New insights into the function of Cullin 3 in trophoblast invasion and migration

Qian Zhang1,4,*, Song Yu2,*, Xing Huang1,5,*, Yi Tan4, Cheng Zhu1, Yan-Ling Wang1, Haibin Wang1, Hai-Yan Lin1, Jiejun Fu3 and Hongmei Wang1

1State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, People’s Republic of China, 2Department of Obstetrics, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, Beijing 100026, People’s Republic of China, 3Key Laboratory of Longevity and Ageing-related Diseases, Ministry of Education, Guangxi Medical University, Nanning 530021, People’s Republic of China, 4Laboratory Animal Center, Chongqing Medical University, Chongqing 400016, People’s Republic of China and 5School of Life Sciences, University of Chinese Academy of Sciences, Beijing 100101, People’s Republic of China

Correspondence should be addressed to H Wang; Email: wanghm@ioz.ac.cn or to J Fu; Email: fujiejun126.com

*(Q Zhang, S Yu and X Huang contributed equally to this work)

Abstract

Cullin 3 (CUL3), a scaffold protein, assembles a large number of ubiquitin ligase complexes, similar to Skp1-Cullin 1-F-box protein complex. Several genetic models have shown that CUL3 is crucial for early embryonic development. Nevertheless, the role of CUL3 in human trophoblast function remains unclear. In this study, immunostaining revealed that CUL3 was strongly expressed in the villous cytotrophoblasts, the trophoblast column, and the invasive extravillous trophoblasts. Silencing CUL3 significantly inhibited the outgrowth of villous explant ex vivo and decreased invasion and migration of trophoblast HTR8/SVneo cells. Furthermore, CUL3 siRNA decreased pro-MMP9 activity and increased the levels of TIMP1 and 2. We also found that the level of CUL3 in the placental villi from pre-eclamptic patients was significantly lower as compared to that from their gestational age-matched controls. Moreover, in the lentiviral-mediated placenta-specific CUL3 knockdown mice, lack of CUL3 resulted in less invasive trophoblast cells in the maternal decidua. Taken together, these results suggest an essential role for CUL3 in the invasion and migration of trophoblast cells, and dysregulation of its expression may be associated with the onset of pre-eclampsia.


Introduction

Trophoblast differentiation and invasion are important events during placentation. Extravillous trophoblasts (EVTs), including interstitial and endovascular EVTs, are motile and highly invasive cells. They infiltrate the endomyometrium to anchor the placenta and penetrate uterine spiral arteries to establish the feto-maternal circulation (Genbacev et al. 1992). Temporal and spatial regulation of trophoblast invasion is mediated in both autocrine and paracrine ways within the trophoblast-endometrial microenvironment. Aberrant trophoblast invasion may lead to gestational diseases, such as miscarriage, intrauterine growth restriction (IUGR), pre-eclampsia (PE), and choriocarcinoma (Stepan et al. 2006, George & Granger 2010).

Two major classes of E3 ubiquitin ligases have been characterized: homologous to E6-APC-terminus domain-containing E3s and really interesting new gene (RING) finger-containing E3s. Cullin-RING ligase multisubunit complexes are the largest E3 ubiquitin ligase family (Petroski & Deshaies 2005, Zimmerman et al. 2010). Cullins are a family of scaffold proteins that assemble RING finger type E3 ligase complexes. Seven members of the Cullin family of proteins, including CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, and CUL7, have been identified in human beings (Petroski & Deshaies 2005). The best-characterized Cullin-based ubiquitin ligase is the Skp1-Cullin 1-F-box protein complex. In contrast, CUL3 associates with Bric-a-brac-Tramtrack-Broad (BTB) complex and Rbx1 to form a BTB-CUL3-Rbx1 (BCR) ubiquitin ligase complex (Canning et al. 2013). Growing evidence has revealed that BCR ubiquitin ligase complex serves as a major regulator of cell cycle and developmental processes. The substrate specificity of the BCR complex is determined by the BTB
domain proteins. For example, CUL3 assembles Keap1, a BTB domain of Kelch-like (KLHL) protein, to control stress responses by targeting Nrf2 for degradation (Kaspary et al. 2012). KLHL10 is a substrate-specific adapter of BCR complex that targets proteins for degradation during spermiogenesis (Wang et al. 2006). Mutations in KLHL3 and CUL3 can cause pseudohypoaldosteronism type II, a condition that mimics hypoaldosteronism to cause hypertension and hyperkalemia with a normal glomerular filtration rate (Boyden et al. 2012).

