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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 EVTs 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 EVTs 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.
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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 EVTs (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 house-keeping 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

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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 (Koslowski *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, Massabbal *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

Placental 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′-AAATTTGG-CAGCTGCCTTCAC-3′; antisense, 5′-TGCTCACATGAGGGT-CACAAG-3′. This fragment was cloned into pGM-T plasmid (Tiangen, Beijing, China) for cRNA probe preparation. The recombinant plasmid was linearized by restrictive endonucleases ApaI or SalI, 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 CM 1950 (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

(Leica RM 2135, Leica Microsystems, Bensheim, Germany). 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% H_2O_2 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 antiserum. Cytokeratine 7 (CK7, 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% CO_2/95% 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′-CAUCUCACUUUCUUGAUAUTT-3′ (sense), 5′-AUAUCAAGAAAGMGAGAUGTT-3′ (antisense); and *PLAC1* siRNA2, 5′-CC AAAGCMGUCUCUCAGGACAUGGU-3′ (sense), 5′-ACCAUGUCCUGAGAGACAGCUUUGG-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′-AAATTTGGCAGCTGCCTTCAC-3′ (sense), 5′-TGATGCCACATTCAGTAACAC-3′ (antisense); *GAPDH* (NM_001256799): 5′-GCCATCAATGACCCCTTCATT-3′ (sense), 5′-TTGACGGTGCCATGGAATTT-3′ (antisense). The data were analyzed using ΔC_t 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, 1×10^5 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 *PLAC1* 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 $CaCl_2$, 1 mM $ZnCl_2$, 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$^+$ Imaging System (Bio-Rad Laboratories).

MTT assay

The HTR8/SVneo cells were seeded at a density of 1×10^4 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-dimethylthiazol-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) (Baczyk *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_2. Twenty-four hours later, the explants were observed and recorded using an inverted microscope system (Nikon Eclipse T*i*, 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

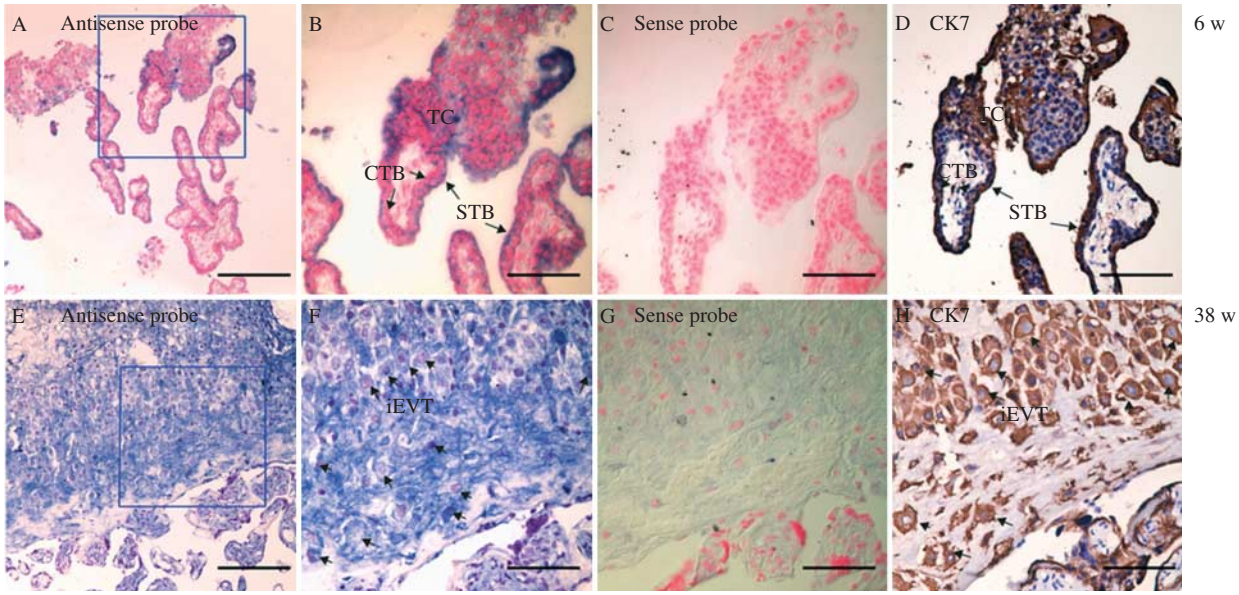

Figure 1 Detection of *PLAC1* mRNA in human first (6 weeks) placenta villi and term placentas by *in situ* hybridization. (A and B) *PLAC1* mRNA was mainly localized in the STB and TC. (C) A slide incubated with sense probe of *PLAC1*. (D) IHC staining was performed to label the TC using CK7 antibody 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.

