

## Original Article

# CUL4A is expressed in human placenta and involved in trophoblast cell invasion

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**Abstract:** CUL4A, a member of the human cullin protein family, plays a crucial role in the degradation of many damaged proteins to maintain the balance of the cellular environment, thus regulating many cellular events, including cell-cycle control, DNA replication and DNA repair. Mounting evidence suggests that cullin family proteins play a critical part in placental development. However, whether CUL4A is involved in the regulation of placentation is not clear. This study aimed to verify the role of CUL4A in human trophoblast cell invasion. We first detected CUL4A expression in first-trimester villi and invasive extravillous trophoblast cells (EVTs) by immunohistochemistry. Then, we further checked CUL4A expression in multiple trophoblast cell lines using Western blotting and reverse transcription polymerase chain reaction (RT-PCR). Furthermore, we tested the invasive capabilities of the HTR8/SVneo cells by CUL4A RNAi. Immunohistochemistry showed CUL4A expression in first-trimester villi and invasive extravillous trophoblast cells (EVTs). CUL4A expression was detected in HTR8/SVneo, B6tert, JAR and JEG-3 cells, during which the highest expression happened in HTR8/SVneo cells of EVT origin. Matrigel invasion assay displayed that the invasive capabilities of the HTR8/SVneo cells were inhibited by CUL4A RNAi. In summary, CUL4A is involved in human trophoblast invasion and may function as a regulator during early pregnancy.

**Keywords:** Cullin4A, placentation, trophoblast cell, invasion/migration

## Introduction

The placenta, composed of villi, amnion and decidua basalis, is critical for embryonic development and a successful pregnancy. The finely programmed processes of trophoblast differentiation and invasion play a decisive role during pregnancy [1]. Placental malformation may contribute to multiple severe pregnancy complications, including spontaneous miscarriage, fetal growth restriction and preeclampsia. Trophoblasts, the major cellular components of villi, play an essential part in placentation [2]. The following three types of trophoblasts exist: progenitor cytotrophoblasts (CTBs) that reside at the basement membrane of the placental villi; fused cytotrophoblasts that are called syncytiotrophoblasts (STBs); and cytotrophoblasts that fuse with extravillous cells to acquire invasive capabilities which are called extravillous

trophoblasts (EVTs) [3, 4]. The STBs form the outer layer of the floating villi, which is the major area of fetal-maternal interface [5], and mediate nutrient transport and hormone production, including the hormones human chorionic gonadotropin (hCG) and human placental lactogen (hPL). EVT's undergo numerous cell processes such as programmed cell death, differentiation, adherence to and degradation of the extracellular matrix, and invasion and interaction with the maternal immune system [1]. There are two EVT subpopulations: interstitial EVT's (iEVTs) and endovascular EVT's (vEVTs). The iEVTs will migrate into the stroma of the maternal deciduas, penetrate the myometrium to form placental bed giant cells and come into close contact with uterine immune cells that aid in vessel remodeling [6, 7]. The iEVTs will appear within the lumen of maternal vessels but there is still controversy on the mode of

invasion of the extravillous trophoblasts in the human placenta [6]. Insufficient invasion by EVT is associated with the development of preeclampsia [8, 9].