Most Cullins are crucial for early embryonic development. Mutations in CUL4B and CUL7 were associated with growth retardation syndromes (Gascoin-Lachambre et al. 2010). CUL1, CUL3, and CUL4A knockout mice fail to develop beyond 7.5 days postcoitum before placenta formation (Li et al. 2002). Embryos from CUL4B null mice show severe developmental arrest and usually die before embryonic day 9.5 (Liu et al. 2012). We have previously reported that CUL7 triggers epithelial–mesenchymal transition of choriocarcinoma JEG-3 cells (Fu et al. 2010). In addition, we have demonstrated that CUL1 promotes the invasion of human trophoblast cells and is significantly downregulated in the placentas from PE patients (Zhang et al. 2013). Combined with the studies from other labs like the implication of Cullin 7 in 3-M syndrome and the Yakut short stature syndrome (Huber et al. 2005, Maksimova et al. 2007), characterized by pre- and post-natal growth retardation, we found that the Cullin family of proteins play very special roles during human placentation. To obtain a whole picture of the function of Cullin family proteins in human placenta development and pregnancy-related diseases, we further studied the roles of other Cullin family members, like CUL3, in placenta development.

In this study, CUL3 was found to be highly expressed in the invasive EVTs of human placenta villi from normal pregnant women, and the expression of CUL3 in the less invasive EVTs from PE patients was significantly lower. CUL3 promoted the invasion and migration of human trophoblast cells in the human EVT cell line HTR8/SVneo or an ex vivo human first trimester villous explant culture model. Decreased pro-MMP9 activity and increased levels of TIMP1 and 2 were the possible mechanisms whereby CUL3 siRNA inhibited the invasion and migration of human trophoblast cells. To further elucidate the function of CUL3 in vivo, we generated a lentivirus-mediated placenta-specific CUL3 knockdown mouse model. Immunohistochemistry and in situ hybridization (ISH) experiments revealed less invasive trophoblast cells in the maternal decidua.

Materials and methods

Tissue collection

All the placentas obtained from pregnant women were under the approval of the Ethics Committee of Beijing Obstetrics and Gynecology Hospital. Samples were used in accordance with standard experimental protocols that were approved by the Ethics Committee of the Institute of Zoology at the Chinese Academy of Sciences. Placentas at first and second trimesters were collected from healthy women undergoing legal abortion, while term placentas (normal or pre-eclamptic) were collected after caesarean birth. All of the samples were obtained under the written consent from each woman who donated her placenta. All of the sample tissues were collected and stored in ice-cold DMEM (HyClone, Thermo Scientific, Dubuque, IA, USA), transported to the laboratory within 60 min after surgery, and washed with ice-cold PBS for fixation (first trimester, n = 5; second trimester, n = 5; normal third trimester, n = 5; PE third trimester, n = 5; PE third trimester, n = 5). Definition of PE was the same as previously reported (Zhang et al. 2013). Briefly, PE was defined as a new onset of hypertension (systolic/diastolic blood pressure ≥140/90 mmHg measured on two occasions of at least 4 h apart) and proteinuria (≥300 mg/24 h) after 20 weeks of gestation. All of the PE patients were 23–35 years old, developed hypertension and proteinuria after gestational age of 32 weeks, and delivered after 36 weeks. Gestational age-matched placentas were collected from normal pregnant women. All of the PE patients involved in the current study were not complicated with chronic hypertension, obesity, gestational diabetes mellitus (GDM), or eclampsia, and two patients were complicated with IUGR. The experimental procedures were performed in accordance with the instructions for the Care and Use of Animals in Research published by Institute of Zoology, Chinese Academy of Sciences.

Cell culture and RNA interference

HTR8/SVneo cell line, widely used as a model for the first trimester EVT invasion and migration, is a kind gift from Dr Benjamin K Tsang (Department of Obstetrics and Gynecology and Cellular and Molecular Medicine, University of Ottawa, ON, Canada). The mouse breast cancer cell line 4T1 was used to detect the interferential efficiency of mouse CUL3 siRNA transfection. The 293T cell line was used for lentivirus packaging. HTR8/SVneo cells were cultured in RPMI-1640 (HyClone), while 4T1 and 293T cells were cultured in DMEM (HyClone) containing 10% fetal bovine serum (FBS; Gibco; v/v), 100 IU/ml penicillin, and 100 μg/ml streptomycin and were incubated under 5% CO2 at 37 °C.

The RNA interference experiments were performed as previously described (Zhang et al. 2013). Briefly, HTR8/SVneo cells and 4T1 cells were transfected with 100 nM CUL3 siRNA with Lipofectamine 2000 (Invitrogen) for 8 h in serum free Opti-MEM (Gibco BRL, Carlsbad, CA, USA) before the medium was changed to the normal medium. Mouse CUL3 siRNA: 5'-AUAAUUGUACUGCAACGAGGCUC-3'; human CUL3 siRNA: 5'-AUAAUUGUACUGCAACGAGGCUC-3' (Invitrogen).

