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Original Article

CUL4A enhances human trophoblast migration and is associated with pre-eclampsia

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Abstract: We previously found that cullin 4A (CUL4A) is able to promote trophoblast invasion. However, the role of CUL4A in other trophoblast behavior such as migration and the association with pregnancy disorder remains unclear. In this study, Immunostaining revealed that CUL4A was relatively lowly expressed in placentas from pre-eclampsia patients compared with the normal controls. Spiral arteries and inadequate trophoblast migration remodeling result in poor placental perfusion, which mediated maternal injury and may lead to pre-eclampsia. To confirm CUL4A's function on trophoblast migration, we employed human villous explants culture and trophoblast cell line HTR-8/SVneo migration and invasion assay. According to our data, knocking-down CUL4A expression inhibited villous explant's outgrowth significantly *in vitro*, and down-regulated migration of HTR8/SVneo cells ($P<0.01$). This effect might be due to reduced matrix metalloproteinases activities, whereas the apoptosis and proliferation of trophoblast cells were not affected. Moreover, CUL4A siRNA increased the levels of both tissue inhibitors of MMPs (TIMP)-1 and -2 significantly. These results suggested that CUL4A may play an essential role in trophoblast cells' migration and dysregulation of CUL4A may be associated with pre-eclampsia.

Keywords: Cullin 4A, pre-eclampsia, explant culture, trophoblast cell, migration

Introduction

The development of mammalian placenta is a fine-tuned process. Proper differentiation of certain trophoblast cell lineages is required for a healthy placentation. Trophoblast cells will differentiate, either into the highly invasive extravillous cytotrophoblast cells (EVTs) or into the syncytiotrophoblast that is formed by cell-cell fusion [1]. Defects of these differentiation processes may lead to sever pregnancy-associated diseases such as pre-eclampsia (PE) or intrauterine growth restriction (IUGR) [2]. Trophoblast migration/invasion is regulated spatially and temporally. Uterine and trophoblastic factors at the fetal-maternal interface have great impacts on this process. For instance, matrix metalloproteinases (MMPs) secreted by cytotrophoblast cells promote while tissue inhibitor of MMP (TIMP) inhibits their migration/invasion [3, 4]. Lots of signaling pathways are involved in regulating trophoblast migration/invasion [5].

Seven mammalian cullin (one subtype of RING-type ubiquitin ligases) proteins including CUL1, 2, 3, 4A, 4B, 5 and 7 have been identified [6]. CUL1, CUL3 and CUL4A, are reported to be essential during early embryonic, as knockout mice of these genes fail to develop 7.5 days postcoitum (dpc) before the formation of placenta [7-9]. Mutations in some cullin genes are reported to be associated with human growth retardation syndromes [10]. CUL7 is a key factor regulating the EMT process of the trophoblast cell lineage [11], and its mutations have also been identified in 3-M syndrome and the Yakuts short stature syndrome [10, 12]. Our previous work has shown that CUL1 and CUL3 promote human trophoblast cell invasion and dysregulation of CUL1 and CUL3 expression are associated with PE [13, 14]. And our published study found CUL4A expression in first-trimester villi and invasive extravillous trophoblast cells (EVTs). CUL4A was able to promote trophoblast invasion [15]. However, whether the expression of CUL4a has any clinical relevance remain elu-

sive. Moreover, the detailed mechanism of how CUL4A regulates the behavior of trophoblast cells needs further investigation.

Considering the fact that CUL4A proteins are expressed in the EVTs and lowly expressed in PE patients, we hypothesized that CUL4A might also regulate trophoblast migration besides invasion. Human early extravillous trophoblast cell line HTR8/SVneo was used to examine CUL4A's function in trophoblast migration. Human first trimester villous explant culture as ex vivo model was used to investigate CUL4A's regulation in EVT cell migration.

Materials and methods

Tissues collection

Human placental tissues from the first trimester (5-9 weeks) were obtained from healthy women that underwent legal abortion for non-medical reasons; term placentas (pre-eclampsia and normal) were collected after caesarean birth. Ethical approval was granted by Ethic Committee of the Xuan Wu Hospital in Beijing. All patients were informed and consent to donate their placentas. All the sample tissues were collected and stored in ice-cold DMEM, transported to the laboratory within half an hour after surgery, and washed with ice-cold PBS for immunostaining study (normal third trimester = 3, pre-eclampsia third trimester = 3) or protein extraction (normal third trimester = 8, pre-eclampsia third trimester = 8).

