Severe damage to the placental fetal capillary network causes mid- to late fetal lethality and reduction in placental size in Peg11/Rtl1 KO mice

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Paternally expressed 11/Retrotransposon-like 1 (Peg11/Rtl1) knockout (KO) mice show mid- to late fetal lethality or late fetal growth retardation associated with frequent neonatal lethality. The lethal phenotype is largely dependent on genetic background and becomes more severe with each succeeding generation in the course of backcross experiments to C57BL/6 (B6). We previously suggested that these lethal and growth phenotypes in the fetal stages were due to severe defects in placental fetal capillaries in the labyrinth layer. In this study, we re-examined KO fetuses and placentas and confirmed that the severe clogging of fetal capillaries was associated with KO fetuses showing mid-fetal lethality with internal bleeding. Importantly, the basal region of the fetal capillary network was specifically damaged, also leading to poor expansion of the labyrinth layer and placental size reduction in the later stage. An apparent down-regulation of transmembrane protein 100 (Tmem100), mesenchyme homeobox 2 (Meox2) and lymphatic vessel endothelial hyaluronan receptor 1 (Lyve1) expression were observed in earlier stage placentas even before apparent size reduction became, suggesting that these genes are involved in the maintenance of fetal capillaries associated with Peg11/Rtl1 during development.

Introduction

The placenta is a specialized organ for fetal growth during gestation in therian mammals: Eutherians have chorioallantoic placentas for long-term gestation, whereas marsupials use yolk sac placentas for a relatively short gestation period, after which altricial marsupial young continue developing in the mother’s pouch by lactation for an extended period. The mouse chorioallantoic placentas comprise three major layers, namely the labyrinth, spongiotrophoblast and trophoblast giant cell layers. The labyrinth layer is an essential part of the placenta, where a large number of fetal capillaries are bathed in maternal blood, exchanging nutrients and gases between the fetus and mother, thus constituting the so-called feto-maternal interface (Rossant & Cross 2001; Watson & Cross 2005).

In the mouse placenta, chorioallantoic attachment occurs at gestational day 8.5 (d8.5), and branching morphogenesis begins at specific sites of chorionic trophoblast cells expressing Gα1 (Anson-Cartwright et al. 2000; Stecca et al. 2002; Cross et al. 2006; Simmons et al. 2008). Then, the chorion forms villi and creates a space into which the fetal blood vessels grow from allantois. Simultaneously, the chorionic trophoblast cells differentiate into two labyrinth cell types, multinucleated syncytiotrophoblast cells surrounding the fetal capillary endothelial cells and mononuclear trophoblast cells lining the maternal blood sinuses. The fetal capillary network of the labyrinth layer is basically completed by d12.5 but becomes larger and more extensively branched until birth (d18.5–19.5) (Watson & Cross 2005), although the placental weight peaks at d16.5. Many genes

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Placental defects in Peg11/Rtl1 KO mice

Placental size during the late stage of development. Peg11/Rtl1 is associated with the mid- to late fetal lethality of the severe damage to the fetal capillaries was not only the study, we re-examined the placental abnormalities of lethality and late fetal growth retardation. In this damage to the fetal capillaries is the cause of fetal rounded trophoblast cells. Thus, we suggested that clogging of the capillaries due to overgrown sur-

endothelial cells from the basement membrane and the KO placentas, showing detachment of the cells and that these cells were severely damaged in specifically expressed in the fetal capillary endothelial cells; Syna and Slk16a1 for syncytiotrophoblast layer I (SynT-I)); Gen1, Synb, Cebpa and Slk16a3 (MCT4) for syncytiotrophoblast layer II (SynT-II)); and Esx1 and Dlx3 (both of the SyTs-1 and 2) as well as Tbpba, Phlha8 (PipG, gamma) and Prl7a2 (PipP) for the spongiotrophoblast cells.

Peg11/Rtl1 is one of the LTR retrotransponson-derived genes specific to eutherian mammals; therefore, its function in the current eutherian developmental system as well as its role in eutherian evolution are issues of interest. We previously reported that Peg11/Rtl1 KO mice showed mid- to late fetal lethality and late fetal growth retardation associated with frequent neonatal lethality upon paternal transmission of the KO allele because Peg11/ Rtl1 is a paternally expressed imprinted gene. The KO fetuses as well as placentas were apparently small at the later stage of development. Their late fetal/ neonatal lethal and late fetal growth retardation phenotypes correspond well to mice with maternal duplication of mouse chromosome 12 (mUPD12), showing that Peg11/Rtl1 is one of the major responsible genes in the imprinting region located in the distal chromosome 12. We found that Peg11/Rtl1 is specifically expressed in the fetal capillary endothelial cells and that these cells were severely damaged in the KO placentas, showing detachment of the endothelial cells from the basement membrane and clogging of the capillaries due to overgrown sur-

rounded trophoblast cells. Thus, we suggested that this damage to the fetal capillaries is the cause of fetal lethality and late fetal growth retardation. In this study, we re-examined the placental abnormalities of the Peg11/Rtl1 KO in detail and confirmed that this severe damage to the fetal capillaries was not only associated with the mid- to late fetal lethality of the Peg11/Rtl1 KO but also caused reduction in the placental size during the late stage of development.