Western blotting

Cell lysates were prepared using whole cell lysis buffer (50 mM HEPES, 1 mM EGTA, 150 mM NaCl, 1.5 mM MgCl2, 100 mM...
NaF, 10 mM sodium pyrophosphate, 10% glycerol (v/v), and 1% Triton X-100 (v/v) containing an inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 μg/ml aprotinin) and kept on ice for 40 min. Culture media were concentrated using Microcon YM-3 centrifugal filter (EMD Millipore Corp., Billerica, MA, USA). Proteins were quantified by the spectrophotometry at 595 nm (Beckman DUS30, Fullerton, CA, USA) using the BCA Protein Assay Kit (Pierce Biotechnology, Thermo Scientific, Rockford, IL, USA). Protein samples were subjected to SDS–PAGE with 10% gel and transferred onto a pure nitrocellulose blotting membrane (Pall Corp., Pensacola, FL, USA). After being blocked with 5% skim milk (w/v) in TBST (10 mM Tris–HCl pH 8.0, 150 mM NaCl, and 0.05% Tween 20 (v/v)) at room temperature for 1 h, the membrane was incubated with primary antibodies against CUL3 (1:500; Rabbit anti-human; A301-109A; Bethyl Laboratories, Inc., Montgomery, TX, USA), TIMP1 (1:500; Rabbit anti-human; sc-5538; Santa Cruz Biotechnology, CA, USA), TIMP2 (1:250; Rabbit anti-human; sc-5539; Santa Cruz Biotechnology), Caspase3 (1:500; Mouse anti-human; sc-7272; Santa Cruz Biotechnology), Cleaved caspase3 (1:800; Rabbit anti-human; 9664; Cell Signaling Technology, Inc., Danvers, MA, USA), and GAPDH (1:8000; Mouse anti-human; ab37187; Abcam, Cambridge, UK) at 4 °C overnight. After being washed three times with TBST, the membrane was incubated with HRP-conjugated secondary antibody at room temperature for 1 h. Signals were detected using enhanced chemiluminescence (Pierce Chemical Company, Rockford, IL, USA). The intensity of the immunoreactive bands was quantified by laser densitometry.

Immunohistochemistry
Five micrometer formalin-fixed and paraﬃn-embedded sections were deparafﬁnized in xylene then rehydrated in a serial gradient of ethanol. Slides were boiled in citrate buffer (10 mM citrate sodium, 10 mM citric acid, pH 6.0) for 15 min to retrieve antigen. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (v/v) in methanol for 10 min. After being blocked with normal goat serum for 20 min, the sections were incubated with primary antibodies against CUL3 (Rabbit anti-human; LS-B359, LifeSpan Biosciences, Inc., Seattle, WA, USA) at a 1:500 dilution, CK7 (Mouse anti-human; ab20206, Abcam, Cambridge, UK) at a 1:200 dilution, and CD31 (Rabbit anti-human; ab28364, Abcam) at a 1:500 dilution overnight at 4 °C. After being washed with PBS, the sections were incubated with biotinylated secondary antibody, and the signals were detected with diaminobenzidine solution (DAB, Zhongshan Golden Bridge Corp., Beijing, China). Normal rabbit serum IgG (011-000-003; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was used as a negative control. The sections were counterstained with hematoxylin and mounted with histomount (Zhongshan Golden Bridge Corp.) and then observed under the microscope (Nikon Eclipse 80i; Nikon, Tokyo, Japan).

Explant culture
Explant culture was performed as described previously (Genbacev et al. 1992). In brief, placental anchoring villi (5–8 w) were identiﬁed by a phase contrast microscope (Leica S6 D Stereozoom; Leica, Heerbrugg, Switzerland), dissected, and placed on the Millicell-CM culture plate inserts (EMD Millipore Corp.) pre-coated with phenol red-free and growth factor-reduced Matrigel (Becton Dickinson, Bedford, MA, USA). The inserts were then placed into a 24-well culture plate (Costar, Cambridge, MA, USA). The explants were cultured in serum-free DMEM mixed 1:1 with Ham’s F-12 (DMEM/F12; HyClone) medium with 500 nM of scrambled control siRNA or CUL3 siRNA (5'-UAACUGUAGCAUG-CAACCAAGGUC-3'). Explants were photographed daily for up to 4 days. The distance from the cell column base to the tip of the outgrowth was measured with SPOT Imaging Software (SPOT Imaging Solutions, Diagnostic Instruments, Inc., Sterling Heights, MI, USA). All the explant experiments were repeated three times and replicated in four separate sets of explants.

Matrigel invasion and transwell migration assay
Cell invasion was performed in transwell inserts (6.5 mm, containing polycarbonate ﬁlters with 8 μm pores size; Costar) coated with growth factor-reduced Matrigel (1 mg/mL; Becton Dickinson) as described previously (Zhang et al. 2013). Briefly, 1 × 10^5 HTR8/SVneo cells (in 200 μL serum-free RPMI-1640 medium) with different treatments were plated onto the upper chamber, whereas medium with 10% FBS was added to the lower well. CUL3 siRNA is the same as used in the explant experiment. Lentivirus carrying human CUL3 shRNA or control shRNA was purchased from Shanghai GenePharma Corp. (Shanghai, China). The sequence of CUL3 shRNA is 5'-ATAATTTGTACATGCAAGCAAGGTC-3'. After an incubation of 24 h, non-invaded cells on the matrigel side of the membrane were removed with a cotton swab and the invaded cells on the other side of the membrane were ﬁxed by methanol for 10 min and stained with hematoxylin and eosin (Zhongshan Golden Bridge Corp.). The numbers of invaded cells were counted under a light microscope (Olympus IX51; Olympus, Tokyo, Japan) in eight random ﬁelds at a magniﬁcation of 100, normalized to the control, and expressed as means of invasion percentage (%) ± S.E.M. Conditional culture medium in the upper chamber was collected for gelatinolytic activity assay. The migratory ability of HTR8/SVneo cells was determined by a transwell cell migration assay, which is similar to the matrigel invasion assay except that the transwell insert was not coated with matrigel.