Cell culture and RNA interference (RNAi)

HTR8/SVneo cell line was widely used as a model for EVT migration during the first trimester. Cells were cultured in RPMI 1640 (Gibco, MA) media containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin, and incubated under 5% CO_2 at 37°C. For concentrating supernatants, cells were cultured in serum free media after siRNA transfection.

The siRNA transfection in HTR8/SVneo cells and explants were performed as described previously [13]. CUL4A siRNA: 5'-UAUAGUCUCUGUCUAUAAGUGACUC-3'.

Western blotting

Proteins were extracted from tissues or cells with whole cell lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 10 mM sodium pyrophosphatate, 1.5 mM $MgCl_2$, 100 mM NaF, 10% glycerol, and 1% Triton X-100) containing an inhibitor cocktail (1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, and 1 mM sodium orthovanadate). Culture media were concentrated using Microcon YM-3 centrifugal filter (Millipore corp.). The protein concentration was measured using the Bradford method by spectrophometry at 595 nm (Beckman DU530, Fullerton, CA). Thirty microgram of denatured protein per well were subjected to SDS-PAGE according to standard protocols. Separated proteins were transferred electrophoretically onto a pure nitrocellulose blotting membrane (Pall Corporation, Pensacola, FL). Blocked with 5% skim milk for 1 h at room temperature, the membrane was sequentially incubated with primary antibodies against CUL4A (A300-739A, Bethyl), TIMP-1 (sc-5538, Santa Cruz, CA), TIMP-2 (sc-5539, Santa Cruz, CA) caspase3 (sc-7272, Santa Cruz, CA) cleaved caspase3 (9664, cell signaling, MA) and GAPDH (ab37187, Abcam, Cambridge, UK) overnight at 4°C, and washed for 10 min with TBST (three times). A subsequent incubation with HRP-conjugated antibodies in 5% skim milk was carried out for 1 hour at room temperature, followed by three times washes with TBST. Immunoreactive bands were detected using enhanced chemiluminescence (Pierce Chemical Co., Rockford, IL).

Immunofluorescence staining

Immunofluorescence was performed on formalin-fixed, paraffin-embedded 5 μm sections. Sections were dewaxed in xylene and rehydrated through gradient of ethanol. Blocked with normal goat serum for 20 min, sections were incubated with primary antibodies against rabbit polyclonal CUL4A (A300-739A, Bethyl) at a 1:500 dilution. Washed in PBS, the sections were incubated with secondary antibody to fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Zhongshan Golden Bridge Crop., Beijing, China) at a 1:100 dilution for 30 min at 37°C. 4',6'-Diamidino-2-phenylindole (DAPI) was used to stain the nuclei.

Explant culture

The explant culture was performed as described previously [13]. In brief; small pieces of tissue (2-3 mm) from tips of first trimester placental villi (5-8 weeks) were dissected. The sam-

