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Lentiviral Vector-Mediated Complementation Restored Fetal Viability but Not Placental Hyperplasia in *Plac1*-Deficient Mice[1]

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ABSTRACT

The X-linked *Plac1* gene is maternally expressed in trophoblast cells during placentation, and its disruption causes placental hyperplasia and intrauterine growth restriction. In contrast, *Plac1* is also reported to be one of the upregulated genes in the hyperplastic placenta generated by nuclear transfer. However, the effect of overexpressed *Plac1* on placental formation and function remained unaddressed. We complemented the *Plac1* knockout placental dysfunction by lentiviral vector-mediated, placenta-specific *Plac1* transgene expression. Whereas fetal development and the morphology of maternal blood sinuses in the labyrinth zone improved, placental hyperplasia remained, with an expanded the junctional zone that migrated and encroached into the labyrinth zone. Further experiments revealed that wild-type placenta with transgenically expressed *Plac1* resulted in placental hyperplasia without the encroaching of the junctional zone. Our findings suggest that *Plac1* is involved in trophoblast cell proliferation, differentiation, and migration. Its proper expression is required for normal placentation and fetal development.

imprinting, intrauterine growth restriction, knockout, lentiviral vector, nuclear transfer

INTRODUCTION

The placenta is a fetomaternal interface that is required for nutrient supply and gas exchange during gestation. The investigation of the function of trophoblast-related genes is important to understand not only placental development but also pregnancy-related diseases. *Plac1* is an X-linked gene that is mainly expressed in the trophoblast lineage, such as trophoblast giant cells and labyrinth trophoblast cells in mice [1]. In humans, *PLAC1* expression is also restricted to trophoblast cell lineage and enriched specifically in the microvillous membrane surface of the syncytiotrophoblast cells [2, 3].

Research literature has implicated the importance of PLAC1 in normal placentation. The Xq26 region of the X-chromosome, including CR59647, GPC-3, HPRT, MGC16121, PHF6, and PLAC1, has been implicated in human placental dysplasia, and *PLAC1* is the only gene that proved to be specifically expressed in placenta [1]. Down-regulation of *Plac1* was also reported in hyperplastic placenta associated with interspecific hybrid placental dysplasia (Ihpd) between *Mus musculus* and *Mus spretus* [4–6]. To directly address *Plac1* function in vivo, Jackman et al. [7] generated *Plac1* disrupted mice and found placental hyperplasia, with the junctional zone encroaching into the labyrinth zone in the conspectuses carrying maternally transmitted mutant X-chromosome. Intrauterine growth restriction and semilethality were observed in these embryos.

By contrast, microarray studies reveal that the *Plac1* gene was consistently upregulated in the hyperplastic placenta of a mouse cloned by nuclear transfer (NT) [8]. It is noteworthy that NT-cloned placenta also had an enlarged junctional zone that encroached into labyrinth zone [9]. One possible interpretation is that *Plac1* upregulation is caused by placental hyperplasia. Another possibility is that placental hyperplasia might be caused by an overexpression of *Plac1*.

In the present study, we first analyzed *Plac1* expression in preimplantation and postimplantation embryos, then generated *Plac1* knockout (KO) mice to examine the gene's importance for embryonic development. Second, we applied the lentiviral (LV) vector-mediated, placenta-specific transgene expression system to complement *Plac1* deficiency in KO mice. Finally, to answer the questions mentioned above, we transgenically expressed *Plac1* in wild-type (WT) placenta and compared the phenotypes with KO and LV-complemented KO placentas. Our results suggest that the proper expression of *Plac1* is required for the normal placentation and fetal development.

MATERIALS AND METHODS

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University. The *Plac1*-deficient mouse line (B6D2F1-Plac1$^{Tm1[KOMP]7Osb}$) and X-linked EGFP mouse line (B6;B6C3-Tg[CAG/Acr-EGFP]CX-FM139Osb) were available to the scientific community through RIKEN BRC (http://www.brc.riken.jp/inf/en/index.shtml).

Reverse Transcription-Polymerase Chain Reaction

Mouse cDNA was prepared from blastocyst (Embryonic Day 3.5 [E3.5]) and early gestational- to midgestational-stage embryos (E6.5∼E7.5). The RT-PCR was performed using 10 ng of cDNA and the following primers: 5′-ATGAACCTTCGCAAGTTCCTGGG-3′ and 5′-TTACATGCTCTTTTGATTGTAGACATAAGGTGG-3′ for *Plac1*, 5′-AAGTGTGACGTTGACATCCG-3′ and 5′-GATCCACATCTGCTGGAAGG-3′ for beta actin (*b-actin*), and 5′-ATCCTCTGCATGGTCAGGTC-3′ and 5′-CGTGGCCTGATTCATTCC-3′ for *lacZ*. [10]. The amplification cycles were 94°C for 30 sec, 65°C for 30 sec, and 72°C for 30 sec, with a final 2 min of extension at 72°C.

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Quantitative RT-PCR

Mouse cDNA was prepared from blastocyst (E3.5) and placenta (E16.5). Each cDNA was subjected to quantitative RT-PCR analysis with a Thermal Cycler Dice TP800 (Takara-Bio) using SYBR Premix Ex Taq II RR820S (Takara-Bio). The relative expression level of each target gene mRNA was normalized to the amount of *b-actin* control. Each reaction was performed in duplicate, and data are presented as the mean plus SD. Primer sets were as follows: 5′-GCGTTACCGAATGTGGCATC-3′ and 5′-CAGCTG CAGTTGGGTTGTTC-3′ for *Plac1*, and 5′-AAGTGTGACGTTGA CATCCG-3′ and 5′-GATCCACATCTGCTGGAAGG-3′ for *b-actin*.

