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PLAC1 is involved in human trophoblast syncytialization

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ABSTRACT

Placenta specific protein 1 (PLAC1) is thought to be important for murine and human placentation because of its abundant expression in placenta; however, the trophoblast subtypes that express PLAC1 at the fetomaternal interface and the major role of PLAC1 in placentation are still unclear. This study investigated the expression pattern of PLAC1 at the human fetomaternal interface and its involvement in trophoblast syncytialization. Localization of PLAC1 at the fetomaternal interface was studied using *in situ* hybridization (ISH) and immunohistochemistry (IHC) assays. Real time RT-PCR and Western Blot were employed to exhibit the expression pattern of PLAC1 during human spontaneous syncytialization of term primary cytotrophoblast cells (CTBs). Spontaneous syncytialization of a primary term CTBs model transfected with siRNA specific to *PLAC1* was used to investigate the role of PLAC1 during human trophoblast syncytialization. The results showed that PLAC1 was mainly expressed in the human villous syncytiotrophoblast (STB) layer throughout gestation, and the expression level of PLAC1 was significantly elevated during human trophoblast syncytialization. Down-regulation of *PLAC1* via specific *PLAC1* siRNA transfection attenuated spontaneous syncytialization of primary term CTBs ($p < 0.05$) as indicated by cell fusion index and the expression patterns of the corresponding markers. These data demonstrate the facilitative role of PLAC1 in normal human trophoblast syncytialization.

1. Introduction

The placenta is a transient but necessary organ for mammalian fetomaternal interaction. Structurally, the typical hemochorial discoid villous type of human placenta is the most highly evolved placental type in mammals [1]. Human placenta villous trees are built by the outer layer of the multi-nuclear syncytiotrophoblast (STB) and the underlying mono-cell layer called cytotrophoblast (CTB). The CTB maintains its proliferative characteristic to achieve expansion and renewal of both CTB layer and STB layer. Placental trophoblast differentiation is tightly associated with its basic physiologic functional aspects, including endocrinology, substance exchange etc. [2–4]. And formation of the STB layer from the CTB is one of the major differentiation pathways of the human trophoblast.

Normal trophoblast syncytialization during human placentation is strictly regulated by some placental specific genes, such as *Gcm1*, *syncytin 1* and *2*, placenta specific protein genes, and other transcription factors that have been reported very important for normal trophoblast syncytialization [5]. Cell fusion is a common biological event in species ranging from lower organisms to mammals and is necessary for normal development and some pathogenetic processes [6,7]. Human trophoblast syncytialization is a highly dynamic cell fusion process. Some specific molecules are

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required to initiate the cell fusion process and are called fusogens, but they are not conserved in different organisms or cell fusion systems [8]. The fusogens involved in human trophoblast syncytialization that have been well characterized are *syncytins 1* and *2* [9]. *Syncytin 1* is reported to be broadly located in the placental villi and in all cell subtypes of extravillous trophoblast (EVT) lineages [10]. However, *syncytin 2* is only observed in the sub-population of cytotrophoblast cells and its sub-cellular location is more frequently the cell membrane at the interface between cytotrophoblastic cells and the syncytiotrophoblast [11].

PLAC1 was firstly suggested as a placenta-specific gene because of its expression pattern in placenta [12,13]; however, subsequently, it was also found in testes and a wide range of cancers [14–16]. The moderate expression of *PLAC1* in EVTs has been reported, and its role in human trophoblast invasion and migration was investigated in our previous work [17]. In our preliminary investigation, the expression of PLAC1 was also observed in the STB layer of human placental villi, which suggests its role in human trophoblast syncytialization. In this study, the localization of *PLAC1* mRNA and protein in human placental villi was examined by *in situ* hybridization (ISH) and immunohistochemistry (IHC). Based on a high level of expression of *PLAC1* in the villous STB layer, changes in its transcription level were detected in a spontaneous syncytialization model with primary term CTB culture. Moreover, regulation of human trophoblast syncytialization by *PLAC1* was studied using *PLAC1*-specific siRNA transfection in the spontaneous syncytialization model.