Wound healing assay
HTR8/SVneo cells transfected with Con shRNA or CUL3 shRNA (the same as in the ‘Matrigel invasion and transwell migration assay’) were cultured in a six-well plate to 80% confluence as a monolayer. The monolayer was scratched with a 1000 μl pipette tip. Cells were washed twice with fresh culture medium and cultured for an additional 24 h. Five randomly selected ﬁelds were photographed at the beginning (0 h) and the end of the culture (24 h).

Cell proliferation assay
HTR8/SVneo cells were plated in a 24-well plate at a density of 1 × 10^3/well and transfected with CUL3 siRNA or the
scrambled siRNA 24 h later. After 20, 44, or 68 h of transfection, the cells were digested and seeded at 5 × 10^4/well in a 96-well plate in fresh medium containing 10% MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Applygen Technologies, Inc., Beijing, China). The MTT reagent was gently removed 4 h later and 100 μl DMEM was added into each well. The optical density of each well was assessed at a wavelength of 570 nm (Beckman DU530, Fullerton, CA, USA). Each time point of the two treatments was performed in triplicates.

Analysis of cell apoptosis by Hoechst staining
Hoechst 33258 staining was performed as previously reported (Chang et al. 2014). In brief, cell suspension was incubated with 10% neutral-buffered formalin (w/v) containing Hoechst 33258 dye (12.5 ng/ml; Sigma). Cells with a shrunken, condensed, or fragmented nucleus were apoptotic cells. The number of apoptotic cells in 200 total cells was counted under a fluorescent microscope (Olympus IX51). Cell counting was performed under a fluorescent microscope (Nikon Eclipse 80i; Nikon).

Gelatin zymography
A conditioned culture medium of HTR8/SVneo cells was diluted with 4× sample buffer (8% SDS (w/v), 0.04% bromophenol blue (w/v), 0.25 M Tris) and incubated at 37 °C for 30 min. Equal amounts of protein were subjected to substrate-gel electrophoresis. The gels were washed twice in 2.5% Triton X-100 and 50 mM Tris–HCl (pH 7.5) for 30 min at room temperature to remove SDS and then incubated in a calcium assay buffer (50 mM Tris, 10 mM CaCl₂, 1 mM ZnCl₂, 1% Triton X-100, pH 7.5) for 24 h at 37 °C. The gels were stained with Coomassie Brilliant Blue R250 and destained in 10% acetic acid (v/v). Clear bands can be visualized to reveal the gelatinase activity.

Animals, lentiviral transduction, and embryo transplantation
All animal use in this study was approved by the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences. CD1 mice, aged 6–8 weeks, were purchased from Vital River Laboratory Animal Technology Corp. (Beijing, China). Female mice were mated with fertile or vasectomized males at night. A successful mating was determined by the presence of the vaginal plug, and the morning of the vaginal plug presence was designated as day 0.5 of pregnancy or pseudogestation. Mice were sacrificed to collect blastocysts. Tissues were snap frozen and stored at −80 °C until use for ISH, western blotting, or fixed in 4% PFA (w/v) at 4 °C for immunohistochemical analysis.

HIV-1 based, self-inactivating, lentiviral vector plasmid pLentiLox 3.7 (pLL3.7) was digested with Xhol and Hpal and then ligated with target shRNA (mouse CUL3 and control) as previously described (Zhou et al. 2009). 293T cells were transfected with pLL3.7-shCUL3 or the control plasmid, packaging plasmid pPRE, pRev, and VSVG expressing plasmid. Virus particles were harvested 2 days after transfection, concentrated 500 times by centrifugation (5000 g, 2 h), and resuspended with potassium-supplemented simplex optimized medium (KSOM). Virus titer was measured by FACS counting of the percentage of GFP positive cells. The titer was then adjusted to a concentration of 8 × 10⁶ TU/ml. WT CD1 females were superovulated by intraperitoneal injection of pregnant mare’s serum gonadotropin (PMSG; five units) followed by human chorionic gonadotropin (hCG; five units) 48 h later and then mated with WT CD1 males. Blastocyst embryos were collected from the females on Day 4. Zona pellucida was removed in acidic Tyrode’s solution (Sigma), and zona-free blastocysts were incubated individually for 8 h in 5 μl of medium containing lentiviral vectors (8 × 10⁶ TU/ml). The transduced blastocysts were washed three times in KSOM and either was transplanted into pseudopregnant CD1 females or continued culturing for 48 h, which was followed by an examination under a confocal microscope (Zeiss).