Cullin family proteins are important regulators of early embryonic development. CUL1, CUL3, CUL4B and CUL7 are crucial for human and murine placental development. CUL4A shares 80% identity with the CUL4B sequence, and CUL4A is involved in cell-cycle regulation and maintaining genomic stability. CUL4A acts as a fundamental character in the degradation of several well-known tumor suppressor genes such as p21, p27, DNA damage-binding protein 2 (DDB2) and p53. Furthermore, CUL4A induces epithelial-mesenchymal transition (EMT) in normal and malignant human mammary epithelial cells, resulting in enhanced *in vitro* growth, migration and invasion and *in vivo* metastasis by regulating zinc finger E-box-binding homeobox 1 (ZEB1) expression [10]. Trophoblast cell invasion shares many similarities with tumor cell invasion and is tightly controlled by a variety of endocrine and paracrine factors [11]. EVTs of the first-trimester placenta are highly invasive. They form trophoblast columns that extend from the endometrium to the proximal third of the myometrium. The placental development requires invasion of endometrium and spiral arteries. This process occurs under low-oxygen conditions, and regulatory factors that are induced under hypoxic conditions contribute in part to invasive trophoblast activation [12]. Trophoblasts synthesize and secrete insulin-like growth factor II (IGF-II), which acts in an autocrine manner. It promotes invasion into the endometrium, whereas decidual cells secrete insulin-like growth factor binding-protein-4 (IGFBP-4), which blocks this autocrine loop [13, 14]. Thus, the extent of trophoblast cell invasion is regulated by matrix degradation regulation and by factors that cause trophoblast migration.

However, the role of CUL4A in trophoblast cell invasion during placental development remains unclear. Our hypothesis is that CUL4A may be involved in this process. In this study, the expression of CUL4A in the placental villi and trophoblast cell lines were analyzed by combining immunohistochemistry, Western blotting and reverse transcription-polymerase chain reaction (RT-PCR). In addition, to confirm whether CUL4A influenced the invasive capabil-

ity of trophoblast cell, we carried out CUL4A RNAi on HTR8/SVneo cells and the matrigel invasion assay further.

### Materials and methods

#### *Tissue collection*

The specimens used in this study were collected from patients following an explanation of the research and the patients' signing of the informed consent form for villi or placenta donation for research use. All of the sample-collection procedures were approved by the Human Ethics Committees of Beijing Obstetrics and Gynecology Hospital, Capital Medical University. Human chorionic villi (6-9 w) and normal-term placentas (37-40 w) were obtained from women who underwent a legal abortion for nonmedical reasons or an elective Caesarean section, respectively. The specimens were transported to the lab immediately (within half an hour after the operation) in ice-cold Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) and then flushed with ice-cold phosphate-buffered saline (PBS). The tissues were fixed for use in immunohistochemistry (first trimester, n = 3; normal term, n = 3) or prepared for Western blotting (first trimester, n = 6; normal term, n = 3). All of the experiments in the current study were conducted following standard guidelines approved by the Human Ethics Committees of Beijing Obstetrics and Gynecology Hospital, Capital Medical University.

#### *Immunohistochemistry*

As previously described [15], the tissues were fixed with formalin and then embedded in paraffin wax before being cut into 5- $\mu$ m sections and subsequently stained with murine biotin-streptavidin-peroxidase (SP) and diaminobenzidine (DAB) (Zhongshan Golden Bridge Corp., Beijing, China) for immunohistochemical analysis. In brief, the sections were deparaffinized in xylene and rehydrated using a graded series of ethanol solutions. The antigen was retrieved by boiling the samples for 15 min in citrate buffer (10 mM citrate sodium, 10 mM citric acid, pH 6.0), while the endogenous peroxidases were inactivated by bathing the samples for 10 min in 3% H<sub>2</sub>O<sub>2</sub> in methanol. The sections were blocked with normal goat serum for 20 min followed by incubation with CUL4A primary anti-

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body (A300-739A, Bethyl Laboratories, Inc., TX, USA) at 1:200 or mouse monoclonal Cytokeratin 7 antibody (ab20206, Abcam, Cambridge, MA, USA) at 1:200 overnight at 4°C. Biotinylated secondary antibody was added after the samples were washed with PBS. The specific marker, cytokeratin 7 (CK7), was used to identify the EVT. The sections were stained using a diaminobenzidine kit. For the negative controls, normal goat serum or immunoglobulin G (IgG) was utilized instead of the primary antibody.