2. Material and methods

2.1. Placenta tissue collection

Placental villi of human first trimester (6 w) and term placental tissues were collected from normal pregnant women undergoing legal abortion or caesarean section at Beijing Shijitan Hospital. The use of clinical placental samples followed the standard experimental protocols approved by the Ethics Committee of College of Veterinary Medicine, Hunan Agricultural University (Case number as HAUCVM20120115). All donors were counseled, and assigned informed consent was obtained from each participant.

2.2. In situ *hybridization (ISH)*

The sequences of the complementary RNA probes for *PLAC1* (NM_021796), the method of preparation of frozen sections and the *ISH* assay protocol were as described previously [17]. Briefly, total RNAs from the first trimester placental villi were isolated and reversed to cDNA, and used as template to amplify a fragment with 879 bp nucleotides, followed by ligation into a plasmid to produce the PLAC1 *ISH* probes. Specific primer pairs used for PCR were as followed: sense, 5′-AAATTTGGCAGCTGCCTTCAC-3′; antisense, 5′-TGCTCACATGAGGGTCACAAG-3′. The sense and antisense *ISH* probes of *PLAC1* were produced using the digoxigenin (DIG) RNA labeling kit (SP6/T7, Roche Molecular Biochemicals, Mannheim, Germany). *ISH* assay was performed on frozen section of the first trimester placental villi which were fixed in 4% neutral paraformaldehyde. Antisense or sense probes of *PLAC1* at a concentration of 2 mg/mL were used for hybridization at 60 °C for 14–18 h after pre-hybridization. Then sections were directly photographed without cell nucleus counter-staining. Tissues from three different individuals were examined.

2.3. Immunohistochemistry (IHC)

Preparation of paraffin sections and IHC staining were performed as described in our previous work [17]. Rabbit anti-human PLAC1 antibody (ab105395; Abcam, Cambridge, UK) and mouse anti-human E-cadherin antibody (sc-71008; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were commercially available. Serial sections incubated with rabbit IgG instead of the primary antibody were employed as negative controls of IHC. Positive signal was conducted by the 3,30-diaminobenzidine tetrahydrochloride (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) and counterstained with hematoxylin. The location of PLAC1 was examined in sections from six different individual human placentae of the first trimester or full term.

2.4. Western blotting

Cell proteins were extracted using lysis buffer (50 mM Tris- HCl pH7.6 and 2% SDS) which was supplemented with proteinase inhibitors (Cocktail, P8340, Sigma-Aldrich Inc., St. Louis, MO, USA). Protein concentration was determined through the BCA (Thermo Fisher, Waltham, MA, USA) method. Protein extracts (50 μg per lane) were subjected to SDS-PAGE and then electrophoretically transferred onto PVDF membranes (Pall Corporation, Pensacola, FL, USA). Membrane were blocked with 5% skim milk for 1 h at room temperature, and sequentially incubated with the primary antibodies overnight at 4 °C, and the HRP-conjugated secondary antibody at room temperature for 2 h. Signals were exhibited by using the Enhanced Chemiluminescence System (Thermo Fisher Scientific). Antibodies against PLAC1 (ab131630; Abcam, Cambridge, UK), GCM1 (hpa011343, Sigma-Aldrich Inc., St. Louis, MO, USA), hCG-β (ab53087, Abcam), Syncytin 2 (AP13018a; Abgent, San Diego, CA, USA) and E-cadherin (sc-71008; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used.