Results

Genetic background influenced lethality of Peg11/ Rtl1 KO

The lethal phenotype of Peg11/Rtl1 KO is largely dependent on genetic background. As previously reported, we generated Peg11/Rtl1 KO mice by mating male chimeras generated by 129/SvEv ES cells having the KO allele with B6 females (Fig. S1 in Supporting information). Peg11/Rtl1 KO mice with this 129/B6 background (F1) were normally born without any lethality but showed growth retardation in the pre- and postnatal periods (Table S1 in Supporting information). However, in the course of a backcross experiment to produce KO mice with the B6 genetic background, fetal and neonatal lethality became apparent at the F3 generation: Half of KO fetuses showed mid- to late fetal lethality from d15.5 to d18.5, whereas the other half developed to term with growth retardation and were born small, approximately 80% of the weight of controls, and most died by the next day. Then, the Peg11/Rtl1 KO lines were maintained basically by maternal transmission to the F8 generation (Sekita et al. 2008).

We repeated the backcross experiment using F5 mice recovered from in vitro fertilization (IVF) using F4 cryopreserved sperm and B6 oocytes to re-examine the fetal and placental abnormalities during the backcross process between the F5 and F10 generations to elucidate what happened with the Peg11/ Rtl1 KO mice (Table 1, F5). We preserved the F4 sperm from three KO males and one KO sperm still retained a high ability to produce viable pups at the F5 generation by IVF, whereas no pups were born from most F4 KO males. Among 11 KO pups, three grew to adulthood at F5 (neonatal lethal rate, 72.7%). In subsequent generations, we selected two or three male KO mice, also with higher reproductive ability. Then, at the F7 generation, the neonatal lethality rate remained approximately 60%. However, two of six KO mice did not produce any viable progeny, and the remaining four mice were used to make the F8 generation of mice (total neonatal lethal rate at F8, 74.6%). When their genetic background became nearly the same as B6 at the F10 generation, the neonatal lethality rate finally reached a maximum (100%), and all the Peg11/Rtl1 KO pups died by the next day of birth (Fig. 1 and Table 1).

In the F10 mice, mid- to late fetal lethality as well as neonatal lethality rates were increased compared with the F5 mice. To examine the fetal and neonatal lethality ratios at the F5 and F10 generations, IVF experiments were conducted using the F4 and F9 sperm (Table 1, F5 and F10). In the IVF experiments, approximately 20 fertilized embryos were transferred back to the uteruses of ICR females. Usually, approximately half were lost immediately after implantation regardless of genotype (WT or KO);
therefore, the KO/WT ratio has no meaning when the litter number examined was very small (less than 3). Thus, we only considered the numbers of live and dead fetuses within the same genotype (WT or KO) to calculate the mid- and late fetal lethality rates, whereas in the case of neonatal lethality, we directly compared the number of WT and KO pups because we had enough data from 11 litters in each case of F5 and F10. No fetal or neonatal lethality was observed in the WT mice at either F5 or F10, whereas each lethality rate in the mid-fetal (d14.5), late fetal (d16.5) or neonatal (P0) stage was increased at F10 compared with F5 in the KO mice (F5 vs F10: 22.2% vs 36.4%, 50.0% vs 66.7% and 72.7% vs 100%, respectively). It should be noted that there were significantly fewer KO pups than WT pups in these IVF experiments (Table 1) compared with the case of natural mating (Fig. 1) (F5: 0.21 vs 0.64 and F10: 0.14 vs 0.46), suggesting the possibility of selection against KO mice under the former artificial conditions, such as IVF followed by embryo transfer to the uterus of a surrogate ICR mother. We confirmed that even in the IVF experiment, mice at the F1-like generation generated by IVF using F9 sperm and 129/Svj (129) oocytes did not show any prenatal lethality, and only one WT and two KO died at the neonatal stage (Table 1, F1-like), which was similar to the result of natural mating in the previous report (Table S1 in Supporting information).

Fetal and placental development was examined in detail at the F10 generation. Even at the F10 generation, Peg11/Rtl1 KO fetuses did not show any apparent abnormal phenotypes until d12.5. Then, some KO fetuses showed mid-fetal lethality at d14.5, although their average weight, including normal-looking but dying fetuses, did not change (94% of WT, not statistically significant) (Fig. 2A). At d16.5, more than half of Peg11 KO fetuses were dead (already resorbing) or dying, and some had apparent internal bleeding (Fig. 2B). Live KO fetuses had a normal appearance, but their average weight was decreased (approximately 85%, significant). The weight fell to 80% (significant) at d19.5, the day of birth (Fig. 2A), and all of them died by the next day, as mentioned above. As shown in Fig. 2B, dead KO fetuses were associated with pale placentas, and even live KO fetuses had slightly pale placentas, suggesting the blood circulation in the placenta was reduced depending on the degree of severity. The placental weight of KO fetuses tended to be decreased, but the difference was statistically significant only at d19.5 (78.5%) (Fig. 2C).

