

Original Article

Notch-1/Dll4 and PI3K/AKT signaling are associated with impaired trophoblast pro-angiogenic regulation in spontaneous abortion

Xiaoli Lv¹, Baozhen Xie², Yuexi Zhou³, Haiwang Wu⁴, Jie Gao⁴, Songping Luo⁵

¹Department of Gynecology, Henan Province Hospital of Traditional Chinese Medicine (The Second Affiliated Hospital of Henan University of Chinese Medicine), Zhengzhou, Henan, China; ²Department of Traditional Chinese Medicine, The Third Affiliated Hospital, Guangzhou Medical University, Guangzhou, Guangdong, China; ³Department of Reproductive Medicine, Wuhan Hospital of Traditional Chinese and Western Medicine, Wuhan, Hubei, China; ⁴Department of Gynecology, The First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, Guangdong, China; ⁵Lingnan Gynecology Research Institute, The First Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou, Guangdong, China

Received May 18, 2026; Accepted July 17, 2026; Epub October 15, 2026; Published October 30, 2026

Abstract: Objective: Impaired trophoblast-vascular coordination at the maternal-fetal interface is closely associated with early pregnancy loss. This study investigated whether Notch-1/Dll4 and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling are associated with angiogenesis-related abnormalities and trophoblast pro-angiogenic behavior in spontaneous abortion (SA). Study design: First-trimester chorionic villous tissues and serum samples from normal early-pregnancy controls (NC) and SA cases were analyzed by immunohistochemistry, RT-qPCR, Western blotting, and ELISA. HTR-8/SVneo cells were treated with Notch inhibitor DAPT or the PI3K inhibitor LY294002. Cell viability, migration, Matrigel invasion, capillary-like network formation, immunofluorescence signals, and angiogenesis-related protein expression were assessed. Results: SA villous tissues showed reduced VEGFA, VEGFR1, VEGFR2, and CD34 expression, together with lower serum VEGF levels. These changes were accompanied by decreased Notch-1/Dll4 expression and reduced p-PI3K/PI3K, p-AKT/AKT, and p-S6/S6 ratios. In HTR-8/SVneo cells, DAPT and LY294002 did not markedly reduce cell viability at the working concentration but suppressed migration, Matrigel invasion, and capillary-like network formation. DAPT decreased Notch-1, Dll4, NICD, p-PI3K/PI3K, p-AKT/AKT, p-S6/S6, VEGFA, VEGFR1, and VEGFR2. LY294002 reduced p-AKT/AKT and p-S6/S6 ratios, decreased Notch-1/Dll4/NICD and VEGF-related protein expression, whereas p-PI3K/PI3K showed no significant change. Conclusion: SA is associated with attenuated VEGF-related angiogenic support and reduced Notch-1/Dll4 and PI3K/AKT signaling at the maternal-fetal interface. These findings suggest that Notch-1/Dll4 and PI3K/AKT signaling contribute to trophoblast pro-angiogenic regulation during early pregnancy loss.

Keywords: Spontaneous abortion, maternal-fetal interface, angiogenesis, Notch-1, PI3K/AKT, trophoblast

Introduction

Early pregnancy loss is a common reproductive complication. In this study, spontaneous abortion (SA) was defined as non-induced pregnancy loss before 28 weeks of gestation with a fetal weight of < 1,000 g. Early pregnancy loss occurs in approximately 10%-15% of clinically recognized pregnancies [1]. Recurrent spontaneous abortion (RSA) represents a smaller yet clinically important subgroup, affecting roughly 1%-5% of couples, and the risk of subsequent pregnancy loss increases with previous miscarriage history [2, 3]. The mechanisms underlying

SA are heterogeneous and may involve embryonic, maternal, placental, immune, endocrine, and vascular factors. An improved understanding of the biological processes involved in SA would enhance the assessment of early pregnancy failure and identify potential targets for intervention.

The maternal-fetal interface is a dynamic site of communication between maternal endometrial tissue and extraembryonic trophoblast-derived structures. During early pregnancy, trophoblast cells invade the maternal decidua, remodel spiral arteries, and participate in the establish-

ment of uteroplacental perfusion [4]. These events promote placental formation and establish the vascular basis for oxygen and nutrient exchange during early embryonic development. Angiogenesis and vascular remodeling within the decidua and placenta are therefore essential for a functional maternal-fetal circulation. Disturbed placental vascular development and reduced uteroplacental perfusion have been implicated in spontaneous abortion [5], underscoring the central role of trophoblast-vascular coordination in maintaining early maternal-fetal interface homeostasis.

Notch signaling is a contact-dependent pathway involved in cell fate determination, differentiation, and tissue remodeling. Ligand-bound Notch receptors undergo proteolytic cleavage mediated by ADAM metalloproteinases and γ -secretase, releasing the Notch intracellular domain (NICD), which then translocates to the nucleus and regulates downstream transcriptional programs [6]. During early gestation, Notch signaling participates in endometrial decidualization, trophoblast differentiation, and placental development [7, 8]. Experimental suppression of Notch signaling has been reported to affect trophoblast syncytialization-related processes [9]. Together, these studies position Notch signaling as a key node in the regulatory network governing embryo-maternal interaction, placental development, and vascular remodeling.

The phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway is another central signaling axis that regulates cell survival, growth, adhesion, motility, invasion, angiogenic responses, and metabolic activity [10, 11]. In trophoblast models, PI3K/AKT signaling has been functionally linked to vascular endothelial growth factor (VEGF)-related responses. Furmento et al. showed that granulocyte colony-stimulating factor promotes VEGF secretion in Swan 71 trophoblast cells through activation of PI3K/AKT and ERK1/2 signaling [12]. The PI3K/AKT pathway is also closely related to angiogenic regulation in vascular systems [13]. These observations support a role for PI3K/AKT signaling in coupling trophoblast functional activity with angiogenesis-related regulation during early placental development.

Although Notch-1 and PI3K/AKT signaling have both been implicated in trophoblast function

and vascular regulation, their association with angiogenesis-related abnormalities at the maternal-fetal interface in SA remains insufficiently defined. In the present study, we examined angiogenesis-related markers, Notch-1/Dll4 expression, and PI3K/AKT pathway phosphorylation in first-trimester chorionic villi from SA cases and normal early-pregnancy controls. We further used DAPT, a γ -secretase inhibitor, to suppress Notch signaling and LY294002, a PI3K inhibitor, to inhibit PI3K signaling in HTR-8/SVneo cells, and then evaluated trophoblast migration, Matrigel invasion, capillary-like network formation, and angiogenesis-related protein expression. LY294002 has been widely used to interrogate PI3K-dependent trophoblast migration and invasion in HTR-8/SVneo cells [14]. This study aimed to define the association between Notch-1/PI3K/AKT signaling and trophoblast pro-angiogenic regulation in the context of SA.

Materials and methods

Participant enrollment and clinical sample collection

The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Guangzhou University of Chinese Medicine (No. K[2019]098). Written informed consent was obtained from all participants before enrollment. Participants were recruited from the First Affiliated Hospital of Guangzhou University of Chinese Medicine between August 2019 and December 2020. The cohort included 40 cases of embryonic arrest and 40 normal early-pregnancy controls (NC) undergoing elective termination. Chorionic villous tissue and peripheral blood samples were collected from each participant. An intrauterine singleton pregnancy at 6-9 weeks of gestation was confirmed by ultrasonography, and fetal cardiac activity was assessed in at least two examinations. Only women with regular menstrual cycles were enrolled; gestational age was determined according to the last menstrual period and verified by ultrasound. After vacuum aspiration, chorionic villi and 4 mL of peripheral blood were collected under sterile conditions. Women were excluded if they had received hormone therapy or had any metabolic disorder within 3 months before enrollment.