2.5. Isolation of term CTBs and development of spontaneous syncytialization model

Primary term CTBs were isolated from normal term placentas as described previously [18]. Briefly, the trophoblastic villous portion of the CTB was retained and digested in DMEM supplemented with 0.125% trypsin and 0.03% DNase-I (Sigma, St. Louis, MO). Then, the CTBs were enriched using Percoll (GE Healthcare, Bio-sciences AB, Uppsala, Sweden) density gradient centrifugation. The isolated CTBs were identified by positive staining of cytokeratin 7 (CK7, clone OV-TL 12/30; DAKO, Carpintera, CA, USA). The identified CTBs were seeded onto 6-well plates with 1.5×10^6 cells per well and cultured in IMDM (Gibco, Invitrogen Corporation, CA, USA) with 10% fetal bovine serum (FBS, Thermo Scientific HyClone, UT, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin in a 5% CO2/95% air incubator at 37 °C. For the differentiation system, the time at which the cells attached to the well bottom was deemed 0 h. The differentiation model was maintained for 72 h. For the cells that were used for siRNA transfection, 1.5 mL of Gibco® Opti-MEM™ Medium (Life technologies) was added to each well prior to siRNA transfection. Sixteen term placentas were examined.

2.6. Real time RT-PCR

Total RNA was extracted and purified using TRIzol® reagent (Invitrogen). Reverse transcription of RNA to cDNA was performed with Superscript II reverse transcriptase (Invitrogen) following the manufacturer's instructions. Real-time PCR was performed using the SYBR Premix Ex Taq kit (Takara, Dalian, China) and the ABI PRISM 7500 Real-time PCR System (Applied Biosystems, Foster City, CA). The specific primers used for amplification were as follows: *hCG-β* (NM_000737): 5′-TGTTGCTGCTGCTGAGCATG-3′ (sense), 5′-CAGAGTGCACATTGACAGCTG-3′ (antisense); *E-cadherin* (NM_004360.3): 5′-AGCCTCTGGATAGAGAACGCATTG-3′ (sense), 5′-GGGTGAATTCGGGCTTGTTGTCAT-3′ (antisense); *PLAC1*

(NM_021796): 5′-AAATTTGGCAGCTGCCTTCAC-3′ (sense), 5′-TGATGCCACATTCAGTAACAC-3′ (antisense); and *GAPDH* (NM_001256799): 5′-GCCATCAATGACCCCTTCATT-3 (sense), 5′-TTGACGGTGCCATGGAATTT-3′ (antisense) were employed for real time RT-PCR. All data were analyzed using $^{\Delta}C_t$ method and normalized to *GAPDH* expression [19,20].

2.7. Transfection of PLAC1 siRNA in primary cultured CTB

The siRNA duplex that specifically targets *PLAC1* mRNA was the same as that described previously [17]. A nonspecific siRNA duplex obtained from Invitrogen was used as a control. Each siRNA duplex was transfected into CTBs using Lipofectamine® 2000 (Invitrogen) to a final concentration of 100 nM according to the manufacturer's instructions.

2.8. Immunofluorescense and trophoblast cell fusion index statistical analysis

Trophoblast cells cultured on glass coverslips were harvested at 0 and 72 h and fixed with neutral PFA at 4 °C for 10 min. After incubation with 1% bovine serum albumin for 30 min, the cells were incubated with primary antibodies against PLAC1 or E-cadherin at 4 °C overnight. On the following day, the cells were washed with 1 × PBS three times and then incubated with the appropriate secondary antibody (goat anti-rabbit-AlexaFluor 555 or goat anti-mouse-Alexa Fluor 488, Life Technologies, USA). Cell nuclei were counterstained with DAPI (Invitrogen) and mounted with anti-fade mounting agent. Staining signals were analyzed in six randomly selected areas using a Carl Zeiss LSM710 laser scanning microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). The cell fusion index was determined using the following formula: index = (N-S)/T (N is the number of nuclei in the syncytia, S is the number of syncytia, and T is the total number of nuclei in each observation area). Sixteen different placentas were used for the CTB syncytialization model for cell fusion index.

