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Original Article

Expression of uPAR in human trophoblast and its role in trophoblast invasion

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Abstract: Placental trophoblast cells differentiate into invasive trophoblasts or syncytiotrophoblasts. Abnormal trophoblast invasion results in pregnancy-associated disease and abortion. uPAR is a cell membrane-bound glycosylated protein, involved in physiological and pathological processes. However, uPAR expression in villi during threatened abortion and its role in trophoblast differentiation are unclear. We determined that, uPAR expression in the villi was reduced in threatened abortion patients than that in normal pregnancy. uPARsiRNA inhibited the potential for trophoblast migration and invasion in explants culture and HTR8/SVneo cells. It also enhanced forskolin-induced fusion of HTR8/SVneo cells. Overall, this study provides a possible reason for abortion.

Keywords: uPAR, trophoblast, abortion, migration/invasion, syncytialization

Introduction

Embryo implantation is a developmental biological event, which requires a mature embryo and a receptive endometrium [1, 2]. The mature embryo invades and penetrates the endometrium in a process called placentation, which is crucial for successful establishment of pregnancy. This is mediated by extra-embryonic cells, termed trophoblasts, present in the placental villi. During placental development, villous trophoblast cells differentiate via 2 pathways. The first involves fusion and differentiation to form syncytiotrophoblasts (STBs), while the other results in extravillous cytotrophoblasts (EVTs) [3, 4]. Inadequate differentiation in any of these pathways causes severe pregnancy-related disorders [5, 6].

STBs are found on the surface of the placental villi and establish a barrier at the fetal-maternal interface. This barrier mediates nutrient, oxygen, and waste exchange between maternal and fetal blood. In addition, STBs secrete hormones such as human chorionic gonadotropin (hCG) and human placental lactogen (hPL), which play a critical role in pregnancy maintenance and fetal development [7, 8]. Inadequate syncytialization, often associated with hormone and cytokine secretion disorders, cause intrauterine growth retardation (IUGR) and even embryo death. For example, low level of hCG is associated with ectopic pregnancy or threatened abortion, while excess hCG secretion often indicates multiple pregnancies or acephalocystis racemosa [9, 10]. Similarly, hPL concentration is related to size of the placenta [11].

EVTs show a highly invasive phenotype, migrating away from the trophoblast cell column. They invade the uterine epithelium and uterine spiral arteries to provide adequate fetoplacental perfusion and establish maternal-fetal blood circulation [12]. Poor EVT migration/invasion and subsequently, poor remodeling of the spiral arteries between the mother and fetus often results in shallow interstitial invasion and inadequate placental perfusion. This in turn causes pregnancy-associated diseases including preeclampsia, fetal intrauterine growth restriction, and even leads to abortion [13]. During embryo implantation, trophoblasts secrete matrix metalloproteinases (MMPs) that degrade the extracellular matrix (ECM), which facilitates

invasion by the blastocysts through the decidua and into the maternal vasculature. Gelatinases (MMP-2) have been implicated in remodeling of ECM in the trophoblast invasion process. Tissue inhibitors (TIMPs) help to balance and tightly control EVT invasion into maternal endomyometrium [14].

The urokinase-type plasminogen activator receptor (uPAR) is a highly glycosylated protein bound to the cell membrane by a glycosyl phosphatidylinositol (GPI) anchor. UPAR-bound and free uPA activate plasmin, which in turn promotes matrix degradation by activating certain MMPs. uPAR is implicated in several cellular processes, ranging from wound repair, inflammation, and tumor metastasis to embryo implantation [15-17]. Swapna et al. reported that uPAR is expressed at all stages of mice decidual tissue development [18]. Naruse et al. found that uPAR is expressed in uterine natural killer cells during early pregnancy, which regulate EVT invasion and spiral artery remodeling [19]. Recent studies have reported that uPAR is expressed on trophoblasts at the invasion/migration front in situ, indicating a causal association of uPAR with migratory and invasive functions of trophoblasts [20]. However, the expression of uPAR on trophoblasts of patients with threatened abortion and the specific role of uPAR during placenta development have not yet been well characterized.

To elucidate the functions of uPAR in embryo implantation, the expression of uPAR in villi during normal early pregnancy and threatened abortion was compared. The role of uPAR in trophoblast differentiation, including invasion and syncytialization was also investigated. We found that uPAR was highly expressed in normal, early-pregnancy trophoblasts as compared to those during threatened abortion. Additionally, using villous explants culture model and trophoblast cell lines, we demonstrated that uPAR significantly promoted trophoblast syncytialization and enhanced CTB outgrowth/invasion.