Generation of Plac1 Gene-Deficient Mice

Plac1 gene-targeting vector (DPGS00170_A_E06) was purchased from the International Knockout Mouse Consortium (IKMC). After linearization with *Asi*SI digestion, the targeting vector was electroporated into EGR-G101 embryonic stem (ES) cells [11]. After positive (G418) and negative (DT) selection, among the 48 drug-resistant clones 14 clones were identified as homologous recombinant clones by PCR screening with primers 5′-CACAACGGGTTCTTCTGTTAGTCC-3′ and 5′-GTCTGGCC TGCTGTGGAGTTGAGAGTCTTG-3′ for the 5′ end, and 5′-ATCCGGGGG TACCGCGTCGAG-3′ and 5′-CAGACAGGACAGAAGCGGTTCTGCA GAG-3′ for the 3′ end. The gene-targeted ES cells were injected into eight-cell-stage ICR mouse embryos to generate chimeric mice. Germ line transmission of mutant *Plac1* allele (X^{-}) was confirmed by PCR with primers Pr1, 5′-GTTCACTCAGATGATCTGAGGAAACCC-3′; Pr2, 5′-ACCCAGG CACCAATGCTAGC-3′; and Pr3, 5′-ATCCGGGGGTACCGCGTCGAG-3′. These mice were mated with B6D2F1 mice and used for further experiments. Maternally and paternally transmitted mutant alleles are referred to as X^{m-} and X^{p-}, respectively.

Blastocyst Collection and Sex Determination

Female mice were superovulated by i.p. injection of a pregnant mare's serum gonadotropin (5 units) followed by human chorionic gonadotropin (5 units) 48 h later, and then they were mated with male mice. Two-cell-stage embryos were collected from the females at 1.5 days after copulation and then incubated in KSOM medium for 2 days to obtain blastocysts. For gender selection, the blastocysts collected from WT female mice mated with $X^{egfp}Y$ (B6;B6C3-Tg[CAG/Acr-EGFP]CX-FM139Osb) male mice were separated into male (EGFP-negative) and female (EGFP-positive) blastocysts under a fluorescence stereomicroscope [12]. The RNA collected from these gender-specific blastocysts was pooled and subjected to RT-PCR for *Plac1* expression analysis. For genotyping experiments, each blastocyst was placed in 20 μl of lysis buffer (20 mM Tris-HCl [pH 8.0], 5 mM ethylene diamine tetraacetic acid, 400 mM NaCl, SDS 0.3%, and 200 μg/ml Proteinase K), and 0.5 μl was subjected to PCR analysis. *Plac1* genotype was performed with the above-mentioned primers, and *Ube1X* primers 5′-TGGTCTGGACC CAAACGCTGTCCACA-3′ and 5′-GGCAGCAGCCATCACATAATCCA GATG-3′ were used for sexing [13].

Tissue Preparation and Histological Analysis

At E3.5 through E16.5, the pregnant mice were killed, and their uteri were dissected to collect embryonic or extraembryonic tissues. For embryos at E6.5 through E9.5, the uteri were removed and conceptuses were used for RT-PCR. For embryos at E12.5 through E16.5, the uteri were removed and individual fetuses were dissected from the uterus with embryonic membranes intact. Placentas were fixed in 4% paraformaldehyde in PBS and were processed for paraffin embedding or frozen section. The 5-μm paraffin sections were stained with periodic acid-Schiff (PAS) and then counterstained with Mayer hematoxylin solution (Wako). The 10-μm frozen sections were stained with X-Gal to detect β-galactosidase (β-gal) activity (LacZ Tissue Staining Kit; InvivoGen), then counterstained with 0.3% eosin for 2 min, rinsed in 70% ethanol, dehydrated in increasing ethanol concentrations, and finally mounted in Entellan new (Merck).

Immunohistochemical Analysis

The sliced sections were incubated with 1% bovine serum albumin in PBS for 1 h to block nonspecific reactions. These were then incubated overnight with the following primary antibodies: anti-Laminin antibody (no. L9393; Sigma-Aldrich) to stain the endothelial basement membrane of fetal capillaries [14], and anti-Ki67 antibody (no. 12202; Cell Signaling Technology) to stain the proliferating cells. After incubation with secondary antibodies conjugated with horseradish peroxidase, we used diaminobenzidine tetrahydrochloride (DAB; Nacalai Tesque) to stain the sliced sections with a hematoxylin counterstain.

Morphometric Quantification of Placenta

The centerlines of each of the maternal blood sinuses in the labyrinth zone of the placenta were manually selected, and the widths were measured. We analyzed 20 sinuses in 3 areas for 3 placentas per group. Each placental layer was manually selected, and the areas were measured. We analyzed three placentas per group. The images were analyzed with ImageJ software (National Institutes of Health).

Preparation of Lentiviral Vectors

The HIV-1-based, self-inactivating lentiviral vector plasmid pLV-*EGFP* was described previously [15]. Mouse *Plac1* cDNA was amplified by RT-PCR using placental tissue with primers 5′-ATCTAGAGCCGCCAT GAACCTTCGCAAGTTCCTGGG-3′ and 5′-AGAATTCTTA CATGCTCTTTTGATTGTAGACATAAGGTGG-3′. A 0.5-kb *Xba*I-*Eco*RI *Plac1* fragment was inserted into lentiviral vector plasmid under the *CAG* promoter (pLV-*Plac1*). The pLV-*Plac1* plasmid was transfected to 293T cells by the calcium phosphate method. Lentiviral vectors were harvested 2 days after transfection, and then they were concentrated 1000 times by ultracentrifugation (19 400 rpm, 2 h; 21 000 rpm, 2 h). After resuspension with Hanks Balanced Salt Solution buffer, the lentiviral vector concentration was determined by measuring p24 gag antigens by ELISA [15].

Lentiviral Transduction for Mouse Blastocysts

Blastocysts were collected as described above. To remove the zona pellucida, the blastocysts were incubated in acidic Tyrode solution (Sigma-Aldrich) for 30–60 sec and washed three times in KSOM. Prepared zona pellucida-free blastocysts were incubated individually for 4 h in medium containing lentiviral vectors. LV-*EGFP* and LV-*Plac1* were used at 1000 ng of p24 per milliliter. The transduced blastocysts were implanted into the uteri of pseudopregnant E2.5 ICR female mice. We transplanted 10 blastocysts into each horn of the uterus.

Statistical Analysis

All values are shown as the mean ± SD of at least three independent experiments. Statistical analyses were performed using Student *t*-test inserted in Excel after the data were tested for normality of distribution.

