Original Paper

Plac1 Expression Pattern at the Mouse Fetomaternal Interface and Involvement in Trophoblast Differentiation

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Key Words

Plac1 • Spatiotemporal expression pattern • Fetomaternal interface • Trophoblast • Differentiation • Placenta

Abstract

Background/Aims: It is well known that *Plac1* is a placenta-specific gene; however, its spatiotemporal expression pattern and exact role at the mouse fetomaternal interface remains unclear. **Methods:** In situ hybridization (ISH) was used to localize the *Plac1* mRNA at the mouse fetomaternal interface. A trophoblast stem cell (TS) differentiation model with *Plac1* shRNA-overexpressing lentivirus was employed to investigate the possible role of *Plac1* in placentation. Real-time RT-PCR was used to detect changes in gene expression. **Results:** *Plac1* was exclusively expressed in the ectoplacental cone (EPC) as well as in 8.5 and 9.5 days post-coitum (dpc) embryos. Subsequently, *Plac1* expression was abundant in the spongiotrophoblast layer and moderately in the labyrinth layer until 13.5 dpc, and declined thereafter. Interestingly, *Plac1* was also expressed by secondary trophoblast giant cells and glycogen trophoblast cells, but not in primary trophoblast giant cells. *Plac1* transcription was increased during the TS differentiation ($P < 0.01$), and knockdown of *Plac1* significantly impaired TS differentiation. **Conclusion:** *Plac1* is abundantly expressed at the fetomaternal interface and in all trophoblast subtypes except in primary trophoblast giant cells. *Plac1* knockdown retarded the progress of TS differentiation, indicating that *Plac1* is necessary for normal trophoblast differentiation into various trophoblast subpopulations.

Introduction

The placenta is a transient mammal-specific organ that controls the exchange of substance between the mother and fetus. Placental mammals originated about 100 million years

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ago, and the placenta ensured a successful pregnancy [1]. During the long-term enveloped process, the placenta has evolved with high diversity both in structure and function across different mammalian species. Primates and rodents showed the least evolutionary distance in terms of placental structure, and have a typical hemochorial placenta, which characterizes the most intimate fetomaternal contact [2]. Thus, mouse and rat models are often used to study placentas as the findings often help understand what happens in human placentas.

The complex diversity of mammalian placentas is determined by their unique regulatory mechanisms of specific genes, which typically have spatiotemporal expression patterns during normal placentation [3-6]. Many studies have investigated these genetic mechanisms, including epigenetic regulation within the placenta, regulatory noncoding RNAs, and classical protein-coding genes, all of which contribute to the structural and functional specificity of the placenta [7-10]. While studies have investigated and provided extensive insights into hemochorial placenta development [4], gaps in knowledge remain, and the precise blueprint of genetic regulating mechanisms for normal placentation remains to be fully understood.

Although Rawn [4] summarized that enveloping the placenta did not involve an entirely new set of genes, the mammalian genome has evolved in concert with placental development and there are some genes that are unique to the placenta alone. A series of genes that were initially reported to be enriched in a placental cDNA library are termed placenta-specific genes (*Placs*). Of these, some genes such as *Plac1* have undergone functional studies and have subsequently been established in mouse models [11]. *Plac1*-mutated mice have been shown to have abnormal placental structure and the viability of postnatal offspring of these mutant mice have been found to be significantly affected. In fact, the precise expression pattern of *Plac1* is still not fully clear; this poses a barrier in fully understanding about how *Plac1* ensures normal placentation. Thus, this study aims to reveal the exact spatiotemporal expression pattern of *Plac1* at the fetomaternal interface in mice. Furthermore, the exact role of *Plac1* in trophoblast differentiation will be investigated.

Materials and Methods

Animals

In this experiment, 10-week-old CD-1 mice were mated and the presence of a vaginal plug the following morning was used to determine the exact developmental age of placentas. The mice were killed by cervical dislocation and the placentas were dissected at different time points. Isolated placentas were directly frozen by liquid nitrogen and stored at -80°C until sectioning. The use of animals in this study was approved by the Ethics Committee of the Shenzhen PKUHKUST Medical Center.