Washed chorionic villous tissue was divided according to downstream analyses. One aliquot

Table 1. RT-qPCR primer information

Name	Primer	Gene sequence
VEGFA	Forward	5'-ACATCACCATGCAGATTATGCG-3'
	Reverse	5'-GGGAGGCTCCTTCCTCTG-3'
VEGFR1	Forward	5'-CTGGACTGACAGCAAACCAAG-3'
	Reverse	5'-CCACAGCTGGAATGGCAGAA-3'
VEGFR2	Forward	5'-TGGTTGTGTATGTCCACCC-3'
	Reverse	5'-TGCTACTGAGACAGCTTGGC-3'
Notch-1	Forward	5'-GACATGCCACGTGGTGGA-3'
	Reverse	5'-GGCAGGATTCCTGACCA-3'
AKT	Forward	5'-AGAGAAGCCACGCTGCTCT-3'
	Reverse	5'-CCGCAGGATAGTTTCTTCCC-3'
β -Actin	Forward	5'-AGAAGAGCTACGAGCTGCCTGACG-3'
	Reverse	5'-GGACTCCATGCCAGGAAGGAA-3'

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

RNA extraction from villous samples was performed with the HiPure Universal RNA Mini Kit (Magenbio, Guangzhou, China), then reverse-transcribed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Amplification used PowerUp™ SYBR™ Green Master Mix on a CFX96™ Real-Time System, with cycling at 95°C for 30 s and 40 cycles of 95°C for 15

was processed for Western blot analysis, a second was stored in RNAlater at -80°C for RT-PCR, and the remaining tissue was fixed in formaldehyde and embedded in paraffin. Peripheral blood was centrifuged at 3,000 rpm for 15 min at 4°C, and the separated serum, approximately 2 mL, was stored at -80°C.

Immunohistochemical analysis

Chorionic villus sections were dewaxed, rehydrated, subjected to EDTA-mediated antigen retrieval, and processed for immunohistochemistry (IHC) after endogenous peroxidase blocking with 3% H₂O₂ and blocking with 10% normal goat serum. Primary antibodies were applied overnight at 4°C: VEGFA (1:300, ab214424, Abcam), VEGFR1 (1:250, ab32152, Abcam), VEGFR2 (1:800, #9698, Cell Signaling Technology), Notch-1 (1:200, 10062-2-AP, Proteintech Group), DII4 (1:200, NB600-892, Novus Biologicals), PI3K (1:300, 67121-1-Ig, Proteintech Group), or AKT (1:300, #4691, Cell Signaling Technology). HRP-conjugated secondary antibodies against rabbit or mouse IgG (Zhongshan, Beijing, China) were added for 30 min at 37°C. DAB chromogenic development was performed with a substrate kit from Zhongshan (Beijing, China) following the supplied protocol. An optical microscope (OLYMPUS IX-7, Tokyo, Japan) was used to perform photomicrographs of 5 discrete fields of view on each slice at high magnification (200×). Mean optical density was obtained from IHC images using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA), calculated as the ratio of integrated optical density to area.

s and 60°C for 15 s. β -actin was used for normalization, primer details are given in **Table 1**, and transcript changes were expressed by the $2^{-\Delta\Delta Ct}$ calculation.

Western blot analysis

Western blotting was performed to assess angiogenesis-related proteins and Notch/PI3K/AKT pathway activation in chorionic villous tissues and HTR-8/SVneo cells. Samples were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors, and total protein concentration was determined using a bicinchoninic acid (BCA) assay kit (Beyotime, China). Equal amounts of denatured protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Ireland). After blocking with 5% skim milk in TBST for 2 h, membranes were incubated overnight at 4°C with primary antibodies against VEGFA (1:1,000, ab214424, Abcam), VEGFR1 (1:1,000, ab32152, Abcam), VEGFR2 (1:1,000, 9698, Cell Signaling Technology), Notch-1 (1:1,000, 10062-2-AP, Proteintech Group), DII4 (1:1,000, NB600-892, Novus Biologicals), NICD (1:1,000, 4147, Cell Signaling Technology), PI3K (1:2,000, 67121-1-Ig, Proteintech Group), p-PI3K (1:1,000, GB150210-100, Servicebio), AKT (1:1,000, 4691, Cell Signaling Technology), p-AKT Ser473 (1:1,000, GB150002-100, Servicebio), S6 (1:800, GB112610-100, Servicebio), p-S6 (1:2,000, GB115635-100, Servicebio), and GAPDH (1:10,000, 10494-1-AP, Proteintech Group). Membranes were washed with

Tris-buffered saline with Tween-20 (TBST) and incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (1:5,000; Proteintech Group, SA00001-2 and SA00001-1). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL) reagent (Fude Biotech, China) and recorded with a Bio-Rad imaging system. Band intensities were quantified using ImageJ. Total protein levels were normalized to GAPDH, whereas phosphorylation-dependent pathway activation was assessed using the ratios of p-PI3K/PI3K, p-AKT Ser473/AKT, and p-S6/S6.

Enzyme-linked immunosorbent assay (ELISA)

For each participant, VEGF in serum separated from peripheral blood was measured with the CUSABIO human VEGF ELISA system (Wuhan, China).

Cell culture and treatment

ATCC-derived HTR-8/SVneo cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified incubator containing 5% CO₂. For inhibitor-based experiments, log-phase cells were seeded into six-well plates at 2 × 10⁵ cells/well and treated with 50 μM DAPT, a γ-secretase inhibitor, or 50 μM LY294002, a PI3K inhibitor, for 24 h. Control cells received the corresponding concentration of DMSO, and the final dimethyl sulfoxide (DMSO) concentration was kept consistent across groups at ≤ 0.1%.

Cell viability assay

HTR-8/SVneo cell viability after DAPT or LY294002 treatment was evaluated using a Cell Counting Kit-8 assay (CCK-8; Servicebio, G4103). Cells were seeded into 96-well plates at 5 × 10³ cells/well in 100 μL of complete medium and allowed to adhere overnight. Cells were then treated with DAPT or LY294002 at 10, 25, or 50 μM for 24 h. Vehicle-treated cells served as the control, and blank wells containing medium without cells were included for background correction. After treatment, 10 μL of CCK-8 reagent was added to each well, followed by incubation at 37°C for 2 h. Absorbance was measured at 450 nm using a microplate reader (BioTek ELx808). Cell viability

was calculated relative to the vehicle control after blank subtraction using the following formula: cell viability (%) = (A_{treatment} - A_{blank})/(A_{control} - A_{blank}) × 100%.

Cell migration and invasion assay

HTR-8/SVneo cell migration and invasion were assessed using Corning Transwell inserts with 8-μm pore membranes. For migration assays, untreated membranes were used. For invasion experiments, the upper surface of membranes were pre-coated with 80 μL Matrigel diluted 1:3 in RPMI-1640 medium. After the indicated treatments, 2 × 10⁴ cells suspended in 100 μL serum-free RPMI-1640 medium were seeded into the upper chamber, while 700 μL RPMI-1640 medium containing 10% FBS was added to the lower chamber as a chemoattractant. After 24 h of incubation, cells remaining on the upper membrane surface were gently removed. Cells that had migrated or invaded to the lower surface were fixed, crystal violet-stained, and imaged under a Nikon microscope at ×100.

Capillary-like network formation assay

Capillary-like network formation was assessed using growth factor-reduced Matrigel as an in vitro readout of trophoblast pro-angiogenic behavior. Each well of a 96-well plate was coated with 60 μL Matrigel (BD Biosciences, USA) and incubated at 37°C for 30 min. After the indicated treatments, HTR-8/SVneo cells were seeded onto the Matrigel surface at 1 × 10⁴ cells/well in 100 μL medium. Capillary-like network structures were imaged after 6 h of incubation using an inverted Nikon microscope at ×100 magnification. Network parameters were quantified using ImageJ based on images from three independent experiments.

Immunofluorescence staining

HTR-8/SVneo cells grown on coverslips were fixed with paraformaldehyde, permeabilized with Triton X-100, and blocked with goat serum. Cells were then incubated overnight at 4°C with primary antibodies against Notch-1 (1:100, 10062-2-AP, Proteintech Group) and AKT (1:200, 4691, Cell Signaling Technology), followed by incubation with a goat anti-rabbit fluorescent secondary antibody (1:200, SA00003-2, Proteintech Group). Nuclear counterstaining and mounting were performed using

Table 2. Clinical profiles of participants in the NC and SA cohorts

Characteristics	NC (n = 40)	SA (n = 40)	t/Z	P
Maternal age (y)	28.75±5.73	31.30±4.16*	-2.277	0.026
Body mass index (kg/m ²)	21.42±2.13	22.11±2.29	-1.403	0.165
Gestational age (wk)	6.50 (6.00, 7.00)	6.00 (6.00, 7.00)	-0.170	0.865
No. of pregnancies (n)	3.00 (2.00, 3.00)	3.00 (2.00, 4.00)	-1.137	0.256
No. of previous live births (n)	1.00 (0.00, 2.00)	1.00 (1.00, 1.00)	-0.804	0.422
No. of previous spontaneous abortions (n)	0.00 (0.00, 1.00)	0.00 (0.00, 1.75)	-1.458	0.145

Data are presented as mean ± SD or median (P25, P75), as appropriate. NC, normal early-pregnancy control group; SA, spontaneous abortion group; BMI, body mass index; P25, 25th percentile; P75, 75th percentile. **P* < 0.05 vs. NC.