### Table 1: Fetal and neonatal lethality of Peg11/Rtl1 KO mice produced by IVF

| Embryonic day | F1-like | WT | KO | Number of litter | WT population size | KO population size | KO lethality (%) | KO/WT | WT population size | KO population size | KO lethality (%) | KO/WT |
|---------------|---------|----|----|-----------------|-------------------|-------------------|-----------------|-------|-------------------|-------------------|-----------------|-------|
| d12.5         | F10     | 8  | 14 | 0               | 2                 | 0                 | 1.75            | 0.75  | 2                 | 0                 | 1.75            | 0.75  |
| d14.5         | F5      | 10 | 12 | 0               | 2                 | 0                 | 0.752           | 0.41  | 2                 | 0                 | 0.752           | 0.41  |
| d16.5         | F10     | 12 | 12 | 0               | 2                 | 0                 | 0.43            | 0.36  | 2                 | 0                 | 0.43            | 0.36  |
| Neonate       | F1-like | 12 | 12 | 0               | 2                 | 0                 | 0.51            | 0.36  | 2                 | 0                 | 0.51            | 0.36  |

In the F1-like and F10 experiments, sperm from the same F9 KO male was used.
Genetic background influenced the lethality of Peg11/Rtl1 KO. Neonatal lethality rate of KO mice in the course of producing mice with B6 background by natural mating. From F6 to F10 generation, the rate gradually increased and reached a maximum (100%) at the F10 generation.

| Generation (F) | 6   | 7   | 8   | 9   | 10  |
|----------------|-----|-----|-----|-----|-----|
| Number of births (WT) | 42 (0) | 39 (1) | 97 (4) | 56 (1) | 35 (0) |
| Number of deaths (KO) | 28 (14) | 30 (18) | 59 (44) | 27 (21) | 16 (16) |
| KO lethality (%) | 50  | 60  | 74.6 | 77.8 | 100 |
| KO/WT | 0.67 | 0.77 | 0.61 | 0.48 | 0.46 |
| Number of litter | 10  | 11  | 26  | 17  | 9   |

Figure 1 Genetic background influenced the lethality of Peg11/Rtl1 KO. Neonatal lethality rate of KO mice in the course of producing mice with B6 background by natural mating. From F6 to F10 generation, the rate gradually increased and reached a maximum (100%) at the F10 generation.

Figure 2 Phenotype of Peg11/Rtl1 KO fetus and placenta. (A) Fetal weight from mid- to late stages of pregnancy. Live fetuses with normal appearance were selected and measured. Growth retardation of KO fetuses became apparent (statistically significant) from d16.5. Black bars: WT, blue bars: Peg11/Rtl1 KO. (B) Fetuses and placentas at d16.5. WT underlined in black (fetal weight: 613.5 mg and 611.7 mg and placental weight: 65.7 mg and 74.1 mg) and Peg11/Rtl1 KO with a blue line (fetal weight: 538.2 mg, 465.0 mg, 244.3 mg and 104.3 mg, corresponding to a live fetus with growth retardation, two dead fetuses with internal bleeding and a resorbing fetus, respectively, and placental weight: 64.8 mg, 61.1 mg, 64.1 mg and 52.3 mg, respectively). Scale bar, 1 cm. (C) Placental weight from mid- to late stages of pregnancy. KO placentas seemed small from d14.5, but the difference was statistically significant only at d19.5. Black bars: WT, blue bars: KO. Numbers of fetuses and placentas measured ((A) and (C)) indicated in parentheses: d12.5 WT (16) and KO (8); d14.5 WT (10) and KO (5); d16.5 WT (4) and KO (4); d19.5 WT (20) and KO (4). *P < 0.05, **P < 0.01 (Student’s t-test). Error bars indicate SD.
Severe damage to fetal capillaries caused mid-fetal lethality and reduction in the labyrinth layer in Peg11/Rtl1 KO placentas

To elucidate what happened with the Peg11/Rtl1 KO placentas, we carried out cytokeratin (a marker of all trophoblast cells) staining and Dolichos biflorus agglutinin (DBA lectin: a marker for endothelium of the fetal capillaries) staining experiments and found that the small placenta was caused by poor growth of the labyrinth layer (Adamson et al. 2002; Favaron et al. 2011). The cytokeratin staining experiment showed that the gross size and morphology of the KO placentas looked normal at d14.5 and d16.5, but the placental size was clearly small at d19.5 (Fig. 3A). Importantly, the dark stained labyrinth layer was selectively thinner (approximately a half depth) than in the WT, whereas the size of the spongiotrophoblast layer was normal (Fig. 3B). Hematoxylin and eosin (HE) staining also showed the clogging of fetal capillaries in d19.5 KO placentas but not in WT and F1 KO placentas, as previously reported (Sekita et al. 2008) (Fig. 3C).