In situ hybridization
ISH was performed using digoxigenin (DIG)-labeled probes. Sections were fixed in 4% PFA for 1 h. DIG labeling was done according to the manufacturer’s instructions (Roche Diagnostics). Hybridization with DIG-labeled cRNA probes was carried out at 55 °C. The hybridization buffer contained 200 mM NaCl, 13 mM Tris, 5 mM NaH₂PO₄, 5 mM Na₂HPO₄, 5 mM EDTA, 50% formamide (v/v), 10% dextran sulfate (w/v), 1 mg/ml salmon sperm DNA, 2% BSA (w/v), and a DIG-labeled probe (final dilution of 1:200 from reaction with 1 μg template DNA). Two washes of 55 °C post-hybridization were carried out (1× SSC, 50% formamide, 0.1% tween-20), which was followed by two washes at room temperature in buffer 1 (150 mM NaCl, 100 mM Tris, pH 7.5). Sections were blocked in buffer I with 2% blocking reagent (Roche; v/v) for 1 h and incubated overnight with anti-DIG antibody (Roche) at a 1:2000 dilution. After being washed four times in buffer I, slides were rinsed in buffer III (100 mM NaCl, 50 mM MgCl₂, 100 mM Tris, pH 9.5) and incubated in NBT/BCIP (Promega Corp.). Slides were counterstained with nuclear fast red, dehydrated, cleared in xylene, and mounted in neutral resin.

Statistical analysis
The bands from western blotting and gelatin zymography were quantified by MetaView Image Analyzing System (Version 4.50; Universal Imaging Corp., Downingtown, PA, USA). Results were presented as means ± s.e.m. The gray densities of immunostaining signals were analyzed by Image J (Version 1.49; Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA). Results were presented as means ± s.d.; t-test was applied in the statistical analyses using the Statistical Package for Social Science (SPSS for Windows package release 10.0; SPSS, Inc., Chicago, IL, USA). P < 0.05 was considered as statistically significant (**P < 0.05; *P < 0.01).

Results
Expression of CUL3 in the human placenta
The spatial and temporal expression pattern of CUL3 in human placental villi at different stages of pregnancy was...
determined by immunohistochemistry. CUL3 was found to be mostly expressed in the nuclei of the villous STB, CTBs and trophoblast cells within the trophoblast column during the first trimester (Fig. 1A). In the second trimester and term placentas, CUL3 was immunologically detected in the nuclei of CTB cells and invasive EVT cells in the maternal decidua (Fig. 1D and G). No obvious CUL3 signaling was detected in the decidua stroma cells (Fig. 1D and G).

Knockdown of CUL3 inhibited the outgrowth of EVTs in an ex vivo placental explant culture model

To investigate the role of CUL3 in the invasion and migration of trophoblast cells, a placental explant culture model was used. Explants obtained from first trimester human placental villi (5–8 w) were cultured on matrigel-coated dishes and incubated with control siRNA or CUL3

Figure 1 Immunohistochemical localization of CUL3 in human placental tissues at different stages of pregnancy. Staining of CUL3 was found in the trophoblast column (TC, region below the red dotted line), cytotrophoblast cells (CTB), and syncytiotrophoblast (STB), as well as extravillous trophoblast (EVT) in the normal human placental villi from the first (5–12 weeks, n = 5), second (13–25 weeks, n = 5), and third (26–40 weeks, n = 5) trimesters placentas (representatively shown in A, D, and G). Representative images of placental villi from 6 w (A, B, and C), 19 w (D, E, and F), and 38 w (G, H, and I) are shown. Negative control images (NEG) in which normal IgG was used to replace the primary antibody showed no specific positive staining (C, F, and I). Cytokeratin 7 (CK7) was included as a marker for CTB and EVT (B, E, and H). MD, maternal decidua; VS, villous stroma; bar = 50 μm.
siRNA for different time durations. EVTs could grow and migrate away from the tip of the villi. The outgrowth distance of the EVTs over the matrigel surface was measured at 24, 48, and 72 h of siRNA transfection. As shown in Fig. 2, CUL3 siRNA did not affect the migratory capacity of EVTs at 24 h after transfection as compared to their corresponding controls. However, at 48 and 72 h of transfection, silencing CUL3 significantly inhibited the outgrowth of the EVTs (P<0.01).

**Knockdown of CUL3 significantly inhibited the invasion and migration of trophoblast HTR8/SVneo cells**

To further confirm the inhibitory effect of CUL3 on the invasion and migration of human trophoblast cells, a matrigel cell invasion and a transwell cell migration model were introduced. More than 90% of HTR8/SVneo cells could be successfully transfected with siRNAs as determined by fluorescence signals in cells transfected with FITC-siRNA (data not shown), while more than 95% of cells were successfully infected with lentivirus carrying shRNA (Supplementary Figure 1A, see section on supplementary data given at the end of this article). Compared with the control siRNA or shRNA, CUL3 siRNA or shRNA significantly decreased the percentage of cells that invaded or migrated to the other side of the filter (Fig. 3A and E, P<0.01). In the third migration model, the wound healing assay, lentivirus carrying CUL3 shRNA or the control shRNA, was transfected to the HTR8/SVneo cell monolayer with a scratch. After 24 h of transfection, the CUL3 shRNA transfected group still remained an ‘unhealed’ wound, while the wound in the control group healed well (Fig. 3D). Knocking down efficiency of CUL3 siRNA or shRNA was shown by western blotting in Fig. 3B and C, as well as in real-time PCR assay in Supplementary Figure 1B.