10 µL DAPI-containing antifade reagent (PO-131, Beyotime, China). Images were acquired using an Olympus fluorescence microscope at ×200 magnification under identical exposure settings within each experiment.

Logistic regression analysis

Logistic regression analysis was performed to evaluate the associations of maternal age and previous spontaneous abortion history with SA status. SA status was used as the outcome variable, with the NC group as the reference. Maternal age was entered as a continuous variable, and previous spontaneous abortion history was entered as a categorical variable, with 0 previous spontaneous abortions as the reference category. Crude and adjusted odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. The adjusted model included maternal age and previous spontaneous abortion history.

Statistical analysis

Statistical analyses were performed using SPSS 23.0, and graphs were generated with GraphPad Prism 8.0. Data distribution was assessed before statistical testing. Normally distributed data are presented as mean ± SD, whereas non-normally distributed data are presented as median (P25, P75). Comparisons between two groups were performed using Student's t-test or the Mann-Whitney U test, as appropriate. For comparisons among multiple groups, one-way ANOVA followed by Bonferroni or Dunnett's post hoc test was used when data met parametric assumptions; otherwise, a non-parametric test was applied. All experiments were repeated independently at least three times unless otherwise indicated. The number of biological replicates, independent experiments, or analyzed fields is specified in the cor-

responding figure legends. All statistical tests were two-sided, and *P* < 0.05 was considered statistically significant.

Results

Attenuated angiogenesis-related factor expression in chorionic villi from SA cases

Villous tissues from 40 SA cases and 40 NC cases undergoing elective termination were first examined for angiogenesis-related markers. Except for higher maternal age in the SA group, the two cohorts were comparable in BMI, gestational age, gravidity, previous live birth number, and prior spontaneous abortion history (**Table 2**). To address this baseline difference, multivariable logistic regression analysis was performed to evaluate the associations of maternal age and previous spontaneous abortion history with SA status. The adjusted results are presented in [Supplementary Table 1](#).

IHC staining showed that VEGFA, VEGFR1, and VEGFR2 immunoreactivity were broadly distributed in villous trophoblast and stromal regions, whereas CD34 staining mainly marked villous endothelial cells and microvessels. Compared with NC villi, SA villous tissues exhibited weaker immunostaining for VEGFA, VEGFR1, VEGFR2, and CD34 (**Figure 1A**). RT-qPCR and Western blot analyses further showed concordant reductions in angiogenesis-related markers, with lower VEGFA, VEGFR1, and VEGFR2 mRNA levels and decreased VEGFA, VEGFR1, VEGFR2, and CD34 protein abundance in SA villous tissues (**Figure 1B, 1C**). In addition, ELISA showed lower peripheral serum VEGF concentrations in the SA group than in the NC group (**Figure 1D**). These findings indicate an attenuated angiogenesis-related profile at the maternal-fetal interface in SA.

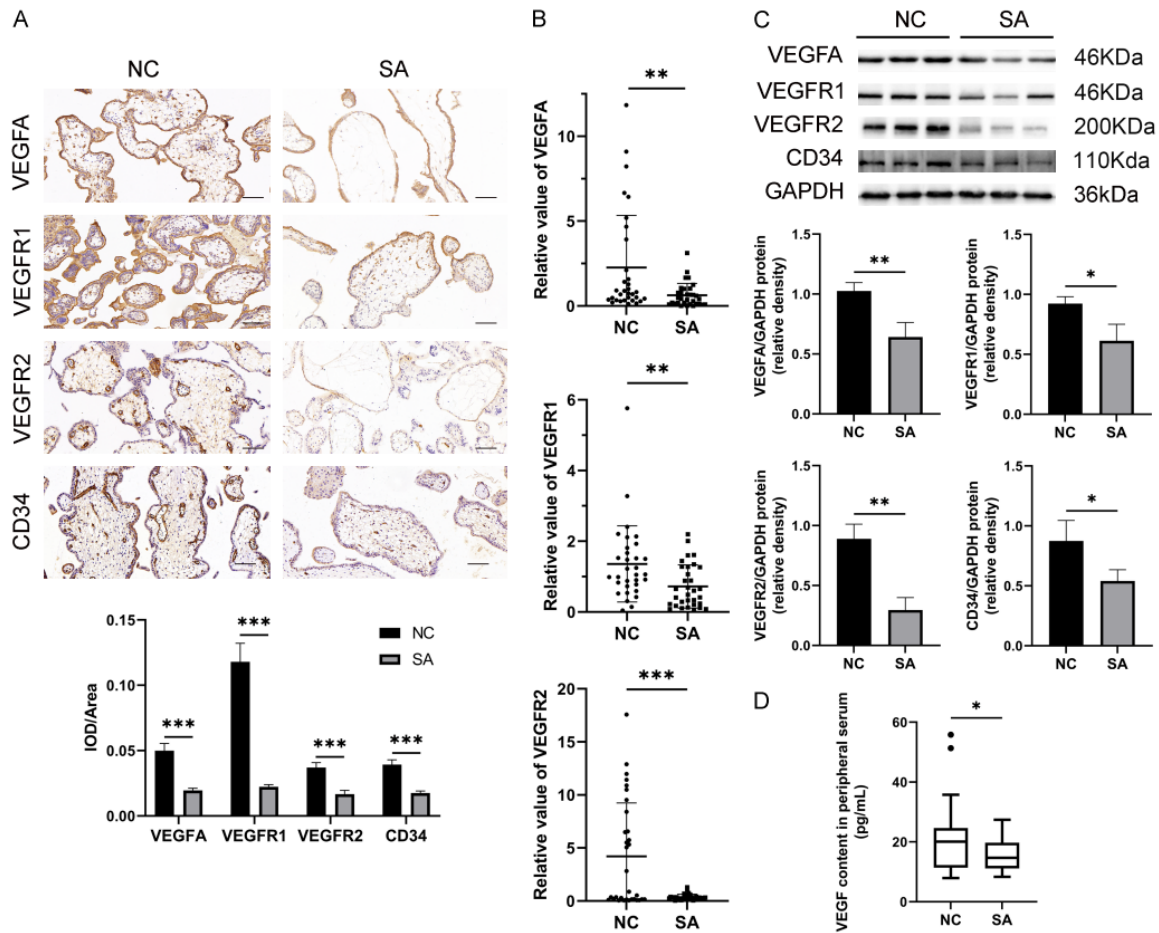


Figure 1. Reduced angiogenesis-related markers in villous tissues and serum from SA cases. A. Representative IHC images and quantification of VEGFA, VEGFR1, VEGFR2, and CD34 in first-trimester villous tissues from NC and SA groups. Scale bar, 100 μ m. B. RT-qPCR analysis of VEGFA, VEGFR1, and VEGFR2 mRNA expression in villous tissues. C. Western blot analysis and quantification of VEGFA, VEGFR1, VEGFR2, and CD34 protein expression in villous tissues. D. ELISA analysis of serum VEGF levels. Data are presented as mean $\pm$ SD or median (P25, P75), as appropriate. $n = 40$ per group for IHC, RT-qPCR, and ELISA; $n = 3$ per group for Western blotting. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NC. NC, normal early-pregnancy control; SA, spontaneous abortion; IHC, immunohistochemistry; RT-qPCR, reverse transcription quantitative polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; VEGF, vascular endothelial growth factor; VEGFA, vascular endothelial growth factor A; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2; CD34, cluster of differentiation 34.

Reduced Notch-1 signaling and PI3K/AKT phosphorylation in chorionic villi from SA cases

We next examined Notch-1/DII4 expression and PI3K/AKT pathway activity in villous tissues. IHC analysis showed lower Notch-1, DII4, PI3K, and AKT staining intensities in SA villi than in NC villi (**Figure 2A**). RT-qPCR further showed reduced Notch-1 and AKT mRNA levels in the SA group (**Figure 2B**). Western blot analysis confirmed decreased Notch-1 protein abundance in SA villous tissues, while DII4 protein showed a downward trend (**Figure 2C**). To further evaluate pathway activation, phosphorylation and total AKT, PI3K, and S6 were examined.

