Role of placenta-specific protein 1 in trophoblast invasion and migration

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Abstract

Placenta-specific protein 1 (PLAC1), a placenta-specific gene, is known to be involved in the development of placenta in both humans and mice. However, the precise role of PLAC1 in placental trophoblast function remains unclear. In this study, the localization of PLAC1 in human placental tissues and its physiological significance in trophoblast invasion and migration are investigated by technical studies including real-time RT-PCR, in situ hybridization, immunohistochemistry, and functional studies by utilizing cell invasion and migration assays in the trophoblast cell line HTR8/SVneo as well as the primary inducing extravillous trophoblasts (EVTs). The results show that PLAC1 is mainly detected in the trophoblast columns and syncytiotrophoblast of the first-trimester human placental villi, as well as in the EVT that invade into the maternal decidua. Knockdown of PLAC1 by RNA interference significantly suppresses the invasion and migration of HTR8/SVneo cells and shortens the distance of the outgrowth of the induced EVT from the cytotrophoblast column of the explants. All the above data suggests that PLAC1 plays an important role in human placental trophoblast invasion and migration.

Introduction

In mammals, the placenta is a transiently developed organ to ensure a successful pregnancy which evolved about 100 million years ago (Springer et al. 2003, Chuong et al. 2010). The primates and rodents possess the typical hemochorial placenta which represents the most intimate fetal–maternal contact (Wildman et al. 2006) and is characterized by highly dynamic trophoblast villous structure and active invasion of extravillous trophoblast (EVTs) into the maternal decidua. In humans, two distinct trophoblast cell types, villous and extravillous trophoblasts, are originated from the trophectoderm in the outer layer of the blastocyst (Bischof & Campana 1997). During human placentation, the trophoblast villous trees are formed and expanded so that there is sufficient trophoblast–uterus interaction surface area for substance exchange to support the fetal growth (Castellucci et al. 1990, Kingdom et al. 2000). These trophoblast cells interact with the uterus and produce functional factors to modify maternal physiological systems, and render enough blood flow and nutrients to the fetal–maternal interface (Burrows et al. 1996, Lyall et al. 1999, Watson & Cross 2005, Osol & Mandala 2009). In the villous tips which contact the maternal decidua, the cytotrophoblast (CTB) progenitor cells can develop and break the syncytiotrophoblast (STB) layer, assemble as the extravillous trophoblast column (TC), and further invade into the decidua to form EVT (James et al. 2005). The proximal portion of TC maintains their proliferative abilities, while trophoblast cells from the distal part invade into the decidua and even deeper into the myometrium, in order to achieve appropriate remodeling of maternal spiral artery and better anchor the fetus to the uterus (Osol & Mandala 2009).

The progress of functional and structural changes in the placental trophoblast is strictly regulated by the housekeeping genes as well as placenta-specific genes (Rawn & Cross 2008). A number of placenta-specific genes, including trophoblast-specific protein (TPBP), syncytin, paternally expressed 10 (PEG10), X-linked homeobox 1 (ESX1), glial cells missing homolog 1 (GCM1), etc., are key molecules in the regulation of trophoblast morphogenesis, invasion and migration, or syncytialization. And these genes would be coincidently important both in murine and human placentation process but they are not...
completely coincident. The corresponding phenotypes revealed by mouse knockout models have been well summarized (Rawn & Cross 2008). For example, Gcm1 mutant fails to form the labyrinth layer of the placenta (Anson-Cartwright et al. 2000). Esx1 deficiency leads to vascularization defects in the labyrinth layer, suggesting a role of Esx1 in normal chorioallantoic morphogenesis (Li & Behringer 1998). Syncytin-A and -B (Rote et al. 2004, Dupressoir et al. 2005) are fusogens which function as key mediators in trophoblast syncytialization. Besides the above genes whose functions in placentation are relatively well defined, functions of a series of genes termed as placenta specific proteins (PLACs), including PLAC1, PLAC2 (later also renamed as tissue differentiation-inducing non-protein coding RNA, TINCR), PLAC3, PLAC8, etc., are yet to be understood.

