Expression of ADAMTS13 in human endometrium and its potential role in recurrent pregnancy loss

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Abstract

Introduction: The mechanisms underlying the pathogenesis of recurrent pregnancy loss (RPL) remain poorly known and effective approaches to treat this disease are lacking. Proteinases of the ADAMTS family play important roles in embryonic growth and development. Our previous study suggested a role of ADAMTS13 during pregnancy. The current study aimed to determine the expression of ADAMTS13 in human endometrium and its association with RPL.

Material and methods: The spatiotemporal expression of ADAMTS13 in human endometrium was examined by immunohistochemistry. Real-time PCR and Western blot were then employed to determine the mRNA and protein expression levels of ADAMTS13 in human endometrium. Proteolytic cleavage of FRETS-VWF73 was performed to determine the activity of ADAMTS13 in plasma and that secreted by human endometrium. ELISA was carried out to measure plasma VWF antigen.

Results: We found that proteolytically active ADAMTS13 is expressed in human endometrium throughout the menstrual cycle and pregnancy. The decidual expression levels of mRNA and protein in women with RPL were significantly lower compared with women with uncomplicated pregnancies ($p < 0.01$, $p < 0.05$, respectively). Furthermore, significantly reduced plasma ADAMTS13 activity (median (range) 69.09% (65.2–93.7%) vs. 93.62% (88.1–115.6%), $p < 0.001$) and elevated plasma VWF antigen levels (median (range) of 125.5% (54.2–262.8%) vs. 91.9% (80.4–138.7%), $p < 0.01$) were detected in RPL patients compared with the control group.

Conclusions: These findings suggest that ADAMTS13 may play a role in embryo implantation and the pathogenesis of recurrent pregnancy loss. Further investigation on ADAMTS13 gene knockout animal models is necessary for understanding the molecular mechanisms of the biological roles of ADAMTS13 during gestation.

Key words: ADAMTS13, VWF, expression, endometrium, decidua, recurrent pregnancy loss.

Introduction

Recurrent pregnancy loss (RPL) currently refers to two or more consecutive spontaneous losses until 20 weeks of gestation, which frustrates 1–5% of childbearing couples [1]. Possible causes of RPL include genetic defects, anatomical abnormalities, immune dysfunctions, infections, endocrine disturbances, thrombotic disorders and unexplained causes [1–3]. Although some couples have benefited from targeted interventions, the specific mechanisms underlying the pathogenesis of RPL remain poorly

understood and there is a lack of effective treatments.

Degradation of the extracellular matrix (ECM) and angiogenesis in the human endometrium are crucial processes necessary for the morphological and functional changes of the endometrium during the menstrual cycle and pregnancy [4, 5]. As a novel family of secreted metalloproteinases, ADAMTS (a disintegrin and metalloproteinase with thrombospondin repeats) is composed of more than 20 genetically distinct subtypes in humans [6]. Accumulating evidence converges to suggest that these novel proteinases play important roles in embryonic growth and development [7] and the cyclic remodeling events that take place in reproductive tissues [8]. Vascular endothelial growth factor (VEGF) is believed to play a key role in angiogenesis in the endometrium [4]. However, as abnormal angiogenesis and aberrant distribution or expression of ECM components in the endometrial stroma have been related to infertility and pregnancy loss [9], it is necessary to determine the full repertoire of proteinases expressed in the endometrium, their distribution and regulation, and, ultimately, their individual role(s) in the development of a uterine environment that is capable of sustaining pregnancy.

ADAMTS13 is mainly synthesized in the liver [10], and its primary function is to cleave von Willebrand factor (VWF) in the circulation, anchored on the endothelial surface, and at the sites of vascular injury. Severe deficiency of plasma ADAMTS13 activity resulting from ADAMTS13 gene mutations or autoantibodies against ADAMTS13 leads to hereditary or acquired (idiopathic) thrombotic thrombocytopenic purpura (TTP) [11, 12]. Recently, ADAMTS13 has been detected in a broad spectrum of human cells including endothelial cells, megakaryocytes, platelets, renal tubular epithelial cells, glomerular endothelial cells and glomerular podocytes at various levels [13]. We previously reported that ADAMTS13 is expressed in human placental tissue, as well as in placental explant and trophoblast cell cultures, and that it can promote angiogenesis and trophoblastic cell development [14]. Significantly reduced levels of placental ADAMTS13 expression were detected in patients with preeclampsia [14]. These findings suggest a role of ADAMTS13 during pregnancy. However, the expression of ADAMTS13 in the human endometrium has never been reported. The changes of plasma ADAMTS13 activity and plasma VWF antigen level in RPL patients are rarely reported. Therefore, our aim was to determine the expression of ADAMTS13 in the endometrium and its association with RPL.

Material and methods

Tissue collection

All study patients were recruited from The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China, between January 2018 and January 2019. This study was reviewed and approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University, and informed consent was obtained from all participants. The endometrial specimens (10 proliferative; 10 secretory) were obtained from premenopausal women undergoing surgery for non-endometrial pathology, such as leiomyomata or benign ovarian cysts. Data from only those tissues that were found to be

normal by microscopic examination are reported here. An experienced pathologist determined the endometrial dating according to the criteria of Noyes *et al*. [15]. Decidual tissues and plasma were obtained from 30 patients undergoing early pregnancy terminations for psychosocial reasons and 25 unexplained RPL patients undergoing therapeutic abortions of early gestations. RPL was defined as the loss of two or more consecutive pregnancies before the 20th week of gestation [1]. Ectopic pregnancy, hydatidiform mole, and biochemical pregnancy were excluded from their pregnancy histories in clinical assessment. All RPL patients underwent karyotype analysis of the aborted embryo. RPL patients with abnormal karyotype, uterine abnormalities, endocrine abnormalities, or immunologic dysfunctions were excluded. The third trimester decidual tissues (n = 10) from normal pregnancies were picked up from the gauze used to scrub the uterus immediately after cesarean section.

In the present study we exclusively used fresh endometrial and decidual tissue samples; any histological signs of tissue damage such as necrosis, old blood clots or infections were excluded from the study. One part was fixed in 4% buffered formalin for subsequent paraffin embedding and the major part was either snap-frozen in liquid nitrogen and stored at –80°C for RNA and protein extraction or used immediately for explant culture. Prior to RNA/protein isolation, tissue samples were cut into sections on a cryostat. One representative section of each sample was stained with hematoxylin to define representative tissues without signs of necrosis. Tissue was selected to obtain approximately equal amounts of stromal tissue, glands and other structures. For decidual fragments, another section was stained immunohistochemically with HLA-G to identify extravillous trophoblast. Only those decidual samples with invasive trophoblasts and no signs of necrosis were chosen for RNA and protein extraction.