Then, we compared the placentas of fetuses of normal-looking, less severe and severe abnormal phenotypes at d14.5 because the latter KO fetuses showed mid-fetal lethality with internal bleeding (Fig. 3D, right). The DBA lectin staining experiment showed that fetal capillary growth was severely damaged in the corresponding KO placentas, although their sizes seemed almost normal. The DBA lectin-positive area was clearly much decreased in the KO placentas with fetuses with internal bleeding compared with less severe fetuses (Fig. 3E, right vs middle), showing that global loss of the fetal capillary endothelial cells occurred even at d14.5 and that its severity was well correlated with the degree of fetal abnormalities. HE staining of the next sections from the same placentas also confirmed that the fetal capillaries were already destroyed in the region where no DBA lectin-positive signal was detected (Figs. 3F and G). Although the clogging at d19.5 (Fig. 3C, and at d15.5 and d18.5 in the previous report) looked like aggregate (or lumps), the clogging at d14.5 occurred at the individual fetal capillary level and thus was not obvious at a glance (Fig. 3H, middle and right). However, much open space in the labyrinth was due to the remaining maternal blood sinuses. We further examined the labyrinth layer by comparing DBA lectin staining (Fig. 3I, K and M, the endothelial cells) to those of cytokeratin staining (Fig. 3J, L and N, syncytiotrophoblast cells). Yellow dotted lines in Fig. 3K-N indicated the same areas between K and L, and, M and N, respectively. The DBA lectin-positive area and cytokeratin-positive area were intermingled in WT (left), whereas no signals of the DBA lectin staining but increased signals of the cytokeratin staining were observed in both the less severe fetus (middle) and internal bleeding fetus (right), indicating that only the syncytiotrophoblast cells but no endothelial cells existed in the labyrinth layer. Therefore, we concluded that the fetal capillary damage is the primary cause of the mid-fetal lethality of Peg11/Rtl1 KO.

Importantly, the fetal capillary endothelial cells in both the basal (Fig. 3E, right, the chorionic membrane side) and peripheral parts (Fig. S2 in Supporting information, right, the spongiotrophoblast layer side) were severely damaged in the KO placentas with dead fetuses, whereas the damage in the peripheral side accompanying the less severe fetuses was correspondingly less severe (Fig. S2 in Supporting information, middle), thus confirming the results of the DBA lectin staining experiment (Fig. 3E). The formation of this part starts at approximately d8.5 when the first branching of villi occurs, and then the fetal capillary network expands to the periphery region. The principal construction of the placenta is completed by d12.5, but the network continues to grow until d18.5–19.5. In the KO placentas, the fetal capillary network was destroyed or being destroyed beginning with this initial part even at d14.5, leading to a small placenta due to the poor expansion of the fetal capillary network in the late stage of KO placentas (d19.5 in this study and d18.5 in the previous report).

Gene expression analyses of Peg11/Rtl1 KO mouse placenta

Through microarray analysis, we found that changes in expression of several placental genes occurred even before the apparent size reduction although most placental cell-specific markers were unchanged. To elucidate genes involved in the maintenance of fetal capillary endothelial cells during the earlier stage of the placenta, we examined the gene expression profiles of the KO placenta at d10.5 and d12.5. As mentioned, no placental growth defects were observed in these stages. We examined several placental cell type-specific markers, such as Pecam1, Mest, Vif and CD34 for the endothelial cells in the labyrinth layer, Slc16a1 for the syncytiotrophoblast layer I (SynT-I)), Gm1, Cebpα, Slc16a3 (MCT4) for
the syncytiotrophoblast layer II (SynT-II), *Eox1* and *Dlx3* for both of the SynTs-I and II, *Prl7a2* (*PpF*) and *Prl8a2* (*PlpC gamma*) for the spongiotrophoblast cells, *Pdh12* and *Prl7b1* for the glycogen trophoblast cells and *Tphpa* for both the spongiotrophoblast cells and glycogen trophoblast cells. They showed only slight changes in expression with no significant difference except one of two CD34 probes showed a 1.6-fold increase in both the d10.5 and d12.5 placentas whereas three genes, *transmembrane protein 100 (Tmem100)*, *mesenchyme homeobox 2 (Mox2)* or *Mox2* and *lymphatic vessel endothelial hyaluronan receptor 1 (Lyve1)*, were commonly down-regulated at both d10.5 and d12.5 (Fig. S3 in Supporting information). Then, we confirmed their expression by RT-qPCR experiment and found that *Tmem100* (0.61 and 0.54-fold), *Lyve1* (0.71 and 0.46-fold) and *Mox2* (0.56 and 0.63-fold) were actually down-regulated in the KO placentas at both d10.5 and d12.5, respectively (Fig. 4); however, no differences were observed in *Gnai2* (1.08 and 1.01-fold) (Fig. S4 in Supporting information).

**Peg11/Rtl1 showed postnatal growth retardation**

All the *Peg11/Rtl1* KO pups at F1 showed postnatal growth retardation, and the same was true for the surviving pups in subsequent generations. As previously reported, *Peg11/Rtl1* KO pups were born small, approximately 80% of the weight of normal controls, but at 1 week their weight was further reduced to 65%. Their weight then recovered to 70% at the weaning period (3 weeks) and reached 90% at 7 weeks. This rate was then maintained. This result was also confirmed in this study (Fig. 5A and B, left). Importantly, we realized that *Peg11/Rtl1* KO mice were actually thinner than WT because they reached almost 90% of the WT length in infancy and caught up in length at 8 weeks (Fig. 5A and B, right). Using microcomputed tomography (Micro-CT) analysis, we analyzed three *Peg11/Rtl1* KO fetuses at d18.5 with an average of 70.3% of the control weight and found that they had 53.0% of the volume of brown fat in the back, 53.1% of the liver, 67.4% of the volume of the thoracic cavity, 72.4% of the kidney and 91.2% of the heart compared with WT (Fig. 5C). Thus, they had organs more or less proportionally reduced in weight except for the heart. We do not know why the KO hearts were larger than the other organs. We confirmed that *Peg11/Rtl1* was not expressed in the d18.5 fetal heart (Fig. 5D), suggesting that it may be caused by secondary effects somehow associated with the growth defects of KO mice.