The p-AKT Ser473/AKT, p-PI3K/PI3K, and p-S6/S6 ratios were all reduced in SA villi compared with NC villi (**Figure 2D, 2E**). These results indicate that SA villous tissues are characterized by reduced Notch-1/DII4-associated signaling and attenuated PI3K/AKT pathway phosphorylation.

DAPT and LY294002 attenuated HTR-8/SVneo trophoblast pro-angiogenic behavior without marked cytotoxicity

To exclude overt viability-related effects under the experimental conditions, HTR-8/SVneo

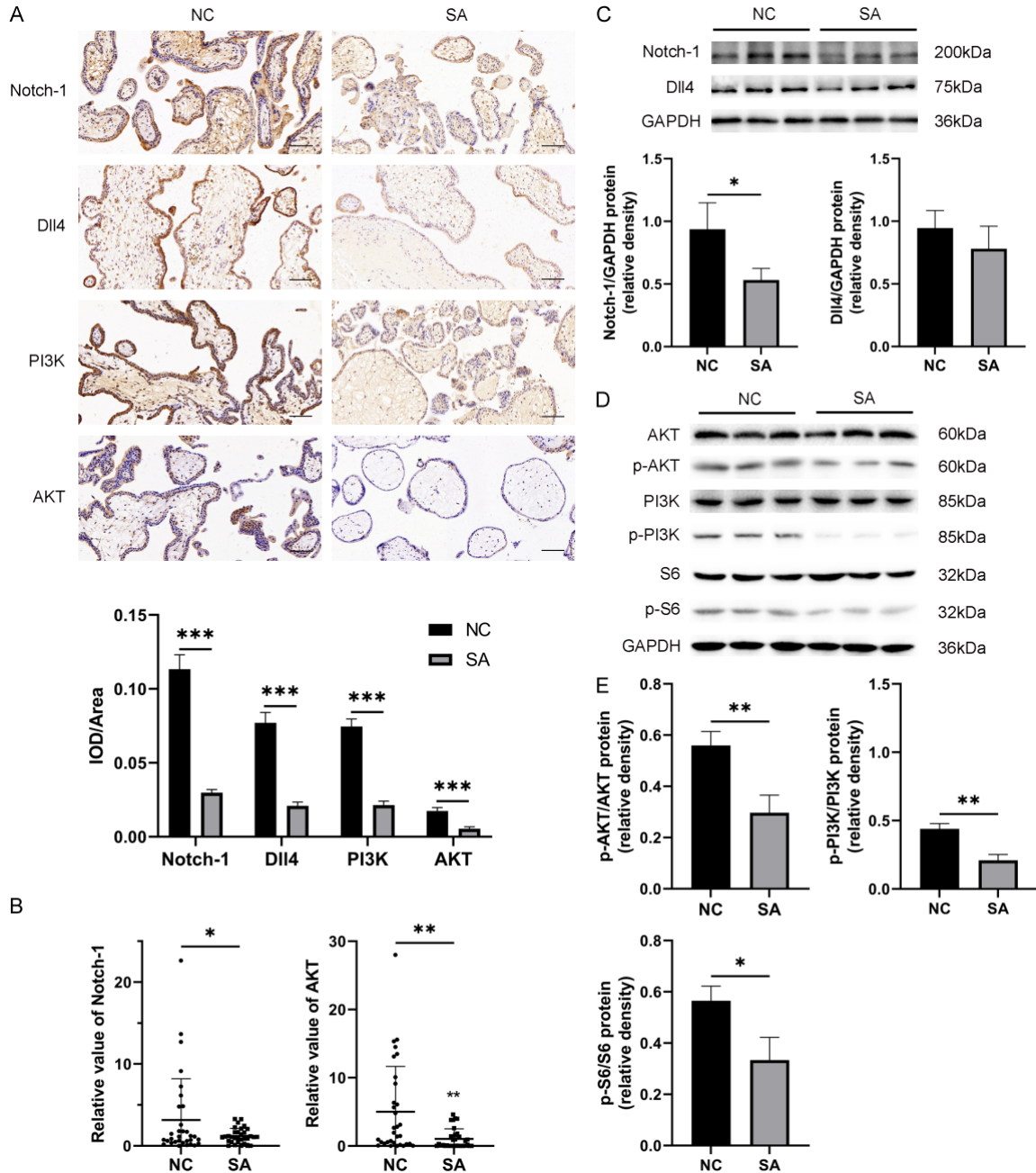


Figure 2. Reduced Notch-1/Dll4 expression and PI3K/AKT pathway phosphorylation in SA villous tissues. **A.** Representative IHC images and quantification of Notch-1, Dll4, PI3K, and AKT in first-trimester villous tissues from NC and SA groups. Scale bar, 100 μ m. **B.** RT-qPCR analysis of Notch-1 and AKT mRNA expression in villous tissues. **C.** Western blot analysis and quantification of Notch-1 and Dll4 protein expression in villous tissues. **D, E.** Western blot analysis of AKT, p-AKT Ser473, PI3K, p-PI3K, S6, and p-S6, with quantification of p-AKT Ser473/AKT, p-PI3K/PI3K, and p-S6/S6 ratios. Data are presented as mean $\pm$ SD or median (P25, P75), as appropriate. $n = 40$ per group for IHC and RT-qPCR; $n = 3$ per group for Western blotting. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NC. NC, normal early-pregnancy control; SA, spontaneous abortion; IHC, immunohistochemistry; RT-qPCR, reverse transcription quantitative polymerase chain reaction; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; p-, phosphorylated; S6, ribosomal protein S6; Dll4, Delta-like ligand 4.

cells were treated with different concentrations of DAPT or LY294002 for 24 h, and cell viability was assessed by CCK-8 assay. Neither inhibitor

markedly reduced cell viability within the tested concentration range (**Figure 3A**). At the working concentration used for subsequent assays,