Normal liver tissue was obtained from the resected livers of patients with liver cancer prior to chemotherapy and/or radiotherapy. Tissue was taken from areas unaffected by the cancer and determined as normal by the pathologist.

Antibodies

Polyclonal rabbit anti-human ADAMTS13 antibody (F481) was produced at the University of Alabama at Birmingham, Birmingham, Alabama using a recombinant human ADAMTS13 variant with the CUB domain deleted (i.e. DelCUB) and recombinant murine full-length ADAMTS13 at the 9:1 ratio (Open Biosystems, Huntsville, AL). Monoclonal mouse anti-human HLA-G antibody and monoclonal rabbit anti-human CD34 antibody were purchased from Abcam (Cambridge, UK). Isotype rabbit IgG and mouse IgG were purchased from Sigma (Aldrich, St. Louis, USA).

Cell culture and transient expression of ADAMTS13

Chinese hamster ovary (CHO) cells were cultured in DMEM/F-12 containing 10% fetal bovine serum (FBS, Gibco BRL, Carlsbad, CA, USA), 50 U/ml penicillin and 50 mg/ml streptomycin in a 5% CO_2 humidified incubator at 37°C. Cells were seeded onto 6-well culture plates and grown to 95% confluence prior to transfection. Transfection was carried out with a plasmid encoding full-length human ADAMTS13 mixed with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Cells were harvested after 48 h of transfection and stored at –80°C until use.

Organ culture

Endometrial tissues and decidual tissues were prepared and cultured as previously described [16, 17]. All dissection was performed in a laminar-flow hood where the tissue was rinsed twice in fresh medium. Decidual tissues were divided into decidua parietalis which was free of fetal tissues and decidua capsularis in which trophoblast cells were attached to the decidua, and the latter were used for culture. Endometrial tissues and decidual tissues were minced into small pieces ~2 mm^3 and then plated to a density of 30–40% of the well in small pieces using 6-well culture plates. Explant culture proceeded in 3 ml of serum-free DMEM/F-12 media, containing 2 mM glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin in a 5% CO_2 humidified incubator at 37°C. After 24 h of incubation, culture medium was collected, centrifuged and concentrated using an Amicon Ultra Centrifugal Filter Unit, 50 kDa (Millipore, Bedford, MA). The supernatant was used for VWF-cleaving activity assay. The tissues were collected and fixed in 4% buffered formalin for subsequent paraffin embedding.

RNA isolation and cDNA preparation

Total RNA from endometrial tissues was prepared with TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. The cDNA was synthesized from total RNA using a reverse transcription kit (TaKaRa, Japan) under the following conditions: 5 min incubation at 65°C for primer annealing, 37°C for 15 min for cDNA synthesis, and 5 min termination at 98°C, and then stored at –80°C until use.

Real-time PCR

Quantitation of ADAMTS13 transcripts was performed by a real-time PCR system (ABI Prism 7000; Applied Biosystems) using the SYBR Premix ExTaq Kit (TaKaRa, Japan) and adjusted to the quantitative expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the corresponding unknowns. The PCR primers used were as follows: ADAMTS13, forward, 5′-TGTG CTGC CCAA ATAC CCTA-3′ and reverse, 5′-AACG GGAA GCAA TCAC CAAC-3′; HLA-G, forward , 5′-GAGGAGACACGGAACACCAAG-3′ and reverse, 5′-GTCGCAGCCAATCATCCACT-3′; CD34, forward, 5′-CTGCCTTCTGGGTTCATGAGT-3′ and reverse, 5′-GGTGGTGAACACTGTGCTGATTAC-3′. GAPDH, forward, 5′-AAGGTCATCCCTGAGCTGAAC-3′ and reverse, 5′-ACGCCTGCTTCACCACCTTCT-3′. Normal liver RNA was used as a positive control [10]. Blank CHO cell RNA was used as a negative control [18].

Immunohistochemistry

The endometrial samples were immediately fixed with 4% neutral paraformaldehyde overnight and then embedded in paraffin wax. ADAMTS13 expression was determined by immunohistochemical staining. Briefly, paraffin-embedded endometrium sections (5 mm) were dewaxed in xylene, and then rehydrated through descending ethanol to PBS. 0.3% hydrogen peroxide in methanol was used to quench the endogenous peroxidase activity in the sample. Antigen retrieval was performed in preheated (95–100°C) 0.05% sodium citrate buffer for 20 minutes. The sections were blocked with goat serum, and then incubated with primary antibodies against ADAMTS13 (20 µg/ml) or HLA-G (10 µg/ml) or CD34 (10 µg/ml) at 4°C overnight. After that, the slides were washed in PBS containing 0.5% Tween 20 (Sigma, Aldrich, St. Louis, MO, USA) before incubating with horseradish peroxidase-labeled secondary antibody (anti-rabbit and anti-mouse, respectively) in a humidor at room temperature for 1 hour. Peroxidase substrate 3, 30-diaminobenzidene tetrahydrochloride (Dako, USA) was used to visualize the specific antibody binding and this was monitored

under a microscope (Olympus IX73, Tokyo, Japan) to ensure proper intensity. Finally, the sections were counterstained with hematoxylin. In the negative control experiments, primary antibodies were omitted or isotype rabbit IgG or mouse IgG (Sigma-Aldrich, St. Louis, MO, USA) was used. The immunostaining was repeated three times on sections from the same sample. Immunostaining scoring of ADAMTS13, HLA-G or CD34 in the sample was evaluated on the basis of staining intensity and positively stained areas by three independent observers as previously described [19].

Western blotting

Expression of ADAMTS13 protein in endometrium tissues was also determined by Western blotting. Total protein was prepared by lysing CHO cells, endometrial tissues with RIPA lysis buffer (Beyotiem, Shanghai, China) containing a protease inhibitor cocktail (Roche Applied Science, Indianapolis, Indian). Protein samples were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membranes were incubated sequentially with primary antibodies against ADAMTS13 (2 µg/ml) and then HRP-conjugated secondary antibody. Proteins were detected by an enhanced chemiluminescent (ECL) detection kit (Amersham, Biosciences, Uppsala, Sweden), and the image was captured using VisionWorksLS image acquisition and analysis software and the EC3 imaging system (UVP LLC, Upland, CA). Lysate from CHO cells transiently transfected with ADAMTS13 plasmid was used as a positive control.