PLAC1 and its orthologous gene in mouse, Plac1, are usually described/represented as placenta-specific gene (Cocchia et al. 2000, Fant et al. 2002), although they have later been found in the testis (Silva et al. 2007), multiple embryonic tissues (Kong et al. 2013), and in a wide range of cancers (Koslowsky et al. 2007, Silva et al. 2007, Dong et al. 2008). In a mutant mouse model, deletion of a large DNA fragment, spanning from 200 to 700 kb on the X chromosome, in which Plac1 was located results in a runty phenotype or causes death at birth due to abnormal placentation (Kushi et al. 1998, Hemberger et al. 1999, Cocchia et al. 2000). Parallel efforts to screen candidate genes involved in trophoblast cell invasion and migration through a CDNA subtraction library between mouse invasive and mature noninvasive trophoblast also indicate that Plac1 is an important trophoblast invasion-associated gene (Hemberger et al. 2000). Localization studies in mouse (Cocchia et al. 2000) and human placentas (Fant et al. 2002, 2007, Massababal et al. 2005, Silva et al. 2007) demonstrate PLAC1 expression primarily in the differentiated trophoblast. The above lines of evidence suggest that PLAC1 is important in placental development in both humans and mice. However, to the best of our knowledge, involvement of PLAC1 in trophoblast cell differentiation has not been shown.

In this study, the specific localization of PLAC1 in TCs of the first-trimester placenta and EVTs of term placenta, examined by in situ hybridization (ISH) and immunohistochemistry (IHC), indicates that PLAC1 is a candidate molecule in the regulation of trophoblast invasion and migration. This was then validated by knocking down PLAC1 in HTR8/SVneo cells and in ex vivo human first-trimester extravillous explant cultures. Both in vitro and ex vivo evidences in this study show that PLAC1 is involved in trophoblast cell invasion and migration.

Materials and methods

Placenta tissues collection

Placenta villi from human first trimester (5–8 weeks), second trimester, and term pregnancy were sampled from normal pregnant women undergoing legal abortion or Caesarean section at term, respectively, in Beijing Obstetrics and Gynecology Hospital. Informed written consent for placenta donation was obtained from all women who donated their placentas. Ethical approval was granted by the Ethics Committee of Beijing Obstetrics and Gynecology Hospital. The utilization of samples is guided by standard experimental protocols approved by the Ethics Committee of Institute of Zoology, Chinese Academy of Sciences. All the placental tissues were collected and stored in ice-cold DMEM (Invitrogen), transported to the laboratory within 1 h of surgery, and washed with ice-cold 1 × PBS three times before fixation or culturing.

In situ hybridization

Total RNAs from 6 weeks placental villi were reversely transcribed, and a fragment of 879 bp nucleotides expanding all three exons of PLAC1 mRNA (NM_021796) was amplified. Specific primer pairs used for PCR were: sense, 5'-AAATTGGAAGCTCTGCTTCCA-3'; antisense, 5'-TGTCACATGAGGTGACCAAG-3'. This fragment was cloned into pGM-T plasmid (Tiangen, Beijing, China) for cRNA probe preparation. The recombinant plasmid was linearized by restrictive endonucleases Apal or Sall, and the linearized plasmid of intact SP6 or T7 promoter was used as the template for in vitro transcription to produce cRNA probes. The sense and antisense cRNA probes of PLAC1 were produced using the digoxigenin (DIG) RNA labeling kit (SP6/T7, Roche Molecular Biochemicals) according to the manufacturers' instruction.