2.9. Statistical analysis

All data are presented as the means ± SEM and were examined by analysis of variance (ANOVA) followed by the paired T-test using the Statistical Package for Social Science (SPSS for Windows package release 10.0, SPSS, Inc., Chicago, IL). Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. PLAC1 has a high expression level in human placental STB layer

E-cadherin and hCG-β antibodies were used to identify the CTB layer and TC (Fig. 1A) and STB layer (Fig. 1B), respectively. During the first trimester of pregnancy, PLAC1 protein was highly

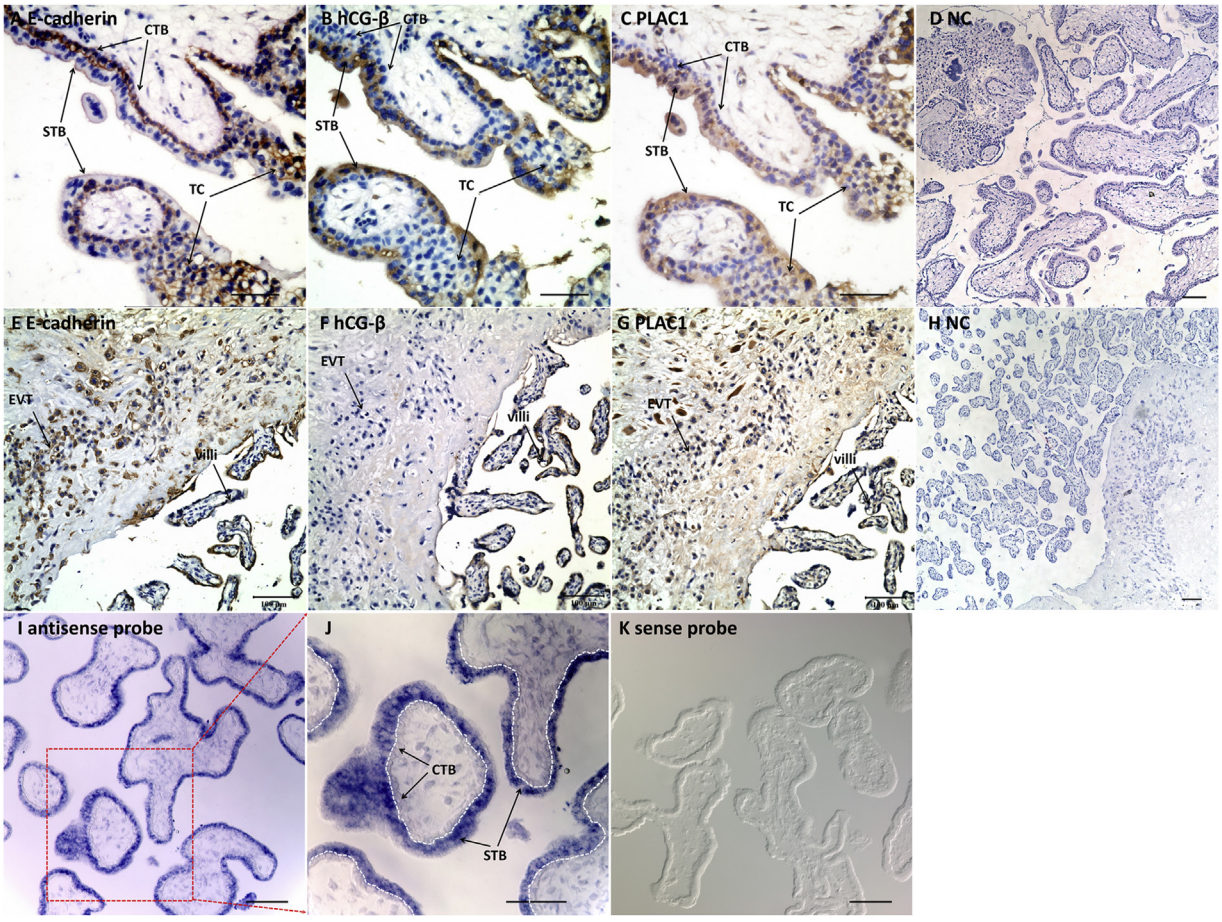

Fig. 1. IHC and ISH methods showed high expression of PLAC1 in the STB layer of human placental villi. (A and B) Antibody against E-cadherin or hCG-β was used to identify the CTB and STB, respectively. (C) A strong positive signal for PLAC1 was mainly observed in the villous STB layer, and a moderate signal was observed in TC (6 w). (E and F) Antibody against E-cadherin or hCG-β to indicates the CTB, EVT, and STB of normal term placenta (37 w), respectively. (G) PLAC1 was highly expressed in term villous STB and EVT in decidua. (D and H) NC sections, the primary antibody was replaced with normal rabbit IgG. (I-J) A strong positive signal was observed in the villous STB layer after incubation with the antisense probe for *PLAC1*. (K) The sense probe was used as a control. IHC, immunohistochemistry; ISH, *in situ* hybridization; NC, negative control; CTB, cytotrophoblast; STB, syncytiotrophoblast; TC, trophoblast column; EVT, extravillous trophoblast. n = 5. Bar represents 100 μm.

expressed in the villous STB layer of placental villi and was moderately expressed in the trophoblast column (TC) (Fig. 1C). In term placenta, EVT and CTB were stained with E-cadherin positive signals (Fig. 1E). And hCG-β was specifically expressed in term villous STB (Fig. 1F). Strong positive signals of PLAC1 were observed in STB and EVT of term placenta (Fig. 1G). No staining signals were observed in the control sections from the first trimester (Fig. 1D) and term placentas (Fig. 1H). The expression pattern of *PLAC1* mRNA was similar to that of PLAC1 protein. A strong positive signal was also observed in the villous STB on slides incubated with the antisense probe, indicating that *PLAC1* mRNA was highly transcribed in the villous STB layer (Fig. 1I and J). The sense probe was used as a negative control for the *ISH* assay (Fig. 1K).

3.2. Expression level of PLAC1 was elevated during the spontaneous syncytialization of primary term CTB