Materials and methods

Cell culture

HTR8/SVneo cells, JAR cells and JEG-3 cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in DMED/F12 (Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C under 5% CO_2 in humidified air according to standard procedures. The growth medium was renewed every 2-3 days.

Tissue samples

The protocols for human study were in accordance with the Institutional Review Board of Dalian Medical University. Samples were obtained from the Secondary Affiliated Hospital of Dalian Medical University from 2012 to 2013. Human villi were obtained from the women at the ages of 25 to 35. The pregnant women and threatened abortion group were confirmed by ultrasound detection at 6 to 10 gestational weeks. Human villi were used for uPAR and keratin 7 (KRT-7) immunohistochemical staining. The fresh human villi tissues were collected from the non-drug abortion women.

Immunohistochemistry and immunofluorescence

Tissue slides were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 15 min. Slides were blocked with 10% normal goat serum for 10 min, and incubated with anti-human uPAR antibody (1:50; Cell Signaling Technology, Beverly, MA, USA) and anti-human keratin 7 antibody (1:50, protein tech, USA) at 4°C for 12 h. The slides were incubated with biotinylated secondary antibody at 37°C for 15 min, and reacted with a streptavidin-peroxidase conjugate at 37°C for 10 min, then used 3, 3'-diaminobenzidine as a chromogen substrate. Meyer's hematoxylin was used as a counterstained dye. A negative control was obtained by replacing the primary antibody with serum IgG from rabbit. Images were captured with the Olympus fluorescence microscope (Olympus, Japan).

Frozen section and trophoblast cells were blocked with goat serum for 2 h at room temperature, the anti-human uPAR antibody (1:50) and anti-human keratin 7 (1:50) were incubated at 4°C for 12 h. Then after 1 h incubation with phycoerythrin (PE)-conjugated anti-rabbit IgG (1:200, Santa Cruz. USA) at 37°C, slides was washed 3 times for 5 min with PBS, and then treated with DAPI (Santa Cruz. USA) for 5 min at 37°C. The slide was photographed with the inverted microscope (Olympus, Japan).

Explant culture

Small pieces of tissues (2-3 mm) from tips of first trimester human placental villi were dissected and explanted in Millicell-CM culture dish inserts (0.4 mm pore size, Millipore, Carrigtwohill, Co., Cork, Ireland) pre-coated with phenol red-free matrigel substrate (Becton Dickinson, Bedford, MA, USA). Inserts were placed into 24-well culture dishes (Costar, Cambridge, MA, USA). The explants were cultured in serum-free DMEM/F12 media with 100 IU/ml penicillin and 100 mg/ml streptomycin at 3% O_2/5% CO_2/92% N_2. Trophoblast cell sprouting and migration from the distal end of the villous tips were recorded for 48 hour. To test the effect of uPAR on the trophoblast cell migration, siRNA specifically targeting uPAR (500 nM), an equal concentration of the control siRNA was introduced into wells of culture media.

Real-time PCR

Cells were treated with RNAiso Plus reagent (TaKara, Japan) for RNA extraction, and PrimeScript™ RT reagent Kit with gDNA Eraser kit (Takara, Japan) was used for synthesizing cDNA. SYBR® Premix ExTaq™ (Takara, Japan) was used for quantitative Real-time PCR. Primers for Realtime PCR were as follows: uPAR: 5-AGGACCCTGAGCTATCGGACTG-3' (forward), and 5'-TGCATTCGAGGTAACGGCTTC -3' (reverse). GAPDH: 5'-GCACCGTCAAGGCTGAGAAC-3' (forward) and 5'-TGGTGAAGACGCCAGTGGA-3' (reverse). The reactions were performed with Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, USA).

Transfection of uPARsiRNA

Cells were plated into 60-mm culture dishes and then transfected with 40 nM of siRNA using lipofectamine 2000 following the manufacturer's instructions. All assays were performed 48 h after transfection. uPARsiRNA sequences were as follows: uPARsiRNA (559): Sense 5'-GCCGUUACCUCGAAUGCAUTT-3', antisense-5' AUGCAUUCGAGGUAACGGCTT-3'. The scrambled control sequences were (5'-UUC UUC GAA CGU GCU ACG UTT-3') and (5'-ACG UGA CAC GUU CGG AGA ATT-3').