Determination of ADAMTS13 activity

The fluorogenic substrate FRETS-VWF73 was purchased from Peptides International (Louisville, KY, USA) and applied for the determination of ADAMTS13 enzyme activity according to the protocol provided by the supplier with minor modifications [20]. Briefly, the test citrated plasma was diluted 1:20 in assay buffer (5 mM Bis-Tris, 25 mM $CaCl_2$, 0.005% Tween 20, pH 6.0), while the culture medium samples were diluted 1:1 with the assay buffer. Then the diluted samples were mixed with 5 µM FRETS-VWF73 substrate solution (20 µl each) in white 384-well plates. Fluorescence was measured at 37°C every 2 min for 1 h in a Chameleon microplate reader (Hidex, Turku, Finland) equipped with a 340 nm excitation and a 460 nm emission filter. The reaction rate was calculated by linear regression analysis of fluorescence over time. A two-fold dilution series of normal human plasma (mixed from citrated plasma samples of 10 healthy blood donors) was applied as a standard curve, 100% ADAMTS13 activity was set at the reaction rate observed in the 1 : 20 diluted plasma sample and the 1 : 1 diluted culture media sample. The intra-assay coefficient of variation (CV) was <5%, and the inter-assay CV was 6–9% (measured at 60 and 100% activity levels).

Determination of plasma VWF antigen levels

VWF antigen (VWF:Ag) levels were measured in citrated plasma by an enzyme-linked immunosorbent assay (ELISA) using commercially available antibodies (Dakopatts, Glostrup, Denmark). Microtiter plates (Immunoplate Maxisorb, Nunc, Roskilde, Denmark) were coated with polyclonal rabbit anti-human VWF antibody diluted 1 : 800 in sodium bicarbonate buffer, pH 9.6. After overnight incubation at 4°C, plates were washed in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). The plates were incubated with plasma samples diluted 1 : 500 or standard control plasma (dilution range 1 : 250 to 1 : 8,000) for 2 h at room temperature. After washes with TBS-T, horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-human VWF antibody was added to the plates and incubated for 1 h, followed by addition of ortho-phenylenediamine (OPD). The optical density was measured at 492 nm. Results are expressed as percentages of a standard composed of pooled human plasma of 10 healthy blood donors.

Statistical analysis

The bands from Western blotting were quantified by the MetaView Image Analyzing System (Version 4.50; Universal Imaging Co., Downingtown, PA, USA) and normalized with respect to the corresponding fragment concentration of the housekeeping gene GAPDH. Normal distribution of data was assessed by the Shapiro-Wilk test. The *t*-test was performed using the SPSS Statistics for Windows package (release 20.0; IBM Corp., USA) and the results shown in the Results section and figure legends. All data were expressed as the mean ±SEM. Statistical significance was set at $p < 0.05$.

Results

Characteristics of patients

The baseline characteristics of the two study groups are presented in Table I. Controls were matched to RPL cases by maternal age and incidence of smoking. Number of previous pregnancies and pregnancy losses were significantly higher in the RPL group than those in the control group.

Table I. Clinical characteristics of the study sample

Characteristic	RPL (n = 25)	Normal (n = 30)	P-value
Maternal age [years]	30.7 ±1.160	29.7 ±0.904	0.475
Previous pregnancies	3.5 ±0.203	2.0 ±0.214	0.000
Pregnancy losses	3.29 ±0.125	0.44 ±0.166	< 0.001
Smoking	2 (8%)	1 (3.3%)	0.585

RPL – recurrent pregnancy loss. Data are shown as mean ±SEM or percentage.

ADAMTS13 mRNA is expressed in human endometrium throughout the menstrual cycle and pregnancy

ADAMTS13 mRNA was detected by real-time PCR in human endometrium throughout the menstrual cycle and pregnancy. Our previous study showed predominant expression of ADAMTS13 in extravillous trophoblast and endothelial cells. We ran the HLA-G probe to evaluate the level of extravillous trophoblast in decidua tissues by real-time PCR. Human liver that is known to express ADAMTS13 [10] was positive, while the blank CHO cell showed no signal in the real-time PCR, suggesting that the amplified ADAMTS13 mRNA signals in the endometrial tissues were specific. GAPDH was used to normalize the total RNA added during the amplification. The results were expressed as the ratio of ADAMTS13 or HLA-G to GAPDH (Figures 1 A, B).

ADAMTS13 protein is synthesized by human endometrium throughout the menstrual cycle and pregnancy

Immunoblots with anti-human ADAMTS13 IgG showed ADAMTS13 proteins of 120 kDa and 190 kDa in the lysates of endometrial tissues during the menstrual cycle and pregnancy (Figure 1 C). No bands were detected in the blank CHO cell lysates as a negative control.