### Cell culture

Four trophoblast cell lines were cultured as previously reported [16]. Briefly, B6Tert cells, which are normal human first-trimester trophoblast cells, were grown in F-12/DMEM supplemented with 1% bovine serum albumin, 200 mM L-glutamine, 1 mg/ml insulin and 1 µg/ml epidermal growth factor. The JAR choriocarcinoma cell line was maintained in Roswell Park Memorial Institute (RPMI) 1640 media (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 g/ml streptomycin. The JEG-3 choriocarcinoma cell line was cultured in F-12/DMEM (1:1) containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The HTR8/SVneo extravillous trophoblast origin cell line, which was a gift from Dr. Benjamin K Tsang (Department of Obstetrics & Gynecology and Cellular & Molecular Medicine, University of Ottawa; Chronic Disease Program, Ottawa Health Research Institute, Ottawa, ON K1Y 4E9, Canada) was used as a model for first-trimester EVT invasion. The culture medium used to culture the HTR8/SVneo cells was the same medium used for the JAR cells. All of the cell lines were grown at 37°C under 5% CO<sub>2</sub>.

### Reverse transcription and semi-quantitative PCR

The total RNA was extracted from the HTR8/SVneo cells, B6Tert cells, JAR cells and JEG-3 cells using TRIzol (Invitrogen) according to the manufacturer's instructions. The RNA (2 µg) was used for cDNA synthesis by reverse transcription as previously reported [16]. Reverse transcription was performed in a 20-µl reaction solution containing Superscript II reverse transcriptase (Invitrogen). The PCR was performed at 42°C for 50 min and terminated by 15 min at 72°C. The forward primer for CUL4A was

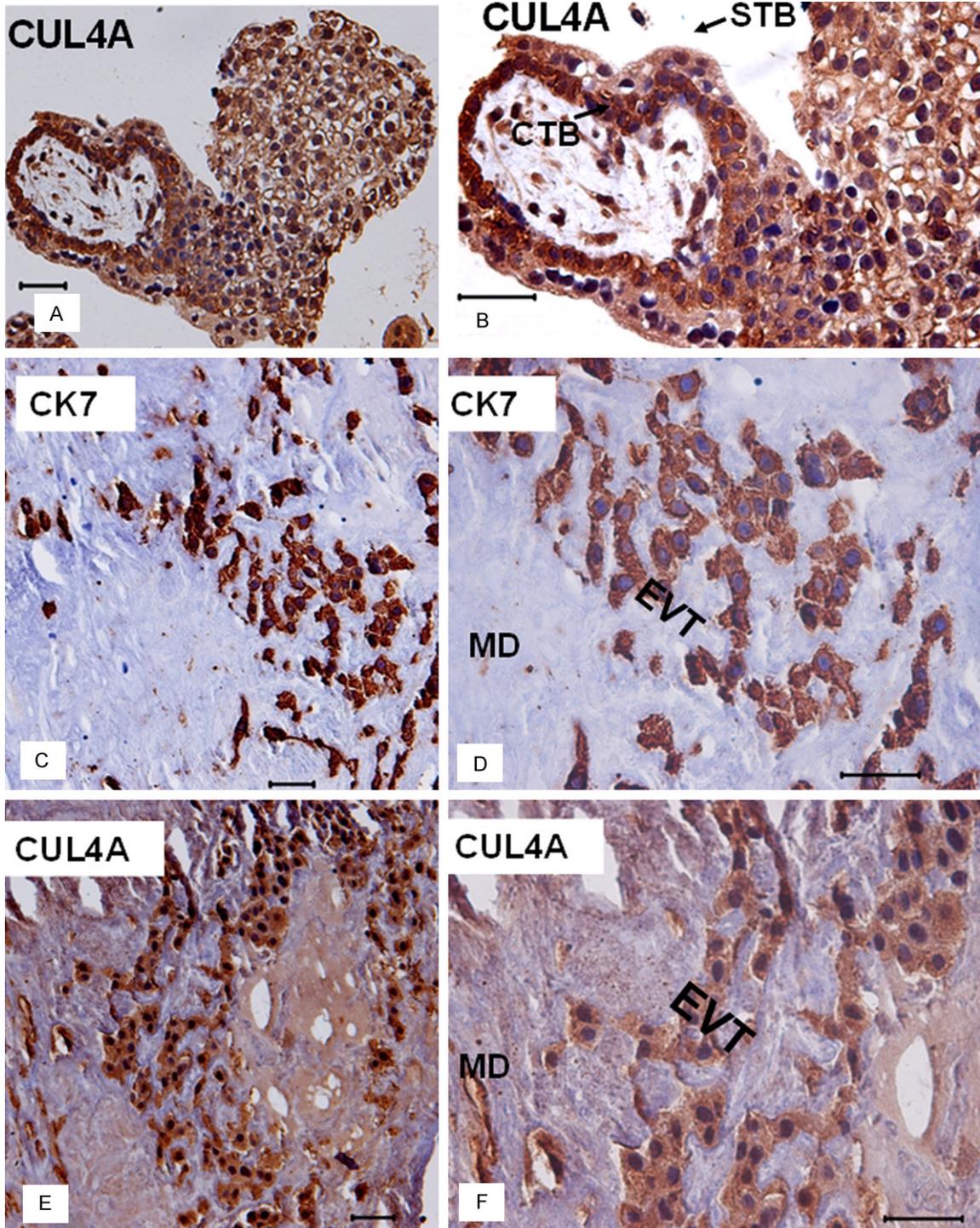
5'-GTGGAAGATGGAGACAAGTTCA-3', and the reverse primer was 5'-GTGTTTCATGAAGGGG-AACCG-3'. The primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were 5'-AGCCACATCGCTCAGACA-3' (forward) and 5'-TGGACTCCACGACGTACT-3' (reverse). The cDNA amplification began at 95°C for 30 s, then 30 thermal cycles of 5 s at 95°C and 34 s at 60°C were employed. The expression of the GAPDH housekeeping gene was used as an internal control.

