cells,^{25,31} we examined P2 subtypes in MDA-MB-231 and HUVECs. qRT-PCR showed that MDA-MB-231 and HUVECs predominantly expressed P2Y2 mRNA (Table S3). To study the role of P2Y2 in ATP-driven YAP signaling activation, we inhibited P2Y2 using its competitive receptor antagonist (AR-C 118925XX). Western blotting showed that ATP-enhanced YAP de-phosphorylation and CTGF upregulation could be blocked by AR-C 118925XX (Figures 4C,D and S4E). In addition, qRT-PCR showed that P2X4 inhibitor 5-BDBD and P2X7 inhibitor KN62 did not block ATP-induced CTGF upregulation (Figure S5A). These results showed that ATP-YAP-CTGF signaling was mediated by the P2Y2 receptor.

Furthermore, we demonstrated that ATP-enhanced migration and angiogenesis could be blocked by Peptide 17 (YAP-TEAD inhibitor 1) and AR-C 118925XX (P2Y2 antagonist) (Figure S5B,C), supporting the role of P2Y2-YAP signaling in ATP-driven migration and angiogenesis.

3.6 | ATP-CTGF axis promotes TNBC cell adhesion to ECs through upregulation of integrin β 1 in TNBC cells and VCAM-1 in ECs

Integrins are the main receptors of secreted CTGF and are closely related to the intercellular connection.³² We examined the expression of integrins by qRT-PCR, and found that mRNA expression of integrins α 1, α 5, α 6, α v and β 1 in TNBC cells was dramatically upregulated upon ATP treatment (Figures 5A and S6A). However, only the upregulation of integrin β 1 could be attenuated by CTGF shRNAs (Figures 5B and S6A). Western blotting verified the upregulation and activation of integrin β 1 by ATP, which could also be attenuated by CTGF shRNAs (Figures 5C and S6B).

It is reported that integrin β 1 in tumor cells interacted with a variety of ligands including fibronectin,³³ zonula occludens-1 (ZO-1),³⁴ angiopoietin-2,³⁵ and vascular cell adhesion molecule-1 (VCAM-1).³⁶ The interaction between integrin β 1 and VCAM-1 mediated cellular adhesion, transmigration, and stimulated tumor cell metastasis.³⁷ To find the ligand for integrin β 1 in ECs, we tested VCAM-1 by qRT-PCR.

Unexpectedly, VCAM-1 was upregulated by more than 10-fold in ECs upon ATP treatment, but this phenomenon disappeared after knockdown of CTGF (Figure 5D). To investigate how the ATP-CTGF axis upregulated VCAM-1 expression in ECs, we tested the receptors of CTGF in ECs and found that the mRNA levels of integrin β 1 were much higher than that of other CTGF receptors (Table S4). Integrin β 1 has been reported to be able to activate the NF- κ B signaling pathway,^{38,39} which is the main regulatory pathway for VCAM-1 expression.⁴⁰ Therefore, our results suggested that ATP-CTGF regulates the expression of VCAM-1 in ECs through integrin β 1.

To investigate the roles of CTGF, integrin β 1 (TNBC cells), and VCAM-1 (ECs) in ATP-mediated adhesion of TNBC cells to ECs, shRNAs against integrin β 1 and inhibitor to VCAM-1 (K-7174) were used in the following experiments (Figures 5E and S6C). We found that ATP-enhanced adhesion of TNBC cells to ECs was attenuated by CTGF shRNAs or integrin β 1 shRNAs or K-7174 (Figures 5F and S6D). In addition, we found that ATP could promote tumor cell transmigration through the EC layer (Figures 5G and S6E), and that this phenomenon could be blocked by AR-C118925XX (P2Y2 inhibitor), CTGF shRNAs, integrin β 1 shRNAs, and K-7174 (VCAM-1 inhibitor) (Figures 5G and S6E). These data indicated that extracellular ATP promoted TNBC cell adhesion to ECs and transmigration through the EC layer by activation of the P2Y2-CTGF-integrin β 1 axis.