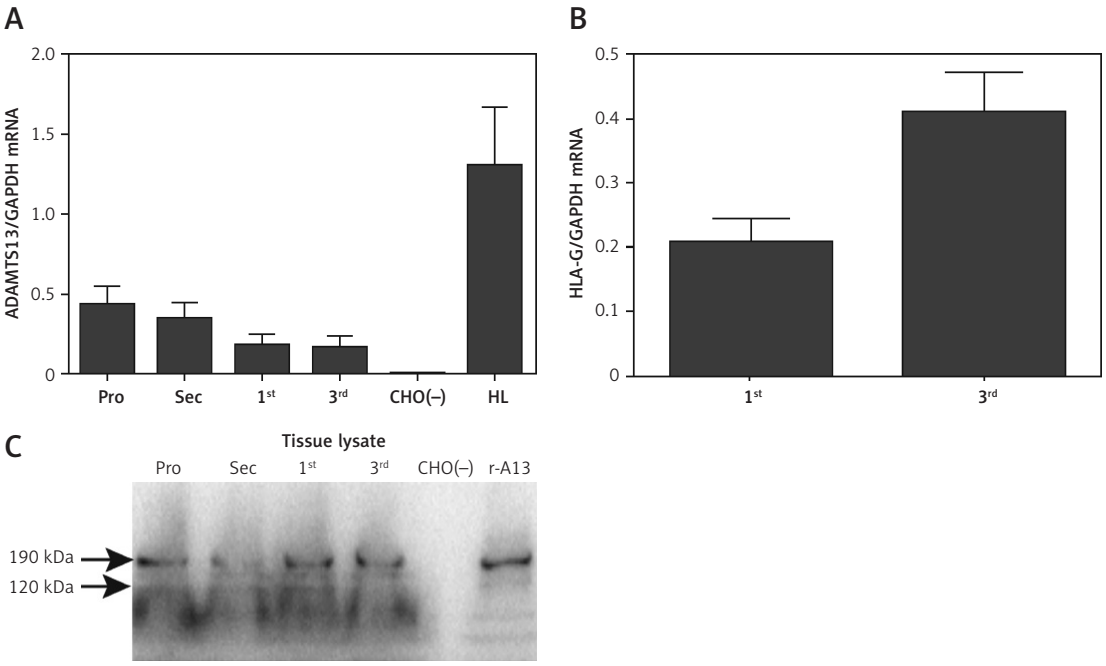

Figure 1. ADAMTS13 activity was detected in the concentrated conditioned media of human endometrium throughout the menstrual cycle and pregnancy. **A** – ADAMTS13 mRNA is detected by real-time PCR in human endometrium throughout the menstrual cycle and pregnancy. Human liver mRNA was used as the positive control and blank CHO cell mRNA was used as the negative control. The data were standardized against the GAPDH gene. Pro, proliferative endometrium (n = 10); Sec, secretory endometrium (n = 10); 1st, first trimester decidua (n = 10); 3rd, third trimester decidua (n = 10); HL, human live. Results are presented as mean ±SEM. **B** – HLA-G mRNA was detected by real-time PCR in human decidual tissues. The data were standardized against the GAPDH gene. **C** – ADAMTS13 is synthesized by human endometrium throughout the menstrual cycle and pregnancy. Lysates from endometrium tissues exhibited bands at 190 and 120 kDa. A full-length recombinant ADAMTS13 (r-A13) secreted from CHO cells transiently transfected with an ADAMTS13 plasmid was used as a positive control and revealed a band at 190 kDa under reducing conditions. No bands were detected in the CHO cell lysates as a negative control (data not shown)

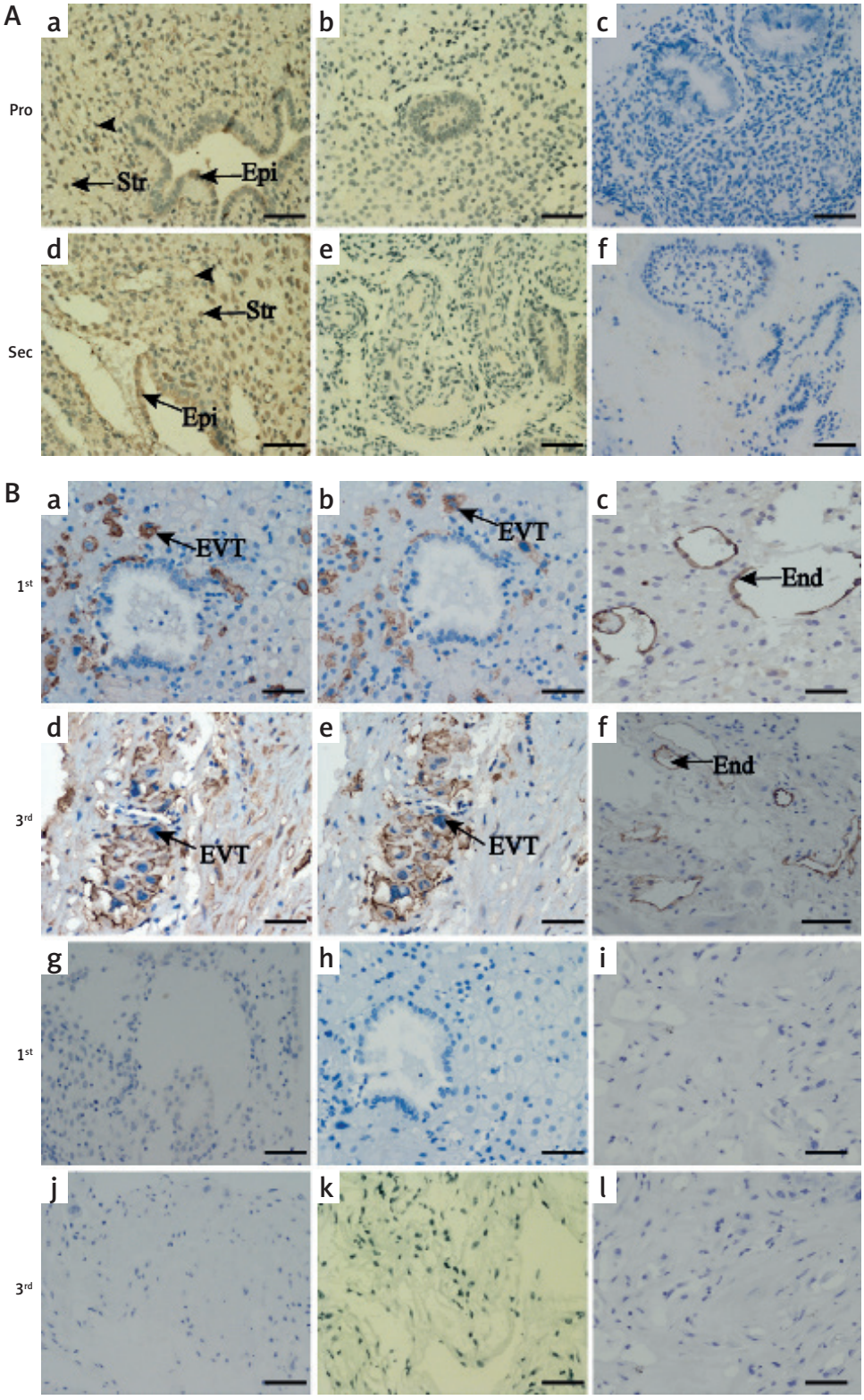

Figure 2. Expression of cellular ADAMTS13 in human endometrium throughout the menstrual cycle and pregnancy. **A a, d** – ADAMTS13 expression in stroma cells (Str), vascular endothelial cells (indicated by arrowhead), and some glandular epithelial cells (Epi) in proliferative endometrium and secretory endometrium. **B a, d** – ADAMTS13 expression in extravillous trophoblasts (EVT) in the decidua. **B b, e** – HLA-G was used as a marker of EVT in the decidua. **B c, f** – CD34 was used as a marker of blood vessel endothelium (End) in the decidua. **A b, e** and **B g, j** – stained with isotype rabbit IgG. **B h, k** – stained with isotype mouse IgG. **A c, f** and **B i, l** – stained only with secondary antibody

Pro – proliferative endometrium, Sec – secretory endometrium, End – endovascular, 1st – first trimester decidua, 3rd – third trimester decidua. Bar = 50 µm.

Localization of ADAMTS13 in human endometrium throughout the menstrual cycle and pregnancy

The cellular localization of ADAMTS13 in human endometrium throughout the menstrual cycle and pregnancy was determined by immunohistochemistry. The results showed that ADAMTS13 protein was detected mainly in stroma cells, vascular endothelial cells, and some glandular epithelial cells in proliferative endometrium and secretory endometrium; ADAMTS13-positive staining was also detected in extravillous trophoblasts in decidua from the first trimester and third trimester. HLA-G was used as a marker of extravillous trophoblasts and CD34 as a marker of endothelial cells in the decidua. Tissue sections stained with isotype IgG or only stained with secondary antibody showed negative staining (Figure 2).