### Western blot analysis

Western blotting was conducted as previously described [17]. Briefly, total protein extracts were prepared in cell lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid (EGTA), 10 mM sodium pyrophosphate, 1.5 mM MgCl<sub>2</sub>, 100 mM sodium fluoride, 10% glycerol and 1% Triton X-100) containing an inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 1 mM sodium orthovanadate). The Microcon YM-3 centrifugal filter (Millipore) was used to condense the culture medium, while the protein concentration was tested using the Bradford method via spectrophotometry at 595 nm (Beckman DU530, Fullerton, CA, USA). A total of 30 µg of the processed protein was subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer onto nitrocellulose membranes. The membranes were first blocked with 5% skim milk at room temperature for 1 h and then incubated with primary antibodies against CUL4A (1:500 A300-739A, Bethyl) and GAPDH (1:20000, ab37187, Abcam) overnight at 4°C. The membranes were then washed with Tris-buffered saline with Tween-20 (TBST) three times, incubated with monoclonal horseradish peroxidase (HRP)-conjugated antibody in 5% skim milk for 1 h at room temperature, and then washed with TBST three times. Immunoreactive bands were developed using the Enhanced Chemiluminescence system (Pierce Chemical Co., Rockford, IL, USA).

### RNA interference

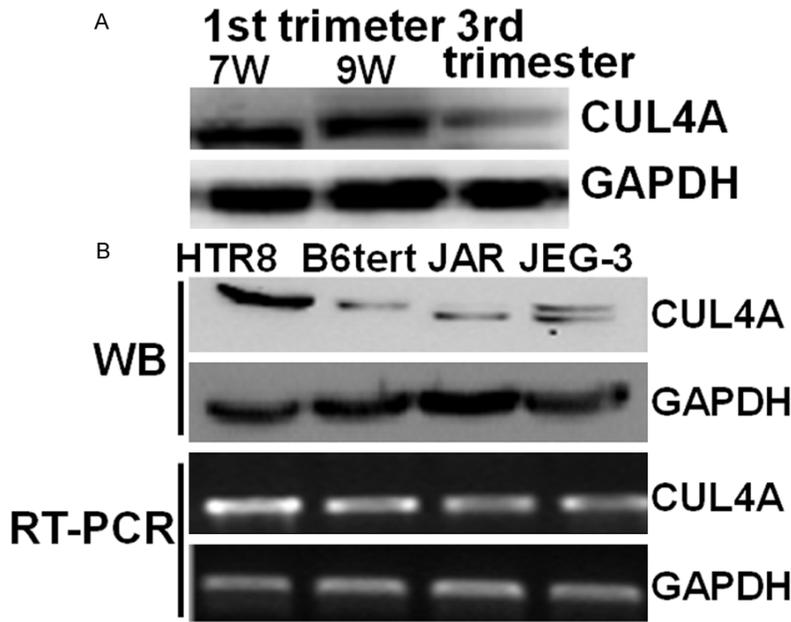
The HTR8/SVneo cells were transfected with 100 nM CUL4A short interfering (si)RNA (5'-UUAUAGUCUCUGUCUAUAAGUGACUC-3'; Invitrogen, MD) or control siRNA (scrambled siRNA,



**Figure 1.** CUL4A was expressed in first-trimester human placenta villi and EVT cells in term decidua. CUL4A immunostaining in first-trimester normal placenta villi (7w, A and B). CUL4A was strongly and exclusively stained in villous CTBs and TCs. EVTs invaded into the maternal decidua at term as shown via CUL4A (E, F) and CK7 (C, D) immunostaining. CTB: cytotrophoblast; STB: syncytiotrophoblast; TC: trophoblast column; EVT: extravillous trophoblast; MD: maternal decidua; Bar = 50  $\mu$ m.

a universal negative control; Invitrogen) with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Fluorescein

isothiocyanate (FITC)-labeled positive control siRNA (Invitrogen) was included to show the efficiency of the transfection, which was great-



**Figure 2.** Expression pattern of CUL4A in human placental villi and trophoblast cell lines. A. CUL4A was expressed in the first- (n = 6) and third- (n = 3) trimester human placenta as shown by Western blotting. B. CUL4A expression in the human trophoblast cell lines. CUL4A was detected in HTR8/SVneo, B6tert, JAR and JEG-3 cells by both Western blotting and RT-PCR analysis.

er than 90%. The cells were collected 24 h after transfection.

*Cell proliferation assay*

The HTR8/SVneo cells were plated in a 6-well plate for incubation at a density of  $2.0 \times 10^5$  cells per well. Transfection with the CUL4A siRNA or the control siRNA was performed with Lipofectamine 2000 as described above. The seeding time was set as 0 hour, and the number of cells was counted every 24 h.

The MTT assay was performed as previously mentioned [15]. In brief, the culture medium was changed to 500  $\mu$ l RPMI 1640 medium without FBS supplemented with 10% MTT reagent (3-(4,5-dimethylthiazol (-z-y1)-3,5-diphenyltetrazoliumbromide; Aplegen Corp. Beijing, China) for 4 h and 500  $\mu$ l DTW (Triton X-100:dimethylformamide (DMF): H<sub>2</sub>O at 1:3:2) for 30 min before being transferred to a 96-well plate for analysis. The optical density of each well was measured at 570 nm (Beckman DU530, Fullerton, CA, USA). The experiments were performed in triplicate.