Notch-1/PI3K/AKT signaling in spontaneous abortion

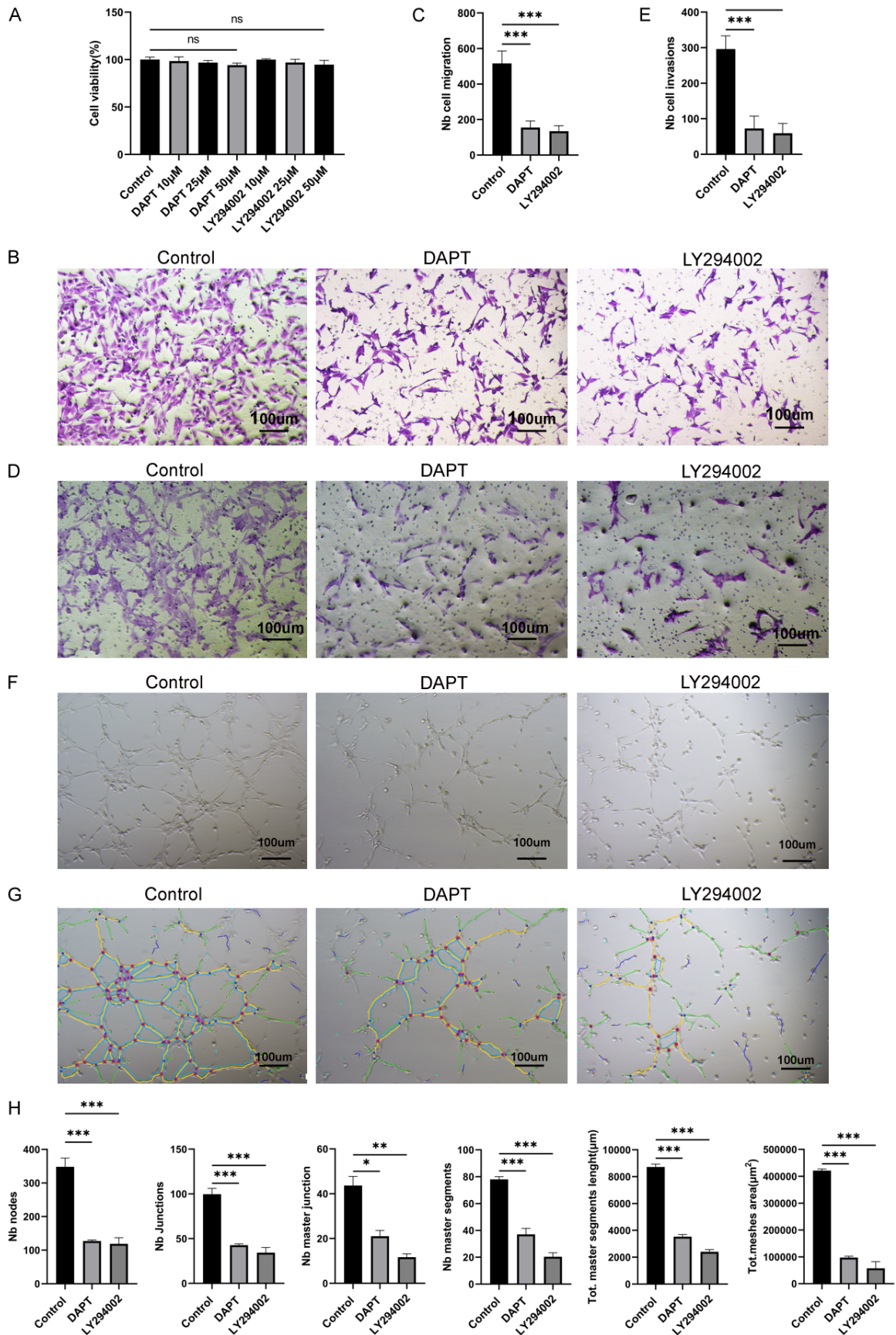


Figure 3. DAPT and LY294002 attenuated HTR-8/SVneo trophoblast pro-angiogenic behavior without marked cytotoxicity. A. CCK-8 assay of HTR-8/SVneo cell viability after treatment with different concentrations of DAPT or LY294002 for 24 h. B, C. Representative Transwell images and quantification of HTR-8/SVneo cell migration after DAPT or LY294002 treatment. D, E. Representative Matrigel-coated Transwell images and quantification of HTR-8/SVneo cell invasion after DAPT or LY294002 treatment. F, G. Representative images and analyzed images of capillary-like network formation by HTR-8/SVneo cells on Matrigel. H. Quantitative analysis of network parameters, including node number, junction number, master junction number, segment number, total segment length, and total mesh area. Scale bar, 100 μ m. Data are presented as mean $\pm$ SD; n = 3 independent experiments. ns, not significant; * P < 0.05, ** P < 0.01, *** P < 0.001 vs. Control. CCK-8, Cell Counting Kit-8; DAPT, γ -secretase inhibitor; LY294002, PI3K inhibitor; PI3K, phosphoinositide 3-kinase; HTR-8/SVneo, human first-trimester extravillous trophoblast cell line.

both DAPT and LY294002 reduced HTR-8/SVneo cell migration through uncoated Transwell membranes and invasion through Matrigel-coated membranes (**Figure 3B-E**). In the Matrigel-based network assay, inhibitor-treated cells formed fewer capillary-like structures than control cells, characterized by fewer nodes, junctions, master junction number, segment number, total segment length, and total mesh area (**Figure 3F-H**). These findings indicate that pharmacological inhibition of Notch or PI3K/AKT signaling attenuates HTR-8/SVneo trophoblast pro-angiogenic behavior in vitro without marked cytotoxicity.

DAPT and LY294002 attenuated Notch-1/PI3K/AKT signaling and angiogenesis-related protein expression in HTR-8/SVneo cells

We next expanded the Western blot analysis to define the signaling changes associated with DAPT- or LY294002-induced impairment of HTR-8/SVneo trophoblast behavior. DAPT treatment reduced Notch-1, DII4, and NICD protein abundance, confirming effective Notch pathway inhibition. This was accompanied by decreased p-PI3K/PI3K, p-AKT/AKT, and p-S6/S6 ratios, together with reduced VEGFA, VEGFR1, and VEGFR2 expression (**Figure 4A, 4B**). LY294002 treatment reduced p-AKT/AKT and p-S6/S6 ratios, whereas p-PI3K/PI3K showed no significant change. LY294002 also decreased Notch-1, DII4, and NICD protein abundance and reduced VEGFA, VEGFR1, and VEGFR2 expression (**Figure 4C, 4D**). These results indicate that pharmacological inhibition of Notch or PI3K signaling converges on reduced trophoblast pro-angiogenic signaling, as reflected by attenuated Notch-1/DII4-associated signaling, downstream PI3K/AKT pathway activity, and angiogenesis-related protein expression.

DAPT and LY294002 decreased Notch-1 and AKT immunofluorescence signals in HTR-8/SVneo cells

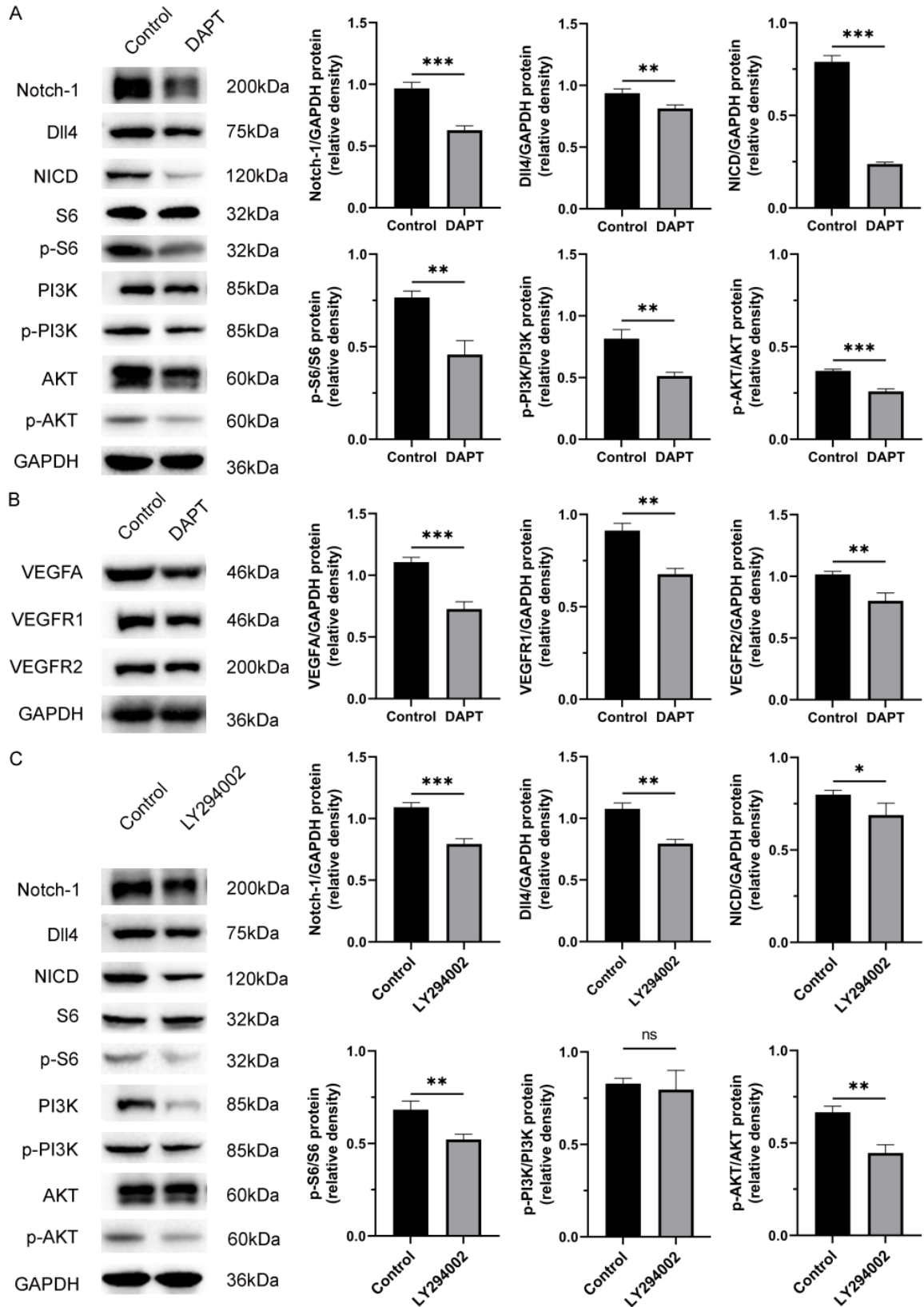
Immunofluorescence staining was performed to visualize Notch-1 and AKT signals in HTR-8/SVneo cells after DAPT or LY294002 treatment. Compared with control cells, both DAPT- and LY294002-treated cells showed reduced Notch-1 fluorescence intensity, as confirmed by quantitative analysis (**Figure 5A**). AKT fluorescence intensity was also decreased after DAPT or LY294002 treatment (**Figure 5B**). These results were consistent with the Western blot data and showed reduced Notch-1 and AKT signals following pharmacological inhibition of Notch or PI3K signaling in HTR-8/SVneo cells.