Integrin β 1 and VCAM-1 often need integrin subunit α 4 or α 9 to interact with each other.⁴¹⁻⁴³ We examined the two integrin subunits, and found that integrin α 4 was not expressed in MDA-MB-231 and that the expression of integrin α 9 was not upregulated by ATP (Figure 5A). However, ATP-enhanced adhesion and integrin β 1-mediated adhesion of TNBC cells to ECs were both attenuated by integrin α 9 siRNAs (Figure S6F,G), indicating the involvement of integrin α 9 in this process.

3.7 | ATP and CTGF promotes xenograft tumor metastasis and angiogenesis in vivo

Finally, we analyzed the effect of the ATP-CTGF axis on metastasis in vivo. BALB/c mice were injected into the mammary fat pad with six million Negative Control (NC) cells (defined as the NC group, $n = 6$), or NC cells followed by apyrase (an ATP hydrolase, 400 U/kg, intraperitoneal injection) treatment (defined as the NC + apyrase group, $n = 6$) or injected with sh-CTGF stably transfected MDA-MB-231 cells (defined as the sh-CTGF group, $n = 6$). Compared with NC inoculated tumors, NC + apyrase and sh-CTGF groups demonstrated a significant decrease in primary tumor volume (Figure 6A).

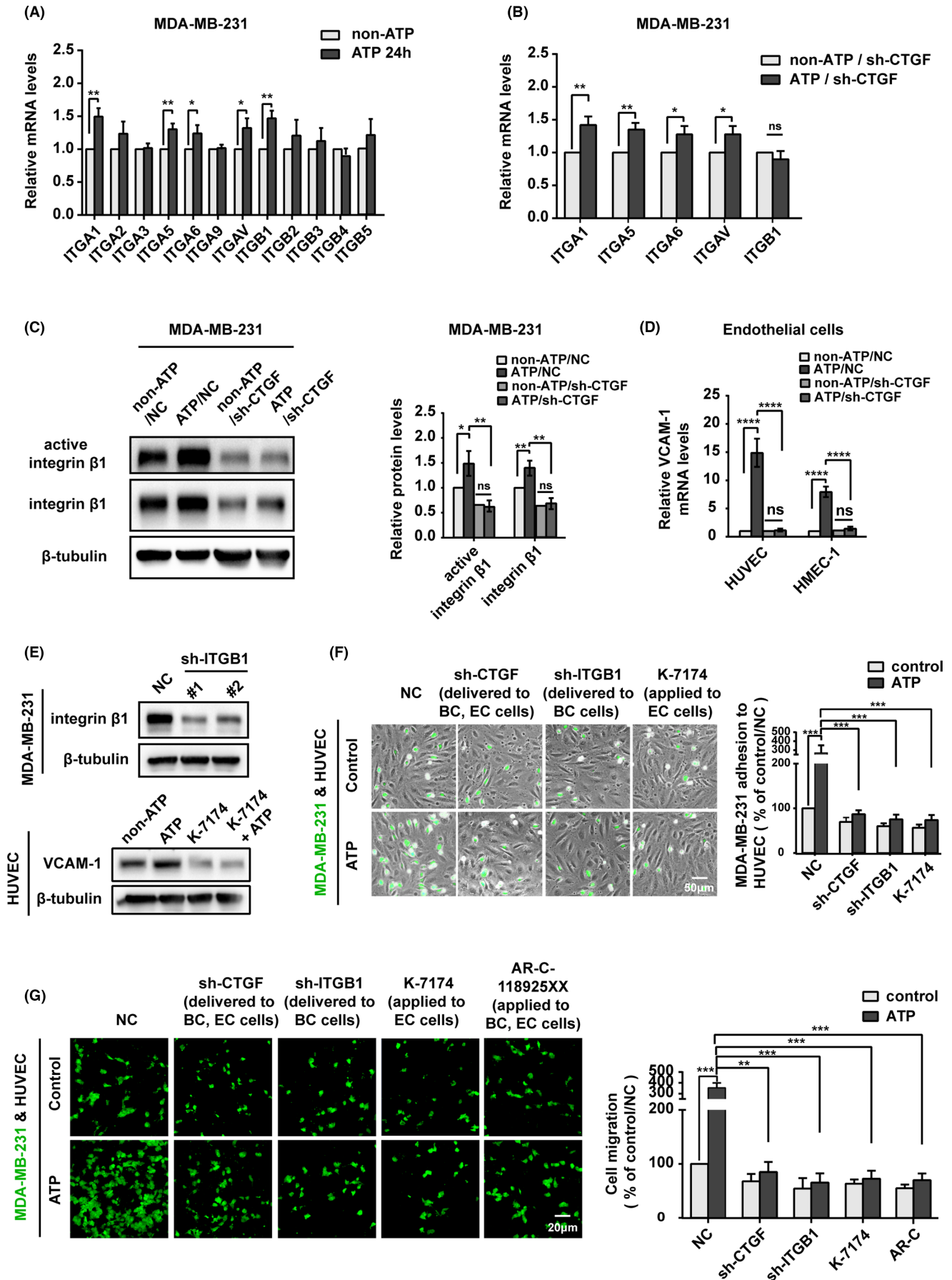


FIGURE 5 (Legend on next page)

FIGURE 5 ATP-driven triple-negative breast cancer (TNBC) cell adhesion to endothelial cells (ECs) is mediated by integrin β_1 in TNBC cells and VCAM-1 in ECs, which are upregulated by connective tissue growth factor (CTGF). (A) Integrin mRNA levels were detected by qRT-PCR in MDA-MB-231 cells. The mRNA level of each integrin subunit without ATP treatment was defined as 1. (B) Integrin mRNA levels were detected by qRT-PCR in MDA-MB-231-sh-CTGF cells with/without ATP treatment for 24 h. (C) MDA-MB-231-NC and MDA-MB-231-sh-CTGF