**Discussion**

We previously reported that the lethal phenotype of *Peg11/Rtl1* KO is dependent on genetic background: KO mice with the 129/B6 F1 genetic background showed only pre- and postnatal growth retardation without any lethality (Table 1, F1-like and Table S1 in Supporting information), whereas fetal and neonatal lethality became apparent from the F3 generation (Sekita et al. 2008). Thus, as far as the lethal phenotype in *Peg11/Rtl1* KO mice is concerned, it is likely that there is some modifier gene(s) independent of the growth retardation phenotype. The identification of this gene or genes is now in progress by quantitative trait loci (QTL) analysis using mating with MSM/Ms (*Mus musculus mollosinus*) and B6 mice.

Not only the neonatal lethality rate but also the mid- to late fetal lethality rate increased in succeeding generations (from F5 to F10). We showed that severe damage to the fetal capillary network corresponded well with the fetuses showing mid-fetal lethality with internal bleeding, suggesting that high blood pressure caused by disrupted fetus-placenta blood circulation is a cause of the sudden death of KO fetuses around the mid-fetal stage, in addition to the effect of the decrease in oxygen and nutrients from the placentas. In both cases, it is concluded that the disruption of the fetal capillary maintenance system is the major factor in the fetal death and fetal growth retardation of *Peg11/Rtl1* KO mice.

As shown in Fig. 3E, the destruction of the fetal capillary network started from the basal part, the initial part, of the network where the first branching formation of the fetal capillaries began just after the chorioallantoic fusion at d8.5 and propagated rapidly thereafter. Although the labyrinth layer continues expanding up to d18.5–19.5 (Watson & Cross 2005), we confirmed that the reduction in the labyrinth layer observed in the KO placentas at d19.5 is due to the severe damage to the fetal capillary network that had already occurred at d14.5.
Placental defects in Peg11/Rtl1 KO mice

Figure 3 (continued)
Then, what is the function of Peg11/Rtl1 in the maintenance of fetal capillaries? How does it regulate the interaction between the endothelial cells and the surrounding syncytiotrophoblast cells? As Peg11/Rtl1 protein possesses a protease domain derived from a retrotransposon Pol protein, it is possible that this
protease activity is important for protecting the endothelial cells from attack by the syncytiotrophoblast cells. The involvement of its protease activity in cancer progression is suggested in liver cancers (Riordan et al. 2013). Importantly, our microarray and RT-qPCR experiments indicated that changes in the labyrinth cells had already occurred at d10.5 and d12.5. Three genes related to the endothelial and syncytiotrophoblast cells showed down-regulation, such as Tmem100, Lyve1 and Meox2.

Tmem100 plays essential roles in arterial endothelium differentiation and vascular morphogenesis downstream of BMP9/BMP10–ALK1 signaling (Somekawa et al. 2012). Importantly, it also plays an important role in the formation of the yolk sac vessel and the early placenta. Lyve1 is expressed in lymphatic vessel endothelial cells and may act as a hyaluronan (HA) transporter. In the human placenta, LYVE1 protein has been detected in the villous core endothelium and syncytiotrophoblast cells, which correspond to the mouse labyrinth cells, suggesting that it functions in the regulation of HA metabolism in the feto-maternal circulation and shows both blood and lymphatic phenotypic characteristics in the fetal endothelium (Gu et al. 2006). Meox2 is also expressed in the syncytiotrophoblast cells of the human placenta and therefore also seems like a good candidate (Quinn et al. 2000). However, it has been reported that no embryonic lethality was observed in Meox2 KO mice (Mankoo et al. 1999); therefore, its involvement in the maintenance of fetal capillaries remains unknown. Thus, it is highly probable that at least Tmem100 and Lyve1 function in the maintenance of fetal capillary endothelial cells, possibly via interaction with Peg11/Rtl1. As all these changes occurred before d14.5, it is likely that at least some genes are directly involved in the interaction between the endothelial and syncytiotrophoblast cells in the absence of Peg11/Rtl1 protein, thus providing an important clue to elucidate how Peg11/Rtl1 functions in the placental fetal capillary proliferation at the biochemical level during the early stages of development.