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 $22.5\pm2.2\%$, 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 $70.6\pm12.2\%$ 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 *PLAC1*siRNA 2) was employed, as shown in Supplementary Figure 2.

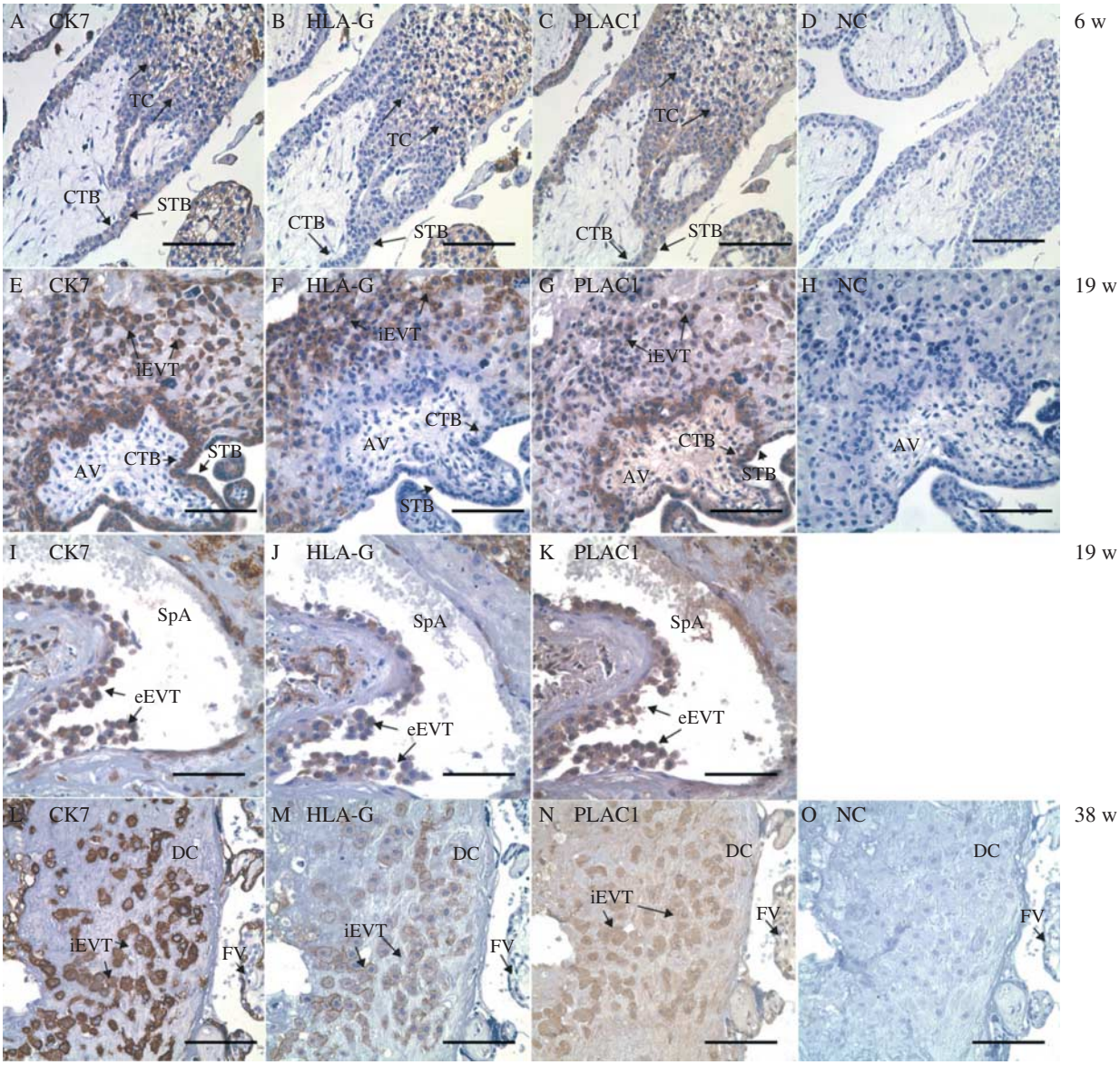

Figure 2 Localization of PLAC1 protein in human placental villi by immunohistochemistry. (C) PLAC1 protein was specifically expressed in the STB and TC at the fetal–maternal interface during the first trimester (6 weeks). In the second-trimester placenta, PLAC1 was highly expressed in CTB and iEVT of anchoring villi (G), as well as in eEVT of spiral artery (K). In the fetal–maternal interface of term placenta (38 weeks), strong expression of PLAC1 in iEVT was also observed (N). CK7 was employed to label the trophoblast in villi and EVTs in decidua respectively (A, E, I, and L). HLA-G antibody incubation was used as the marker for EVT (B), iEVT (F and M), and eEVT (J). No positive staining was observed in the corresponding control sections of the first trimester (D), second trimester (H), and term placenta (O). CTB, cytotrophoblast; STB, syncytiotrophoblast; TC, trophoblast column; iEVT, interstitial