cells were treated with/without 100 μ M ATP. Active integrin β_1 and total integrin β_1 expression levels were measured by western blotting. (D) VCAM-1 mRNA levels were detected by qRT-PCR in ECs with/without ATP treatment for 24 h. (E) Integrin β_1 expression in MDA-MB-231 cells and VCAM-1 expression in HUVEC cells after the indicated treatment were measured by western blotting. K-7174, inhibitor of VCAM-1. (F) Representative images and quantification of adhesion assay. MDA-MB-231-sh-CTGF, HUVEC-sh-CTGF, and MDA-MB-231-sh-ITGB1 cells were used in this assay, or the expression of VCAM-1 in HUVEC was inhibited by K-7174 before this assay. MDA-MB-231 cells were labeled with FITC. A cell adhesion assay was used to detect the number of MDA-MB-231 cells attached to HUVEC. (G) Representative images and quantification of cancer cell transendothelial migration assay. MDA-MB-231 cells (green) were seeded onto HUVEC cells (100% confluency). After 24 h, the number of MDA-MB-231 cells passing through HUVEC cells was measured. Bar graphs represent means \pm SD from triplicates. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001

Knockdown of CTGF by shRNA and intraperitoneal injections of apyrase both could dramatically inhibit lung and liver micrometastasis, and decrease the invasive ability into the neighboring tissues (Figure 6B), which indicated a less malignant phenotype. Western blotting and qRT-PCR showed that both NC + apyrase and sh-CTGF groups expressed lower levels of CTGF, nuclear YAP, pFAK, ITGB1, active ITGB1, and VCAM-1 than the NC group (Figure 6C,D). Immunohistochemical assay demonstrated the downregulation of tumor angiogenesis in both apyrase and sh-CTGF tumor tissues compared with the NC group (Figure 6E).