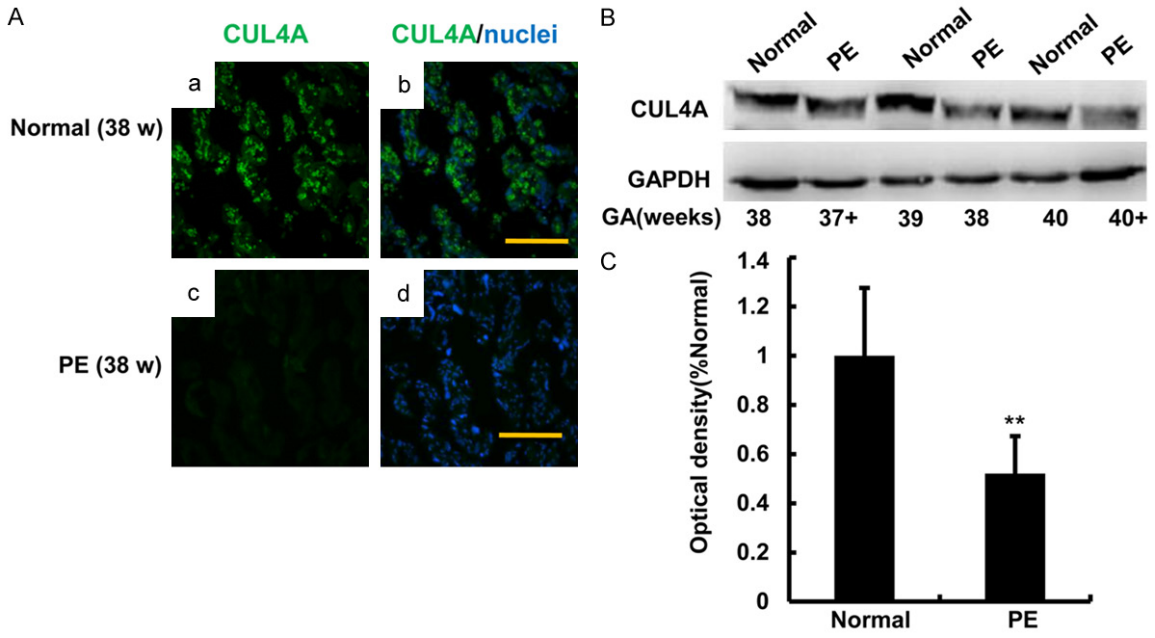


Figure 1. CUL4A is significantly down-regulated in the human placental villi from PE patients. (A) Immunofluorescent staining using CUL4A antibody (green) shows that the expression of CUL4A protein is lower in PE patients (c) as compared with normal controls (a) at the same exposure time. (b, d) DAPI staining of sections (a and c) to visualize the nuclei. Bar = 50 μm. (B) Western blotting showing the expression of CUL4A proteins in the placental villi from normal pregnant (n=3) or pre-eclamptic (n=3) women. (C) Graphic depiction of the Western blotting results. Three groups was shown, representing the total 8 groups utilized in this study. (*t*-test; **, $P<0.01$).

ples of dissected villi were explanted in Millivell-CM culture dish inserts (0.4 μm pore size, Millipore, Carrigtwohill, Co. Cork, Ireland) precoated with phenol red-free matrigel substrate (Becton Dickinson, Bedford, MA). Inserts were placed into 24-well culture dishes (Costar, Cambridge, MA). The explants were cultured in serum-free DMEM/F12 media with 500 nM siRNA, 100 IU/ml penicillin and 100 μg/ml streptomycin, at 5% O_2/5% CO_2/90% N_2. For up to four days, EVT sprouting and migration from the distal end of the villous tips were recorded every day. The extent of migration (*i.e.* the distance from the cell column base to the tip of the outgrowth) was measured at defined positions with SPOT software. All explant experiments with cultured villi were repeated three times and were replicated in four separate sets of explants.

Transwell migration assay

As previously reported, the migratory ability of HTR8/SVneo cells was determined by their ability to cross the 8 μm pores on the filter of the migration chambers [13].

Hochest 33258 staining

The Hochest 33258 staining was performed as described previously [13]. In brief, cell suspension (around 0.5×10^4 cells) was incubated with neutral-buffered formalin (10%) containing Hoechst 33248 dye (12.5 ng/ml, Sigma). The shrunken, condensed cells and fragmented nuclear were identified as apoptotic cells. The number of apoptotic cells in 200 total cells was counted under a fluorescent microscope (Olympus IX51, Japan).

Cell proliferation assay

HTR8/SVneo cells transfected with CUL4A siRNA or the control siRNA for 24 h were plated in a 6-well plate at a density of 1.5×10^5 cells per well. The plate was put in an incubator and the number of cells was counted every 24 hours.

Gelatin zymography

Analysis of gelatinolytic activity was performed using 10% (w:v) polyacrylamide gels containing 0.5 mg/ml gelatin (Difco Laboratories, Detroit,