*Matrigel invasion assay*

Cell Matrigel (Becton Dickinson)-coated transwell inserts (6.5 mm, Costar) containing poly-

carbonate filters with 8- $\mu$ m pores were used for the invasion assay as previously reported [18]. Briefly, 50  $\mu$ l of 1 mg/ml Matrigel matrix was pre-coated onto the inserts at 37°C for 4 h for gelling. The HTR8/SVneo cells ( $1 \times 10^5$  in 200  $\mu$ l of serum-free medium) transfected with siRNA were plated in the upper chamber, whereas the medium in the lower well contained 10% FBS. After 24 h incubation, the cells on the Matrigel side of the insert were scraped off gently with a cotton swab. The inserts were fixed in methanol for 10 min at room temperature and were later stained with hematoxylin and eosin (Zhongshan Golden Bridge Corp.). Light microscopy was used to

count the number of invaded cells (Olympus IX51, Japan) in five random fields at a magnification of  $\times 200$ . The procedure was conducted in triplicate. The level of cell invasion was compared with the control and described as an average of invasion percentage (%)  $\pm$  SD.

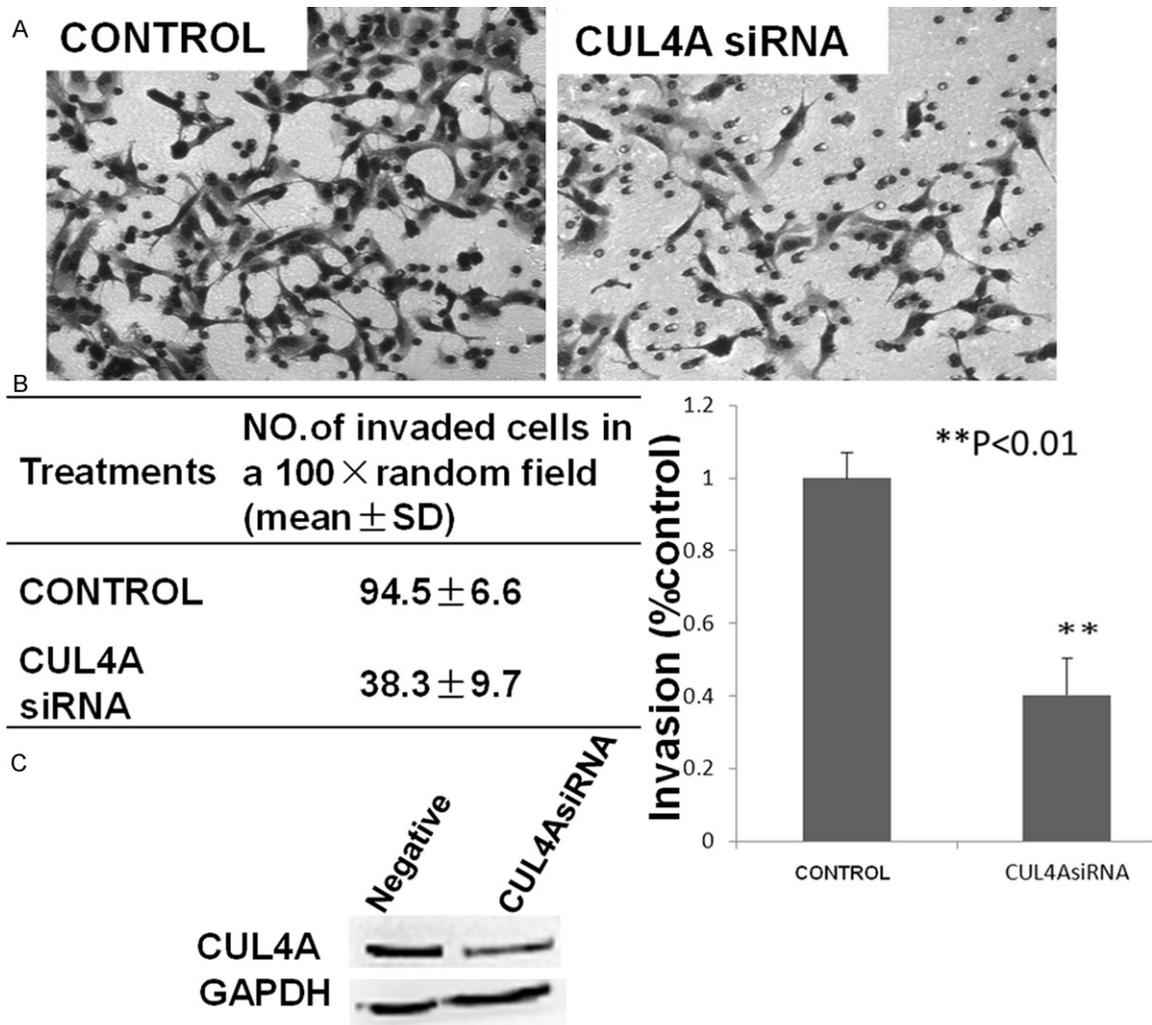
*Statistical analysis*

The results are presented as the mean  $\pm$  standard error of the mean (SEM) of at least three independent experiments. Statistical analyses were performed with the Statistical Package for Social Science (SPSS for Windows package release 10.0; SPSS Inc., Chicago, IL, USA). The quantitative data were compared using Student's t-test between the two groups.  $P < 0.05$  was considered statistically significant.

**Results**

*CUL4A is highly expressed in first-trimester villi and invasive extravillous trophoblast cells (EVTs)*

To estimate the expression of CUL4A in the different compartments of the villi at different gestational ages, CUL4A expression was first



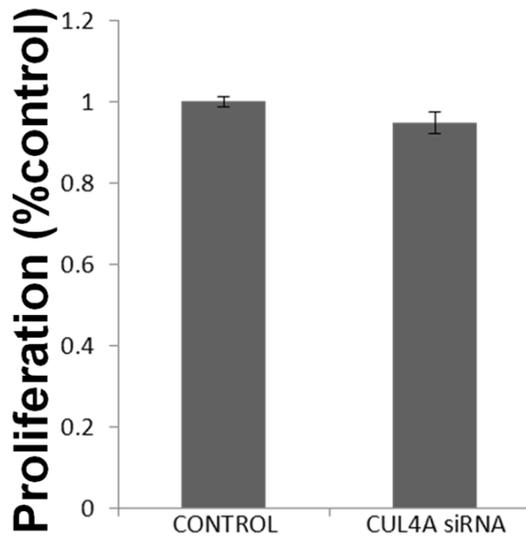