The important question remains of whether Peg11/Rtl1 functions directly in fetuses and neonates beyond the placenta. As mentioned, Peg11/Rtl1 KO mice showed neonatal lethality and postnatal weight reduction. Although it is possible that all these phenotypes are secondary effects originated by the placental failures discussed, it is also possible that Peg11/Rtl1 also plays a role by interacting with other genes concerning postnatal growth (eating behavior, hormones and metabolism including the brain and digestive system or tract). The phenotypes observed in the Peg11/Rtl1 KO mice are quite similar to the phenotypes of mUPD12 mice as well as human maternal duplication of chromosome 14 (upd(14)mat) (Georgiades et al. 2000; Kotzot 2004). Therefore, we previously concluded that Peg11/Rtl1 is a major gene responsible for abnormal phenotypes observed in mUPD12 mice as well as upd(14)mat patients (Sekita et al. 2008). In addition, phenotypes of mice with paternal duplication of the same region (pUPD12)
are also very similar to the ones observed in human paternal disomy of chromosome 14 (upd(14)pat) patients (Georgiades et al. 2000; Kagami et al. 2005, 2015). The upd(14)pat patients showed typical bell-shaped thorax and abdominal muscle failure, and these factors seem to be the cause of neonatal lethality (Kagami et al. 2005, 2015). We also previously reported that its severity correlates with the overproduction of PEG11/RTL1 (Kagami et al. 2008). Recently, the involvement of Peg11/Rtl1 in muscle development has been suggested by transgenic mice over-expressing Peg11/Rtl1 (Xu et al. 2015). Therefore, it will be very important to know whether Peg11/Rtl1 is directly involved in fetal and postnatal development and growth in addition to the placentas to understand all the roles of Peg11/Rtl1, an LTR retrotransposon-derived, eutherian-specific gene.

**Experimental procedures**

**Mice**

All animals and experimental procedures were approved by the Animal Ethics Committees of Tokyo Medical and Dental University. Peg11/Rtl1 KO mice were generated using ES cells (CCE) of 129/SvEv mouse origin, as previously described (Sekita et al. 2008). Peg11/Rtl1 KO lines were maintained by continuous crossing with C57BL/6J females (WT), and mice in the F1, F5, F7-9 and F10 generations were used in this study.

**In vitro fertilization (IVF)**

Seven-week-old C57BL/6j or 129Svj female mice and sperm from the F4 or F9 generation of Peg11 KO male mice were used for IVF. Superovulation was induced by injecting 7.5 IU of pregnant mare’s serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG; after 48 h later injected PMSG) into the female mice. At 16–17 h after injection, the females were euthanized and the oviducts removed. Up to eight cumulus–oocyte complex (COC) masses were released into individual drops of CARD MEDIUM (100 μL/drop; KYUDO) covered with mineral oil (Sigma), and the COCs were kept in a 37 °C, 5% CO2 incubator for 30–60 min before insemination. The mouse spermatozoa (frozen straw) were thawed in a 37 °C water bath. After 10 min, the sperm suspension was transferred into a drop of FERTIUP (pre-incubation medium: PM; KYUDO) covered with mineral oil (Sigma), and the COCs were kept in a 37 °C, 5% CO2 incubator for 30–60 min before insemination. The mouse spermatozoa (frozen straw) were thawed in a 37 °C water bath. After 10 min, the sperm suspension was transferred into a drop of FERTIUP (pre-incubation medium: PM; KYUDO) covered with mineral oil (Sigma), and the COCs were kept in a 37 °C, 5% CO2 incubator for 30–60 min before insemination. The mouse spermatozoa (frozen straw) were thawed in a 37 °C water bath. After 10 min, the sperm suspension was transferred into a drop of FERTIUP (pre-incubation medium: PM; KYUDO) covered with mineral oil (Sigma), and the COCs were kept in a 37 °C, 5% CO2 incubator for 30–60 min before insemination. The mouse spermatozoa (frozen straw) were thawed in a 37 °C water bath. After 10 min, the sperm suspension was transferred into a drop of FERTIUP (pre-incubation medium: PM; KYUDO) covered with mineral oil (Sigma), and the COCs were kept in a 37 °C, 5% CO2 incubator for 30–60 min before insemination. The mouse spermatozoa (frozen straw) were thawed in a 37 °C water bath. After 10 min, the sperm suspension was transferred into a drop of FERTIUP (pre-incubation medium: PM; KYUDO) covered with mineral oil (Sigma), and the COCs were kept in a 37 °C, 5% CO2 incubator for 30–60 min before insemination. The mouse spermatozoa (frozen straw) were thawed in a 37 °C water bath. After 10 min, the sperm suspension was transferred into a drop of FERTIUP (pre-incubation medium: PM; KYUDO) covered with mineral oil (Sigma), and the COCs were kept in a 37 °C, 5% CO2 incubator for 30–60 min before insemination. The mouse spermatozoa (frozen straw) were thawed in a 37 °C water bath. After 10 min, the sperm suspension was transferred into a drop of FERTIUP (pre-incubation medium: PM; KYUDO) covered with mineral oil (Sigma), and the COCs were kept in a 37 °C, 5% CO2 incubator for 30–60 min before insemination. The mouse spermatozoa (frozen straw) were thawed in a 37 °C water bath. After 10 min, the sperm suspension was transferred into a drop of FERTIUP (pre-incubation medium: PM; KYUDO) covered with mineral oil (Sigma), and the COCs were kept in a 37 °C, 5% CO2 incubator for 30–60 min before insemination. The mouse spermatozoa (frozen straw) were thawed in a 37 °C water bath. After 10 min, the sperm suspension was transferred into a drop of FERTIUP (pre-incubation medium: PM; KYUDO) covered with mineral oil (Sigma), and the COCs were kept in a 37 °C, 5% CO2 incubator for 30–60 min before insemination. The mouse spermatozoa (frozen straw) were thawed in a 37 °C water bath. After 10 min, the sperm suspension was transferred into a drop of FERTIUP (pre-incubation medium: PM; KYUDO) covered with mineral oil (Sigma), and the COCs were kept in a 37 °C, 5% CO2 incubator for 30–60 min before insemination. The mouse spermatoza and spermatozoa were incubated for 3 h in an incubator. After incubation, the oocytes were washed three times in fresh mHTF covered with mineral oil, avoiding transfer of the CARD MEDIUM. At 6 h after insemination, parthenogenetic oocytes with only one pronucleus were removed. After overnight culture of the oocytes, two-cell-stage embryos were removed to a new
drop of mHTF, and approximately 20 embryos were transferred to recipient females (ICR). To deliver pups by cesarean section at d19.5, 1.25 mg of progesterone (Mochida) was injected into each pregnant female mouse at both d17.5 and d18.5 (Nakagata & Takeshima 1992; Nakagata et al. 1995; Nakagata 1996, 2011; Okamoto et al. 1998; Takeo et al. 2008; Takeo & Nakagata 2010, 2011).