3.8 | The ATP–CTGF axis is associated with clinical breast cancer survival

Kaplan–Meier OS analyses for patients with BC (<http://gepia.cancer-pku.cn/index.html>) and TNBC (<http://kmplot.com/analysis/>) were performed. The results showed that high levels of CTGF and ITGB1 were associated with lower OS rates in BC patients (Figure 7A), as well as in TNBC patients (Figure 7B), although VCAM-1 was not significantly associated with OS. Through correlation analysis (<https://xena.ucsc.edu/welcome-to-ucsc-xena/>), we found that the expression levels of CTGF in breast cancer were positively correlated with vascular-related molecules (FGF1, FGF2, ANGPT1, ANGPT2, CD34, PECAM1) (Figure 7C). The GEPIA database showed that the expression levels of ITGB1 and VCAM-1 were positively correlated with CTGF in breast cancer (Figure 7D). These BC patients' prognostic data supported our described experimental results.

4 | DISCUSSION

Extracellular ATP plays an important part in the tumor microenvironment. Under normal conditions, the extracellular ATP concentration ranges from 20 to 100 nm. When the cells in the tumor tissues are under conditions of ischemia and hypoxia, a large amount of intracellular ATP will be released, making the extracellular ATP concentration reach several hundred micromoles.⁵ In the 1990s, our laboratory demonstrated that extracellular ATP

promoted the invasion of prostate cancer cells by activating PI3K/ATK and ERK1/2 signaling pathways.^{7,31} In further in-depth studies, we found that ATP could activate purine receptors P2Y2 and P2X7, then activate β -catenin and JAK1-STAT3-SOX9 signaling pathways and promote the invasion and metastasis of breast cancer cells.^{24,25}

Previous studies have mainly focused on the effect of extracellular ATP on tumor cells themselves, while various components in the tumor microenvironment, such as ECs, fibroblasts, and inflammatory cells, also play important roles in the process of tumor invasion and metastasis.^{44,45} Using function assays, we found that ATP could promote cell migration and angiogenesis. Using cDNA microarray and RNA-seq, we found that ATP could significantly increase the expression levels of multiple migration- and angiogenesis-related genes, which further confirmed this finding. Evgenia V Gerasimovskaya et al.⁴⁶ found that extracellular ATP dramatically increased the rearrangement of isolated vasa vasorum ECs into tube-like networks on Matrigel, which supported our discovery. But the exact mechanism remains unclear. We found that CTGF expression was dramatically increased in TNBC cells and ECs after ATP treatment. Pai-Sheng Chen et al.¹⁶ found that CTGF enhances the motility of breast cancer cells via S100A4 upregulation. Wang LH et al.²³ reported that CTGF could regulate angiogenesis in osteosarcoma through regulation of angiopoietin-2, which explained our findings. Accordingly, we also found that ANGPT2 in ECs was upregulated after ATP treatment (data not shown).

We found that the expression of CTGF was induced by ATP in only TNBC cells and ECs via P2Y2-YAP signaling, but not normal cells or non-TNBC cells. Lamar JM et al.⁴⁷ reported that TAZ/YAP-TEAD complexes directly promoted the expression of CTGF. As a critical part of the complexes, nuclear TAZ was found to be conspicuously overexpressed in TNBC tissues, but not in other types of breast cancer tissues.⁴⁸ Skibinski A et al. revealed that 44% of basal-like breast tumors exhibited some degree of TAZ copy-number amplification, compared with only 10% and 20% of luminal A and luminal B tumors, respectively. The high expression of TAZ in basal-like tumors is likely to result from copy-number amplification.⁴⁹ The TNBC cells MDA-MB-231 and HS578T we used may also have TAZ copy-number amplification, which may be the possible reason for our findings.