In situ hybridization

The *Plac1* probe sequences were cloned into the pGM-T plasmid (Tiangen, Beijing, China) with the T7 and SP6 promoters flanking both sides of the insertion site. The following forward and reverse primers were used to amplify a 333-bp *Plac1* fragment (NM_019538): 5'-TTGAAGGCTAAGGGACCTGC-3', and 5'-TCTGTCGAGCACAGCACATT-3', respectively. The fragment was then cloned to the plasmid as a template to generate the probe. The probe was labeled using a digoxigenin (DIG) RNA labeling kit (SP6/T7) (Roche, Mannheim, Germany).

The mouse placenta was embedded by Tissue-Tek® OCT compound (Sakura, Tokyo, Japan). Briefly, 8- μm -thick sections were prepared from the frozen placental samples using a Leica CM 1950 cryostat (Wetzlar, Germany), and mounted on RNase-free glass slides. The sections were fixed by neutral paraformaldehyde (PFA; pH 7.4) for 1 h at 4°C . ISH was performed as previously described [12]. Briefly, 2 $\mu\text{g}/\text{mL}$ of DIG-labeled cRNA probe of *Plac1* was used for hybridization at 60°C for 16 h. The sections were then counterstained by nuclear fast red solution (N3020, Sigma). The sense probe was incubated with the serial sections and deemed as the negative control. The marker gene trophoblast-specific protein α (*Tpbbp*) was used as the antisense probe to label the EPC or spongiotrophoblast layer [13]. Tissues from five different female mice were employed at each stage of pregnancy.

TS cell establishment and differentiation

TS cells were isolated from embryonic day (E) 3.5 mouse blastocysts according to the procedure described previously [14]. The established TS cell line was maintained in a proliferative state in mixed medium (70% embryonic fibroblast conditioned medium and 30% TS cell medium). The medium also contained 25 ng/mL FGF4 and 1 mg/mL heparin. The TS cell medium containing CHIR99021 (Biovision, Milpitas, CA) and without bFGF, heparin, and embryonic fibroblast preconditioning was used for TS differentiation culture.

Plac1 knockdown by specific shRNA lentivirus

TS cells were previously infected with *Plac1*-specific shRNA-overexpressing lentivirus (pLL3.7) before the induced differentiation. A scrambled shRNA lentivirus was applied to TS cells as the control. The *Plac1* shRNA sequence was 5'-TGCCACCTTATGTCAATCAAA-3'. The lentiviral titer used was in line with the rule that each cell has the chance of being infected by ten lentivirus particles.

Real-time RT-PCR

Total RNA was extracted and purified from the TS differentiation group cultured for 0, 2, 4, and 6 days. A corresponding TS control group was maintained according to the manufacturer's instructions of TRIzol® reagent (Invitrogen, Carlsbad, CA). The RNA concentration was examined by a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE). RNA was reverse transcribed to cDNA using a PrimeScript™ RT reagent kit (Takara, Dalian, China) with 1 µg of total RNA per sample.

The relative transcript levels were quantified on a Real-time PCR System (ABI PRISM 7500 Real-time PCR System, Applied Biosystems, Foster City, CA) using a SYBR Premix Ex Taq kit (Takara, Dalian, China). Applied PCR was carried out according to the manufacturer's instructions of the SYBR Premix Ex Taq kit. *Plac1* transcription levels were analyzed using the ΔC_t method and normalized to the expression of *Gapdh* (NM_001289726) [15]. The primer pairs used for mouse *Gapdh* were 5'-GGTAAGGTCGGTGTGAACA-3' (forward) and 5'-CTCGCTCCTGGAAGATGGTG-3' (reverse). The primer pair for *Plac1* (NM_019538) was 5'-TGCTCCACACGGAGAGAACA-3' (forward) and 5'-TGATTGGGAGGGCAACCAAG-3' (reverse). The marker genes used to indicate TS cell differentiation included *Tpbpa*, *Pl-1*, *Esrrb*, and *Eomes*, and the primers used for these genes were as described previously [16].