Histological analysis

Placentas were collected and fixed in 4% paraformaldehyde (PFA) in PBS, then soaked in graded sucrose solution (10% and 25% in PBS) at 4 °C overnight before being embedded in OCT compound (SAKURA). OCT blocks were sectioned at 10 μm thickness using a cryostat (MICROME), mounted on super-frost microslide glasses (Matsunami Glass) and then stored at −80 °C. Sections were rehydrated in PBS, stained with hematoxylin for 2 min and washed in tap water for 2 min. After staining with eosin for 1 min, the sections were then immersed in 70% and 80% ethanol for a few seconds each, 100% ethanol three times for 2 min, dehydrated in xylene three times for 3 min and mounted with malinol (MUTO).

Immunostaining

Sections were rehydrated in PBS, post-fixed in 4% PFA for 10 min and washed in PBS three times for 5 min. To retrieve the antigen, the sections were boiled in 10 mm citrate buffer pH 6.0 at 98 °C for 40 min and immersed in cold methanol at −30 °C for 30 min. After air-drying, the sections were blocked with 10% goat serum, 1% bovine serum albumin (BSA), 0.1% Triton X-100 and 0.05% NaN$_3$ in PBS at room temperature for 30 min. They were then incubated with primary antibody prepared in 1% BSA, 0.1% Triton X-100 and 0.05% NaN$_3$ in PBS at room temperature for 2 h. Polyclonal rabbit anticytokeratin antibody (DAKO) was used as the primary antibody, prepared in PBS. This primary reaction was developed with biotinylated goat anti-rabbit IgG secondary antibody (1:200; Vector Laboratories) for 30 min. For DBA lectin staining, the sections were incubated with anti-DBA lectin biotin conjugate antibody (Sigma) for 30 min. After biotin blocking, they were incubated with alkaline phosphatase (AP) complex (1:200; Vectastain ABC-AP kit; Vector Laboratories) for 30 min. The histochemical detection of the alkaline phosphatase activity was carried out using BICP/NBT (PRO-MEGA) in NTMT Buffer (Fig. 3A, B and E) or BICP/NBT (Vectastain) in 100 mm Tri-HCl pH 9.8 (Fig. 3i–n). Nuclei were stained using Nuclear Fast Red (Vector Laboratories) for 2 min and washed in tap water for 1 min, then immersed in 30%, 50%, 70% and 90% ethanol for 1 min each and 100% ethanol twice for 2 min, dehydrated in xylene three times for 3 min and mounted with malinol (MUTO). Images were captured using a VB-7000 Digital Camera System (Keyence) with an ECLIPSE E600 microscope (Nikon) (Fig. 3A, C and E) or BIOREVO (Keyence) (Fig. 3F–N).

Microarray analysis

Total RNA samples were prepared from d10.5 and d12.5 placentas using TRizol reagent (Life Technologies). The labeled cRNA was synthesized from 50 to 100 ng of total RNA using the Low Input Quick Amp Labeling Kit (Agilent). The amplified cRNA samples were purified using the RNeasy Mini Kit (Qlagen). After quantification of the cRNA, the fragmentation size was 1500 nm and hybridization buffer (Agilent) added. Each hybridization sample was applied on the gasket. SurePrint G3 Mouse GE 8 × 60 K (Agilent) was added and hybridized at 65 °C for 17 h at 10 rpm. The slides were then washed with gene expression wash buffer and acetonitrile. After air-drying, the slides were scanned using the Agilent SureScan Microarray Scanner (Agilent). The output file of feature extraction (Agilent Technologies) was normalized by qspline method (Workman et al. 2002). Genes showing a fold change >2.0 and P-value <0.05 (Student’s t-test) were considered significantly different. Microarray data are available at the NCBI Gene Expression Omnibus (accession number was as follows: d10.5, GSE88849; d12.5, GSE88891; d10.5 and d12.5, GSE88892).