Discussion

In this study, SA villi showed a coordinated attenuation of angiogenesis-related signaling, Notch-1/DII4 expression, and PI3K/AKT pathway phosphorylation. The decreases in VEGFA, VEGFR1, VEGFR2, CD34, and serum VEGF suggest compromised villous vascular support at the maternal-fetal interface, while the parallel reductions in Notch-1/DII4 and PI3K/AKT phosphorylation suggest that this vascular alteration occurs within a broader change in trophoblast-associated signaling. These findings are consistent with studies linking placental vascular insufficiency and angiogenic imbalance to pregnancy complications [15, 16], and align with the established central role of VEGF-related signaling in early embryonic and placental vascular development [17-21]. Together, the tissue results suggest that early pregnancy loss is associated with a broader disturbance of the trophoblast-vascular microenvironment.

Notch signaling is closely integrated into cell-cell communication within the placental villous

Notch-1/PI3K/AKT signaling in spontaneous abortion



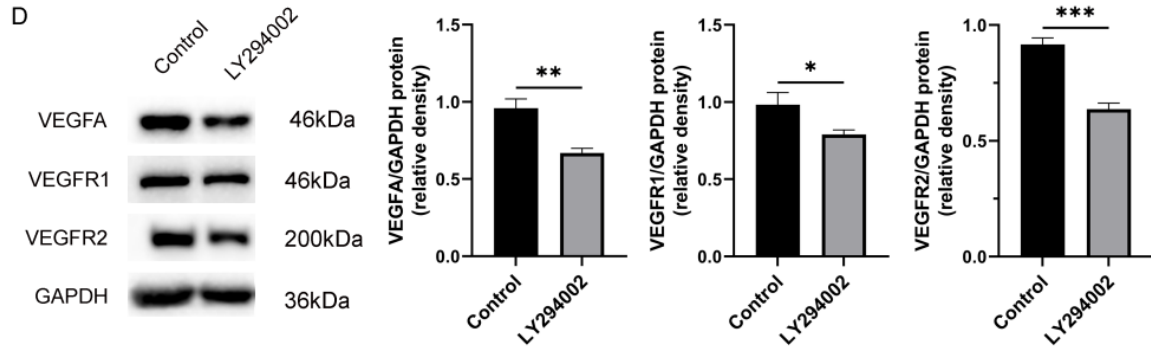


Figure 4. Effects of DAPT and LY294002 on Notch-1/PI3K/AKT signaling and angiogenesis-related proteins in HTR-8/SVneo cells. A. Western blot analysis and quantification of Notch pathway components and PI3K/AKT pathway phosphorylation after DAPT treatment. B. Western blot analysis and quantification of VEGFA, VEGFR1, and VEGFR2 expression after DAPT treatment. C. Western blot analysis and quantification of Notch pathway components and PI3K/AKT pathway phosphorylation after LY294002 treatment. D. Western blot analysis and quantification of VEGFA, VEGFR1, and VEGFR2 expression after LY294002 treatment. Total protein expression was normalized to GAPDH, and phosphorylation levels were quantified as p-PI3K/PI3K, p-AKT/AKT, and p-S6/S6 ratios. Data are presented as mean $\pm$ SD; $n = 3$ independent experiments. ns, not significant; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. Control. NICD, Notch intracellular domain; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; p-, phosphorylated; VEGFA, vascular endothelial growth factor A; VEGFR1, vascular endothelial growth factor receptor 1; VEGFR2, vascular endothelial growth factor receptor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

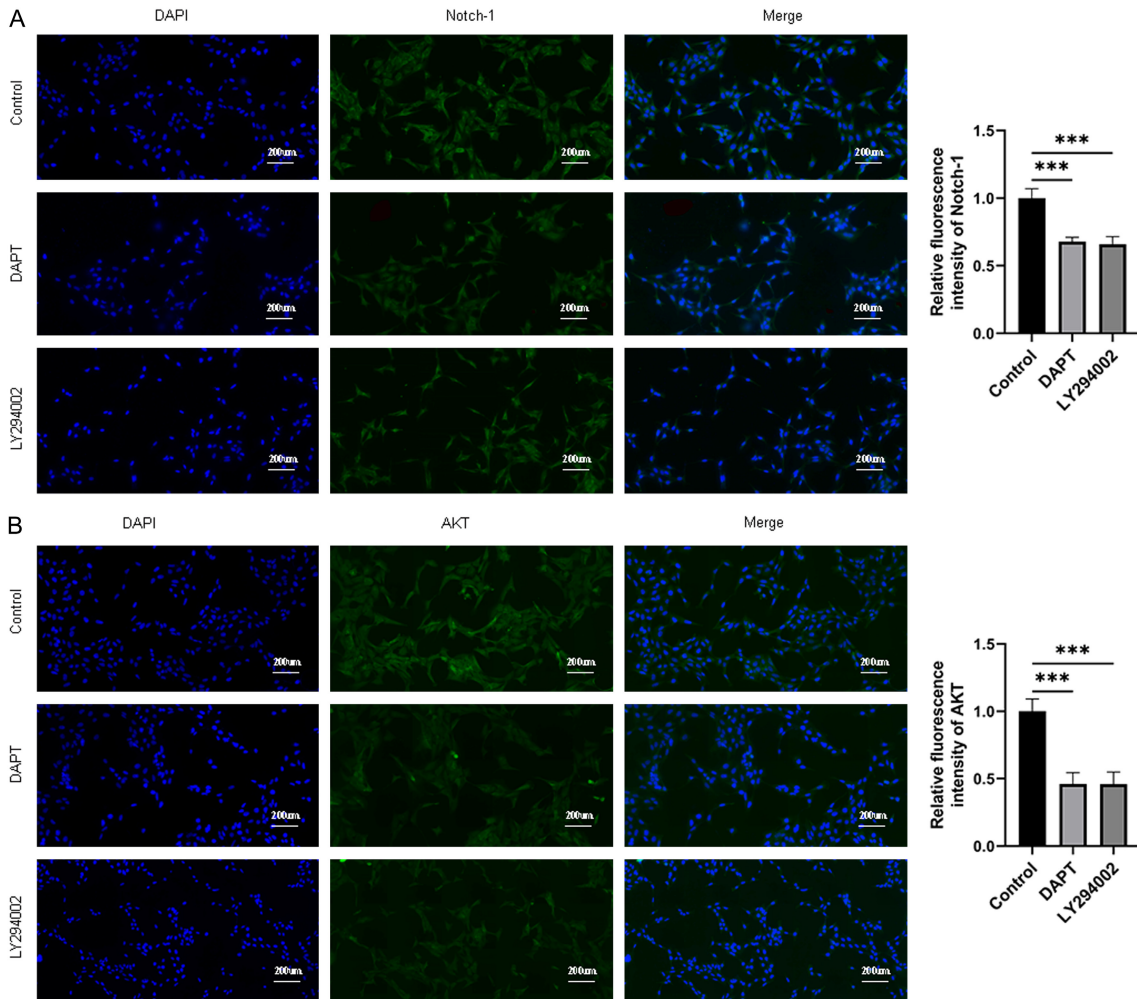


Figure 5. DAPT and LY294002 decreased Notch-1 and AKT immunofluorescence signals in HTR-8/SVneo cells. A. Representative Notch-1 immunofluorescence images and quantitative analysis in HTR-8/SVneo cells following DAPT or LY294002 treatment. Notch-1 is shown in green, and nuclei counterstained with DAPI are shown in blue. B. Representative immunofluorescence images and quantitative analysis of AKT under the same treatment conditions. AKT is shown in green, and DAPI-labeled nuclei are shown in blue. Scale bar, 200 μ m. Data are presented as mean $\pm$ SD; n = 3 independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. Control. DAPT, γ -secretase inhibitor; LY294002, PI3K inhibitor; AKT, protein kinase B; DAPI, 4',6-diamidino-2-phenylindole.

microenvironment. Notch signaling has been widely implicated in cell proliferation, differentiation, and vascular remodeling across disease contexts [22-26]. In the placenta, this pathway is embedded in villous cell-cell communication, with Notch receptors and ligands expressed across trophoblast, endothelial, stromal, and immune-related compartments [27]. Notch-related signaling has also been implicated in trophoblast proliferation, migration, and invasion in models of placental disorders [28]. The Notch-1/Dll4 axis is closely associated with vascular remodeling and angiogenic patterning [29-32]. In trophoblast models, Notch-1 signaling has been linked to migratory and invasive behavior [33], and Notch activity is also required for trophoblast endovascular invasion and placental vascular adaptation [34]. In this cohort, the reduction of Notch-1 and Dll4 occurred together with decreased VEGF-related markers, placing Notch attenuation within the altered angiogenesis-related profile of SA villi. Fang et al. reported dysregulated Dll4/Notch and HIF-1 α -VEGF signaling in missed abortion, characterized by reduced VEGF expression but increased Notch1 expression [35]. The different direction of Notch1 change between Fang et al. and the present study suggests that Notch signaling in pregnancy loss may depend on disease stage, villous cellular composition, and compensatory responses within the local hypoxic-angiogenic environment.