Western blot

Villi and decidua tissues were washed in phosphate-buffered saline (PBS) for three times before incubation with lysis buffer (PBS with 1% NP-40 and 1 mmol/L PMSF) at 4°C for 2 h. The tissue lysates were clarified by centrifugation at 8000 ×g for 10 min, and boiled with 5× loading buffer (250 mmol/L Tris-HCl (pH 6.8, 10% SDS, 50% glycerol, 5% β-mercaptoethanol) for 10 min. Total protein was quantified using with the Coomassie Protein Assay Reagent (Bio-Rad, USA) using bovine serum albumin (BSA) as a standard. Total proteins (30 μg) were separated by 12% SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and blocked with 5% fat-free milk powder diluted in Tris-buffered saline with 0.05% Tween 20 (TTBS) for 2 h, and incubated with rabbit anti-human uPAR antibody (1:1000, Cell Signaling Technology, USA), TIMP-1 (1:1000, Bioworld, USA), TIMP-2 (1:500, Bioworld, USA), Caspases 8 (1:500, Protein Tech, USA), Caspases 9 (1:500, Protein Tech, USA), β-hCG (1:500, Protein Tech, USA)and GAPDH (1:5000, Protein Tech, USA) in TTBS overnight at 4°C. After washing three times the next day, membranes were incubated with horseradish peroxidase (HRP) -conjugated goat anti-rabbit IgG antibody (1:1000, Santa Cruz. USA) for 40 min at room temperature, and immunoreactive proteins were visualized with enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Piscataway, NJ).

Matrigel cell invasion and transwell cell migration assay

For Matrigel cell invasion assay, transwell inserts (6.5 mm, Costar, Cambridge, MA, USA) containing polycarbonate filters with 8 mm pores were precoated with 50 ml of 1 mg/ml Matrigel matrix. For cell migration assay, the inserts were not precoated with matrigel. 1.0×105 of HTR8/SVneo cells in serum-free medium were plated in the upper chamber, whereas medium with 10% FBS was added to the lower chamber. After incubating for 24 h, the cells on the Matrigel side of the inserts were removed by cotton swab. The inserts were fixed in methanol and stained with Crystal Violet. The number of invaded or migrated cells attached to the other side of the insert was