RESULTS

Allele-Specific Expression of Plac1 Gene

Whereas maternally imprinted *Plac1* expresses after implantation and *Plac1* KO mice tend to succumb somewhere between late gestation and weaning [7], *Plac1* expression and its physiological role have not been examined in preimplantation embryos. Thus, we examined *Plac1* expression in blastocyst-stage embryos. When we mated WT female mice with transgenic male mice carrying the *EGFP*-expressing transgene on the X chromosome (X^{egfp}) [12], blastocysts could be separated into fluorescent female blastocysts (XX^{egfp}) and nonfluorescent male blastocysts (XY) under a fluorescent stereomicroscope. By RT-PCR, we found that *Plac1* expression in female blastocysts was higher than in the male blastocysts, indicating paternally imprinted gene expression (Fig. 1, A and B). Soon after implantation, maternal *Plac1* started expression, and both sexes of embryos expressed equivalent amounts of *Plac1*, implicating silencing of paternal *Plac1* expression (Fig. 1, A and B).

Generation of Plac1 Mutant Mice

To characterize *Plac1* gene expression and its function, we generated *Plac1* mutant mice with the gene-targeting

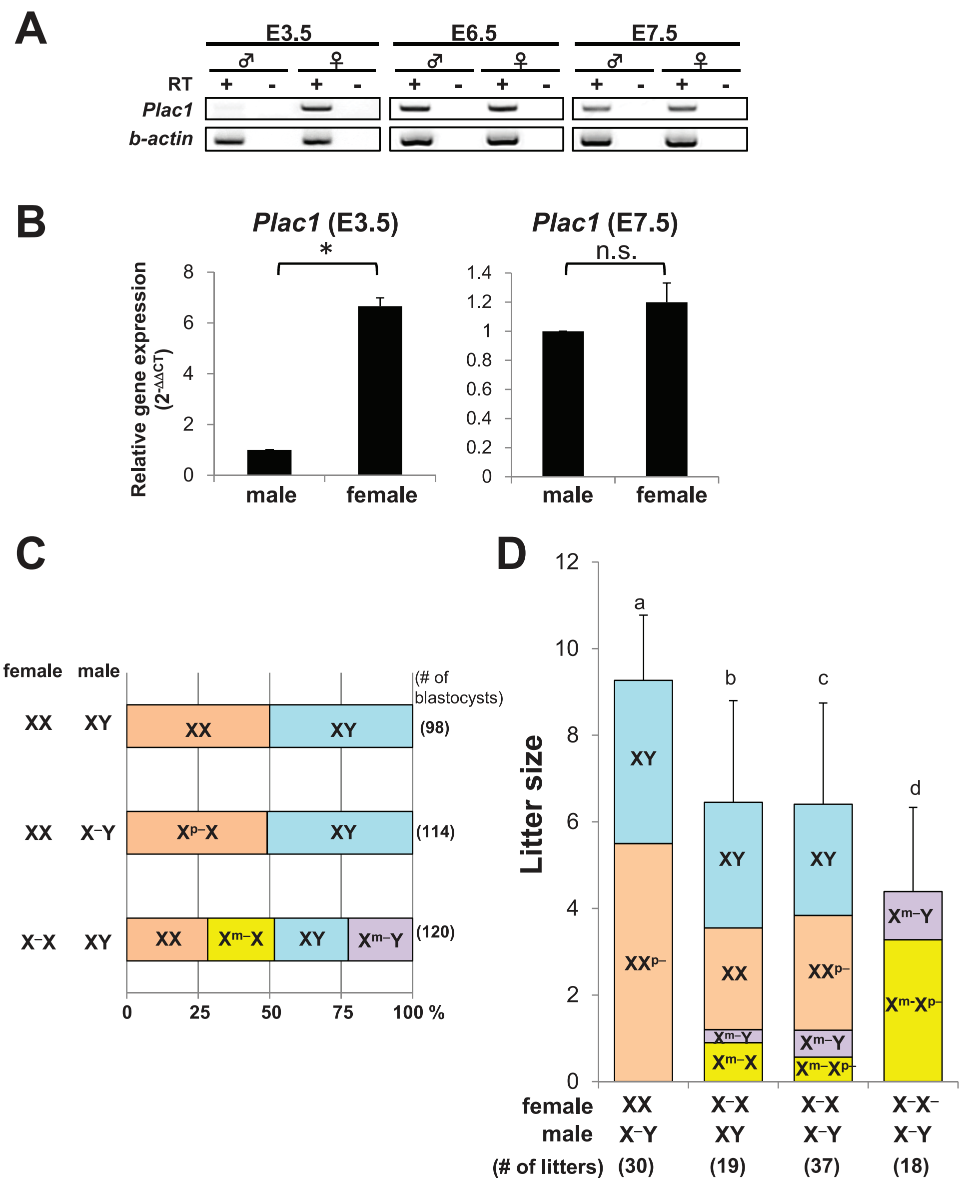

FIG. 1. The developmental viability of *Plac1* mutant embryos. **A**) RT-PCR analysis of *Plac1* using preimplantation and postimplantation embryos. *Plac1* mRNA was dominantly detected in female blastocyst (E3.5). After implantation, *Plac1* expression was observed in both sexes (E6.5 and E7.5). **B**) The expression level of *Plac1* was compared between female and male embryos (E3.5 and E7.5) by quantitative RT-PCR to compare. The *Plac1* mRNA level in female blastocysts was approximately seven times greater than in male blastocysts (E3.5; $^*P < 0.05$). Comparable expression levels of *Plac1* mRNA were found in E7.5 female and male embryos. n.s., no significant difference. **C**) The proportion of mutant embryos at blastocyst stage. The blastocysts were genotyped and sexed by PCR. The mutant embryos were obtained at the expected Mendelian ratio. **D**) Fecundity of *Plac1* mutant mice. *Plac1* mutant females (X^-X or X^-X^-) were mated with XY or X^-Y males, and newborn pups were genotyped by PCR (a vs. b, c, d $P < 0.05$).

vector designed by IKMC (Supplemental Fig. S1A; Supplemental Data are available online at www.biolreprod.org). The ORF region was replaced with a *lacZ* reporter transgene to visualize *Plac1* gene expression and disrupt *Plac1* gene function simultaneously. We confirmed the homologous recombination in ES cells and germ line transmission by PCR analysis (Supplemental Fig. S1B). Whereas *LacZ* expression in the $X^{p-}X$ blastocyst was observed, *Plac1* expression in the $X^{p-}X$ blastocyst was lower than in the control XX blastocyst (Supplemental Fig. S1C). When we stained the placenta with X-gal at E11.5, *LacZ*-positive cells appeared only in the placenta carrying the maternally transmitted mutant allele. Positive cells were mainly distributed in the junctional zone, and some were in the labyrinth zone (Supplemental Fig. S1D). At E14.5, *LacZ*-positive signals were strongly detected in the encroached junctional zone (Supplemental Fig. S1E). The expression of *Plac1* from the maternally inherited allele during placentation was consistent with previous reports [7].