Statistical analysis

Data were illustrated as the means $\pm$ SEM. Data were calculated by analysis of variance using Statistical Package for Social Science software (SPSS for Windows package release 10.0, SPSS, Inc.) followed by the *t* test. Statistical significance was accepted as $P < 0.05$. The experimental results involved in statistical calculations were performed in triplicate.

Results

The *Tpbpa* antisense probe was applied for ISH with serial frozen sections to identify the EPC of 8.5- and 9.5-dpc mouse placenta (Fig. 1, first row, left), or the spongiotrophoblast layer of a being formed placenta which observed at 10.5, 11.5 and 13.5 dpc (Fig. 1, first row, right). As illustrated by the performance of the *Plac1* antisense probe, *Plac1* mRNA levels were abundant at the EPC of 8.5- and 9.5-dpc placentas (Fig. 1, second row, left). The complete spongiotrophoblast and labyrinth layers could be observed begin from 10.5 dpc, and the *Plac1* expression levels remained high at the spongiotrophoblast layer but moderate at the labyrinth layer (Fig. 1, second row, middle). From 13.5 dpc, *Plac1* expression in both these cell layers obviously decreased (Fig. 1, second row, right). In fact, *Plac1* expression was not completely specific to the placenta. *Plac1* expression in the fetus was also very high from 8.5 to 10.5 dpc, and decreased at 11.5 dpc and thereafter. *Plac1* expression level in the maternal decidua was weak (Fig. 1, second row). The sense probe incubated with serial frozen sections of mouse placenta at different developmental stages was employed as the negative control of ISH (Fig. 1, third row).

The fetomaternal interface consists of multiple components of cell subtypes and structures. At the fetomaternal interface, *Plac1* exhibited trophoblast-specific expression,

Fig. 1. An overview of *Plac1* localization at the mouse fetomaternal interface at different stages of pregnancy. Frozen sections of placenta obtained at five different stages of placentation (8.5, 9.5, 10.5, 11.5, and 13.5 dpc) were employed. The *Tpbpa* antisense probe was a marker to label the EPC (8.5 and 9.5 dpc) and the Spo layer of formed placenta (from 10.5 dpc). *Plac1* mRNA was observed in the EPC and Emb in the 8.5- and 9.5-dpc specimens, or the Spo, Lab, and UC and Emb in the 10.5-dpc specimens. From 11.5 dpc, a positive signal could be observed at the maternal side, which appeared to be infiltrated by invading trophoblast cells. *Plac1* expression level at the Emb side was obviously declined and restricted to some specific areas starting from 11.5 dpc. Furthermore, *Plac1* expression levels were obviously decreased in Lab but remained high in Spo, the artery, and the Dec. EPC, ectoplacental cone. Dec, decidua. Emb, embryo. UC, umbilical cord. Spo, spongiosotrophoblast, Lab, labyrinth. n = 3. Bar = 200 μ m.

and was exclusively detected in the EPC of 8.5-dpc placentas, and it was expressed in both the spongiosotrophoblast and labyrinth layers from 10.5 dpc (Fig. 2, line 1). However, *Plac1* expression in both these layers began to decline from 13.5 dpc. When focusing on the maternal side, no obvious positive signals for the antisense probe was observed at any of the pregnancy stages studied (Fig. 2, line 2). A positive signal was observed at the decidual cells adjacent to the spongiosotrophoblast layer at 11.5 dpc, which may have been produced by glycogen trophoblast cells or by giant cells that remodel the spiral artery (Fig. 2, 11.5 and 13.5 dpc, line 2).

Trophoblast giant cells and glycogen trophoblast cells are the invasive trophoblast subpopulations at the mouse fetomaternal interface. All glycogen trophoblast cells and some trophoblast giant cells were positive for the *Plac1* antisense probe. There are two major types of trophoblast giant cells: primary trophoblast giant cells, which arise at 6.5 dpc and secondary trophoblast giant cells, which emerge at 8.5 dpc. As illustrated in Fig. 2 (line 3), *Plac1* expression was absent in primary giant cells (black star) but strongly present in secondary giant cells (blue star).