Placental villi from the first trimester and term were fixed in 4% neutral paraformaldehyde (PFA, pH 7.4) for 1 h, washed with RNase-free PBS three times at 5-min interval, and dehydrated with gradient glucose from 5 to 30% at 4 °C within 12 h. The tissues were then embedded with Tissue-Tek OCT compound (Sakura Finetek, Zoeterwoude, The Netherlands) and stored at −80 °C. Frozen sections (8 μm) were prepared by Leica CM1950 (Leica Microsystems, Wetzlar, Germany) and mounted on RNase-free 3-aminopropyltriethoxysilane pre-coated slides. ISH was performed as previously described with small modifications (Zhao et al. 2012). Briefly, after pre-hybridization treatment, DIG-labeled cRNA probes of PLAC1 at a concentration of 2 μg/ml were used for hybridization at 60 °C for 14–18 h. The sections were directly photographed after counterstaining with nuclear fast red solution (Sigma-Aldrich) using a phase-contrast microscope. A sense probe was used as a control for ISH of PLAC1. Three different normal placental villi of the first trimester and term placenta respectively were utilized to examine the localization of PLAC1 mRNA. Serial sections were also stained with CK7 antibody as a marker to label the TCs and EVTs in decidua of term placenta.

Paraffin section and IHC

After three washes with sterile cold PBS, the placental tissues were fixed with 4% neutral PFA (Sigma-Aldrich) at 4 °C (1 h for first-trimester placental villi and 6 h for term-placental tissues). The tissues were dehydrated with gradient ethanol, embedded in paraffin, and sectioned at 5 μm by using rotary microtome.
The sections were deparaffinized and rehydrated. IHC was performed using the Streptavidin– Peroxidase Histostain-Plus Kit (Zhongshan Golden Bridge Corp., Beijing, China) as previously reported (Fu et al. 2009, Yang et al. 2013). Endogenous peroxidase activity was eliminated by incubating the section with 3% H2O2 in the dark for 10 min at room temperature. The sections were then incubated with the primary antibody, rabbit anti-human PLAC1 (ab105395; Abcam, Cambridge, UK) overnight at 4°C after antigen retrieval in citrate antigen retrieval solution (pH 6.0) and blockage with goat serum. Cytokeratine 7 (C7, clone OV-TL 12/30; DAKO, Carpintera, CA, USA) was used as the trophoblast marker and HLA-G (SC-21799, 4H84, Santa Cruz Biotechnology) was used as the marker for EVTs. A serial section incubated with mouse or rabbit IgG instead of the primary antibody was included as a negative control. On the following day, the sections were incubated with a peroxidase-conjugated secondary antibody, stained with 3,3'-diaminobenzidine tetrahydrochloride (Zhongshan Golden Bridge Corp.) and counterstained with hematoxylin. Localization of molecules was determined on sections from three different placentas of the same gestational stage.

Cell culture

The trophoblast cell line HTR8/SVneo (Graham et al. 1993), a gift from Dr Benjamin K Tsang (Department of Obstetrics & Gynecology and Cellular & Molecular Medicine, University of Ottawa, Canada), was maintained in the RPMI 1640 medium (Invitrogen) with 10% fetal bovine serum (FBS, Thermo Scientific Hyclone, Logan, UT, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured in a 5% CO2/95% air incubator at 37°C.

RNA interference

Two fragments of small interfering RNA (siRNA) duplexes specifically targeted to PLAC1 mRNA denoted as siRNA1 and siRNA2 in this study were designed and synthesized by GenePharma (Shanghai, China) and Invitrogen respectively. The sequences were as follows: PLAC1 siRNA1, 5'-CAUCUUCUUUCUUUGAUAUTT-3' (sense), 5'-AUUAUCAGAAAAGM-GAGAUGGT-3' (antisense); and PLAC1 siRNA2, 5'-CC AAAGCMGUCUCUCAGGACAUUGU-3' (sense), 5'-ACCAU-GUCCUGAGAGACGCUCUUGG-3' (antisense). A scrambled siRNA duplex from Invitrogen was used as a non-specific control. siRNA duplex was transfected into the trophoblast cell line by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions with a final concentration of 100 nM. The efficiency of knockdown was examined by real-time RT-PCR.