To explore possible mechanisms by which knockdown of CUL3 inhibits trophoblast invasion, we examined the gelatinolytic activities of MMP2 and MMP9 by gelatin zymography and the expression levels of their tissue-specific inhibitors TIMP2 and TIMP1 by western blotting. Cell lysates were prepared and supernatants were collected 48 h after siRNA transfection in HTR8/SVneo cells. Compared with the control siRNA-transfected group, knockdown of CUL3 significantly decreased the activity of pro-MMP9 but not of pro-MMP2 (Fig. 3G; P<0.01). The expression levels of TIMP1 (P<0.05) and 2 (P<0.01) were significantly increased in the CUL3 siRNA-transfected group as compared with the control group (Fig. 3F).

To exclude the possibility that the decrease in cell invasion and migration after CUL3 siRNA transfection is due to the decrease in cell proliferation or the increase in cell apoptosis after CUL3 siRNA transfection, the effect of CUL3 siRNA on the proliferation and apoptosis of HTR8/SVneo cells was examined. Although CUL3 siRNA transfection did show an inhibitory effect on cell proliferation after 48 (P<0.05) and 72 h (P<0.01) of transfection based on an MTT assay, at 24 h of siRNA transfection, a time point when invasion and migration assay was finished, there was no significant difference in the cell number between CUL3 knockdown cells and the control ones (Fig. 4A and B; P>0.5). Apoptosis of a cell population was evaluated by Hoechst 33258 staining. No significant difference in apoptosis was found between CUL3 siRNA-transfected cells and the controls at 24 h of transfection (Fig. 4D; P>0.05). The cells transfected with control siRNA or CUL3 siRNA also did not exhibit significantly different levels of apoptotic protein Cleaved caspase3 by western blotting (Fig. 4C).

**CUL3 was downregulated in the placental villi from PE patients**

Inadequate trophoblast migration/invasion and spiral artery remodeling result in poor placenta perfusion,
which mediates maternal injury such as the onset of PE. Placental villi from pre-eclamptic patients and normal pregnant women at close gestational stages were dissected for protein extraction and immunoblotting. Western blotting (Fig. 5A) and statistical analysis (Fig. 5B; \( P < 0.05 \)) showed that there was a significantly lower level of CUL3 in the placental villi from pre-eclamptic patients than in the corresponding control placentas. Immunohistochemical staining also showed weak positive signals for CUL3 in the EVTs from PE placentas compared with the strong CUL3 staining in the EVTs from normal placentas (Fig. 5C).

**Placenta-specific knockdown of CUL3 inhibited the migration and invasion of mouse trophoblast cells**

To examine the expression pattern of CUL3 mRNA in the mouse placenta, ISH was carried out. CUL3 mRNA was highly expressed in the mouse placenta compartments including spongiotrophoblasts, trophoblast giant cells (TGCs), and glycogen trophoblasts in the labyrinth layer but was weakly expressed in the maternal decidua (Fig. 6A, a, b and c). CD31, a marker for vascular endothelial cells, indicated the labyrinth layer (Fig. 6A, d). The spongiotrophoblast layer was identified by Tpbp (Fig. 6A, e).

To study the function of CUL3 in mouse placenta development, a lentivirus-mediated placenta-specific knockdown model was established. CUL3 shRNA was generated based on an efficient CUL3 siRNA (Fig. 6D). The knockdown efficiency of CUL3 shRNA was determined in mouse 4T1 cells by western blotting (Fig. 6E). Zona-free blastocysts were infected with constitutive GFP-expressing lentiviruses carrying CUL3 shRNA or control shRNA for 8 h (Fig. 6B) and observed under fluorescence microscope, which showed that only the trophectoderm, but not the inner cell mass, was infected by the lentiviruses. After transplantation to the WT recipient mice for 13 days, mice were sacrificed and