Plac1 *Deficiency and Preimplantation Development*

To determine whether paternally expressed *Plac1* plays an important role during preimplantation development, we collected and genotyped blastocysts from WT XX females mated with *Plac1* mutant X^-Y males, and *Plac1* mutant X^-X females mated with WT XY males. In either combination, the expected number of blastocysts carrying *Plac1* mutant allele

was obtained (Fig. 1C). There were no morphological or developmental abnormalities observed in the mutant blastocysts (data not shown). These results indicate that the *Plac1* gene is not required for preimplantation embryonic development.

Plac1 *Deficiency and Postimplantation Development*

To evaluate the effect of *Plac1* deficiency in postimplantation development, *Plac1* mutant female mice were mated with mutant male mice as shown in Figure 1D. Whereas the intercrossing with *Plac1* XX female and $X^{-}Y$ male mice showed normal litter size (number of pups: $X^{p-}X$, 5.5 ± 1.7; XY, 3.8 ± 1.3; litter size, 9.3 ± 1.5; n = 30), *Plac1* mutant females ($X^{-}X$) showed a decreased litter size, with reduced numbers of the maternally mutated embryos ($X^{-}X \times XY$ mating: number of pups: XX, 2.4 ± 1.3; XY, 2.9 ± 1.4; $X^{m-}X$, 0.9 ± 1.2; $X^{m-}Y$, 0.3 ± 0.6; litter size, 6.5 ± 2.4; n = 20; $X^{-}X \times X^{-}Y$ mating: number of pups: XY, 2.6 ± 1.4; $X^{p-}X$, 2.6 ± 1.7; $X^{m-}Y$, 0.6 ± 1.0; $X^{m-}X^{p-}$, 0.6 ± 0.7; litter size, 6.4 ± 2.3; n = 37; Fig. 1D). We analyzed the proportion of embryos produced by the cross between $X^{-}X$ females and XY males in midgestational stages (E11.5~E14.5; n = 15) through late-gestational stages (E15.5~E17.5; n = 6). Although the number of WT embryos was comparable (midgestation, 3.9 ± 1.0; late gestation, 3.7 ± 2.3; $P = 0.7813$), X^{m-} mutant embryos tended to decrease during late-gestational stages (midgestation: X^{m-} mutant, 4.7 ± 1.8; late-gestation: X^{m-}mutant, 3.0 ± 1.3; $P = 0.0494$). These results reconfirmed that maternally expressed PLAC1 protein plays an important role in postimplantation embryonic development [7]. Notably, the intercrossing with *Plac1* $X^{-}X^{-}$ and $X^{-}Y$ mice gave 3.7 times more maternally mutated pups (number of pups: $X^{m-}Y$, 1.1 ± 0.7; $X^{m-}X^{p-}$, 3.3 ± 1.5; litter size, 4.4 ± 1.9; n = 18) than heterozygous intercrosses (maternally mutated pups, 1.2 ± 0.96 in $X^{-}X \times XY$ and 1.2 ± 0.86 in $X^{-}X \times X^{-}Y$ mating; Fig. 1D).

Placental Hyperplasia and Intrauterine Growth Restriction in Plac1 *Mutant Mice*

To understand when the defects appear during gestation, we weighed fetuses and placentas obtained from the intercrossing of *Plac1* $X^{-}X$ female with XY male mice at the indicated embryonic days (Supplemental Fig. S2, A and B). Placentas carrying the X^{m-} mutation showed weights comparable with those of WT placentas (WT, 70 ± 23 mg; n = 14; X^{m-}, 83 ± 37 mg; n = 12) at E12.5, then significantly increased their weight during midgestation (E14.5: WT, 104 ± 18 mg; n = 16; X^{m-}, 190 ± 33 mg; n = 20; E16.5: WT, 86 ± 21 mg; n = 16; X^{m-}, 203 ± 55 mg; n = 12; Supplemental Fig. S2A). Whereas X^{m-} mutated fetal weight was comparable with that of the WT fetus during E12.5 through E14.5, X^{m-} mutated fetal weight was lower than that of the WT fetus at E16.5 (E12.5: WT, 83 ± 12 mg; n = 14; X^{m-}, 70 ± 23 mg; n = 12; E14.5: WT, 267 ± 42 mg; n = 16; X^{m-}, 246 ± 38 mg; n = 20; E16.5: WT, 770 ± 52 mg; n = 16; X^{m-}, 670 ± 119 mg; n = 12; Supplemental Fig. S2B).

Histological Analysis of Plac1 *Mutant Placentas*

We then performed the histological analysis of *Plac1* mutant placentas by PAS staining, which detects polysaccharides, such as glycogen and mucosubstances, such as glycoproteins, glycolipids, and mucins. Whereas no apparent abnormality was found in maternally *Plac1* mutated (X^{m-}) placentas at E12.5 (Supplemental Fig. S2C), mutant placentas showed overgrowth with an expanded junctional zone that encroached into the labyrinth zone at E13.5 (Fig. 2A). No overt developmental abnormalities were found in paternally *Plac1* mutated (X^{p-}) placentas (Fig. 2A). Whereas the areas of the decidua and labyrinth zone showed no difference among WT, X^{p-}, and X^{m-} placentas, the area of the junctional zone significantly increased in the X^{m-} placenta (Fig. 2B). When we stained placentas with anti-Ki67 (cell proliferation marker) antibody, more signals were observed in the junctional zone of the X^{m-} placenta (Supplemental Fig. S3). It is noteworthy that morphological abnormalities appeared in the X^{m-} mutant placental labyrinth zone by laminin immunostaining (Fig. 2C). Although the fetal blood vessel formation of X^{m-} mutant placenta was observed, adjacent maternal blood sinuses were dilated (Fig. 2, C and D).