The primary CTBs from term human placentas spontaneously entered the syncytialization differentiation pathway when they were cultured *in vitro* under normal conditions; and this is a valuable *ex vivo* cell model for investigating molecules involved in trophoblast syncytialization. The transcriptional activation of *hCG-β* was used as an indicator of successful trophoblast syncytialization. As shown in Fig. 2A, the transcription level of *PLAC1* was significantly increased by nearly 600-fold during syncytialization of the primary term CTBs ($P < 0.01$) after 72 h of culture, and this trend was also observed for *hCG-β* mRNA. Expression of PLAC1 protein was also significantly increased in the spontaneous syncytialization model (Fig. 2B). Syncytin 2, hCG-β, GCM1, and E-cadherin are usually used as molecule markers to evaluate spontaneous syncytialization. In this model, elevation of hCG-β, Syncytin 2 and GCM1, and decrease of E-cadherin were observed, which indicated the success of spontaneous syncytialization.

3.3. PLAC1 knockdown attenuated the human trophoblast spontaneous syncytialization

To study the regulation role of PLAC1 in spontaneous CTB syncytialization, *PLAC1*-specific siRNA was transfected into CTBs after they had completely attached to the well bottom, and that time point was designated 0 h. After 72 h of transfection, glass coverslips were collected for the ICC assay to determine the cell fusion index, and the cells in the wells were harvested for total RNA extraction to examine the expression levels of corresponding genes. *PLAC1* was significantly silenced by *PLAC1*siRNA with an efficiency of ~75% compared to normal universal siRNA ($P < 0.05$, Fig. 3C). In the present study, the transcription of *hCG-β*, an indicator of trophoblast syncytialization, was also significantly decreased (~70%) after transfection with *PLAC1* siRNA for 72 h. Furthermore, *E-cadherin* expression was decreased during the process of trophoblast fusion. *E-cadherin* expression was increased by transfection with PLAC1 siRNA compared to transfection with the control siRNA ($p < 0.05$, Fig. 3C). The trophoblast fusion index was significantly increased after 72 h of culture, and knockdown of *PLAC1* decreased cell syncytialization (Fig. 3A and B). Similar expression change for PLAC1 protein was also obtained (Fig. 3D). When the expression of PLAC1 was knockdown, the increase of positive markers (including Syncytin 2, GCM1 and hCG-β) was attenuated during syncytialization. Meanwhile, the decrease of the negative marker E-cadherinwas inhibited during the trophoblast syncytialization. All these data indicate that PLAC1 is necessary for normal trophoblast syncytialization.