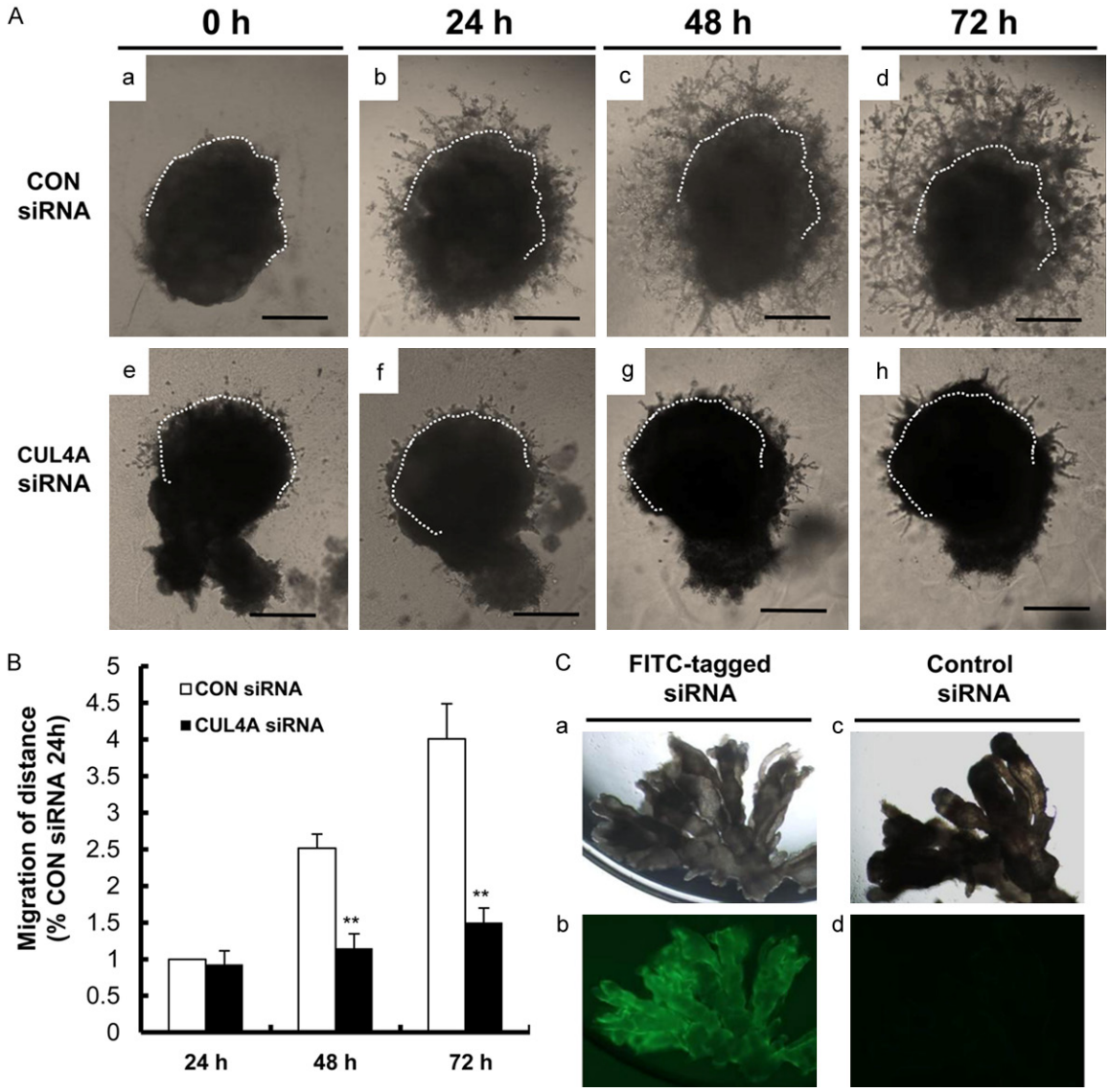
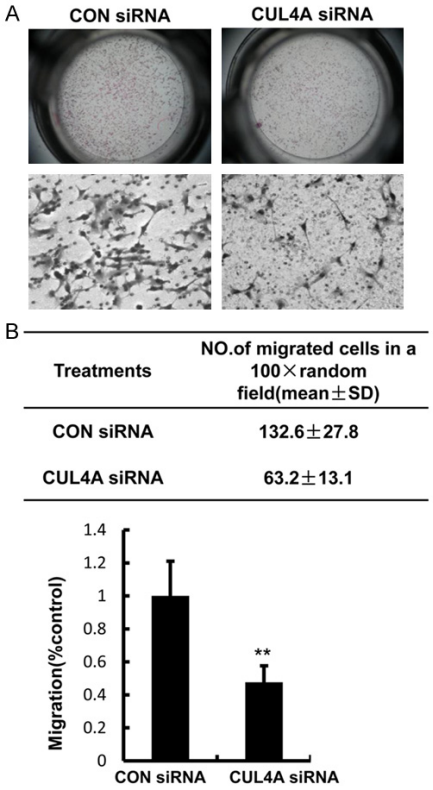