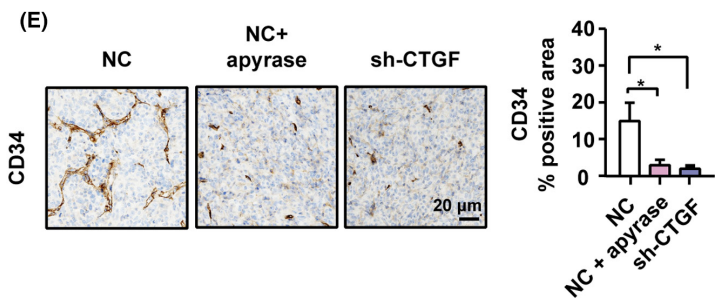
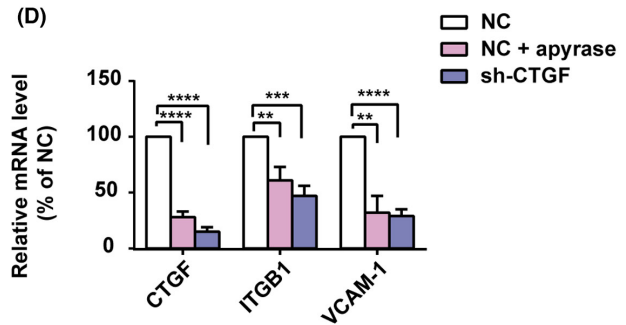
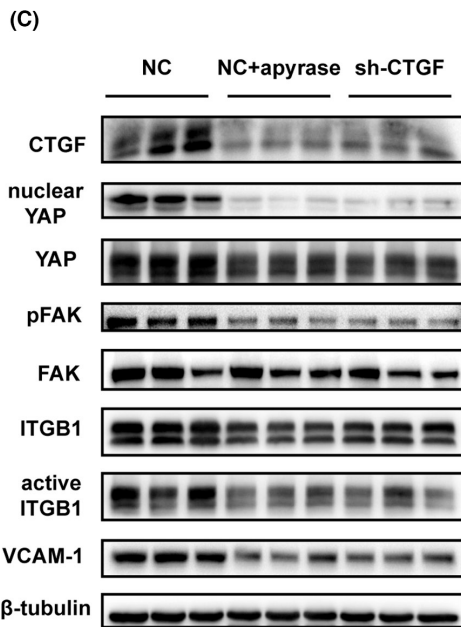
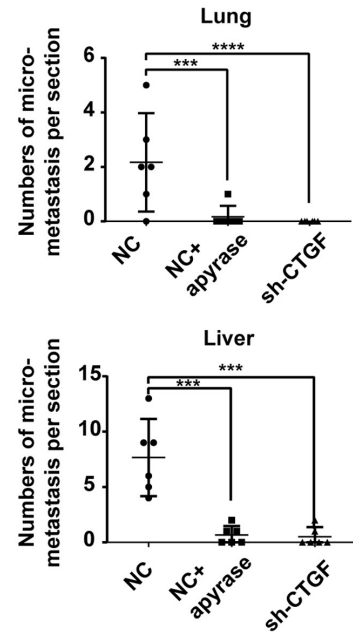
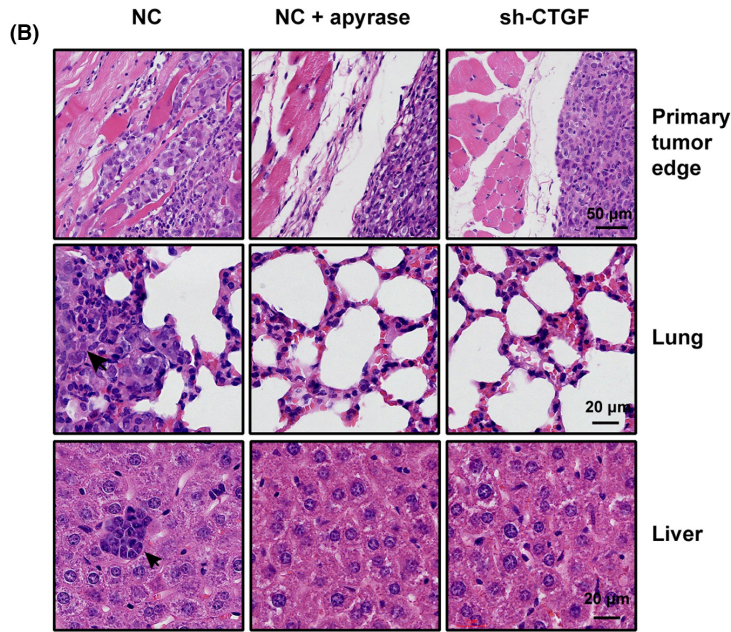
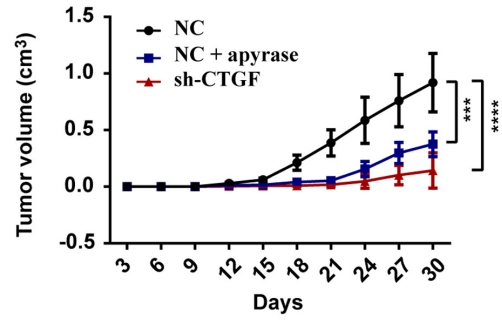
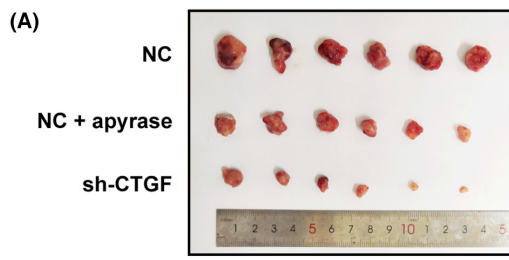