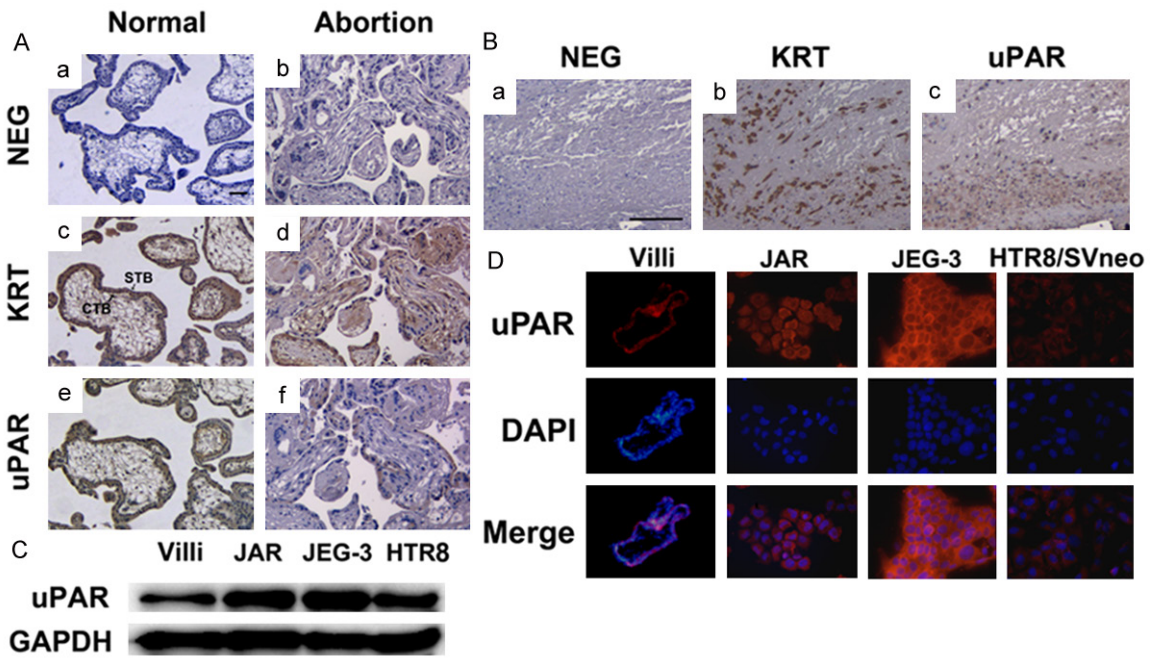

Figure 1. Expression of uPAR in human villi at the normal pregnant and the threatened abortion patients and cell lines. A. Immunostaining of keratin 7 (KRT7) as a marker of CTB (c, d), uPAR (e, f) in normal pregnancy and threatened abortion villi from the first trimester. Negative control (NEG): serum IgG from rabbit was used in place of primary antibody (a, b). CTB: cytotrophoblast; STB: syncytiotrophoblast. Bar=50 mm. B. EVT invaded into the maternal decidua in normal pregnancy. uPAR express in EVT and maternal decidua cell (c), and KRT7 as a marker of EVT (b). Bar=200 mm. C. Expression of uPAR in villi and different trophoblast cell lines by Western blot. D. Representative immunofluorescence of uPAR in 7 weeks of first-trimester villi and different trophoblast cell lines.

counted under a light microscope (Olympus, Japan) in five random fields at a magnification of ×100. Three independent experiments were performed. Numbers of invasive or migrated cells under different treatments were normalized to the control and expressed as a means of invasion or migration percentage (%) ± S.D.

Fusion of HTR8/SVneo cells after forskolin treatment

HTR8/SVneo cells were treated with forskolin at different concentrations (0 mM, 50 mM, 100 mM) after being starved. To test whether uPAR siRNA has a role to play during forskolin-induced fusion of HTR8/SVneo cells, uPAR siRNA or control siRNA were transfected into HTR8/SVneo cells for 24 h before being treated with forskolin for 48 h. To assess fusion, we performed multinucleated cell counting following E-cadherin immunostaining.

Gelatin zymography assay

MMP-2 activity was detected using the gelatin zymography assay. The supernatants were collected and used as the samples. Cells were also collected, and proteins were extracted using lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.02% NaN3 and 1% NP-40) for 30 min. The protein concentration was determined using the Bradford assay. A concentration of 40 mg of total proteins of supernatants were loaded per lane and electrophoresed on 10% SDS-polyacrylamide gels copolymerized with 1% gelatin. After electrophoresis, the gels were washed five times in 2.5% Triton X-100 (20 min each) and two times in buffer without Triton X-100 to remove Triton X-100, and then incubated in 50 mmol/l Tris-Cl, pH 7.6, and 5 mmol/l $CaCl_2$ (18 h, 37 1C). The gels were stained with 0.1% Coomassie blue R250 and destained in 10% isopropanol and 10% acetic acid in H_2O. MMP-2 was detected as transparent bands on the blue background of a Coomassie blue-stained gel.

Statistical analysis

All experiments were performed at least 3 times, and statistical analyses were carried out