**Figure 3.** HTR8/Svneo cell invasion was inhibited after CUL4A silencing. **A.** HTR8/Svneo cell invasion was inhibited after CUL4A silencing. **B.** Left table: the number of invaded cells in a 100 random field; Right graph: statistical bar graphs representing the summary of three independent experiments (t-test; \*\*,  $P < 0.01$ ). **C.** RNAi of CUL4A was confirmed by Western blotting.

examined using immunohistochemistry. As presented in **Figure 1A** and **1B**, in first-trimester villi, CUL4A was highly expressed in the cytotrophoblast cells (CTBs) and the trophoblast column (TC). CUL4A expression was strikingly stronger in the EVT<sub>s</sub> (**Figure 1E** and **1F**) that had invaded into maternal decidua and were marked by cytokeratin 7 (CK7) (**Figure 1C** and **1D**) compared with the maternal tissues in the term placentas. The CUL4A expression pattern analyzed via immunohistochemistry was further confirmed by Western blotting (**Figure 2A**) in which CUL4A expression was also detected in both first-trimester villi and term placentas. CUL4A expression in the first-trimester villi was

markedly higher than in the term placentas (**Figure 2A**).

*CUL4A is expressed in human trophoblast cell lines*

To further verify the expression pattern, CUL4A expression was examined in HTR8/SVneo, B6Tert, JAR, and JEG-3 cells, all of which are of human trophoblast cell origin. As shown in **Figure 2B**, CUL4A protein was detected in all four of the cell lines by Western blotting, with higher CUL4A expression in HTR8/SVneo cells and relatively lower expression in the B6Tert, JAR, and JEG-3 cells. The CUL4A mRNA assess-



**Figure 4.** The effect of CUL4A siRNA on the proliferation of HTR8/SVneo cells at 48 h. The proliferation of HTR8/SVneo cells transfected with the indicated siRNAs showed no statistically significant difference compared with controls (t-test; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

ment was consistent with the protein content (**Figure 2B**).