Transgenic Complementation of Plac1 *Deficiency Using Lentiviral Vectors*

To confirm that PLAC1 deficiency is the principal cause of the phenotype, we restored *Plac1* expression by using lentiviral (LV) vector-mediated, placenta-specific gene expression system [15]. Blastocysts collected from *Plac1* $X^{-}X$ females mated with WT male mice were transduced with the LV vectors expressing PLAC1 (LV-*Plac1*) and transplanted into pseudopregnant mice (Fig. 3A). Placenta-specific genomic integration of the LV-*Plac1* was confirmed by PCR (Fig. 3B). When we compared the amount of *Plac1* mRNA by quantitative RT-PCR, LV-*Plac1*-transduced X^{m-} mutant placentas showed about five times more *Plac1* mRNA than LV-*EGFP*-transduced WT placenta (Supplemental Fig. S4). When we transduced the maternally *Plac1*-mutated blastocysts ($X^{m-}X$ and $X^{m-}Y$) with LV-*Plac1*, the proportion was improved compared with that of the control LV-*EGFP* (X^{m-} mutant pups ratio was 40.4% with LV-*Plac1* compared with 22.9% with control LV-*EGFP*; Fig. 3C). The fetal body weight of LV-*Plac1*-transduced mutant placentas was also improved (LV-*EGFP*-treated X^{m-} mutant: 649 ± 128 mg; n = 19; LV-*Plac1*-treated X^{m-} mutant: 740 ± 88 mg; n = 21; Fig. 3D, right). Unexpectedly, LV-*Plac1*-transduced mutant placentas remained hyperplastic (LV-*EGFP*-treated X^{m-} mutant: 202 ± 55 mg; n = 12; LV-*Plac1*-treated X^{m-} mutant: 276 ± 74 mg; n = 15; Fig. 3D, left). Histological analysis revealed that dilation of maternal blood sinuses in the labyrinth zone was improved, although the hyperplastic junctional zone remained (Fig. 3, E and F).

*LV-*Plac1 *Transduction in WT Placentas*

Whereas *Plac1* deficiency caused placental hyperplasia, overexpression of *Plac1* was also reported in hyperplastic placentas from the NT-cloned mouse [8]. Therefore, we transduced LV-*Plac1* in WT placentas to examine its function. There was no difference in fetal development ability when we observed at E16.5 (live fetuses:transplanted blastocysts were 15:40 [37%] and 14:40 [35%] with LV-*EGFP* and LV-*Plac1*, respectively). Placental weight significantly increased in the LV-*Plac1* treated group (LV-*EGFP*-treated WT placenta: 85.6 ± 21 mg; n = 16; LV-*Plac1*-treated WT placenta: 129 ± 27.5 mg; n = 24; Fig. 4A). Although the fetal weight of the LV-*Plac1*-treated group tended to be smaller than that of the LV-*EGFP*-treated controls (LV-*EGFP*-treated WT fetus: 770 ± 51.9 mg; n = 16; LV-*Plac1*-treated WT fetus: 732 ± 65 mg; n = 24; Fig. 4B), it was not significant ($P = 0.055$). Histological analysis revealed that the LV-*Plac1*-transduced labyrinth zone did not show the dilation