Real-time RT-PCR

Total RNA was extracted and purified from fresh human placental villi or cultured cells using TRizol reagent (Invitrogen). RNA concentration was determined using a NanoDrop 2000 u.v.–Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Reverse transcription of RNA to cDNA was carried out with Superscript II reverse transcriptase (Invitrogen) from 2 μg of total RNA as the template in a volume of 20 μl reaction system. Real-time PCR was carried out by using the SYBR Premix Ex Taq Kit (Takara, Dalian, China) with the Real-Time PCR System (ABI PRISM 7500 Real-time PCR System, Applied Biosystems). Specific primers used for PCR were as follows: PLAC1: 5'- AAATTTGGCGAGTGCCTTCAC-3' (sense), 5'-TGATGCCACATTAGTAACAC-3' (antisense); GAPDH (NM_001256799): 5'-GCCATCAATGACCCCTTCATT-3' (sense), 5'-TTGACGGTGCCATGGAATT-3' (anti-sense). The data were analyzed using ΔCt method and normalized to GAPDH expression (Livak & Schmittgen 2001, Schmittgen & Livak 2008).

Matrigel cell invasion and transwell cell migration assays

Matrigel cell invasion and transwell cell migration assays were carried out as described previously (Zhou et al. 2009). Briefly, trophoblast cells were transfected with PLAC1-targeted siRNA (the universal scrambled siRNA from Invitrogen was used as a control). Twenty-four hours after siRNA transfection, 1 × 10^4 HTR8/SVneo cells in 200 μl RPMI 1640 medium (without FBS) were seeded onto the filter of the transwell chamber (8.0 μm pore size for transwell cell migration assay; Corning Life Sciences, Acton, MA, USA), or the filter pre-coated with matrigel (1 mg/ml for matrigel cell invasion assay), and a medium supplemented with 10% FBS was added to the lower chamber. After 24 h, cells on the upper side of the filter were removed with a cotton swab, and those on the opposite side were fixed with iced methanol for 10 min, stained with hematoxylin and eosin, and counted under a light microscope (Olympus IX51, Olympus Corporation). Eight random fields at a magnification of ×200 were recorded. The experiment was carried out in triplicate. The conditional medium (from the matrigel invasion assay) in the transwell chamber was collected for gelatinolytic activity assay. The remaining cells transfected with RNA siRNA or scrambled siRNA were used for RNA extraction after transfection for 48 h to detect the gene-silencing efficiency by real-time RT-PCR.

Gelatin zymography

The gelatinolytic activities of matrix metalloproteinase (MMP) 2 and 9 secreted by HTR8/SVneo cells were detected by gelatin zymography as described previously (Wang et al. 2006). The conditioned medium collected from the upper chamber of the transwell was mixed with 4× SDS sample loading buffer (8% SDS (w:v), 0.04% bromophenol blue (w:v), 0.25 M Tris–HCl, pH 7.6) and incubated at 37°C for 30 min. Fixed volumes of samples (20 μl per sample) were loaded and separated on a 12% polyacrylamide gel containing 0.5 mg/ml gelatin (Difco Laboratories, Detroit, MI, USA). After electrophoresis, the gel was renatured with renaturation buffer (2.5% Triton X-100, 50 mM Tris–HCl, pH 7.5) for 30 min at room temperature and incubated with developing buffer (50 mM Tris–HCl, pH 7.5, 10 mM CaCl2, 1 mM ZnCl2, 1% Triton X-100) for 24 h at 37°C. The gel was subsequently stained with 0.5% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid
for 1 h at room temperature and then destained in 10% acetic acid for 6 h to visualize the zymogen bands. The gelatinolytic activities of MMP2 and 9 were qualified by using Molecular Imager Gel Doc XR\textsuperscript{+} Imaging System (Bio-Rad Laboratories).

**MTT assay**

The HTR8/SVneo cells were seeded at a density of $1 \times 10^5$ cells per well onto a 24-well plate and transfected with PLAC1 siRNA or scrambled siRNA 24 h later. The culture medium was replaced with a fresh medium with 10% 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Apllygen Corp., Beijing, China) at 20, 44, or 68 h after transfection respectively. The MTT reagent was gently removed 4 h later and 500 μL DMSO was added into each well to dissolve the formed formazan. The OD values were measured at 570 nm wavelengths (Beckman DU530, Beckman Coulter, Inc., Fullerton, CA, USA). The experiment was carried out in triplicate.