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Figure 3 Knockdown of CUL3 inhibits the invasion and migration of HTR8/SVneo cells by decreasing the gelatinolytic activity of pro-MMP9 and increasing the expression of TIMP1/2. (A) Upper panels: representative images of transwell membranes carrying invaded cells in matrigel invasion assay and transwell migration assay; lower panels: statistical bar graphs summarizing the results as representatively shown in the upper panels (\( t \)-test; **\( P < 0.01 \); \( n = 3 \)). Confirmation of the knockdown efficiency of CUL3 siRNA (B) or shRNA (C) was shown by western blotting. GAPDH is a loading control. (D) Wound healing assay of HTR8/SVneo cells under indicated treatments (\( n = 3 \)). (E) Transwell migration assay (left panel: representative images of transwell membranes carrying invaded cells; right panel: statistical analysis) showed a decrease in cell migration after CUL3 was knocked down by CUL3 shRNA (\( t \)-test; **\( P < 0.01 \); \( n = 4 \)). (F) Left panel: western blotting of whole cell lysates from HTR8/SVneo cells under indicated treatments to show the expression of CUL3 and TIMP1 and 2. GAPDH is a loading control; right panel: statistical analysis of western blotting showing the significant increase of TIMP1 and 2 in CUL3 knockdown HTR8/SVneo cells compared to the control (\( t \)-test, *\( P < 0.05 \) and **\( P < 0.01 \); \( n = 4 \)). (G) Gelatin zymography assay (left panel) and the statistical analysis (right panel) showing the zymographic activities of pro-MMP9 and 2 under indicated treatments (\( t \)-test; **\( P < 0.01 \); \( n = 4 \)).
The effect of CUL3 siRNA on the proliferation and apoptosis of HTR8/SVneo cells. (A) Proliferation assay of HTR8/SVneo cells transfected with indicated siRNAs over 3 days of culture (t-test; *P<0.05; **P<0.01; n=4). (B) Statistical analysis shows no significant difference in proliferation after 24 h of culture (n=4). (C) Western blotting results using indicated antibodies. GAPDH is a loading control. (D) Ratio of apoptotic cells in a HTR8/SVneo cell population transfected with indicated siRNAs.

Discussion

Emerging evidence shows that Cullin-RING ligase multisubunit complexes play critical roles in regulating placental development and function. However, how Cullin proteins regulate placental development is largely unknown. In this study, we reported a brand new role of CUL3 in the trophoblast invasion and migration in both humans and mice based on the following lines of evidence. First, CUL3 was strongly expressed in the highly invasive human EVT cells. Second, silencing CUL3 resulted in an inhibition of cell migration and invasion in a well-established invasive EVT cell line, HTR8/SVneo. Third, in a villi explant culture system, an excellent model for studying migratory events during the process of placentation, the outgrowth and spreading of EVT cells in the placenta were significantly inhibited by CUL3 siRNA. And finally, in a mouse lentiviral-mediated placenta-specific knockdown system, knocking down the expression of CUL3 exclusively in the placenta led to less invasive glycogen trophoblast cells in the maternal decidua.

It has been reported that in the E7.5 mouse embryo, the most intense nuclear CUL3 staining is present in the extraembryonic ectoderm and TGCs (Singer et al. 1999). Indeed, TGCs are analogous to extravillous cytotrophoblast (EVT) cells in the human placenta. Both types of cells are known to be highly invasive and to contribute to the process of placentation, the outgrowth and spreading of EVT cells in the placenta were significantly inhibited by CUL3 siRNA. And finally, in a mouse lentiviral-mediated placenta-specific knockdown system, knocking down the expression of CUL3 exclusively in the placenta led to less invasive glycogen trophoblast cells in the maternal decidua.

The placenta and fetuses were collected and observed under fluorescence microscope. The placenta, but not the fetuses, exhibited green fluorescence (Fig. 6C). The control placentas and the placentas with knocked down CUL3 were then processed for cryosection and the morphology of the placenta was illustrated by the ISH result of Tpbp. The invasive glycogen trophoblasts in CUL3 knockdown placentas were less than those in the control placentas (Fig. 6F; P<0.01), indicating that CUL3 plays an important function in mouse trophoblast invasion and migration into maternal decidua.