Although *Plac1* was termed a placenta-specific gene, we observed a strong positive signal of *Plac1* at the chorioallantois (Fig. 2, line 4) and at the umbilical cord (Fig. 1). Furthermore, *Plac1* expression levels in the chorioallantois and umbilical cord increased from 8.5 dpc, peaked at 10.5 dpc in the chorioallantois and at 11.5 dpc in the umbilical cord, and decreased at 13.5 dpc. Similar expression patterns for *Plac1* were also observed in the fetus, with the peak expression in the fetus ranging from 8.5–10.5 dpc, with an obvious decline from 11.5 dpc observed together with restricted localization (Fig. 1, and line 5 of Fig. 2).

Fig. 2. The exact spatiotemporal expression pattern of *Plac1* at the mouse fetomaternal interface. The signal produced by the *Plac1* antisense probe incubation was mainly observed at the SpA and Lab as well as at the trophoblast component of the fetomaternal junction zone (first panel). *Plac1* expression was the highest at the Spo of 10.5-dpc and 11.5-dpc specimens, respectively. The *Plac1* mRNA level obviously declined at Spo and Lab from 13.5 dpc onwards. The decidua and SpA located in the decidua were both negative for the *Plac1* antisense probe (at 8.5 and 10.5 dpc, included in the second panel). However, *Plac1* mRNA was observed in the decidua after 10.5 dpc because of the invasion of trophoblast giant cells (in the SpA wall) and glycogen trophoblast cells adjacent to the Spo (11.5, 13.5, and 10.5 dpc sections included in the second panel). *Plac1* expression exclusively detected in trophoblast components, except primary giant cells (black star indicates primary giant cells, and blue star indicates secondary giant cells). A strong positive signal for *Plac1* mRNA was also observed in the chorioallantois (third panel). Strong *Plac1* expression was detected in the embryo at an early stage of pregnancy (8.5- and 10.5-dpc sections; fifth panel), and the expression of *Plac1* obviously declined from 11.5 dpc. Meanwhile *Plac1* expression showed an obvious reduction and was restricted to specific areas of the embryo (fifth panel). EPC, ectoplacental cone. Dec, decidua. Emb, embryo. UC, umbilical cord. Spo, spongiotrophoblast. Gly, Glycogen trophoblast. Lab, labyrinth. Ut, uterus. Ch-Al, chorioallantois. SpA, spiral artery. n = 3. Bar = 100 μ m.

An *in vitro* TS cells differentiation model was employed to indicate the possible role of *Plac1* in trophoblast differentiation. The differentiation status of TS cells and control cells were indicated by transcriptional changes in the following marker genes: *Tbp*, *Pl-1*, *Esrrb*, and *Eomes* (Fig. 3a–d). *Plac1* transcription was absent in TS cells, but it was dramatically elevated along with the TS differentiation process (Fig. 3e). After 6 days of differentiation, the *Plac1* level showed an increase of more than 500-fold ($P < 0.01$; Fig. 3e).

Here, an *in vitro* TS cell differentiation model with lentiviral *Plac1* knockdown was employed to discriminate the exact role of *Plac1* in TS differentiation. The changes in the transcriptional levels of marker genes relative to the control were used to determine the differentiation level of TS. The corresponding changes in gene expression were determined by real-time RT-PCR. Similar to the results in Fig. 3, *Eomes* and *Esrrb* expression levels significantly decreased in the negative control lentivirus treatment with progression of TS differentiation (Fig. 4a and b). In contrast, although *Eomes* and *Esrrb* expression in the *Plac1* knockdown group also significantly decreased after six days of differentiation, *Eomes* and *Esrrb* expression levels declined to a lesser extent relative to the control. The *Eomes* and *Esrrb* mRNA levels decreased by 100 $\times$ and 3 $\times$ the corresponding levels in the negative

Fig. 3. Quantitative RT-PCR analysis of changes in *Plac1* expression during the differentiation process of TS cells in vitro. TS differentiation was indicated by the downregulation of *Eomes* (a) and *Esrrb* (b) and the upregulation of *Pl-I* (c) and *Tpbpa* (d). *Plac1* mRNA level increased in a time-dependent manner and showed an increase of more than 500-fold after TS cells underwent differentiation for 6 days. TS, trophoblast stem cells. n = 3. *, p<0.05. **, p<0.01.

control respectively (Fig. 4c and d). The silencing efficiency for *Plac1* shRNA lentivirus was approximately 80% (Fig. 4e).