Figure 2. Silencing of CUL4A suppresses the outgrowth of extravillous explant cultures. (A) Serial pictures of human placental explants that were incubated with CUL4A siRNA or control (CON) siRNA were taken under the light microscope. Bar = 100 μm. Outgrowth cells from the villi tip were above the white dotted line. Bar = 100 μm. (B) A statistical analysis of the migration distance of EVTs from villous tips. (*t*-test; *, $P<0.05$; **, $P<0.01$). (C) Placental Villi transfected with FITC-tagged siRNA (a and b) or control siRNA (c and d) were observed under light microscopy (a and c) or fluorescent microscopy (c and d) to show the transfection efficiency (b) or the background fluorescence of villi.

MI) as previously described [16]. Briefly, conditioned medium was diluted in 4X sample buffer (8% SDS (w:v), 0.04% bromophenol blue (w:v), 0.25 M Tris-HCl) and incubated at 37°C for 30 min, and equal amounts of protein were subjected to substrate-gel electrophoresis. After electrophoresis, the gel was washed twice in 2.5% Triton X-100 (v:v) and 50 mM Tris-HCl (pH 7.5) for 30 min at room temperature, and incubated in calcium assay buffer (50 mM Tris, 10 mM $CaCl_2$, 1 mM $ZnCl_2$, 1% Triton X-100, pH 7.5) for 24 h at 37°C. The gel was then stained with Coomassie Brilliant Blue R250 and de-stained in 10% acetic acid to reveal zones with gelatinase activity.

Statistical analysis

Each experiment was performed at least three times. The bands from Western blotting and gelatin zymography were quantified by Meta-View Image Analyzing System (Version 4.50; Universal Imaging Corp., Downingtown, PA). Results were presented as means ± SD. Statistical analyse (*T*-test) was performed using the Statistical Package for Social Science



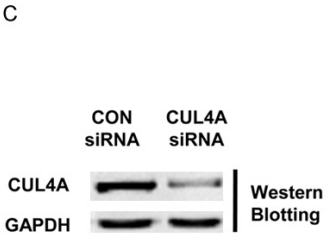


Figure 3. CUL4A siRNA inhibits migration of HTR8/SVneo cells. A: Whole and representative images of transwell filters containing migrated cells from migration assay. B: The number of migrated cells in a 100X random field (top). The statistical bar graphs showing the summary of three independent experiments (bottom). (*t*-test; **, *P*<0.01). C: Examination of CUL4A knockdown efficiency after by Western blotting. GAPDH was used as a loading control.

(SPSS for Windows package release 10.0; SPSS Inc., Chicago, IL) and were shown in Results and Figure legends. P<0.05 was considered as statistically significant. *, *P*<0.05. **, *P*<0.01.

Results

CUL4A is down regulated in placental villi from PE patients

Placental villi from pre-eclamptic patients and normal pregnancy controls of matched gestational stages were dissected for protein extraction and tissue sections. As shown in **Figure 1A**, the expression of CUL4A protein was lower in PE patient as compared with normal ones at the same exposure time by immunofluorescent staining. Western blotting (**Figure 1B**) and the statistical analysis (**Figure 1B**) showed that CUL4A was significantly lower in the placental villi from pre-eclamptic patients, compared with control placentas.