ADAMTS13 activity was detected in explant culture medium of human endometrium throughout the menstrual cycle and pregnancy

Explant culture medium of endometrial tissues throughout the menstrual cycle and pregnancy were harvested and concentrated. Immunohistochemistry was performed to evaluate the expression of HLA-G and CD34 in cultured decidual tissues (Figure 3 A). The results showed that HLA-G and CD34 expression levels in 1st trimester decidua were significantly lower than those in 3rd trimester decidua (Figures 3 B, C). Real-time PCR showed that the expression level of HLA-G mRNA was obviously lower in 1st trimester decidua than that in 3rd trimester decidua (Figure 3 D). FRETS-VWF73 substrate was used to determine the proteolytic activity as described in the *Material and methods*. The ADAMTS13 activity in the conditioned medium of these explants was 4.61% (range 2.6–6.4%), 5.6% (range 3.4–8.1%), 4.82% (range 2–7.4%) and 5.88% (range 3.9–7.8%), respectively (Figure 3 E).

Plasma ADAMTS13 activity is reduced and VWF antigen levels are increased in RPL patients

There was a significant difference in plasma ADAMTS13 activity between the RPL group and the control group (median (range) 69.095 (65.2–93.7%) vs. 93.62% (88.1–115.6%), $p < 0.001$) (Figure 3 F). Furthermore, plasma VWF antigen levels

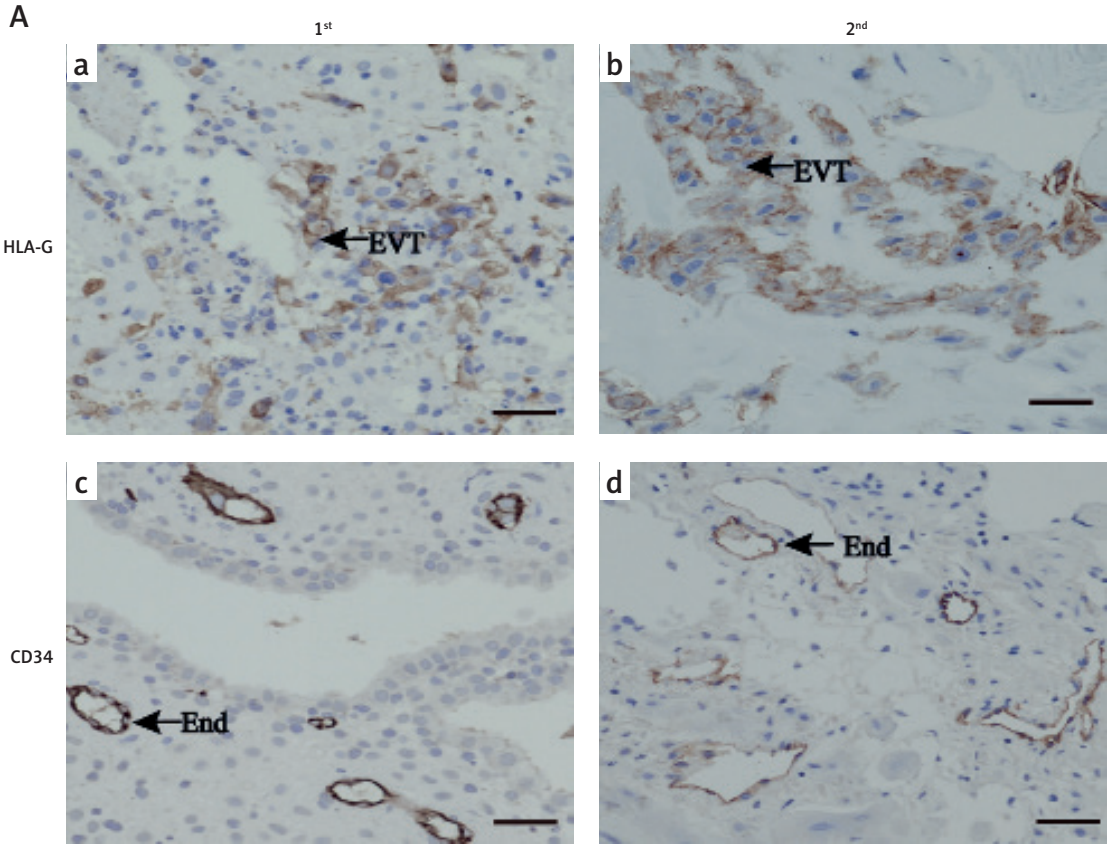

Figure 3. ADAMTS13 activity was detected in the concentrated conditioned media of human endometrium throughout the menstrual cycle and pregnancy. Tissues after culture for preparation of conditioned media were immunohistologically evaluated. **A a, b** – HLA-G was used as a marker of extravillous trophoblasts (EVT) in the decidua. **A c, d** – CD34 was used as a marker of blood vessel endothelium (End) in the decidua