Discussion

Plac1 first attracted the attention of researchers due to a chromosomal deletion of 200–700 kb around the mouse *Hprt* locus, which is the location of the *Plac1* gene, resulted in fetal growth retardation and early death [17]. Recently, *Plac1* has been found to be highly expressed in the main trophoblast lineage [18]. *Plac1* mutation resulted in mouse placental defects, presented as expansion and excessive invasion of the labyrinth by the spongiotrophoblast layer [11]. Meanwhile, knockdown of *Plac1* has been found to be associated with a lethal form of hydrocephalus [11, 19]. The corresponding orthologous gene in humans, *PLAC1*, is expressed in a wide range of carcinoma and cancer cell lines and facilitates the invasion process [20]. Furthermore, several studies have reported the association between abnormal *PLAC1* expression and adverse pregnancy outcomes [21–24]. This is the first study to identify the spatial and temporal expression pattern of *Plac1* at the mouse fetomaternal interface.

As illustrated in our study, *Plac1* is highly expressed in the EPC of 8.5 dpc mice. Another study focusing on human trophoblasts have shown that *PLAC1* is very important for the invasion of extravillous trophoblasts [12] and syncytialization [25]. *Plac1* is abundantly expressed in mice, and is widely distributed in different trophoblast subpopulations. There

Fig. 4. *Plac1* knockdown significantly retarded the progression of TS cell differentiation. As indicated by the downregulation of the *Eomes* (a) and *Esrrb* (b) transcript levels and the upregulation of the *Pl-I* (c) and *Tpbpa* (d) transcript levels, knockdown of *Plac1* expression (e) by lentivirus significant impaired the TS differentiation level although it could not completely block it. TS, trophoblast stem cells. n = 3. *, p<0.05. **, p<0.01.

are three main invasive trophoblastic subpopulations at the fetomaternal interface of mice: primary trophoblast giant cells, which are observed from 6.5 dpc; secondary trophoblast giant cells, which emerge at 8.5 dpc; and glycogen trophoblasts, which can clearly be distinguished at 11.5 dpc. *Plac1* is strongly expressed in secondary trophoblast giant cells and glycogen trophoblast cells but not in primary trophoblast giant cells.

Although *Plac1* was initially suggested as a placenta-specific gene [18], it has been reported to be abundantly expressed in the fetus [11, 19], coinciding with the observation that *Plac1* is not only essential for normal placentation but also for embryonic development. The results of our ISH also illustrated that the early embryo (8.5–10.5 dpc) had the highest and most widespread distribution of *Plac1* expression. Then, the *Plac1* expression level decreased and its distribution became restricted. *Plac1* knockout in the placenta was found to be attributed to the abnormal structure of spongiotrophoblasts, and knockout of *Plac1* in the embryo has been shown to induce hydrocephalus [11], which can be rescued by *Plac1* overexpression [26]. Furthermore, the possible function of *Plac1* in the chorioallantois, umbilical cord, and uterus during pregnancy deserves our attention because its abundance and lasting expression in the chorioallantois, umbilical cord, as well as the uterine epithelium from 13.5 dpc was observed in our study.

Plac1, which is typically enriched in the placental cDNA library, is considered to play a significant role in pregnancy by participating in trophoblast differentiation. This was further reinforced by our study with the observation of significant transcription activation for *Plac1* in the TS cell differentiation process. Although the *Plac1* knockout mouse model was fertile, compromised fecundity has been previously observed [11]. Lentiviral knockdown of *Plac1*

could significantly retard the progress of TS cell differentiation. In conclusion, our research suggested that *Plac1* is very important for trophoblast differentiation and plays a unique role facilitating normal pregnancy in mice.

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Disclosure Statement

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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