extravillous trophoblast; eEVT, endovascular extravillous trophoblast; SpA, spiral artery; AV, anchoring villi; FV, floating villi. Scale bar=100 μm. All the 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

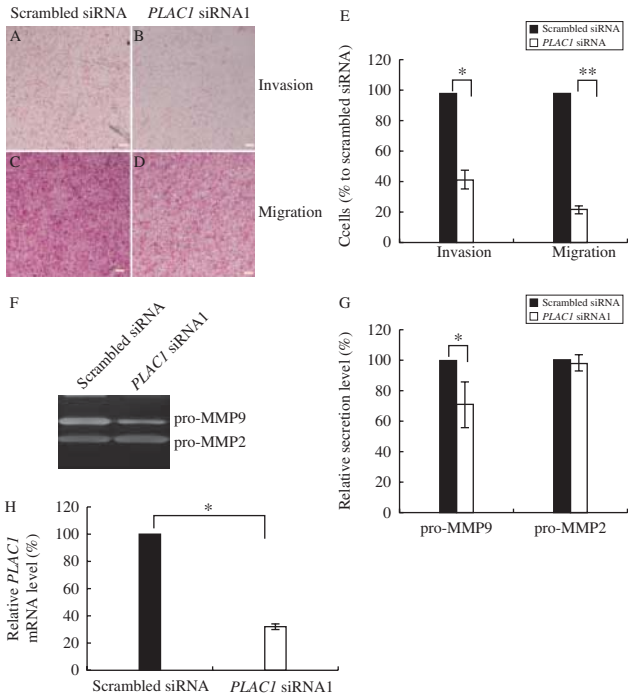

Figure 3 Knockdown of *PLAC1* significantly impaired the invasion and migration of human trophoblast HTR8/SVneo cells. (A, B, C, and D) Images of filters carrying invaded or migrated cells transfected with indicated siRNAs in matrigel cell invasion assay or transwell cell migration assay. (E) Statistical analysis of changes in invasion or migration after *PLAC1* siRNA treatment ($n=3$; $^{*}P<0.05$; $^{**}P<0.01$). (F) *PLAC1* siRNA decreased zymographic activities of pro-MMP9; (G) statistical analysis of zymographic activities of pro-MMP9 and pro-MMP2 as shown in (F) ($n=3$; $^{*}P<0.05$); (H) the silencing efficiency of *PLAC1* siRNA was detected by real-time RT-PCR after the cells were transfected for 48 h ($^{*}P<0.05$). Scale bar=200 μm.

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 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 spongiotrophoblast layer in their placenta and mild intrauterine growth