The PI3K/AKT phosphorylation results further link the villous vascular phenotype with trophoblast functional signaling. Previous trophoblast studies have shown that PI3K/AKT signaling participates in migration, invasion, autophagy, hormone secretion, and survival-related responses [36-38], and PI3K-dependent signaling has also been linked to VEGF expression and angiogenic regulation in endothelial systems [39]. In the present study, SA villi showed reduced p-PI3K/PI3K, p-AKT Ser473/AKT, and p-S6/S6 ratios, further extending the findings from reduced total protein levels to suppressed

phosphorylation-dependent signaling. The concurrent reduction of PI3K/AKT phosphorylation and VEGF-related markers indicates that weakened trophoblast signaling and impaired villous vascular support are closely coupled in SA.

The HTR-8/SVneo experiments further linked these pathway changes to trophoblast behavior. DAPT-mediated Notch inhibition and LY294002-mediated PI3K inhibition reduced trophoblast migration, Matrigel invasion, and capillary-like network formation, while the CCK-8 assay showed no marked cytotoxicity at the working concentration. Because HTR-8/SVneo cells are trophoblast-derived cells, the Matrigel network assay reflects trophoblast pro-angiogenic behavior in vitro. At the molecular level, inhibition of either pathway reduced angiogenesis-related protein expression and was accompanied by coordinated changes in Notch-1/Dll4 abundance and PI3K/AKT phosphorylation. These findings position Notch-1/Dll4 signaling and PI3K/AKT phosphorylation as components of a shared trophoblast pro-angiogenic signaling program that supports migration, invasion, capillary-like network formation, and angiogenesis-related protein expression.

Several aspects of this study warrant further investigation. The clinical tissue analysis was based on first-trimester villous samples and revealed a consistent association between SA and altered angiogenesis-related signaling, but the observational design precludes definitive conclusions about causality. HTR-8/SVneo cells were used to model trophoblast behavior in vitro; although this model is useful for assessing migration, invasion, and pro-angiogenic responses, it cannot fully recapitulate the cellular heterogeneity of early villous tissues. In addition, capillary-like network formation on Matrigel reflects trophoblast pro-angiogenic behavior rather than direct in vivo angiogenesis. Future studies using primary trophoblasts, villous explants, genetic gain- and loss-of-function approaches, and pathway rescue experi-

ments will help clarify how Notch-1/Dll4 and PI3K/AKT signaling are functionally organized during SA-associated villous vascular dysfunction.

In conclusion, this study shows that SA villi are characterized by reduced VEGF-related angiogenic support, attenuated Notch-1/Dll4 expression, and decreased PI3K/AKT pathway phosphorylation. Inhibition of Notch or PI3K signaling impaired HTR-8/SVneo cell migration, Matrigel invasion, and capillary-like network formation, together with reduced angiogenesis-related protein expression. These findings indicate that Notch-1/Dll4 and PI3K/AKT signaling participate in trophoblast pro-angiogenic regulation at the maternal-fetal interface and provide a molecular basis for understanding impaired villous vascular support in early pregnancy loss.

Acknowledgements

This work received support from the National Natural Science Foundation of China (81873336) and the Doctoral Research Start-up Fund of Henan Province Hospital of Traditional Chinese Medicine (The Second Affiliated Hospital of Henan University of Chinese Medicine) (2022BSJJ05).

Disclosure of conflict of interest

None.

Address correspondence to: Jie Gao, Department of Gynecology, The First Affiliated Hospital of Guangzhou University of Chinese Medicine, No. 12 Jichang Road, Baiyun District, Guangzhou 510405, Guangdong, China. Tel: +86-13760605046; Fax: +86-020-36588217; E-mail: gaojie1769@gzucm.edu.cn; Songping Luo, Lingnan Gynecology Research Institute, The First Affiliated Hospital of Guangzhou University of Chinese Medicine, No. 12 Jichang Road, Baiyun District, Guangzhou 510405, Guangdong, China. Tel: +86-13005156625; Fax: +86-020-36588217; E-mail: luosongping@gzucm.edu.cn

References

- [1] Rai R and Regan L. Recurrent miscarriage. *Lancet* 2006; 368: 601-611.
- [2] Green DM and O'Donoghue K. A review of reproductive outcomes of women with two consecutive miscarriages and no living child. *J Obstet Gynaecol* 2019; 39: 816-821.

- [3] Ticconi C, Pietropolli A, Specchia M, Nicastri E, Chiamonte C, Piccione E, Scambia G and Di Simone N. Pregnancy-related complications in women with recurrent pregnancy loss: a prospective cohort study. *J Clin Med* 2020; 9: 2833.
- [4] Ji L, Brkić J, Liu M, Fu G, Peng C and Wang YL. Placental trophoblast cell differentiation: physiological regulation and pathological relevance to preeclampsia. *Mol Aspects Med* 2013; 34: 981-1023.
- [5] Rizov M, Andreeva P and Dimova I. Molecular regulation and role of angiogenesis in reproduction. *Taiwan J Obstet Gynecol* 2017; 56: 127-132.
- [6] Faló-Sanjuan J and Bray SJ. Decoding the Notch signal. *Dev Growth Differ* 2020; 62: 4-14.
- [7] Afshar Y, Jeong JW, Roqueiro D, DeMayo F, Lydon J, Radtke F, Radnor R, Miele L and Fazleabas A. Notch1 mediates uterine stromal differentiation and is critical for complete decidualization in the mouse. *FASEB J* 2012; 26: 282-294.
- [8] Haider S, Pollheimer J and Knöfler M. Notch signalling in placental development and gestational diseases. *Placenta* 2017; 56: 65-72.
- [9] Xue Y, Sun R, Zheng W, Yang L and An R. Forskolin promotes vasculogenic mimicry and invasion via Notch-1-activated epithelial-to-mesenchymal transition in syncytiolization of trophoblast cells in choriocarcinoma. *Int J Oncol* 2020; 56: 1129-1139.
- [10] Glaviano A, Foo ASC, Lam HY, Yap KCH, Jacot W, Jones RH, Eng H, Nair MG, Makvandi P, Georger B, Kulke MH, Baird RD, Prabhu JS, Carbone D, Pecoraro C, Teh DBL, Sethi G, Cavalieri V, Lin KH, Javidi-Sharifi NR, Toska E, Davids MS, Brown JR, Diana P, Stebbing J, Fruman DA and Kumar AP. PI3K/AKT/mTOR signaling transduction pathway and targeted therapies in cancer. *Mol Cancer* 2023; 22: 138.
- [11] Samakova A, Gazova A, Sabova N, Valaskova S, Jurikova M and Kyselovic J. The PI3k/Akt pathway is associated with angiogenesis, oxidative stress and survival of mesenchymal stem cells in pathophysiologic condition in ischemia. *Physiol Res* 2019; 68 Suppl 2: S131-S138.
- [12] Furmento VA, Marino J, Blank VC and Roguin LP. The granulocyte colony-stimulating factor (G-CSF) upregulates metalloproteinase-2 and VEGF through PI3K/Akt and Erk1/2 activation in human trophoblast Swan 71 cells. *Placenta* 2014; 35: 937-946.
- [13] Karar J and Maity A. PI3K/AKT/mTOR pathway in angiogenesis. *Front Mol Neurosci* 2011; 4: 51.