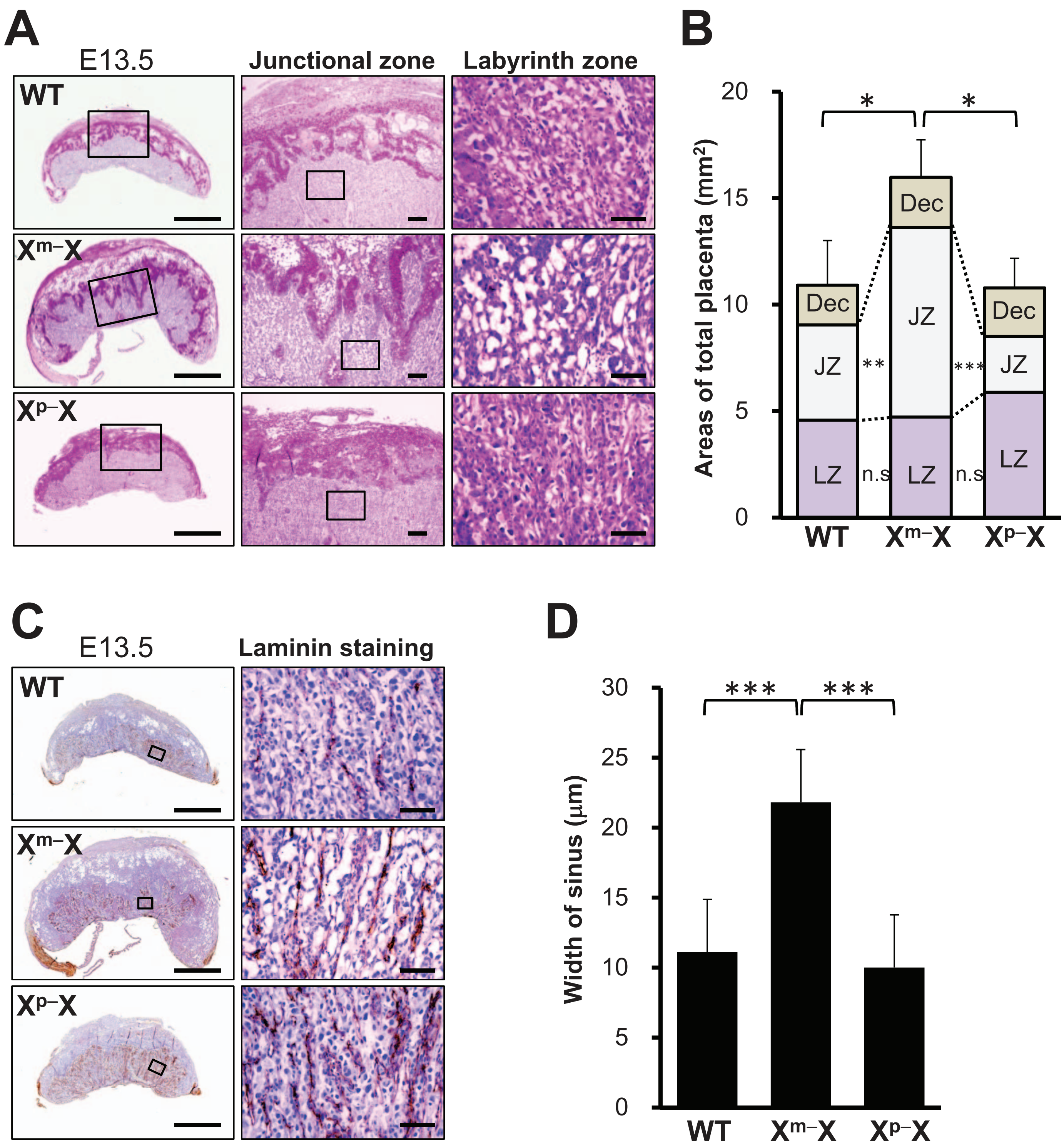

FIG. 2. Histological analysis of hyperplastic placenta in *Plac1* X^{m-} mice. **A**) PAS staining of placenta. At E13.5, *Plac1* X^{m-} placenta showed hyperplasia, expanded the junctional zone, and showed morphological abnormalities in the labyrinth zone. *Plac1* X^{p-} placenta was normally formed. The enlargements of the leftmost images are shown in the middle images, and the enlargements of the middle images are shown in the rightmost images. Bars = 1 mm (left), 200 µm (center), and 50 µm (right). **B**) Morphometric analysis of placenta. The area of the junctional zone significantly increased in X^{m-} mutant placenta at E13.5 (n = 3 per group; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; n.s., no significant difference). **C**) Laminin immunohistochemistry of placenta (DAB staining). Whereas fetal capillaries in the labyrinth zone were observed, neighboring maternal blood sinuses were dilated in *Plac1* X^{m-} placenta. Right images are magnified images of the boxes indicated in the left figures. Bars = 1 mm (left) and 50 µm (right). **D**) Width of maternal blood sinus in the labyrinth zone at E13.5 (n = 180 from 3 placenta per group; $***P < 0.001$).

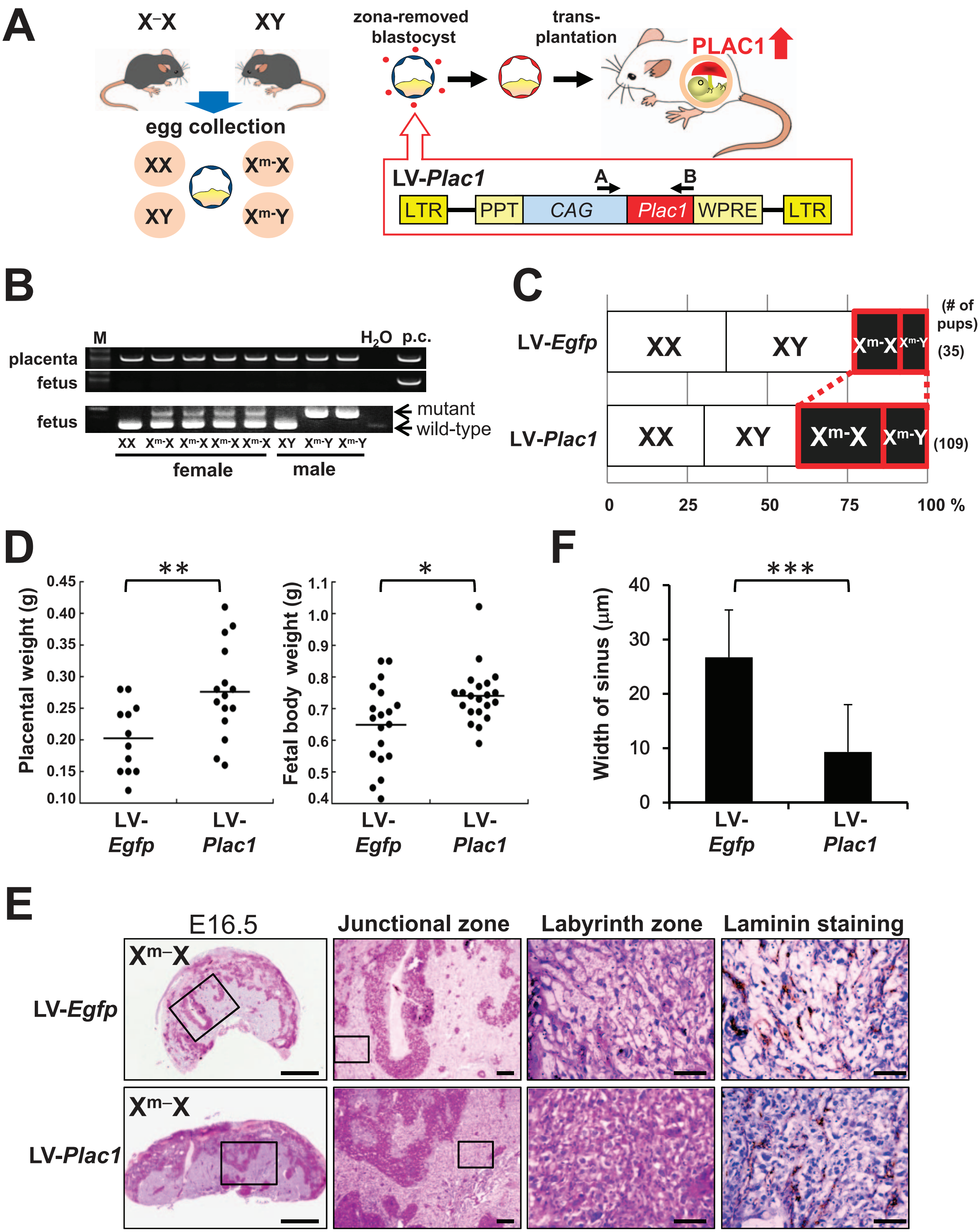

FIG. 3. Transgenic complementation of *Plac1* mutant mice by lentiviral vector-mediated, placenta-specific *Plac1* gene transduction. **A**) Scheme for LV vector-mediated transgenic complementation. *Plac1* Xm–X and Xm–Y blastocysts were collected from *Plac1* X⁻X mothers. Zona pellucida-free blastocysts were transduced with a lentiviral vector expressing *Plac1* (LV-*Plac1*) and were transplanted into pseudopregnant females. Primers A (5′-GCAACGTGCTGGTTGTTGTGCTGTCTCATC-3′) and B (5′-TTACATGCTCTTTTGATTGTAGACATAAGGTGG-3′) were used to detect LV-*Plac1* transgene. **B**) Placenta-specific *Plac1* transgene integration. Genomic DNA was extracted from fetuses or placentas at E17.5 and subjected to PCR analysis using the