Quantitative PCR assay

Quantitative real-time PCR was carried out using five nanograms of cDNA in THUNDERBIRD SYBR qPCR mix (TOYobo). The cycle conditions were 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 10 s using the LightCycler 480 apparatus (Roche). Gene expression levels were normalized with Gapdh. The primer sequences used for this study were as follows: Gnat2, 5'-GGACGTGGCCTCTTCAGAG-3' and 5'-AAATCTTAGAGCCAGAGGAG-3'; Timem100, 5'-GTGGCCCTCTCGGTAATGGA-3' and 5'-CATGTTACAGGTTGGAG-3'; Lyve1, 5'-TCAGAGACAGGTTCCAGGT-3' and 5'-CTCAGTCCACAGCTCCTTG-3'; G6PD, 5'-AATGCGAGGATCTGACGTCT-3' and 5'-AGCCAAAGCAACATCCA TC-3'; G6pdh, 5'-CACTTCTCAACCTCGATGC-3' and 5'-CTCTTGTCTCAGTGTCCTTG-3'.

Micro-CT analysis

Micro-CT analysis was conducted by the method described in previous studies (Degenhardt et al. 2010 and Tamura et al. 2013), with some modification. Mouse fetuses were scanned using a SCAMMATE-E090S scanner (Comscantech) at a tube voltage peak of 40 kVp and a tube current of 100 μA. Samples were rotated 360° in steps of 0.24°, generating 1500 projection images of 640 × 480 pixels. Before scanning, the d18.5 fetuses were fixed in Bouin’s solution (WAKO) and soaked in a contrast agent consisting of 10% phosphotungstate acid (TAAB): 100% EtOH = 3: 7 at 4 °C for 1 week and then embedded in 1% agarose (Tamura et al. 2013). Images were reconstructed using the OsiriX (www.osirix-viewer.com) software program.
**A** Weight

![Graph showing weight over time](image)

**B** KO/WT (%)

![Graph showing KO/WT over time](image)

**C** Images of WT and Peg11/Rtl1 KO

![Images showing differences in tissue distribution](image)

|                | Weight (mg) | Thoracic cavity (mm³) | Heart (mm³) | Fat (mm³) | Liver (mm³) | Kidney (mm³) |
|----------------|-------------|------------------------|-------------|-----------|-------------|--------------|
| WT             | 1358.3      | 9.23                   | 5.71        | 2.97      | 9.52        | 6.07         |
| Pat-KO         | 955.1       | 6.22                   | 5.21        | 1.58      | 5.06        | 4.4          |
| Pat-KO/WT (%)  | 70.3        | 67.4                   | 91.2        | 53.0      | 53.1        | 72.4         |

**D**

| 76 | Peg11/Rtl1 KO | Positive control |
|----|---------------|------------------|
|    |               | d18.5 WT placenta|

*Peg11/Rtl1* x 30 cycles

*Gapdh*
Figure 5 Postnatal growth abnormality of Peg11/Rtl1 KO mice (at F8 generation). (A) Growth curve of WT and KO mice from 1 week after birth to 35 weeks. KO mice (blue) were constantly lighter (left) than WT (black) but caught up in length (right) at approximately 8 weeks. Weight (left) and length (right) were measured every 1 week. WT: n = 9, KO: n = 8. *P < 0.05, **P < 0.01 (Student’s t-test). Error bars indicate SD (B) Difference in postnatal growth between WT and KO. KO mice were 65% and 70% of the weight of WT at 1 and 3 weeks (weaning period), respectively, and 90% of the weight at adulthood. The KO mice were almost 90% of the length of WT mice in infancy and caught up shortly thereafter. (C) Micro-CT images of d18.5 fetuses of WT (left) and KO (right) mice. Heart, thoracic cavity and brown fat in the back are indicated. Volumes of thoracic cavity and kidney were approximately 70–75%, proportional to the fetal weight, whereas the fat and liver were almost half the size of WT by volume. Compared with these organs, the heart seemed larger than expected. WT: n = 3, KO: n = 3. (D) Expression of Peg11/Rtl1 in WT and KO hearts at d18.5. No apparent bands were detected in either WT or KO by RT-PCR, as described in Experimental procedures.

Expression analysis of Peg11/Rtl1

Genomic DNA and total RNA samples were prepared from d16.5 embryos using TRIzol reagent (Life Technologies). The methods of RT-PCR for 3′-RACE of Peg11/Rtl1 followed by cDNA synthesis have been previously described (Sekita et al. 2006).

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Author contributions

T.K.-I. and F.I. conceived and designed the experiments; M.K. and M.T. carried out the experiments; M.K. analyzed the KO mice and carried out the immunofluorescent analysis and microarray analysis; M.K. and M.T. carried out the micro-CT analysis; M.K., T.K.-I. and F.I. analyzed the data and wrote the paper.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Construction of Peg11/Rtl1 KO mice.

Figure S2 HE staining of d14.5 placentas.

Figure S3 Microarray analysis of Peg11/Rtl1 KO mouse placentas.

Figure S4 Quantitative RT-PCR analysis of Gna12 in d10.5 and d12.5 placentas.

Table S1 Neonatal lethality of Peg11/Rtl1 KO mice at F1 generation by natural mating

Table S2 Microarray analysis of Peg11/Rtl1 KO mouse placentas

Table S3 Microarray analysis of Peg11/Rtl1 KO mouse placentas (UP)

Table S4 Microarray analysis of Peg11/Rtl1 KO mouse placentas (DOWN)