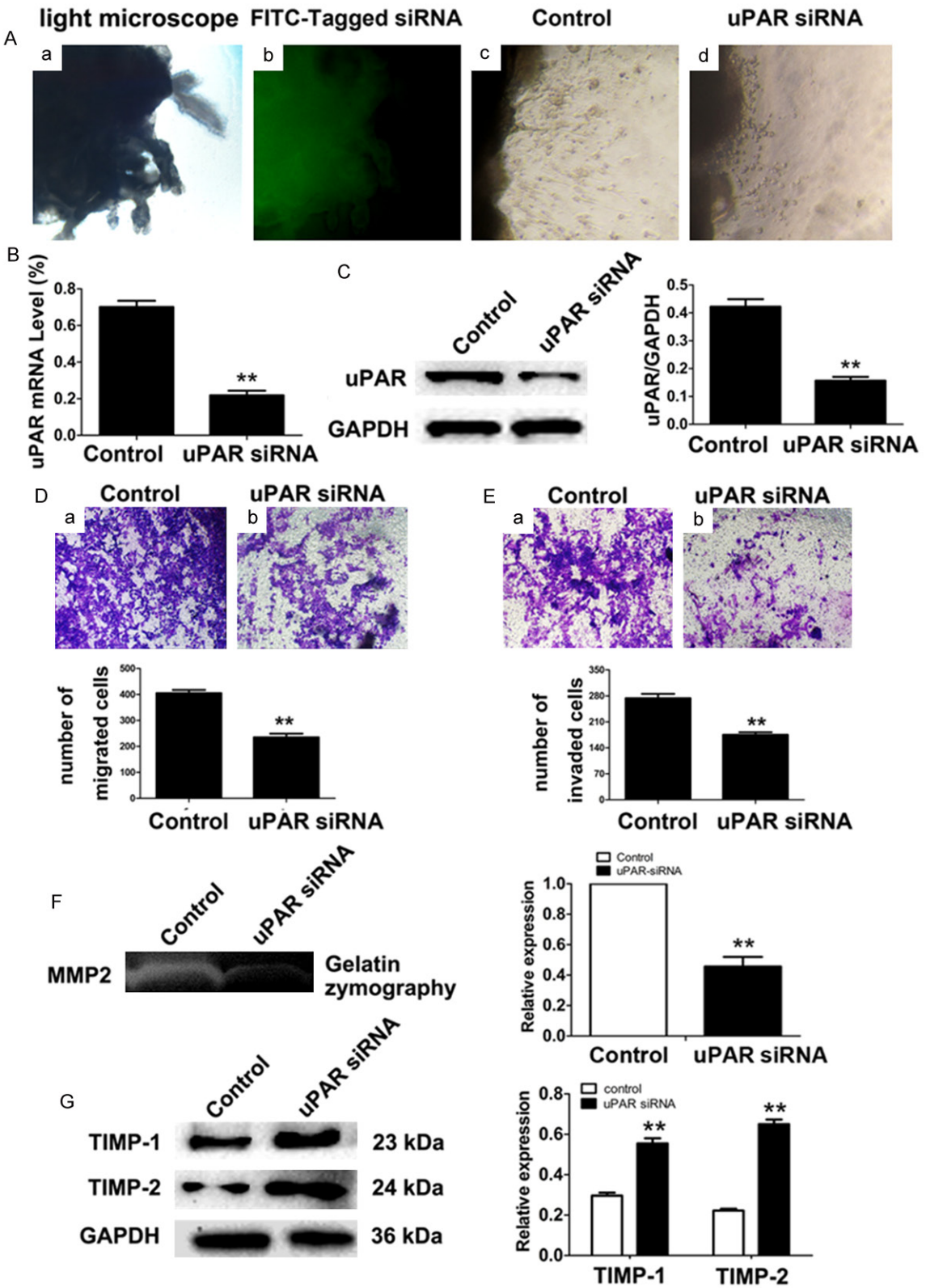

Figure 2. Silencing uPAR suppresses trophoblast outgrowth in extravillous explants cultures, migration and invasion of HTR8/SVneo cells. A. Extravillous explants from 7 weeks of gestation maintained in culture on matrigel were incubated with uPARsiRNA (d) or control siRNA (c). Representative pictures of explants were taken under the microscope (10×) after 48 h of culture in vitro. Villi transfected with FITC-tagged siRNA, showing the transfection efficiency (b). B,

C. Expression of uPAR in HTR8/SVneo cells after control or uPARsiRNA transfection by Real-time PCR and Western blot. D, E. Representative images of the HTR8/SVneo cells treated with control or uPARsiRNA in transwell migration assay and matrigel invasion assay (100×). The statistical bar graphs showed the summary of three independent experiments (**P<0.01). F. Gelatin zymography assay of MMP-2 in the culture medium collected from HTR8/SVneo cells which were treated with control siRNA or uPARsiRNA. G. Western blot analysis of TIMP-1 and TIMP-2 in HTR8/SVneo cells transfected with uPARsiRNA or control siRNA.

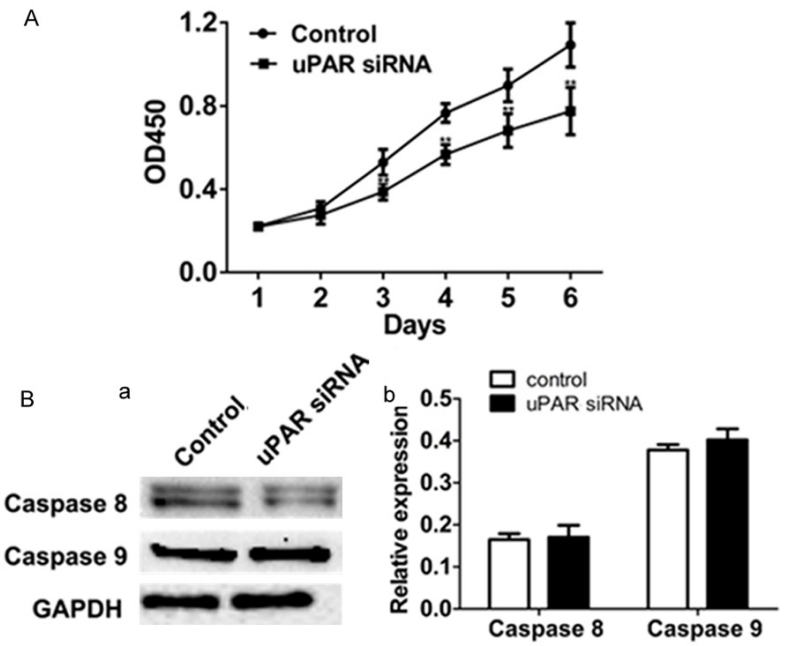

Figure 3. Effect of uPARsiRNA on HTR8/SVneo cells proliferation and apoptosis. A. Effects of uPARsiRNA on HTR8/SVneo cells proliferation by CCK-8 assay. B. Expression of caspase 8, 9 in HTR8/SVneo cells after uPARsiRNA transfection.