CUL4A siRNA inhibits migration of EVTs in a villous explant culture model

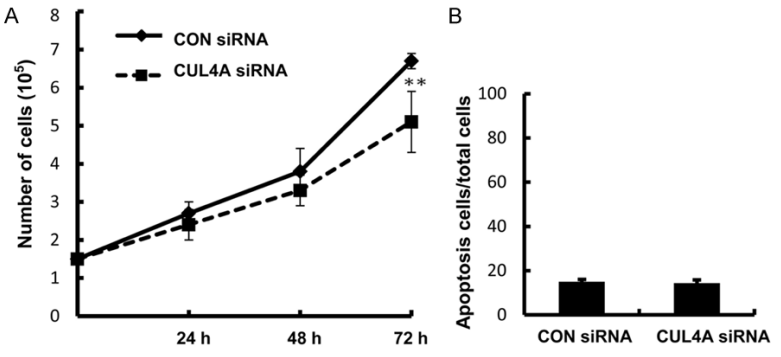
Inadequacy of trophoblast migration/invasion and spiral artery remodeling result in poor placenta perfusion often occurs during early pregnancies, which lead to maternal injury such as the onset of pre-eclampsia [17, 18]. Given the evidence that the expression of CUL4A in pre-eclamptic placentas was lower than normal ones, we then investigated whether CUL4A affect the migratory capacity of trophoblast cells. Villous explants obtained from first trimester human placental villi (5 w-8 w) were cultured on matrigel-coated dishes and incubated with control or CUL4A siRNA. As a result of EVT migration, the outgrowth distance over the matrigel surface was measured at 24, 48, and 72 h. As the data shown in **Figure 2**, CUL4A siRNA treated groups did not affect the migratory capacity of EVTs at 24 h as compared to controls (*t*-test; *P*>0.05) (**Figure 2Ab** and **2Af**, **2B**). However, at 48 and 72 h of *in vitro* culture, silencing CUL4A significantly inhibited the outgrowth of EVTs (**Figure 2Ac**, **2Ag**, **2Ad**, **2Ah**, **2B**; 48 h: *P*<0.01; 72 h: *P*<0.01). Effective introduction of siRNA into the explant was determined by transfecting with a fluorescent (FITC)-labeled siRNA (**Figure 2C**).

Knocking down CUL4A significantly inhibits migration of HTR8/SVneo cells

To further confirm the role of CUL4A in trophoblast migration, transwell cell migration models were performed in triplicate. The expression level of CUL4A protein in HTR8/SVneo cells significantly decreased after transfection of CUL4A siRNA by western blotting (**Figure 3C**). HTR8/SVneo cells treated with CUL4A siRNA or control siRNA after 24 hours, were plated onto pure filter for following migration measurement 24 hours later. Comparing the control siRNA, CUL4A siRNA significantly reduced migration based on the percentage of cells that migrated to the other side of the filter (**Figure 3B**; *t*-test; *P*<0.01).

Reduced expression of CUL4A has no obvious effect on the proliferation and apoptosis of HTR8/SVneo cells

We examined the proliferation and apoptosis of HTR8/SVneo to exclude the possibility that the



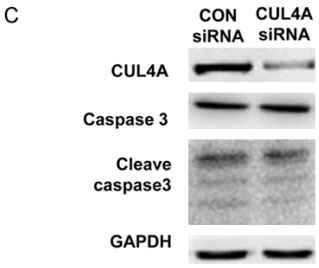
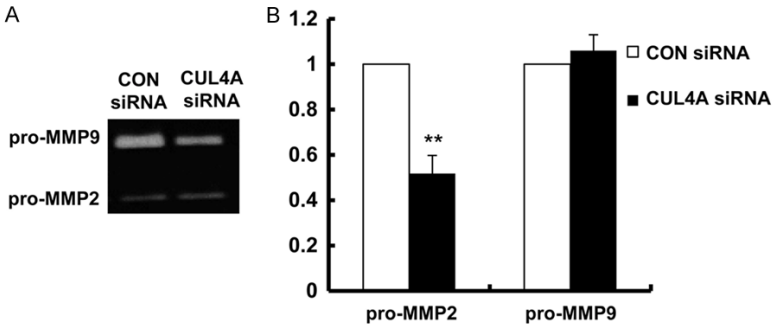


Figure 4. The effect of CUL4A siRNA on proliferation and apoptosis of HTR8/SVneo cells. A: Proliferation assay of HTR8/SVneo cells transfected with indicated siRNAs (*t*-test; **, *P*<0.01). B: Ratio of apoptotic cells in HTR8/SVneo cells transfected with indicated siRNAs (*t*-test; *P*>0.05). C: Western blotting results using indicated antibodies. GAPDH is a loading control.