**Hoechst 33258 staining for apoptosis assay**

HTR8/SVneo transfected with PLAC1 siRNA or scrambled siRNA for 48 h were harvested, pelleted, and re-suspended in neutral buffered formalin (10%)-containing Hoechst 33258 dye (12.5 ng/ml; Sigma–Aldrich). The cell suspensions were dropped onto slides and covered by a coverslip. The apoptotic cells were counted based on nuclear morphology (nuclear shrinkage, condensation, and fragmentation) under a Zeiss fluorescence microscope (Carl Zeiss, Oberkochen, Germany). For each slide, at least five randomly chosen fields (each field has 100–200 cells) were counted blindly to avoid experimental bias.

**Human first trimester ex vivo extravillous explant culture**

Human placental villi from the first-trimester of pregnancy (5–8 weeks, $n=16$) were collected and kept in cold DMEM with 100 U/ml penicillin and 100 μg/ml streptomycin before dissection (Hunkapiller & Fisher 2008). Anchoring villi identified by a phase-contrast microscope (Leica S6 D Stereozoom, Leica Microsystems AG, Heerbrugg, Switzerland) were dissected into explants of 2–5 mm of diameter. The explants were implanted onto Millicell-CM culture dish inserts (Millipore Corporation, Bedford, MA, USA) pre-coated with growth factor-reduced Matrigel (BD Biosciences, Bedford, MA, USA) (Baczkyn et al. 2009). After the implantation of explants into the matrigel (usually takes 8–10 h), serum-free DMEM mixed 1:1 with Ham’s F-12 (DMEM/F12; HyClone) was added into the culture dish insert chamber. Then 500 μL DMEM/F12 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 100 μg/ml gentamycin, and 2.5 μg/ml fungizone was added to the lower well. The explants were cultured at 3% oxygen and 5% CO\textsubscript{2}. Twenty-four hours later, the explants were observed and recorded using an inverted microscope system (Nikon Eclipse Ti, Tokyo, Japan). Only the explants with a successful initiation of trophoblast cell outgrowth were deemed as viable and kept for the following experiment. The explants from the same placental villi were divided into two groups, one group being treated with PLAC1 siRNA and the other with scrambled siRNA. siRNA was added to culture medium directly at a final concentration of 300 nM, and the conditional medium was replaced every 24 h. The outgrowth of explants was monitored and recorded every 24 h.

**Whole-mount fluorescent IHC**

To characterize outgrown EVTs from the explanted villi, whole-mount double fluorescent immunocytochemistry was performed. The explanted villi on matrigel were fixed using 4% neutral PFA at room temperature for 30 min after being cultured for 72 or 96 h. After three washes in 1× PBS with 1% Triton, the explanted villi were sequentially incubated with blocking buffer (10% FBS) for 1 h, primary antibody against HLA-G or PLAC1 at 4 °C for 2 days, secondary antibody for 2 h, and DAPI for 20 min to label the nuclei. The negative control experiment was carried out by replacing the primary antibody with goat serum. The images were taken using a confocal microscope (Zeiss LSM780, Carl Zeiss AG).

**Statistical analysis**

The results were presented as means±s.d. Statistical analysis was performed by paired-sample t-test, which was performed by using the Statistical Package for Social Science (SPSS for Windows package release 10.0, SPSS, Inc.). $P<0.05$ is considered as statistically significant (*$P<0.05$; **$P<0.01$).

**Results**

**Localization of PLAC1 mRNA in first-trimester and term-human placentas**

In the first-trimester human placenta villi (6 weeks), PLAC1 mRNA was intensely and specifically expressed in the STB and TC as exhibited by ISH (Fig. 1A and B). No positive or very weak staining signals were observed in the CTB (Fig. 1B) or on sections hybridized with the PLAC1-sense probe (Fig. 1C). The TC was labeled using CK7 antibody in the serial sections (Fig. 1D). In term placenta, strong positive signal for PLAC1 mRNA was observed in the decidua side which was mainly in the iEVTs (Fig. 1E and F), same as labeled by CK7 antibody on serial sections (Fig. 1H). No positive signal for the sense probe in term placenta was observed (Fig. 1G).