FIGURE 6 (Legend on next page)

FIGURE 6 Apyrase (ATP hydrolyzing enzyme) and knockdown connective tissue growth factor (CTGF) inhibit xenograft tumor metastasis and angiogenesis in vivo. (A) Stably transfected MDA-MB-231 cells (NC, sh-CTGF) were inoculated orthotopically onto the mammary fat pad of 6-week-old female BALB/c mice ($n = 6$). Primary tumor size was measured and quantified every 3 days for 30 days. (B) Representative images of primary tumors and metastatic tumors, which were H&E stained. Numbers of metastatic lesions in lung and liver were quantified. Expression levels of CTGF and its related genes were examined by western blotting (C) and qRT-PCR (D) in inoculated tumor tissues of each mouse. (E) Vascular density was examined by CD34 immunohistochemistry (IHC) staining, which was calculated using ImageJ software. Bar graphs represent means \pm SD. For each analysis, $n = 6$ in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

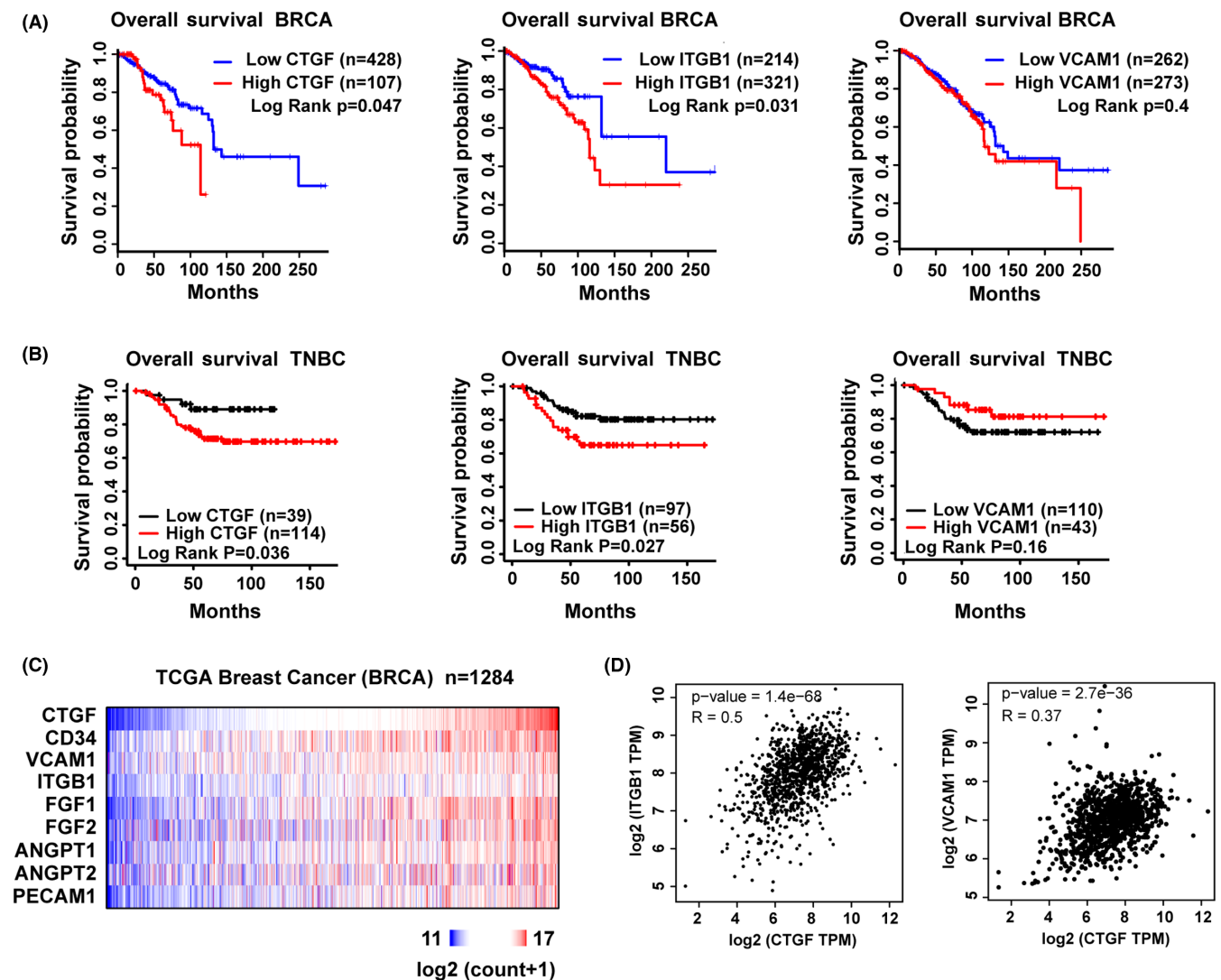


FIGURE 7 The ATP-connective tissue growth factor (CTGF) axis is associated with clinical breast cancer survival. (A) Kaplan–Meier survival analysis (<http://gepia.cancer-pku.cn/index.html>) shows the negative correlations between survival time of breast cancer patients and expression levels of CTGF and integrin β_1 in breast cancer. For each gene, the mRNA expression level over the median is defined as high expression, and lower than the median is defined as low expression. (B) Kaplan–Meier survival analysis (<http://kmpplot.com/analysis/>) shows the negative correlations between overall survival time of triple-negative breast cancer (TNBC) patients and expression levels of CTGF and integrin β_1 . (C, D) correlation analysis (<https://xena.ucsc.edu/welcome-to-ucsc-xena/>) shows that the expression levels of CTGF in breast cancer were positively correlated with vascular-related molecules

Integrins are the main receptors for CTGF.¹³ Downstream of the integrin pathways, FAK plays an important role in cell migration and angiogenesis promoted by CTGF. FAK has been reported to mediate keratinocyte and human chondrosarcoma cell migration through integrin $\alpha_5\beta_1$ and $\alpha_v\beta_3$, respectively.^{50,51} FAK was also reported to mediate angiogenesis in intrahepatic cholangiocarcinoma

and gliomas through integrin $\alpha_v\beta_3$ and CD93–MMRN2–integrin β_1 respectively.^{52,53} All of these findings supported the function of FAK. In our study, we also found that FAK could be activated by ATP via CTGF.