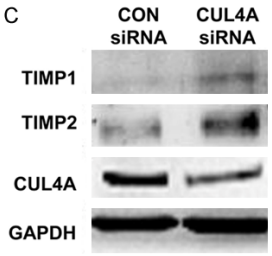


Figure 5. CUL4A siRNA inhibits the gelatinolytic activities of pro-MMP9 and enhances the expression of TIMP-1, -2. A: Serum-free culture medium was collected after 48 h siRNA transfection for gelatin zymography assay analysis. B: Statistical analysis of the zymographic results in A (*t*-test; **, *P*<0.01). C: Serum-free culture medium or the whole cell lysates of HTR8/SVneo cells transfected with indicated siRNAs were used for western blotting.

reduction in cell migration after CUL4A siRNA transfection is due to the decrease in cell proliferation or the increasing cell apoptosis. The results showed that the siRNA had no obvious effect on cell proliferation at both 24 hours and 48 hours of transfection (**Figure 4A**; *t*-test; *P*>0.05). But the significantly reduced proliferation of HTR8/SVneo cells after 72 hours of culture were observed (**Figure 4A**; *t*-test; *P*<0.01). Due to the migration assay was finished after 48 h of siRNA transfection, there was no significant difference in the cell number between CUL4A knockdown cells and the control group.

Silencing of CUL4A markedly decreased the activity of proMMP-9 and increased the secretion of TIMP-1,2

Migratory trophoblast cells express MMPs especially MMP9, MMP2 to degrade extracellular matrix (ECM) to promote migration/invasion. In order to explore mechanisms underlying which knockdown of CUL4A inhibits trophoblast migration, we examined the gelatinolytic activities of MMP2 and MMP9 by gelatin zymography (**Figure 5A**). The results showed that CUL4A interference significantly reduced pro-MMP-9 but not pro-MMP-2 activities in the supernatants of HTR8/SVneo on 48 hours after transfection (**Figure 5B**; *t*-test; *P*<0.01). Moreover, we observed that the protein concentrations of their tissue specific inhibitors TIMP-1 and -2 augmented significantly in supernatants of CUL4A siRNA transfection as compared with control siRNA (**Figure 5C**).

Discussion

One major cause of the onset of pre-eclampsia, is proposed to be due to insufficient trophoblast invasiveness/migration resulting in poor spiral arterial remodeling and consequently inadequate placental perfusion [19, 20]. Previously we have shown that CUL4A are able to promote trophoblast invasion [15]. However, whether CUL4A is associated with pregnancy disorder remains unclear. In this study, we found the level of CUL4A was relatively lower in the placentas from PE patients as compared with controls (**Figure 1**), suggesting that CUL4A may be disrupted under pathological condition, which highlights the importance of CUL4a during pregnancies. Besides the ability to promote invasion of trophoblast cells, in our present study we have

further demonstrated that CUL4A promotes trophoblast migration, based on the following lines of evidence First, CUL4A siRNA significantly inhibited trophoblast outgrowth and EVT migration in an *ex vivo* extravillous explant culture model. Second, CUL4A enhanced migration of trophoblast HTR8/SVneo cells as revealed by RNAi experiment. Finally, down-regulation of pro-MMP-9 and up-regulation of TIMP-1 and -2 were accompanied by the reduction in migration after CUL4A siRNA administration.

In human placenta, degradation of ECM is involved during extravillous trophoblast cells migration/invasion. In this study, CUL4A siRNA was demonstrated to be able to decrease the gelatinolytic activities of MMP-9 in the media of HTR8/SVneo and explants culture. Our results showed that secretion of both TIMP-1 and -2 were elevated in CUL4A-knockdown cells. These indicate that CUL4A might promote trophoblast invasion and migration by enhancing the positive regulators and inhibiting the negative regulators. The precise mechanism through which CUL4A controls the levels or activities of MMPs or TIMPs, and whether these enzymes are direct targets for CUL4A, require further investigation.

Previously it has been reported that CUL4A regulates biological events such as DNA replication and repair, chromatin remodeling, spermatogenesis, hematopoiesis, cell-cycle progression and cancer cell metastasis [21, 22]. Additionally, a recent study showed that CUL4A induces EMT, which is critical in placentation [21, 23]. Our study further highlights the essential role for CUL4A during human placentation.

In summary, in combination with our previous report, our present study suggests potential association between CUL4A and the onset of preeclampsia, since lack of CUL4A impairs the invasion and migration of human trophoblast cells.

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Disclosure of conflict of interest

None.

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