using the SPSS statistical software 17.0. One-way analysis of variance (ANOVA) was used to compare multiple groups. P<0.05 were considered statistically significant.

Results

uPAR is highly expressed in first trimester woman placental villous and trophoblast cells than in patients with threatened abortion

To analyze the expression of uPAR in the first trimester of a normal pregnancy and in patients with threatened abortion, immunohistochemistry was carried out (**Figure 1A**). Trophoblast cells were identified by cytokeratin 7 staining on a separate adjacent section (**Figure 1Ac** and **1Ad**). uPAR could be detected in the villous trophoblast during the first trimester. In addition, the expression levels of uPAR were significantly lower in the villi of patients with threatened abortion than in the villi of women with normal pregnancy (**Figure 1Ae** and **1Af**).

In the normal maternal compartment of placenta, EVT was identified by cytokeratin 7 staining from the decidualized stromal cells (**Figure 1Bb**). And uPAR staining was found not only on EVT, but also in human decidualized stromal cells (**Figure 1Bc**).

Expression of uPAR in different human trophoblast cell lines was confirmed by immunofluorescent staining and western blot analysis (**Figure 1C** and **1D**). The fluorescent image showed that uPAR is expressed in human villi (**Figure 1D**). Immunofluorescence microscopy revealed that uPAR was expressed in HTR8/SVneo, JAR and JEG-3 cells (**Figure 1D**). The results of the western blot further confirmed that both human villi and trophoblast cells express poFUT1 (**Figure 1C**).

uPARsiRNA significantly inhibits trophoblast outgrowth, migration, and invasion

To explore the role of uPAR in trophoblast invasion, extravilloua explants and trophoblast cell model were utilized. Extravillous explants from first trimester human villi were transfected with uPARsiRNA and cultured on matrigel-coated dishes. After 48 h of culture, the outgrowth of trophoblast significantly decreased in uPAR siRNA treated group (**Figure 2A**). The successful transfection of siRNA into explants was determined by FITC-tagged Control siRNA, which exhibit green fluorescence (**Figure 2Ab**).

To further confirm the role of uPAR in rophoblast migration and invasion, trophoblast cell line, HTR8/SVneo cells, was explored. The HTR8/SVneo cells transfected with uPARsiRNA