*CUL4A siRNA significantly inhibits HTR8/SVneo cell invasion*

Recent studies have shown that CUL4A regulates many critical processes such as cell-cycle progression, DNA replication, DNA repair and cancer cell invasion and metastasis [10, 19]. To determine the character of CUL4A in HTR8/SVneo cell invasion, Matrigel assays were performed in triplicate. The HTR8/SVneo cells were plated onto Matrigel or a pure filter to measure invasion or migration followed by transfection with CUL4A siRNA or the negative control for 24 h. The CUL4A content significantly decreased in the HTR8/SVneo cells after transfection with CUL4A siRNA for 24 h (**Figure 3C**). Compared with the negative control siRNA, CUL4A siRNA significantly decreased the HTR8/SVneo cell invasion and migration (**Figure 3A and 3B**,  $P < 0.05$ ). The cell proliferation assay 48 hours after siRNA transfection and indicated that no significant difference existed between the CUL4A siRNA group and the control, suggesting that CUL4A has no effect on HTR8/SVneo cell proliferation (**Figure 4**).

**Discussion**

Trophoblast differentiation and invasion are critical events during placentation and are tightly regulated in the maternal-fetal environment. CUL4A deletions cause embryonic lethality, demonstrating that this protein is essential for embryonic development [20]. CUL7 induces EMT and promotes trophoblast migration and invasion by up-regulating the expression of ZEB1 and Slug, two transcriptional repressors of E-cadherin [16, 17]. CUL1 knockdown decreases the gelatinolytic activities of matrix metalloproteinase 9 (MMP-9) and increases tissue inhibitor of metalloproteinase (TIMP) 1/2 expression. In addition, CUL4A is involved in cancer cell migration and invasion.

In the present study, we demonstrated that CUL4A participated in trophoblast cell invasion and placentation. First, CUL4A was strongly expressed in first-trimester villi CTBs and TCs and in invasive EVT at term, all of which possess high invasive potential, suggesting that CUL4A may be related to their invasiveness. Moreover, compared with term placentas, CUL4A was highly expressed in the first-trimester villi (when trophoblast cells exert high invasiveness). Furthermore, CUL4A expression was also detected in HTR8/SVneo cells of EVT origin, B6Tert cells of normal human first-trimester trophoblast cell origin, and JAR and JEG-3 cells of choriocarcinoma origin. The expression of CUL4A in the HTR8/SVneo cells was more intense compared with its expression in the other three cell lines. We investigated the effect of CUL4A in trophoblast invasion using the Matrigel invasion assay. CUL4A knockdown by RNAi significantly decreased HTR8/SVneo cell invasion. These findings strongly suggest that CUL4A is involved in trophoblast cell invasion and placentation.

CUL4A, as a cullin protein, takes part in pleiotropic cellular activities, including cell-cycle regulation and maintaining genomic stability. CUL4A is imperative for the ubiquitination of some well-defined tumor suppressor genes such as p21 [21], p27 [22] and p53 [23]. Changes in CUL4A potentially exhibit diverse effects that alter cellular functions including differentiation, proliferation, and apoptosis. Maybe these are the reasons why CUL4A was not exclusively expressed in EVTs in term pla-

centas (**Figure 1E** and **1F**), but also moderately expressed in the background cells.

In addition, a recent study showed that CUL4A induces EMT, which is critical in trophoblast cell differentiation, invasion and placentation [10, 24]. This finding highlights a potentially essential element for CUL4A in this process. Whether CUL4A plays a role in trophoblast cell EMT remains to be determined.

In summary, this study is the first to determine the expression and functional role of CUL4A in the human placenta and EVT and suggests that CUL4A may be involved in the early stages of EVT differentiation and function.

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### Disclosure of conflict of interest

None.

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