4. Discussion

PLAC1 was first identified as a placenta-specific gene through data mining of amouse placenta cDNA library [12]. Later, its high level of transcription in human placental villi was reported [13]. In

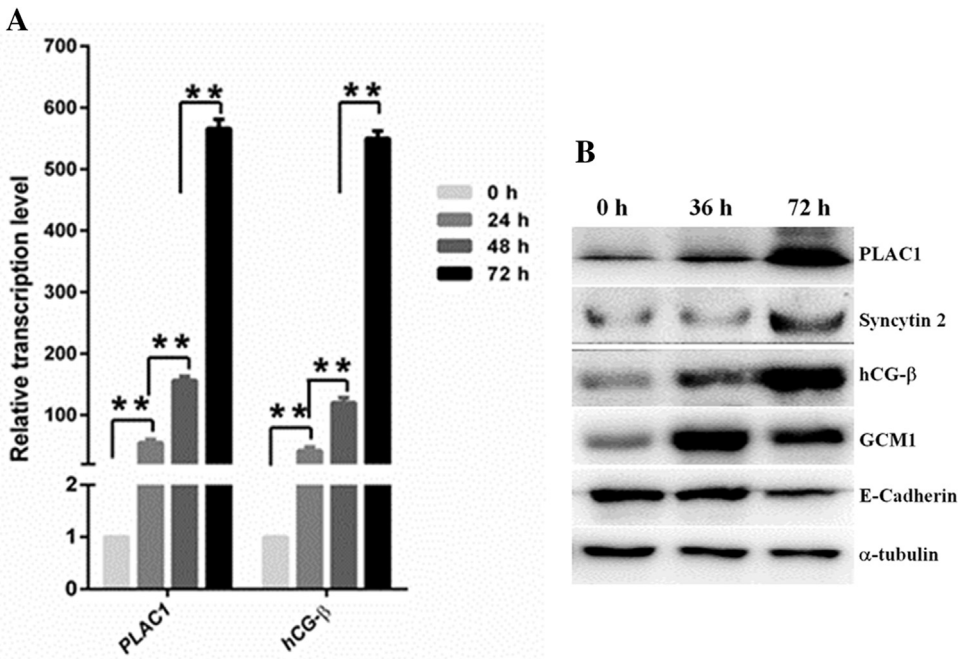

Fig. 2. Expression pattern of PLAC1 in human trophoblasts undergoing spontaneous syncytialization. Both (A) Transcription and (B) protein of *PLAC1* were significantly elevated in normal primary term CTBs undergoing spontaneous syncytialization. Expression of syncytin 2, hCG-β and GCM1 were increased, and E-Cadherin was decreased in spontaneous syncytialization model. CTB, cytotrophoblast. n = 16. ** means $p < 0.01$.