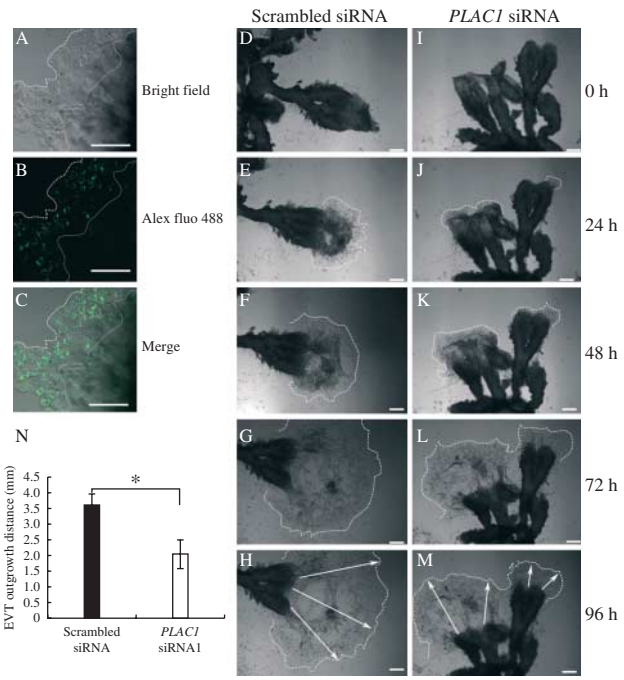

Figure 4 Silencing of *PLAC1* restricted the outgrowth of induced EVTs in the placental villous explant culture model. (A, B, and C) The bright (A) and fluorescent (B) field views showing that small interfering RNA labeled with Alexa Fluor 488 successfully penetrated into the EVT outgrowth; (C) a merged microscopy picture. (D, E, F, G, H, I, J, K, L, and M) Migration (dotted line circled area) of induced EVTs from the placental villi treated with scrambled or *PLAC1* siRNA for indicated time duration. (N) Statistical analysis of the outgrowth distance of induced EVTs treated with scrambled or *PLAC1* siRNA for 96 h ($^{*}P<0.05$, $n=16$). Scale bar=100 μm.

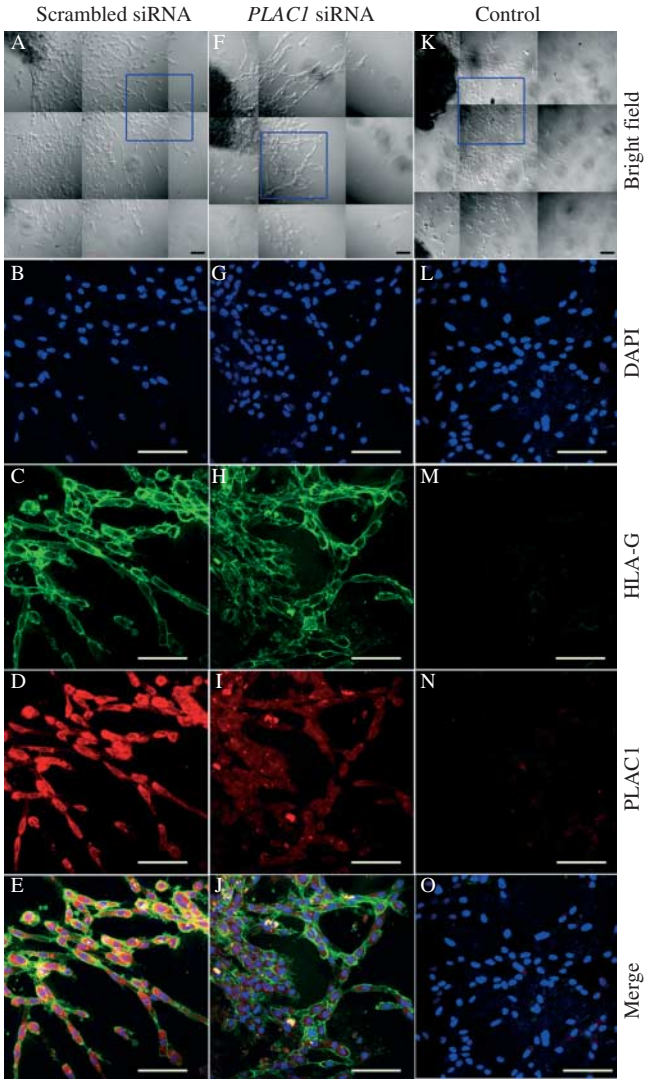

Figure 5 Whole-mount immunofluorescence assay showed the silencing efficiency of *PLAC1* siRNA in EVTs outgrowth. (A, F, and K) Bright field images. Immunofluorescent staining of the boxed area in (A), (F), and (K) using HLA-G or PLAC1 antibodies is shown in (C), (D), (H), and (I). (K, L, M, N, and O) 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.

retardation (Jackman *et al*. 2012). 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 pathophysiological 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.

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.

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