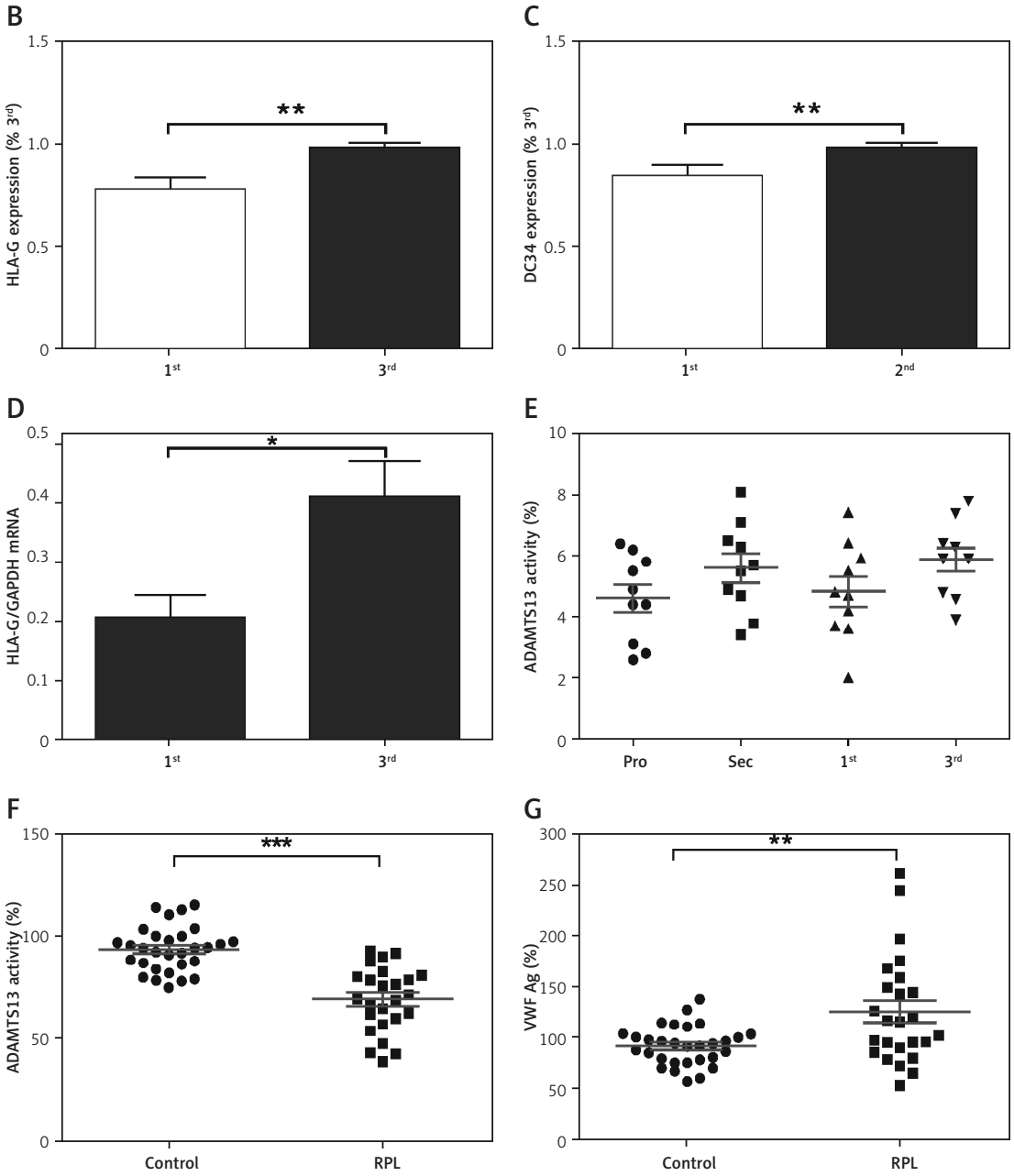

Figure 3. Cont. **B, C** – HLA-G or CD34 in the sample was evaluated on the basis of staining intensity and positively stained areas. HLA-G and CD34 expression levels in 1st trimester decidua were significantly lower than those in 3rd trimester decidua.*p values < 0.05, ** p values < 0.01. **D** – Expression level of HLA-G mRNA was obviously lower in 1st trimester decidua than that in 3rd trimester decidua. The data were standardized against the GAPDH gene. *p value of < 0.05. **E** – The proteolytic activity of ADAMTS13 was determined by FRETS-VWF73 substrate. Tissue media from proliferative endometrium (Pro, n = 10), secretory endometrium (Sec, n = 10), 1st trimester decidua (1st, n = 10) and 3rd trimester decidua (3rd, n = 10) exhibited activity, with mean values of 4.61% (range 2.6–6.4%), 5.6% (range 3.4–8.1%), 4.82% (range 2–7.4%) and 5.88% (range 3.9–7.8%) respectively. **F** – There was a significant difference in plasma ADAMTS13 activity between the RPL (n = 25) and the control groups (n = 30) (median (range) 69.09% (65.2–93.7%) vs. 93.62% (88.1–115.6%), p < 0.001). **G** – Plasma VWF antigen levels were significantly higher in RPL (n = 25) women than in control women (n = 30) (median (range) of 125.5% (54.2–262.8%) vs. 91.9% (80.4–138.7%), p < 0.01). **p values <0.01, ***p values < 0.001

were significantly higher in RPL patients than in control women (median (range) of 125.5% (54.2–262.8%) vs. 91.9% (80.4–138.7%), p < 0.01) (Figure 3 G).

ADAMTS13 mRNA and protein expression levels are reduced in deciduae of RPL patients

Immunostaining of the deciduae did not reveal a difference in the ADAMTS13 expression pattern

on extravillous trophoblasts between RPL patients and controls (Figures 4 A a, b). ADAMTS13 (Figure 4 B) and HLA-G (Figure 4 E) expression levels in RPL decidua were significantly lower than those in control decidua, while the CD34 (Figure 4 H) expression level showed no significant difference between the two groups. The levels of ADAMTS13 mRNA in decidual tissues were determined by real-time PCR. As shown, decidual tissues had lower levels of ADAMTS13 mRNA in patients with RPL compared with those from normal pregnancy (Figure 4 C). This may be explained by the obviously reduced presence of extravillous trophoblast as indicated by the decreased expression of HLA-G mRNA in RPL tissue samples (Figure 4 D). Consistent with real-time PCR, immunoblot showed obviously reduced levels of ADAMTS13 protein in the deciduae from