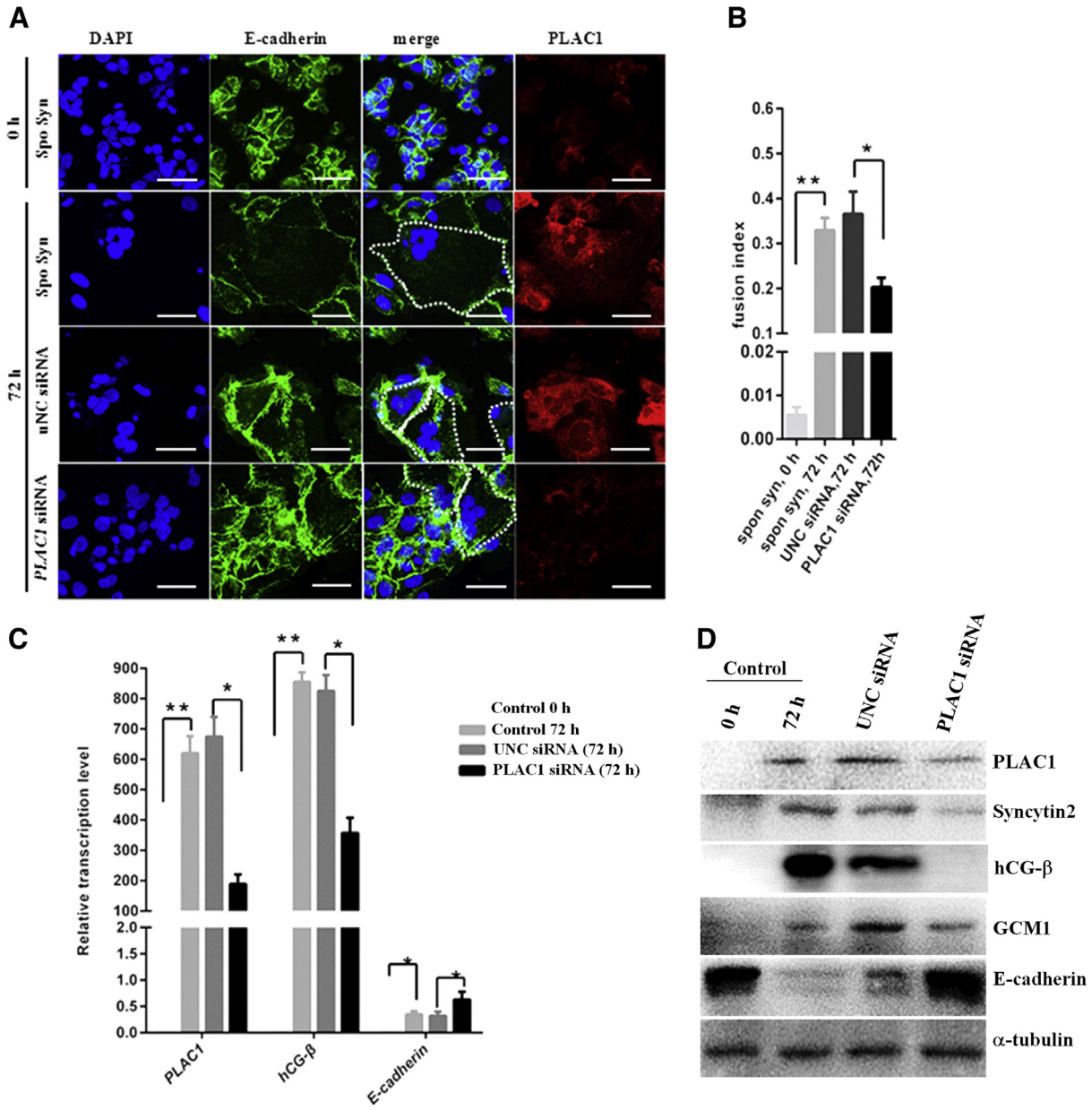

Fig. 3. Transfection of PLAC1 specific siRNA in the primary term CTBs impaired spontaneous syncytialization. (A and B) Syncytium formation was observed in the spontaneous syncytialization model after 72 h of culture and was attenuated by the transfection with *PLAC1* siRNA. (C) Expression of *PLAC1*, *hCG-β* and *E-cadherin* genes during spontaneous syncytialization with or without transfection of *PLAC1* siRNA. (D) Transfection of *PLAC1* siRNA attenuated the increase of the corresponding markers (GCM1, Syncytin2 and hCG-β) for spontaneous syncytialization and inhibited the decrease of E-cadherin. CTB, cytotrophoblast; Spo Syn, spontaneous syncytialization; uNC siRNA, universal negative control siRNA. n = 5; *, $p < 0.05$; **, $p < 0.01$. Scale Bar = 100 μm.