- [14] Chaudhary P, Babu GS, Sobti RC and Gupta SK. HGF regulate HTR-8/SVneo trophoblastic cells migration/invasion under hypoxic conditions through increased HIF-1 α expression via MAPK and PI3K pathways. *J Cell Commun Signal* 2019; 13: 503-521.
- [15] Alfaidy N, Brouillet S, Rajaraman G, Kalionis B, Hoffmann P, Barjat T, Benharouga M and Murthi P. The emerging role of the prokinetins and homeobox genes in the vascularization of the placenta: physiological and pathological aspects. *Front Physiol* 2020; 11: 591850.
- [16] Cho S, Sohn YD, Kim S, Rajakumar A, Badell ML, Sidell N and Yoon YS. Reduced angiogenic and increased inflammatory profiles of cord blood cells in severe but not mild preeclampsia. *Sci Rep* 2021; 11: 3630.
- [17] Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ and Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 1996; 380: 439-442.
- [18] Carmeliet P, Lampugnani MG, Moons L, Breviaro F, Compernelle V, Bono F, Balconi G, Spagnuolo R, Oosthuysen B, Dewerchin M, Zanetti A, Angellilo A, Mattot V, Nuyens D, Lutgens E, Clotman F, de Ruiter MC, Gittenberger-de Groot A, Poelmann R, Lupu F, Herbert JM, Collen D and Dejana E. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* 1999; 98: 147-157.
- [19] Subi TM, Krishnakumar V, Kataru CR, Panigrahi I and Kannan M. Association of VEGF and p53 polymorphisms and spiral artery remodeling in recurrent pregnancy loss: a systematic review and meta-analysis. *Thromb Haemostasis* 2022; 122: 363-376.
- [20] Binder NK, Evans J, Gardner DK, Salamonsen LA and Hannan NJ. Endometrial signals improve embryo outcome: functional role of vascular endothelial growth factor isoforms on embryo development and implantation in mice. *Hum Reprod* 2014; 29: 2278-2286.
- [21] Hiratsuka S, Kataoka Y, Nakao K, Nakamura K, Morikawa S, Tanaka S, Katsuki M, Maru Y and Shibuya M. Vascular endothelial growth factor A (VEGF-A) is involved in guidance of VEGF receptor-positive cells to the anterior portion of early embryos. *Mol Cell Biol* 2005; 25: 355-363.
- [22] Wu Y, Chen X, Dong W, Xu Z, Jian Y, Xu C, Zhang L, Wei A, Yu X, Wang S, Wang Y, Liu G, Sun X and Wang S. ST3Gal IV mediates the growth and proliferation of cervical cancer cells in vitro and in vivo via the Notch/p21/CDKs pathway. *Front Oncol* 2021; 10: 540332.
- [23] Perez-Fidalgo JA, Ortega B, Simon S, Samartzis EP and Boussios S. NOTCH signalling in ovarian cancer angiogenesis. *Ann Transl Med* 2020; 8: 1705.
- [24] Zhang N, Ji J, Zhou D, Liu X, Zhang X, Liu Y, Xiang W, Wang M, Zhang L, Wang G, Huang B, Lu J and Zhang Y. The interaction of the senescent and adjacent breast cancer cells promotes the metastasis of heterogeneous breast cancer cells through Notch signaling. *Int J Mol Sci* 2021; 22: 849.
- [25] O'Brien R and Marignol L. The Notch-1 receptor in prostate tumorigenesis. *Cancer Treat Rev* 2017; 56: 36-46.
- [26] Zhang S. Cancer stem cells promote tumorigenesis and progression of pancreatic cancer through Notch-1 pathway. *Cancer Invest* 2026; 44: 26-38.
- [27] Herr F, Schreiner I, Baal N, Pfarrer C and Zygmunt M. Expression patterns of Notch receptors and their ligands jagged and delta in human placenta. *Placenta* 2011; 32: 554-563.
- [28] Jia X, Yang S, Wang X, Ruan J and Huang W. HOXB3 promotes trophoblast cell proliferation, invasion, and migration to alleviate preeclampsia via mediating the Notch/Wnt/beta-catenin pathway. *Eur J Pharmacol* 2023; 960: 176015.
- [29] Wu ZQ, Rowe RG, Lim KC, Lin Y, Willis A, Tang Y, Li XY, Nor JE, Maillard I and Weiss SJ. A Snail1/Notch1 signalling axis controls embryonic vascular development. *Nat Commun* 2014; 5: 3998.
- [30] Hainaud P, Contrerès JO, Villemain A, Liu LX, Plouët J, Tobelem G and Dupuy E. The role of the vascular endothelial growth factor-Delta-like 4 ligand/Notch4-ephrin B2 cascade in tumor vessel remodeling and endothelial cell functions. *Cancer Res* 2006; 66: 8501-8510.
- [31] Zhu G, Lin Y, Liu H, Jiang D, Singh S, Li X, Yu Z, Fan L, Wang S, Rhen J, Li W, Xu Y, Ge J and Pang J. Dll4-Notch1 signaling but not VEGF-A is essential for hyperoxia induced vessel regression in retina. *Biochem Biophys Res Commun* 2018; 507: 400-406.
- [32] Krebs LT, Shutter JR, Tanigaki K, Honjo T, Stark KL and Gridley T. Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. *Genes Dev* 2004; 18: 2469-2473.
- [33] Yu Y, Wang L, Tang W, Zhang D and Shang T. RNA interference-mediated knockdown of Notch-1 inhibits migration and invasion, down-regulates matrix metalloproteinases and suppresses NF-kappaB signaling pathway in trophoblast cells. *Acta Histochem* 2014; 116: 911-919.
- [34] Hunkapiller NM, Gasperowicz M, Kapidzic M, Plaks V, Maltepe E, Kitajewski J, Cross JC and Fisher SJ. A role for Notch signaling in tropho-

- blast endovascular invasion and in the pathogenesis of pre-eclampsia. *Development* 2011; 138: 2987-2998.
- [35] Fang Y, Yu S, Ma Y, Sun P, Ma D, Ji C and Kong B. Association of Dll4/Notch and HIF-1 α -VEGF signaling in the angiogenesis of missed abortion. *PLoS One* 2013; 8: e70667.
- [36] Xu J, Xia Y, Zhang H, Guo H, Feng K and Zhang C. Overexpression of long non-coding RNA H19 promotes invasion and autophagy via the PI3K/AKT/mTOR pathways in trophoblast cells. *Biomed Pharmacother* 2018; 101: 691-697.
- [37] Li M, Cheng W, Luo J, Hu X, Nie T, Lai H, Zheng X, Li F and Li H. Loss of selenocysteine insertion sequence binding protein 2 suppresses the proliferation, migration/invasion and hormone secretion of human trophoblast cells via the PI3K/Akt and ERK signaling pathway. *Placenta* 2017; 55: 81-89.
- [38] Chen J, Yue C, Xu J, Zhan Y, Zhao H, Li Y and Ye Y. Downregulation of receptor tyrosine kinase-like orphan receptor 1 in preeclampsia placenta inhibits human trophoblast cell proliferation, migration, and invasion by PI3K/AKT/mTOR pathway accommodation. *Placenta* 2019; 82: 17-24.
- [39] Jiang BH, Zheng JZ, Aoki M and Vogt PK. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. *Proc Natl Acad Sci U S A* 2000; 97: 1749-1753.

Notch-1/PI3K/AKT signaling in spontaneous abortion

Supplementary Table 1. Logistic regression analysis of the associations of maternal age and previous spontaneous abortion history with SA status

Variable	Category	Crude OR (95% CI)	Crude <i>P</i> value	Adjusted OR (95% CI)	Adjusted <i>P</i> value
Age	Per 1-year increment	1.108 (1.010-1.216)	0.029	1.109 (1.008-1.220)	0.033
Previous spontaneous abortions	0 times (reference)	1.00	-	1.00	-
	1 time	1.312 (0.490-3.517)	0.589	1.062 (0.379-2.977)	0.909
	≥ 2 times	2.625 (0.748-9.210)	0.132	2.480 (0.678-9.074)	0.170

Data are presented as odds ratios (ORs) with 95% confidence intervals (CIs). Logistic regression analysis was performed with SA status as the outcome variable and the NC group as the reference. The adjusted model included maternal age and previous spontaneous abortion history. Previous spontaneous abortion history was entered as a categorical variable, with 0 occurrences as the reference category. SA, spontaneous abortion; NC, normal early-pregnancy control; OR, odds ratio; CI, confidence interval.