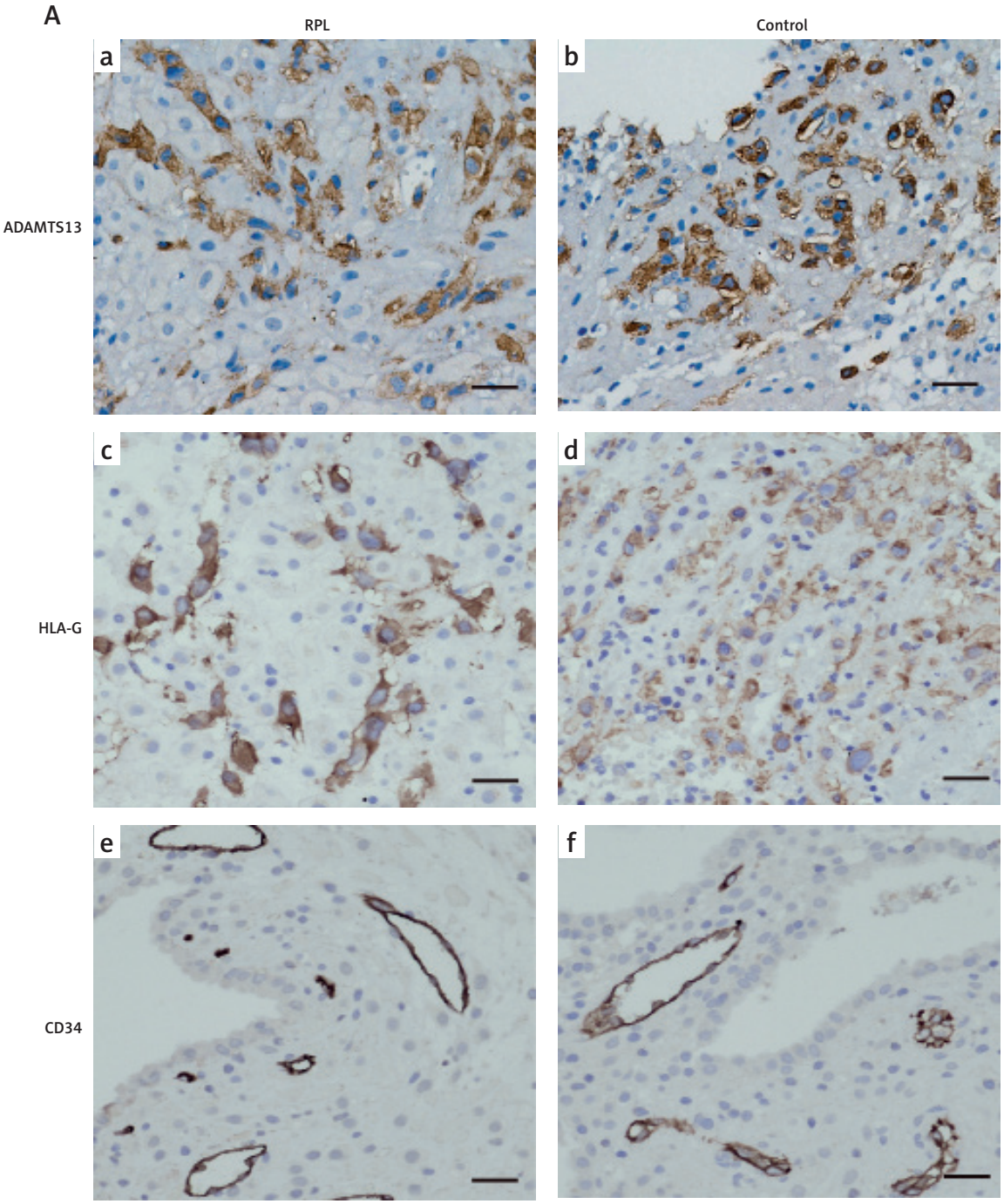

Figure 4. ADAMTS13 expression is down-regulated in RPL decidua. **A a, b** – A13 staining on extravillous trophoblasts was present in a similar pattern in RPL and control groups. Human leukocyte antigen (HLA)-G was used for labeling for extravillous trophoblasts ADAMTS13 (**A c, d**) and CD34 for blood vessel endothelium in decidua (**A e, f**)

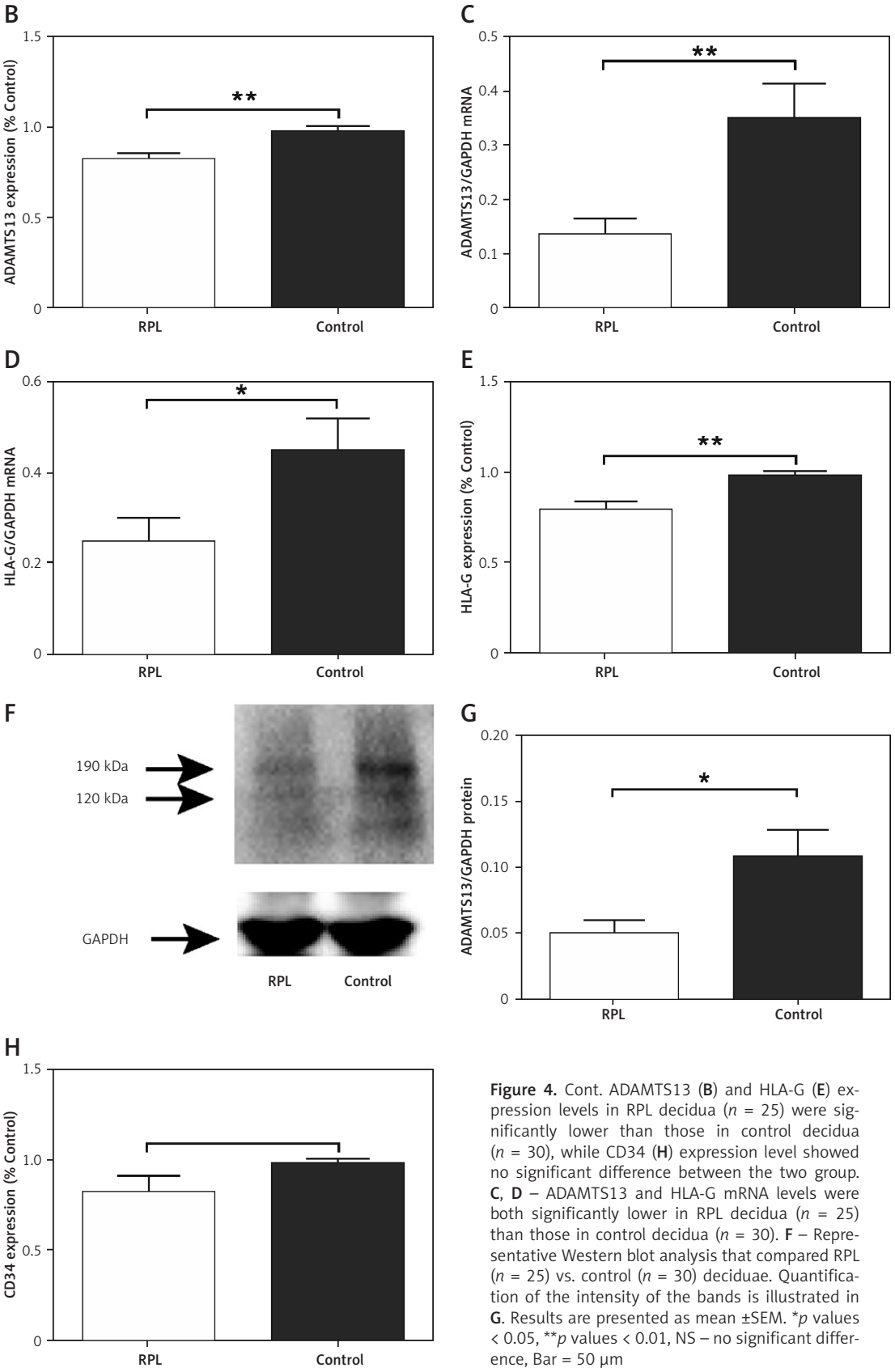

Figure 4. Cont. ADAMTS13 (**B**) and HLA-G (**E**) expression levels in RPL decidua (n = 25) were significantly lower than those in control decidua (n = 30), while CD34 (**H**) expression level showed no significant difference between the two group. **C**, **D** – ADAMTS13 and HLA-G mRNA levels were both significantly lower in RPL decidua (n = 25) than those in control decidua (n = 30). **F** – Representative Western blot analysis that compared RPL (n = 25) vs. control (n = 30) deciduae. Quantification of the intensity of the bands is illustrated in **G**. Results are presented as mean ±SEM. *p values < 0.05, **p values < 0.01, NS – no significant difference, Bar = 50 µm

RPL patients compared with those in the control (Figure 4 F, G).