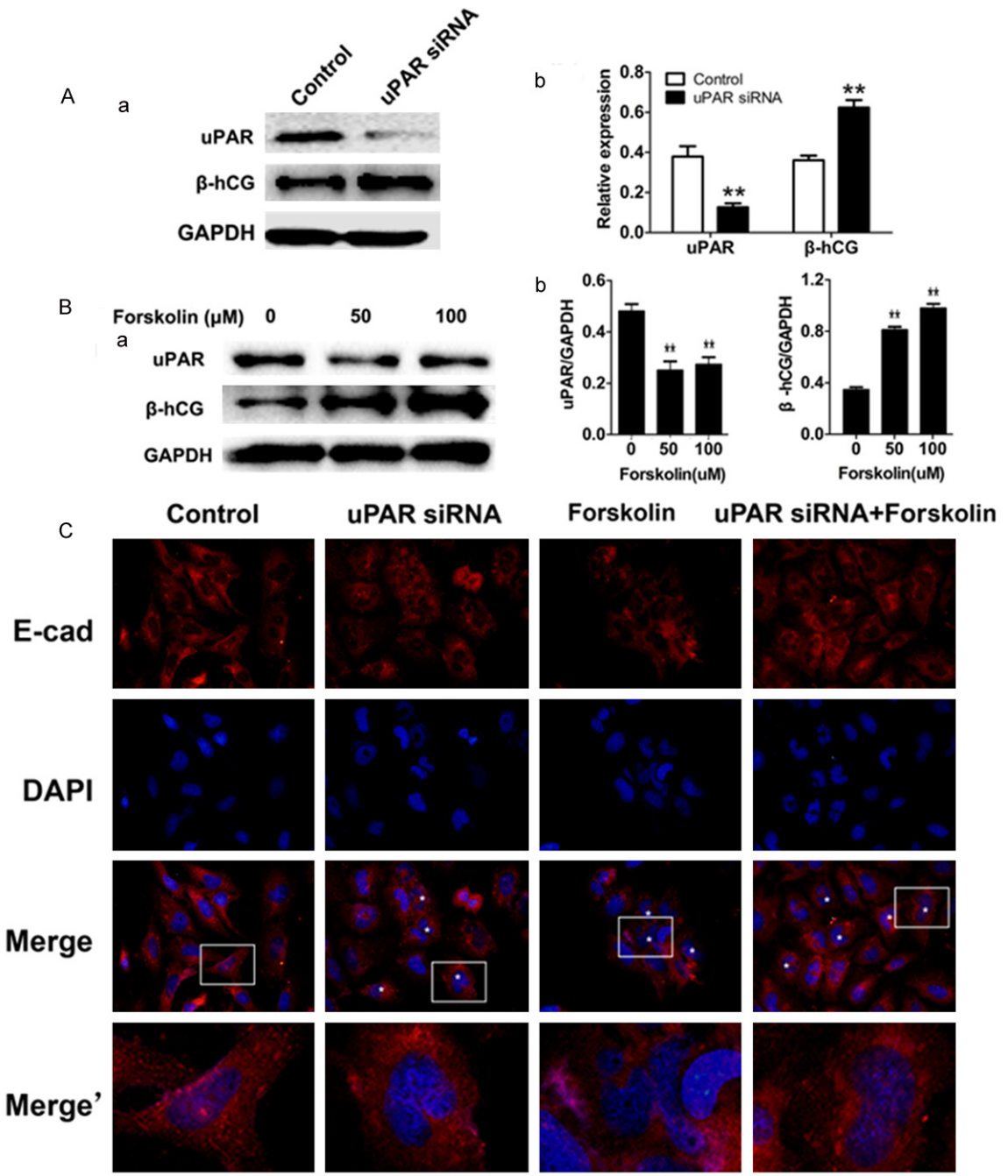

Figure 4. Effect of uPAR on trophoblast syncytialization. A. uPAR and β-hCG expression were detected after uPAR siRNA transfection in HTR8/SVneo cells by Western blot. B. Forskolin inhibits uPAR and β-hCG expression in HTR8/SVneo cells. C. 24 hours after transfection with uPARsiRNA or control siRNA, HTR8/SVneo cell treated with forkolin, multinucleated cell were observed. Cells were immunostained with E-cadherin (red), and nuclei (blue) were stained show multinucleated syncytia. Multinucleated syncytia are indicated with white stars. Representative immunofluorescence image (100×, 400×) showed the fusion of HTR8/SVneo cells in different treated group.

had significantly lower expression levels of uPAR mRNA and protein than those transfected with scramble RNA (Control), which was used as the control (**Figure 2B** and **2C**). Transwell migration and matrigel invasion assays were used to evaluate the HTR8/SVneo cells migration and invasion ability, respectively. Compared with the Control siRNA, uPARsiRNA group sig-

nificantly decreased the percentage of cells that showed migration (**Figure 2D**; $^{**}P<0.01$) or invasion (**Figure 2E**; $^{**}P<0.01$).

The remodeling of extracellular matrix is crucial during trophoblast invasion. Here, we evaluate the activity of MMP-2 and the levels of TIMP-1 and -2 using gelatin zymography and western blot, respectively. As illustrated in **Figure 2F**, the suspension medium from uPARsiRNA treated cells exhibited much lower levels of MMP-2 activity than that from control siRNA treated cells. TIMP-1 and -2 levels were significantly higher in uPARsiRNA transfected cells than in those transfected with control siRNA (**Figure 2G**).

The impace of uPAR on HTR8/SVneo cells of apoptosis and proliferation were assessed in parallel. Compared with control siRNA, uPAR siRNA had no obvious effect on the expression of pro-apoptotic cleaved caspase 8 and 9 in HTR8/SVneo cells (**Figure 3B**). However, uPAR siRNA significantly decreased proliferation of HTR8/SVneo cells by CCK-8 assay (**Figure 3A**).

uPARsiRNA inhibit trophoblast syncytialization

To investigate the role of uPAR in trophoblst cell syncytialization, the level of β-hCG, which seemed as the marker of trophoblast formation of multinucleated syncytium, was measured. The data showed that uPARsiRNA increase the β-hCG secretion (**Figure 4A**), with the promoted multinucleated syncytium formatted in HTR8/SVneo cells (**Figure 4C**). Furthermore, forskolin, which could induce fusion of trophoblast cell, caused decreased of uPAR protein, meanwhile significantly increased the number of multinucleated cells (**Figure 4B** and **4C**).