Figure 5 CUL3 is significantly downregulated in the placental villi from PE patients. (A) Representative western blotting showing the expression of CUL3 proteins in the placental villi from normal pregnant (n=5) or pre-eclamptic (PE; n=5) women at similar gestational age (GA). GAPDH is a loading control. (B) Statistical analysis of the western blotting results as representatively shown in (A) (t-test; *P<0.05; n=5). (C) Immunostaining of CUL3 in the EVTs from normal and PE placentas at same gestational age (a and d). CK7 is used as a marker for EVTs (b and e). Negative control images (NEG) in which normal IgG was used to replace the primary antibody showed no specific positive staining (c and f). Mean gray density of randomly chosen EVTs in (a) and (d) were analyzed (g; t-test; **P<0.01; n=50). Bar=100 μm.
Figure 6 Decrease of invasive trophoblasts appeared in placenta-specific CUL3 knockdown mouse. (A) The localization of CUL3 mRNA in the mouse placentas at E13.5 ((a), in situ hybridization of CUL3 mRNA in the E13.5 mouse placenta; (b), the magnification of the framed region in (a); (c), the magnified image of the framed region in (b); (d), immunostaining of CD31, a marker of vascular endothelial cells, in the mouse placenta at E13.5; (e), in situ hybridization of Tpbp in the E13.5 mouse placenta; Sp, spongiotrophoblast; La, labyrinth). (B) Blastocysts with GFP positive signals show a successful infection of lentivirus restricted to the trophectoderm. (C) Placentas and embryos in the CUL3 shRNA-treated group were photographed at E13.5, and the placentas but not the embryos were infected by lentivirus, based on the expression of GFP. (D) Western blotting results show that CUL3 siRNA (used for shRNA vector construction) successfully knocked down the expression of CUL3 in mouse 4T1 cells. α-Tubulin is a loading control. (E) Western blotting shows the knockdown efficiency of CUL3 shRNA in mouse 4T1 cells. α-Tubulin is a loading control. (F) Left panel: In situ hybridization of Tpbp mRNA in the control shRNA-infected (a) and CUL3 shRNA-infected placentas (b). (c) and (d) are the magnifications of the framed regions in (a) and (b), respectively (Gly, glycogen trophoblast; Sp, spongiotrophoblast; red dotted line indicates the boundary of Gly and Sp); right panel: statistical analysis of the relative square of glycogen trophoblast area in E13.5 CUL3 knockdown mouse placenta and the control (t-test, **P<0.01; n=3).
inherently invasive and interact with maternal blood vessels (Parast et al. 2001, Hu & Cross 2010). By using placentas from later stages of pregnancy, we found CUL3 was mainly expressed in the spongiotrophoblast layer and invasive glycogen trophoblasts in E13.5 mouse placentas, further supporting a role of CUL3 in trophoblast invasion. CUL3−/− embryos at E7.5 exhibit abnormally developed trophoderm, and TGCs are more sparsely represented than in WT embryos. Furthermore, an accumulation of cyclin E is observed in the trophoderm and extraembryonic ectoderm of CUL3−/− embryos. It is speculated that there is a cell type-specific and gestational stage-specific effect of CUL3 on S-phase regulation. Loss of CUL3 leads to an increased number of cells in S phase in the extraembryonic ectoderm, whereas loss of CUL3 in the trophoderm imposes a block to entry into S phase and, thus, the endoreduplication cycles in the trophoderm are blocked (Singer et al. 1999). In our study, we generated a placenta-specific CUL3 knockdown mouse model and found that knocking down CUL3 led to less invasive glycogen trophoblast cells in the maternal decidua. The previous evidence suggests multiple functions of CUL3 during mouse placental development.

A number of cytokines and growth factors have been shown to increase the invasiveness of trophoblastic cells through regulating the expression of MMPs and TIMPs (Cohen et al. 2006, Luo et al. 2012, Li et al. 2014). In this study, CUL3 siRNA was shown to decrease the gelatinolytic activities of MMP9 in the spent media of HTR8/SVneo cells. On the other hand, the expression of both TIMP1 and 2 was increased in CUL3 knockdown cells. The precise mechanisms whereby CUL3 controls the levels or activities of MMPs or TIMPs remain to be determined.

The migration/invasion of trophoblast is often related to that of the malignant tumor cells during tumorigenesis, although the capacity of trophoblasts is manipulated. It has been shown that CUL3 is associated with the aggressiveness of bladder cancer, breast cancer, and lung cancer (Haagenson et al. 2012, Grau et al. 2013). CUL3/BACURD complex has been shown to mediate the degradation of RhoA, which is involved in regulating the actin cytoskeleton dynamics and cell movement. Loss of a functional CUL3/BACURD complex inhibits migration potential of cultured mouse MEF cells and human HeLa cells. Knockdown of CUL3 led to severe migration defects that could be rescued by simultaneous knockdown of RhoA (Chen et al. 2009). These findings suggest that the CUL3/BACURD complex controls cell migration potential by manipulating the cellular protein level of RhoA. Whether or not these identified target genes of CUL3 are involved in human placental development remains to be determined.

PE is a critical pregnancy-related disease complicated by hypertension and proteinuria and a major cause of maternal mortality, morbidity, perinatal death, preterm birth, and IUGR (Stepan et al. 2006). Impaired trophoblast invasion/migration and subsequent defects in spiral arterial remodeling have been implicated in the pathogenesis of PE (Hawfield & Freedman 2009, George & Granger 2010). Besides the in situ observation of the shallow interstitial invasion and limited incorporation into endothelium of spiral arteries, extracted primary CTBs from PE patients also show abnormal abilities of differentiation and invasion, as well as the transcriptomic differences in various genes related to PE (Zhou et al. 2013). Cullin family proteins have been suggested to be involved in the development of this disorder (Gascoin-Lachambre et al. 2010). Our recent study also suggests that CUL1 might be involved in the pathogenesis of PE (Zhang et al. 2013). In this study, we found that the level of CUL3 in the placentas from PE patients was lower than their age-matched normal placentas. Thus the less invasive capacity would lead to an insufficient remodeling of maternal spiral arteries and result in placental ischemia/hypoxia. Nevertheless, the molecular basis remains to be further investigated in order to clarify the mechanism of the downregulation of CUL3 in pre-eclamptic placentas.

In conclusion, our data provided the first insight into the function of CUL3 in human and mouse placenta trophoblast invasion and migration, at least in part, through activation of MMP9 and reduction of TIMP1/2, and suggested a possible pathological implication in PE.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-15-0126.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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