Discussion

The current study demonstrates for the first time that proteolytically active ADAMTS13 is present in human endometrium tissues throughout the menstrual cycle and pregnancy. What is more, ADAMTS13 expression is significantly reduced in the deciduae from RPL women. In addition, we were the first to discover the reduction of plasma ADAMTS13 activity and elevation of plasma VWF antigen level in RPL patients.

The presence of many members of the ADAMTS family has been demonstrated in human endometrial tissues [21–25], many albeit at low levels, indicating that these metalloproteinases may play important roles in implantation and placentation. We have previously observed that proteolytically active ADAMTS13 is expressed in human placenta throughout the pregnancy; in the placenta, ADAMTS13 is mainly expressed by trophoblast and fetal blood vessel endothelium; ADAMTS13 synthesis is significantly reduced in placental tissues from PE patients and that exposed to hypoxic conditions; what is more, recombinant full-length ADAMTS13 promotes proliferation, migration, invasion and network formation of trophoblasts [14]. Taken together, these observations suggest important roles of ADAMTS13 in pregnancy and likely the pathogenesis of preeclampsia. The current study demonstrated for the first time the expression of ADAMTS13 mRNA and protein in human endometrium tissues throughout the menstrual cycle and pregnancy.

Other ADAMTS members, known as aggrecanases, including ADAMTS-1, -4, -5, -8, -9, have also been detected in human deciduae [21, 23–25]. Mice null-mutant for ADAMTS-1 or ADAMTS-5 subtype are capable of undergoing normal decidualization [26] or proceed to develop large endometrial cysts during the reproductive cycle [25], indicating a uterine environment capable of supporting pregnancy; therefore the role(s) of these subtypes in the process of the highly regulated, multimolecular complex structures present in the endometrial ECM under normal and pathological conditions remains to be elucidated. ADAMTS13 gene-knockout mice are viable and fertile [27, 28], suggesting that the establishment and maintenance of pregnancy are not dependent upon the regulated expression of ADAMTS13 in the placenta or decidua and that other local ADAMTS members may play overlapping/compensatory roles in this multi-step reproductive process. Such redundancy is usually seen in gene families [29].

While the well-established biological role of ADAMTS13 is to regulate the process of hemostasis and thrombosis, ADAMTS13 may also be involved in inflammation, angiogenesis and tissue remodeling. Apart from the typical matrix proteins of ECM, such as fibronectin, laminin, and fibrin-collagen, fibrin and fibrinogen are found deposited in fibrosis tissue, indicating that coagulation factors may also be constituents of ECM. VWF is the only known substrate of ADAMTS13. While VWF is secreted by endothelial cells in arteries into both the lumen and the subendothelial space [30], ADAMTS13, as a metalloprotease, has been speculated to be related to ECM degradation. VWF is found upregulated in the microvascular endothelial cells in the area of fiber-like structure and necrosis during liver injury [31]. Therefore, it is reasonable to presume that ADAMTS13 released at the time of tissue injury may prevent excessive ECM constituent deposition [32] and development of fibrosis in the liver [33]. Likewise, ADAMTS13 expressed by podocytes and tubular cells may possibly be involved in tissue remodeling in situ [34, 35].

There has been an increased interest in the role of ADAMTS13 in angiogenesis. Recently, Lee *et al*. have demonstrated that ADAMTS13 might exert either pro-angiogenic or anti-angiogenic effects depending on the cellular environments [36]. In the absence of excessive VEGF, ADAMTS13 promotes angiogenesis by phosphorylation of VEGFR2 and upregulating VEGF [36, 37]. In the presence of excessive VEGF, ADAMTS13 appears to inhibit angiogenesis depending on the C-terminal TSP1 repeats, by competing with VEGF for binding to its receptor VEGFR. Collectively, ADAMTS13 expressed in deciduae has the potential to contribute to the development of a uterine environment capable of supporting a pregnancy via the degradation of the decidual ECM and/or the extensive vascular changes that occur in this dynamic tissue during implantation and placentation.

RPL is a complicated problem related to chromosomal abnormalities, autoimmune disorders, endocrine dysfunction, infectious processes, environmental toxins, advanced maternal and paternal age, and congenital or structural uterine anomalies [1, 38]. However, until now none of them have provided a clear explanation about its etiology and pathogenesis. Invasion of extravillous trophoblast, vascular remodeling, and decidualization are the prominent processes for successful embryo implantation [39]. Decidualization involves the reorganization of the decidual ECM. Angiogenesis is a fundamental process occurring during embryonic development and reproductive cycles [40]. Deficiency in decidualization and angiogenesis due to abnormal protein expression involved in this process might lead to aberrant growth of the fetus or, in severe cases, pregnan-

cy loss. Interestingly, in the current study both the mRNA and protein expression levels of ADAMTS13 were lower in decidua samples of RPL patients than those of normal controls. This suggests that decidua ADAMTS13 may play a significant role in the modulation of the uterine environment, preparing the uterus for implantation of the developing conceptus and formation of a functional placenta during the establishment of pregnancy. However, further investigation in ADAMTS13 gene knockout animal models is necessary to understand the molecular mechanisms of the biological roles of ADAMTS13 during gestation.

Maternal thrombophilia has been suggested as an important risk factor for RPL [1, 38]. In this study, the activity of ADAMTS13 was found to be lower in RPL patients than in controls. The presence of increased amounts of VWF in RPL patients can probably be ascribed to the decreased levels of ADAMTS13 activity. Owing to the decrease of ADAMTS13 activity, ultralarge VWF multimers, which are biologically more active, circulate in RPL patients, and this may lead to maternal thrombophilia. These data indicate that reduction in the levels of ADAMTS13 may play a role in the onset of RPL. Our previous study showed predominant expression of ADAMTS13 in extravillous trophoblast and endothelial cells and low or undetectable ADAMTS13 in nearby decidual stromal cells. Therefore, we speculate that the lower expression of ADAMTS13 in tissue extract and supernatant is a reflection of impaired trophoblast invasion and reduced presence of extravillous cytotrophoblast in the RPL tissue sample, which was demonstrated by the decreased immunohistochemical staining intensity of extravillous trophoblast in RPL decidual tissue.

In summary, we determined the expression of ADAMTS13 in human endometrium tissues and found that ADAMTS13 expression was significantly reduced in the deciduae from RPL patients. In addition, we observed the decrease of plasma ADAMTS13 activity and increase of plasma VWF antigen level in RPL patients. Collectively, these findings serve as the basis for future studies on the expression and function(s) of ADAMTS13 in the human endometrium under normal and pathological conditions.

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Ethics approval

This study was reviewed and approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University, and informed consent was obtained from all participants.

Conflict of interest

The authors declare no conflict of interest.

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