Discussion

Successful embryo implantation depends on complete placentation. Differentiation of CTBs into STBs and EVTs is a critical process required for the development of a functional placenta [21]. Impaired trophoblast differentiation often leads to abortion or pregnancy-related complications and diseases, such as fetal uterine growth restriction, pre-eclampsia, and the HELLP syndrome [22]. Here, we detected uPAR expression in human placental villi in normal pregnant women and patients with threatened abortion, using immunohistochemistry. The results showed that uPAR was expressed in the trophoblast column and EVT cells that invaded into maternal deciduae (**Figure 1A**). Additionally, uPAR was expressed in trophoblast cells to a greater extent in the first trimester than that in the patients with threatened abortion (**Figure 1A**). Based on these results, we hypothesize that uPAR may participate in the regulation of trophoblast differentiation.

uPAR is a GPI-anchored protein on the cell plasma membrane and is highly expressed in several human cancers such as breast cancer, melanoma, and hepatoma. uPAR expression correlates with cell proliferation, migration, invasion angiogenesis, and the malignant phenotype of cancer [15, 23-25]. Recent studies have shown that uPAR is expressed on the trophoblasts during the first trimester. Margreet et al. reported that uPAR was detected in the decidual tissues and extravillous trophoblasts [20]. Fenga et al. reported that uPAR mediates trophoblast invasion and plays a significant role in angiogenesis during early macaque implantation [26]. However, little information is available regarding the role of uPAR in trophoblast differentiation, especially compared with normal pregnant women and threatened abortion. The present study demonstrated that uPAR is expressed on human trophoblast cells of placental villi. Additionally, silencing uPAR not only significantly inhibited trophoblast outgrowth in the extravillous explants culture model, but also suppressed the migration and invasion potential of cultured trophoblast cells (**Figure 2**). The present study thus, identifies a new role of uPAR in placentation.

Using an in vitro extravillous explants culture model, uPAR was shown to inhibit the outgrowth of trophoblasts. EVT cells are highly invasive and degrade ECM, which facilitates embryo invasion into maternal decidua. MMP-2 and TIMPs are also associated with cell invasiveness and metastasis potential [27]. In this study, we found that silencing uPAR inhibited trophoblast cell invasion by down-regulating MMP-2 activity, while up-regulating the expression of TIMP-1 and TIMP-2, which are inhibitors of MMP activity in tissues (**Figure 2**). On the other hand, since trophoblast cell outgrowth was reduced, we explored if this inhibition was a result of decreased trophoblast cell proliferation. Cell proliferation assay showed that uPAR

siRNA significantly decreased HTR8/SVneo cell proliferation until 48 h of culture (**Figure 3**), but had no influence on cell apoptosis. The results indicate that uPAR regulates trophoblast outgrowth not only by altering MMP-2 section, but also by influencing trophoblast cell proliferation.

Compared with normal pregnant women, uPAR expression on the villi of patients with threatened abortion decreased in both, cytotrophoblasts and syncytiotrophoblasts. Additionally, uPAR was not detectable in all the syncytiotrophoblasts in normal pregnant women villi using immunohistochemistry. We further explored if uPAR played a role in trophoblast syncytialization. Cytotrophoblasts fused to form syncytiotrophoblasts, with multiple nuclei, which are responsible for gaseous and nutrient exchange between maternal and fetal blood outside the villi [28]. Several molecules including vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF)-I and its receptors (IGF1R), cAMP-responsive element binding protein, and CUL1 have been shown to regulate trophoblast cell fusion [6, 29, 30]. Our results demonstrate that uPARsiRNA promote fusion of HTR/SVneo cells (**Figure 4**). Silencing uPAR increased levels of hCG, which is commonly accepted as an STB differentiation marker. We also found that uPAR siRNA promoted HTR/SVneo cell syncytialization and that uPAR expression decreased during forskolin-induced HTR/SVneosyncytialization. Although evidence suggests that uPAR may be involved in trophoblast syncytialization, the detailed mechanism needs further investigation.

In summary, we provided valuable information for the role of uPAR at the fetal-maternal interface. uPAR expression is higher in normal pregnant women than that in patients with threatened abortion. uPAR promotes trophoblast migration and invasion during embryo implantation as well as plays a role in trophoblast differentiation into syntrophoblasts. Thus, the results from this study provide a possible cause for abortion.

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Disclosure of conflict of interest

None.

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