PLAC1 protein was also expressed in the first-trimester human placenta villi (6 weeks) in a similar pattern, as illustrated by IHC (Fig. 2). The trophoblast including TC of first-trimester placenta villi was labeled by CK7 antibody (Fig. 2A). The HLA-G antibody was used to figure out the EVTs (Fig. 2B). PLAC1 was highly expressed in the STB, and with modest levels in the TC (Fig. 2C) and parts of CTB of the first-trimester placenta villi. No positive staining signals were observed in the control sections (Fig. 2D). In the second-trimester
placenta (19 weeks), CK7 was used as antibody labeling the CTB of an anchoring villi (Fig. 2E). The iEVTs in decidua and endovascular extravillous trophoblasts (eEVTs) invading into spiral artery were labeled both by using CK7 and HLA-G antibodies (Fig. 2E and I for CK7, Fig. 2F and J for HLA-G respectively). PLAC1 was highly expressed in the CTB and original iEVTs of the anchoring villi of second trimester (Fig. 2G). The strong expression of PLAC1 in eEVT was also observed in the second trimester (Fig. 2K) and no staining signals were observed in the control sections (Fig. 2H). In term placentas (38 weeks), high expression levels of PLAC1 protein were also observed in the villous trophoblast cells and iEVTs invading into the maternal decidua (Fig. 2N). The serial sections were stained with CK7 (Fig. 2L) and HLA-G (Fig. 2M) to label the iEVTs in decidua of term placenta. No positive staining was exhibited in the control for term placenta (Fig. 2O).

**PLAC1 siRNA diminished the invasion and migration ability of trophoblast cell line HTR8/SVneo**

To investigate the role of PLAC1 in trophoblast cell invasion and migration, the human first-trimester extravillous trophoblast cell line HTR8/SVneo was employed. As shown in Fig. 3A, B, C, and D, compared with scrambled siRNA, PLAC1 siRNA1 significantly decreased the invasion (P<0.05) and migration (P<0.01) of HTR8/SVneo cells. The invasion and migration abilities of HTR8/SVneo cells were decreased to 42.2 ± 6.3 and 22.5 ± 2.2%, respectively, after PLAC1 knockdown when normalized to scrambled siRNA-transfected cells (Fig. 3E).

MMPs, such as MMP2 and MMP9, play important roles in degrading extracellular matrix which in turn facilitates the trophoblast cell invasion process. Therefore, gelatinolytic activities of MMP2 and MMP9 were detected using gelatin zymography assay in the above-mentioned matrigel invasion model. Results (Fig. 3F) and corresponding statistical analysis (Fig. 3G) revealed that pro-MMP9 was decreased to 70.6 ± 12.2% in the supernatant of cells transfected with PLAC1 siRNA1, when normalized to the scrambled siRNA group (P<0.05, n=3). No obvious changes of pro-MMP2 were detected (Fig. 3F and G). The knockdown efficiency of PLAC1 siRNA1 was determined by real-time RT-PCR. As shown in Fig. 3H, PLAC1 siRNA1 significantly decreased the expression of PLAC1 mRNA in HTR8/SVneo cells with an efficiency of around 73%.

Meanwhile, PLAC1 siRNA1 had no significant effect on the proliferation and apoptosis of HTR8/SVneo cells, as shown by MTT assay and Hoechst33258 staining (Supplementary Figure 1, see section on supplementary data given at the end of this article). These data confirm that the decrease in invasion and migration of HTR8/SVneo cells by PLAC1 knockdown is not a result of a decrease in cell proliferation or an increase in cell apoptosis. Similar results were obtained for the matrigel invasion and transwell migration assay when another specific PLAC1 siRNA (designated as PLAC1sRNA 2) was employed, as shown in Supplementary Figure 2.