In addition to promoting tumor angiogenesis, we found ATP could increase the adhesion of tumor cells to ECs and promote cancer cell

transmigration through the EC layer. Galina F et al.⁹ found that ATP plays important roles in polymorphonuclear (PMN) adhesion to ECs, supporting our findings. As the main receptors of secreted CTGF, integrins were also reported to be closely related to the intercellular connection.³² We then tested the expression of integrins in TNBC cells. We discovered that ATP upregulated the expression of integrin β_1 by secreted CTGF in TNBC cells. Riley KG et al.⁵⁴ found CTGF could upregulate integrin β_1 expression in β -cells, which supported our discovery. The bioinformatics analysis in our study also supported our findings. We found that integrin β_1 played an important role in ATP-driven TNBC cell adhesion to ECs and transmigration through the EC layer. More and more reports have shown that integrins facilitate cancer cell adhesion to ECs and tumor metastasis. ATN-161 ($\alpha_5\beta_1$ inhibitor) was found to be able to diminish cancer metastases, reduce angiogenesis, and improve survival in a mouse model and human phase I trial.^{55,56}

As integrin and cell adhesion were studied, we further detected the ligand of integrin β_1 , VCAM-1.⁵⁷ We found that VCAM-1 in ECs was also upregulated by the ATP-CTGF axis. Rice GE et al.⁵⁸ first reported the importance of VCAM-1 for melanoma adhesion to the endothelium. In addition to adhesion, VCAM-1 on ECs also mediates transendothelial migration of tumor cells.^{59,60} These reports supported our findings that upregulation of VCAM-1 by the ATP-CTGF axis participates in cancer cell adhesion to ECs and that TNBC cells transmigrate through the EC layer.

Our mouse model showed that both apyrase (an ATP hydrolyzing enzyme) and CTGF shRNAs could decrease angiogenesis and inhibit the metastasis of inoculated tumors. Morrone FB et al.⁶¹ reported that a rat glioma became less invasive and proliferative after apyrase treatment. Schumacher D et al.⁶² demonstrated that mice deficient in P2Y2 (ATP receptor) or lacking ATP secretion from platelets showed strongly reduced tumor metastasis. Hutchenreuther J et al.²¹ found that mice deleted for CTGF in CAFs showed impaired vasculogenic mimicry of subcutaneously injected B16-F10 cells in vivo. All these reports supported our findings.

In summary, our study demonstrated that extracellular ATP promoted angiogenesis via upregulation of CTGF. In addition, ATP stimulated TNBC cell adhesion to ECs and transmigration through the EC layer via CTGF by upregulation of integrin β_1 on TNBC cells and VCAM-1 on ECs (refer to the graphical abstract). The findings in this study have significant implications regarding our understanding of extracellular ATP in TNBC progression and metastasis. The pleiotropic effects of ATP in angiogenesis and cell adhesion suggested that extracellular ATP or CTGF could be an effective target for TNBC therapy.

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DISCLOSURE

The authors have no conflict of interest.

AUTHOR CONTRIBUTIONS

Yan-Ting Zhou designed the studies and wrote the manuscript. Yan-Ting Zhou, Yu-Qing Yu, and Hui Yang performed the experiments and analyzed data. Han Yang, Yan-Fei Huo, and Yang Huang helped with the experiments. Xin-Xia Tian and Wei-Gang Fang suggested experiments and revised the manuscript. All authors contributed to manuscript and reviewed and approved the manuscript.

ETHICAL APPROVAL

Approval of the research protocol by an Institutional Review Board: The tissue microarrays for breast cancer patients were purchased from the National Human Genetic Resources Sharing Service Platform 2005DKA21300 (Shanghai Outdo Biotechnology Company Ltd., China). The research protocols were approved by the Ethics Committee of Shanghai Outdo Biotech Company (no. SHYJS-CP-1807025) and were conducted in accordance with the Declaration of Helsinki. Animal Studies: All procedures and protocols for animal studies were approved by the Institutional Animal Care and Use Committee of Peking University (no. LA2018190).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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