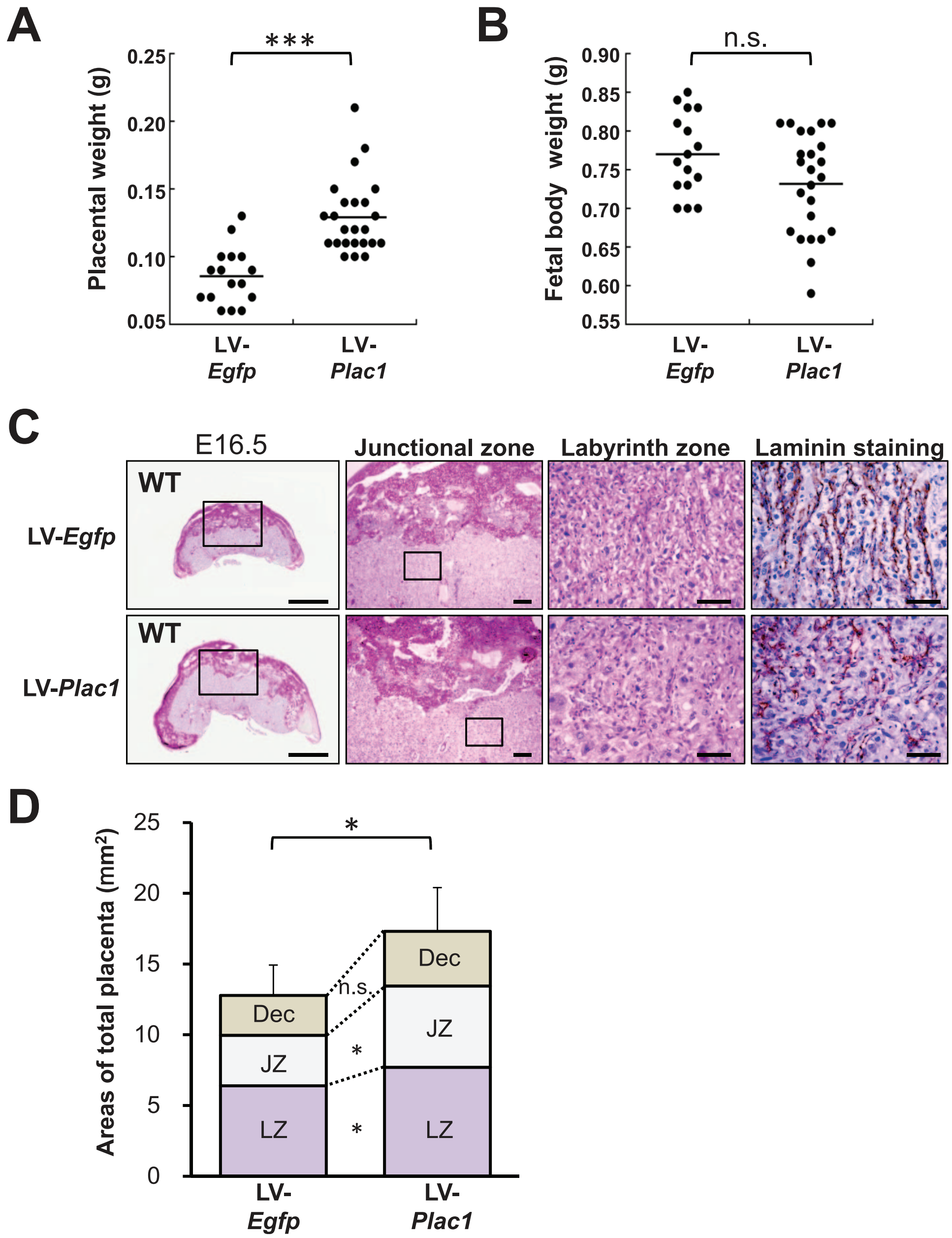

FIG. 4. LV-*Plac1* transduction of WT placenta resulted in hyperplasia. **A**) The placental weight of LV-*Plac1*-transduced WT embryos at E16.5. LV-*Plac1*-treated placentas were larger than those in the LV-*EGFP*-treated group (****P* < 0.001). **B**) The fetal weight of LV-*Plac1*-transduced WT embryos at E16.5. There was no significant difference between the two groups. **C**) Histological analysis by PAS staining and laminin immunostaining of LV-*Plac1*-transduced WT placentas at E16.5. There was no apparent abnormality in the junctional and labyrinth zones. Bars = 1 mm, 200 μm, 50 μm, and 50 μm, respectively from the left. **D**) Morphometric analysis of placenta. The area of junctional and labyrinth zones significantly increased in LV-*Plac1*-transduced WT placentas at E16.5 (n = 3 per group; **P* < 0.05). n.s., no significant difference.