Figure 1 Detection of PLAC1 mRNA in human first (6 weeks) placenta villi and term placentas by in situ hybridization. (A and B) PLAC1 mRNA was mainly localized in the STB and TC. (C) A slide incubated with sense probe of PLAC1. (D) IHC staining was performed to label the TC using CK7 antibody on a serial section. (E and F) The PLAC1 mRNA was mainly expressed in iEVTs of term placenta. iEVTs on a serial section of term placenta was figured out by CK7 antibody. (G) No signal for the sense probe incubation in term placenta was observed. CTB, cytotrophoblast; STB, syncytiotrophoblast; TC, trophoblast column; iEVT, interstitial extravillous trophoblast; w, weeks. Scale bar = 100 μm. Experiments were repeated in triplicate and representative images are shown.
PLAC1 siRNA inhibited the invasion and migration of EVTs in an extravillous explant culture model

To further explore the role of PLAC1 in trophoblast cell invasion and migration, an ex vivo extravillous explant culture model was employed. In this model, extravillous explants from first-trimester human placental villi were cultured on matrigel, and EVTs can migrate from the tip of the TC and infiltrate into the matrigel after 1–4 days of culture. Introduction of siRNA into the EVTs outgrowth was first determined by a transfection with the scrambled siRNA labeled with Alexa Fluor 488 (Fig. 4A, B, and C).

Next, the effect of PLAC1 siRNA on the outgrowth distance of EVTs from the TC was investigated. The results showed that PLAC1 knockdown limited the outgrowth distance of EVTs (Fig. 4D, E, F, G, and H for the scrambled siRNA group vs Fig. 4I, J, K, L, and M for PLAC1 siRNA1 group). Statistical analysis (Fig. 4N) indicated that the outgrowth distance of EVTs was...
human placenta villi during the first trimester, as well as in EVTs invading into the maternal decidua at term. Second, PLAC1 siRNA significantly inhibited the invasion and migration of trophoblast HTR8/SVneo cells, accompanied by the decrease in gelatinolytic activity of pro-MMP9. Third, PLAC1 siRNA significantly compromised the EVT outgrowth in an ex vivo extravillous explant culture model.

Earlier studies have indicated the expression of Plac1/PLAC1 during mouse and human placental development. In mice, Plac1 expression is restricted to all cells of the trophoblast lineage in ectoplacental cone, giant cells, and labyrinth trophoblasts from 7.5 days postcoitum (dpc) to 14.5 dpc (Cocchia et al. 2000). The expression of the human orthologous gene PLAC1 in placenta is also trophoblast specific at all stages of gestation (8–41 weeks; Fant et al. 2002). Immunohistochemical studies demonstrate that PLAC1 protein is localized to the STB as well as CTBs throughout gestation (Massabbal et al. 2005, Fant et al. 2007, Silva et al. 2007). More recently, it has been reported that Plac1 knockout mice exhibit an expanded spongiosotrophoblast layer in their placenta and mild intrauterine growth significantly reduced when the explants were cultured for 96 h (outgrowth distance 2.01 ± 0.61 mm for PLAC1 siRNA1 group vs 3.70 ± 0.52 mm for the scrambled siRNA group, \( P < 0.05, n = 16 \)).

The efficiency of PLAC1 knockdown in this ex vivo model was further validated by a whole-mount immunofluorescence assay (Fig. 5). The bright fields in Fig. 5A and F showed the EVT outgrowth of explants treated with scrambled siRNA and PLAC1 siRNA1 respectively. Significant knockdown of PLAC1 (PLAC1 siRNA1 vs scrambled siRNA, Fig. 5D vs I) was validated. HLA-G was a marker used to indicate EVTs (Fig. 5C and H). Antibody specificity was confirmed by incubating the slides with goat serum (Fig. 5K, L, M, N, and O).

**Discussion**

In this study, we have demonstrated that PLAC1 siRNA treatment inhibits trophoblast cell invasion and migration based on the following lines of evidence. First, PLAC1 was specifically localized in the TC of
In this study, we utilized both ISH and IHC to investigate the expression profile of PLAC1 in human placenta during the first trimester and term pregnancy. In addition to the STB of the first trimester villi, we report strong positive PLAC1 staining in the TC of the first-trimester placenta and EVTs invading into maternal decidua of term placenta for the first time. Therefore, our data together with previous reports implicate an important function of PLAC1 in the process of human trophoblast differentiation, and the notable intense expression in the TC and EVTs prompted us to investigate whether PLAC1 is involved in trophoblast cell invasion and migration.