our previous work, moderate expression of *PLAC1* was observed in invasive EVTs and its involvement in trophoblast invasion and migration was also investigated [17]. In the present study, the abundent expression of *PLAC1* was observed in the villous STB layer of the fetomaternal interface, which suggests that it has a role in human trophoblast syncytialization.

Genes specifically expressed in placenta that initiate the cell fusion process are called placenta-specific fusogens. The trophoblast specific genes *syncytin 1* and *2* are the only two known fusogens involved in human placentation [3]. In addition to the key regulatory roles of fusogens in controlling trophoblast syncytialization, other genes and signaling pathways that interact with fusogens are also important in the trophoblast fusion process. GCM1 is a critical factor in controlling placental cell fusion, and it acts by targeting syncytin 1 and 2 [21,22]. The ERK1/2 and p38 MAPK pathways have also been reported to activate molecules downstream of adenylyl cyclase and to regulate *hCG* secretion and the expression of specific fusogenic genes [23].

Identification of genes with specific expression patterns in the process of trophoblast fusion could be an efficient approach for screening fusogenic candidates. In the present study, using a spontaneous syncytialization model of term CTB, the transcription level of *PLAC1* was found to be elevated nearly 600-fold after 72 h of culture, and a similar level of expression had been reported by a previous study [24]. To further investigate the regulation of PLAC1 in trophoblast syncytialization, *PLAC1*-specific siRNA was

employed to silence *PLAC1* transcription, and it significantly attenuated the cell fusion process by altering the cell fusion index and the expression levels of hCG-β, GCM1, Syncytin 2 and E-cadherin. PLAC1 is not only involved in EVT invasion and migration [17], based on the results of the current study, it also has a significant role in human trophoblast syncytialization, similar to the dual role of GCM1 in human trophoblast syncytialization and trophoblast invasion and migration during placentation [25,26].

Multiple studies have shown that PLAC1 is associated with several clinical conditions [27–32]. In preeclampsia, dysfunctional trophoblast invasion reduces placental perfusion and/or creates an intermittent flow [33]. Furthermore, abnormal placentation was reported in a *Plac1* mutated mouse model [28]. The typical pathological features of preeclampsia placenta are shallow invasion and migration of interstitial EVT in the maternal decidua and insufficient artery remodeling of the endovasculature [34]. However, whether abnormal trophoblastic syncytialization is also a major contributor to preeclampsia is still unclear. Recent studies have demonstrated that the appearance of many placental micro-particles in the maternal circulation may contribute to circulating soluble fms-like tyrosine kinase 1 in women with preeclampsia [35–37]. The observed changes in the levels and locations of these placental micro-particles indicated that the structure and metabolism of the villous STB layer were dynamically altered in women with preeclampsia. Several reports suggest that PLAC1 is a biomarker of preeclampsia that is present in maternal serum, and cell-free mRNA of *PLAC1* in maternal plasma is elevated in women with preeclampsia [30,38]. The plasma concentration of *PLAC1* mRNA is higher in early-onset than late-onset preeclampsia [39]. Therefore, increased *PLAC1* mRNA in maternal serum might be associated with syncytial aggregates ejected into the intervillous space or to the activation of its transcription in placental STBs. However, the exact mechanism of PLAC1 in the development of the PE and its implications in abnormal trophoblast differentiation and placental villi structural changes need to be further explored.

Conflict of interests

The authors have no conflict of interest.

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