◀

primers indicated in **A**. p.c. = pLV-*Plac1* plasmid. **C**) The proportion of *Plac1* mutant pups after LV-*Plac1* transduction. The pups developed from the LV-*Plac1*-transduced blastocysts were genotyped by PCR at birth. **D**) The placental and fetal body weights of *Plac1* X^{m-} mutant mice at E16.5. The fetuses developed from LV-*Plac1*-transduced mutant blastocysts were larger than those of the LV-EGFP control. The LV-*Plac1*-transduced mutant placenta remained hyperplastic (**P* < 0.05, ***P* < 0.01). We present the placental weight and fetal body weight of WT controls in Figure 4, A and B (LV-*EGFP*-treated WT placenta, 85.6 ± 21 mg; n = 16; LV-*EGFP*-treated WT fetus, 770 ± 51.9 mg; n = 16). **E**) PAS staining and laminin immunostaining (DAB staining) of LV-transduced placenta at E16.5. Hyperplastic junctional zone that encroached into the labyrinth zone was observed. The laminin immunostaining shows that fetal capillaries and maternal blood sinus were formed normally in LV-*Plac1* complemented placenta. Bars = 1 mm, 200 μm, 50 μm, and 50 μm, respectively from the left. **F**) Width of maternal blood sinus in the labyrinth zone of E16.5 LV-treated *Plac1* X^{m-} mutant placenta (n = 180 from 3 placenta per group; ****P* < 0.001). Dilation of maternal blood sinuses was restored in LV-*Plac1*-transduced X^{m-} mutant placenta.

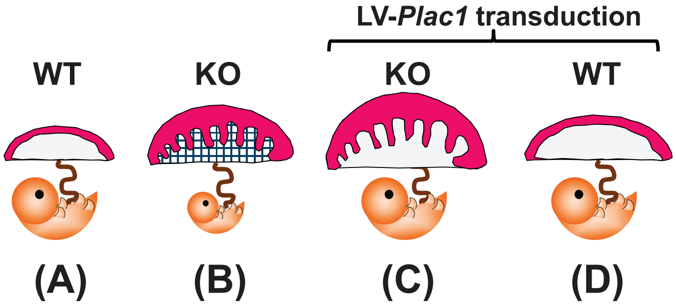

FIG. 5. Summary of placental dysplasia found in *Plac1* mutant mice. Phenotypes of *Plac1*-manipulated placentas. **A**) WT control. The junctional and labyrinth zones are indicated in red and gray, respectively. **B**) *Plac1* mutant placenta showed hyperplasia with expanded junctional zone, encroaching into the labyrinth zone. Impaired maternal blood sinus structures were observed in the labyrinth zone. **C**) LV-*Plac1*-complemented mutant placenta remained hyperplasic with an encroaching junctional zone, whereas abnormality in the labyrinth zone was improved. **D**) LV-*Plac1*-transduced WT placenta also showed hyperplasia, but the junctional and labyrinth zones were normally formed.

of maternal blood sinus that was observed in *Plac1* KO placenta (Fig. 4C). In addition, the areas of the junctional and labyrinth zones significantly increased in LV-*Plac1*-transduced placentas (Fig. 4D).

DISCUSSION

Plac1 is an X-chromosome-linked and reported to be maternally expressed gene that plays important roles in both placental and embryonic development [7]. In the present study, by separating male and female blastocysts using an X^{egfp} transgenic strain, we demonstrated that *Plac1* gene is paternally expressed before implantation and then switched to be maternally expressed after implantation. Recently, transient imprinting and aberrant allele switching have been implicated in developmental and disease-related phenotypes [16, 17]. For example, during mammalian development, many loci maintain parent-of-origin DNA methylation only briefly after fertilization [16]. Antisense noncoding RNA has also been implicated in the imprint switch mechanism in breast cancers [17]. Because Gm35988 is listed as intragenic lincRNA from the *Plac1* locus, it may play a role in both preimplantation and postimplantation gene expression. Although *Plac1* is not essential for preimplantation development, further studies might elucidate the transition mechanism of gene expression and its physiological functions.

After implantation, *Plac1* is maternally expressed, and its disruption causes impaired placentation and subsequent fatal problems in embryos [7]. In the present study, we also confirmed that maternal *Plac1* deficiency causes fatality during pregnancy. Unexpectedly, more than twice as many maternally mutated pups were obtained from the intercrossing with *Plac1* X^-X^- and X^-Y mice compared with those obtained from heterozygous intercrosses (Fig. 1D). Because all of the offspring from the $X^-X^- \times X^-Y$ cross became *Plac1* null mice, the possibility of *Plac1* reactivation can be ruled out. In general, litter size and fetal body size are usually inversely correlated. Because the litter size from the X^-X^- and the X^-Y cross was reduced, we assumed that the embryos received more nutrients from the mother. In other words, placental functions are more critical for fetal development in competitive circumstances.

Notably, there are some similarities and differences among WT placenta, *Plac1* mutant placenta, LV-*Plac1* complemented mutant placenta, and LV-*Plac1* transduced WT placenta (Fig. 5). From the histological analysis, we observed defects in *Plac1* mutant placenta: the encroaching junctional zone, and the morphological impairment of maternal blood sinuses in the labyrinth zone. Lentiviral vector-mediated, placenta-specific *Plac1* expression restored the morphology of labyrinth zone but not the placental hyperplasia with the encroaching junctional zone. With the recent report that *Plac1* is also expressed in the fetus proper [18], the contribution of fetal *Plac1* expression should be taken into account for placental size regulation and compartmentation of placental layers. This is also supported by work in which WT tetraploid complementation could not restore the placental phenotypes of NT-cloned mice [19].

Although the *Plac1* gene is upregulated in NT-cloned placentas, *Plac1*-deficient placentas showed phenotypes similar to NT-cloned placentas. We transduced LV-*Plac1* in placentas specifically in WT embryos. Unexpectedly, these placentas showed hyperplasia with normal junctional and labyrinth zone formation. The expression level of the *Plac1* transgene is approximately five times greater than that of the endogenous gene, implicating that *Plac1* overexpression caused the placental hyperplasia. Because LV-*Plac1*-treated KO and WT placentas showed comparable amounts of *Plac1* mRNA in the placenta (five times more than endogenous level), the histological difference observed in these placentas may be attributed to the difference in fetal *Plac1* expression. With the fact that NT-cloned placental defects were normalized with WT fetus [19], our results reinforce the importance of fetal *Plac1* expression for normal placentation.

In summary, we used gene knockout mice and a lentiviral vector-mediated, placenta-specific gene expression system to elucidate the *Plac1* function in vivo. Our findings demonstrate the importance of the fine tuning of *Plac1* expression for proper formation of the junctional and labyrinth zones. These findings augment our limited knowledge of how *Plac1* gene expression is developmentally regulated and how it influences placental formation and functions. In addition, our results further support prior work showing the critical importance of the fetoplacental proportion in determining total intrauterine growth.

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