Using a human trophoblast cell line, HTR8/SVneo, we found that PLAC1 siRNA inhibited cell invasion and migration. Moreover, silencing PLAC1 significantly compromised the outgrowth capacity of EVTs in the extravillous explant culture model. In human placenta, EVT invasion into maternal decidua involves the degradation of ECM, and MMP expression/activation in migratory trophoblasts is a pre-requisite (Bischof et al. 1995). In this study, we found that the decrease in invasion and migration resulting from PLAC1 siRNA transfection was accompanied by a decrease in the gelatinolytic activity of pro-MMP9. These data support the conclusion that PLAC1 promotes the invasion and migration of trophoblast cells, which may be partially mediated by the secretion of pro-MMP9. The underlying mechanisms through which PLAC1 regulates the expression and activation of MMP9 require further investigation.

Besides being expressed in the placenta, PLAC1 is also found to be highly expressed in a wide range of cancer cells (Koslowski et al. 2007, Silva et al. 2007, Dong et al. 2008), which share some common biological characteristics with trophoblast cells in their capacity of invasion and migration (Silva et al. 2007). Indeed, knockdown of PLAC1 in breast cancer cell lines MCF-7 and BT-549 also markedly impairs cell invasion and migration ability (Koslowski et al. 2007). Interestingly, placenta- and cancer-specific expression of PLAC1 involves activation of two distinct promoters by transcriptional factors such as SP1, C/EBPβ, ESR1 (ERα), RXRα, and LXR (Koslowski et al. 2009, Chen et al. 2011). Therefore, PLAC1 function may have been co-opted by various cancer cells, and the regulatory mechanism governing the restricted expression of PLAC1 in placenta deserves to be further elucidated.

One cause of pre-eclampsia, a pregnancy-related complication which remains a leading cause of maternal and perinatal morbidity worldwide, is impaired trophoblast invasion and migration, resulting in poor spiral arterial remodeling and inadequate placental perfusion (Redman & Sargent 2005). Studies on cell-free placental mRNAs in maternal plasma intended to reflect pathological alterations in pre-eclampsia have revealed elevated PLAC1 mRNA by different groups (Fujito et al. 2006, Purwosunu et al. 2007, Kodama et al. 2011). In this respect, whether the expression of PLAC1 in pre-eclamptic placenta is dysregulated and whether it is related to the etiology of pre-eclampsia await confirmation.

In summary, this study suggests that PLAC1 promotes trophoblast cell invasion and migration, and further studies are needed to elucidate the underlying mechanism and its implication in pregnancy-related diseases, such as pre-eclampsia.

Figure 5 Whole-mount immunofluorescence assay showed the silencing efficiency of PLAC1 siRNA in EVTs outgrowth. (A, F, and K) Bright field images. Immunofluorescent staining of the boxed area in (A), (F), and (K) using HLA-G or PLAC1 antibodies is shown in (C), (D), (H), and (I). (K, L, M, N, and O) Negative control experiments were carried out by replacing the primary antibody with normal goat serum. (B, G, and L) Staining the nuclei of the cells in the boxed area by DAPI in (A), (F), and (K) respectively. Scale bar = 100 µm. Experiments were repeated in triplicate and representative images are shown.
Role of PLAC1 in trophoblast invasion and migration

Funding
This work was supported by the National Key Basic Research Program of China (2011CB944440 and 2012CB126310), the Strategic Priority Research Program of Chinese Academy of Sciences (XDA01020102), and grants from the Natural Science Foundation of China (81225004, 31271603, and 31171438).

Acknowledgements
The authors sincerely thank Prof. Yu Zhang at Department of Immunology, Peking University Health Science Center for his valuable suggestions and for generously providing PLAC1 antibodies.

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Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-14-0052.

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.


Received 31 January 2014
First decision 24 February 2014
Revised manuscript received 4 June 2014
Accepted 2 July 2014