Complementary roles of genes regulated by two paternally methylated imprinted regions on chromosomes 7 and 12 in mouse placentation

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Imprinted genes have prominent effects on placentation; however, there is limited knowledge about the manner in which the genes controlled by two paternally methylated regions on chromosomes 7 and 12 contribute to placentation. In order to clarify the functions of these genes in mouse placentation, we examined transcription levels of the paternally methylated genes, tissue differentiation and development and the circulatory system in placentae derived from three types of bi-maternal conceptuses that contained genomes of non-growing (ng) and fully grown (fg) oocytes. The genetic backgrounds of the ng oocytes were as follows: one was derived from the wild-type (ng⁷WT) and another from mutant mice carrying a 13 kb deletion in the H19 transcription unit including the germline-derived differentially methylated region (H19-DMR) on chromosome 7 (ng⁷ch7). Another set of oocytes was derived from mutant mice carrying a 4.15 kb deletion in the intergenic germline-derived DMR (IG-DMR) on chromosome 12 (ng¹ch12). Although placental mass was slower in the ng⁷WT/fg placentae compared with that in the WT placentae, it was recovered in the ng⁷ch7/fg placentae, but not in the ng¹ch12/fg placentae. The ng⁷ch7/fg placental growth improvement was associated with severe dysplasia such as an expanded spongiotrophoblast layer and a malformed labyrinthine zone. In contrast, the ng¹ch12/fg placentae retained the layer structures with expanded giant cells, but their total masses were smaller with a normal circulatory system in order. Our findings demonstrate that the genes controlled by the two paternally methylated regions, H19-DMR and IG-DMR, complementarily organize placentation.

INTRODUCTION

In mammals, imprinted genes, wherein only one of the two parental chromosome copies is expressed, are regulated by epigenetic modifications, including DNA methylation. Acquisition of methylation at key regional controlling elements occurs in the parental germ lines, during male or female gametogenesis. Most imprinted genes are regulated by maternally derived methylation, whereas only three have been identified as genes regulated by paternally derived methylation (1). The latter include H19-Igf2 and Gtl2-Dlk1, which are regulated by a differentially methylated region (DMR) on chromosomes 7 (H19-DMR) and 12 (IG-DMR), respectively, and Rasgrf1 on chromosome 9 (2–4). H19-Igf2 and Gtl2-Dlk1 are regulated by paternally derived methylation; however, Igf2 and H19 have opposite expression patterns as do Dlk1 and Gtl2. Imprinted genes also have prominent effects on placentation (5). A series of experiments have demonstrated that in the mouse placenta, Igf2 and Peg1/Mest appear to be involved in growth enhancement, whereas Grb10 and Phlda2 (Ipl, Tssc3) have restraining effects on growth processes. Imprinted genes on chromosome 12 have also been shown to play a role in regulating placental size and organization (6,7). Additionally, Mash2 and many genes linked to the X chromosome play critical roles in placentation (8–14).

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Normal parthenotes containing two maternal genomes from matured oocytes showed poorly developed extraembryonic tissues, contributing to their death by E9.5 (embryonic day 9.5) (15,16). The failure of the placenta in parthenotes is due to the inappropriate expression of imprinted genes. This mainly occurs because the maternal epigenotype of the imprinted domain has been imposed on both the two haploid sets resulting in the absence of expression of paternally expressed imprinted genes or the overexpression of maternally expressed imprinted genes (17–19). However, when non-growing (ng) oocytes of WT mice (ngWT), which have erased their maternal imprints and thus are considered to be naive with respect to most of the maternal imprinting process, were combined with fully grown (fg) oocytes, we observed that ngWT/fg bi-maternal conceptuses could develop to E13.5. Such ngWT/fg bi-maternal conceptuses formed placentae that consisted of three layers, namely the trophoblastic giant cells, spongiotrophoblast and labyrinthine layers. This suggests that failure to acquire maternal imprints in the ng oocyte rescues the placenta to some extent. Further, we have shown that ng/fg bi-maternal conceptuses are infrequently able to develop to term, when the embryos are reconstructed with ng oocytes (ngCh7) of mutant mice carrying a 13 kb deletion in the H19 transcription unit with its DMR (20–23). This more successful outcome associated with the provision of Igf2 indicates that its absence in normal parthenotes is a contributory factor to their developmental failure. In addition, in these bi-maternal conceptuses, the IGF-DMR on chromosome 12 of the ngCh7/fg bi-maternal conceptuses that developed to term became unexpectedly methylated. Expression studies confirmed that the switch from the maternal to the paternal epigenotype in the imprinted domain at the distal regions of chromosomes 7 and 12 resulted in the appropriate transcription of Igf2-H19 and Dlk1-Gtl2 from the ng allele, which in turn enables further development of bi-maternal conceptuses. However, placenta in ng/fg bi-maternal conceptuses has not been investigated to date.

Investigating placenta in ng/fg bi-maternal conceptuses may help in elucidating the roles that paternally methylated imprinted genes play in mouse placenta in three ways. First, our system provides the only way of manipulating the expression of multiple imprinted genes, that can be compared with the extremes of full parthenogenesis and androgenesis. Secondly, we can monitor the development of placenta that exclusively possessed the maternal genomes during gestation; that is, we can investigate the phenotypes of the placenta in which all paternally methylated imprinted genes are absent by default. In the case of the ngWT/fg placenta, maternal methylation imprinting was globally modified, differing from the imprinting that is observed in the case of normal parthenotes because one set of maternal imprints are erased and neither maternal methylation nor a second set of maternal methylation imprints are imposed. Thirdly, the evaluation of the ngCh7/fg placenta enables an understanding of the relative contributions of paternally methylated imprinted genes on chromosome 7 to the development of the placenta. Furthermore, in this study, we examined the ngCh2/fg placenta by using ng oocytes of mice heterozygously carrying a 4.15 kb deletion of the IG-DMR on chromosome 12 (ngCh12) (4). It is known that the deletion of the IG-DMR from the maternally inherited chromosome causes loss of imprinting of all genes in the 1 Mb cluster that carries the maternally repressed genes, Dlk1, Dio3 and Rtl1, and the maternally expressed non-coding RNAs, including Gil2, and microRNAs (4). In the absence of the IG-DMR on the maternal chromosome, Dlk1, Dio3 and Rtl1 are activated and the non-coding RNAs repressed. As expected, the ngCh12/fg placenta did not show appropriate expression of imprinted genes on chromosome 7 and exhibited normal expression of imprinted genes on chromosome 12. On the basis of this, it is be possible to address and compare the contributions of paternally methylated imprinted genes on chromosomes 7 or 12 to mouse placenta in a ng/fg bi-maternal epigenetic background.

Morphometric and histological analyses revealed that correction of the expression of paternally methylated imprinted genes on chromosome 7 affected not only the increase in placentomal mass but also the normal differentiation of giant cells. However, ngCh7/fg bi-maternal conceptuses formed placentae with severe dysplasia such as an anomalously expanded spongiotrophoblastic layer and a malformed labyrinthine layer with an anomalous circulatory system. This indicates that these phenotypes are caused by the absence and/or overexpression of other imprinted genes in these placentae. Through the correction of the expression of paternally methylated imprinted genes on chromosome 12, we demonstrated that ngCh12/fg bi-maternal conceptuses could develop into at least 18.5-day-old bi-maternal conceptuses, with the placentae comprising the three layers in order. We further observed that the circulatory system of the ngCh12/fg placenta was comparable to that of the WT placenta. However, the placental weight barely increased, and the giant cells were abnormally expanded. This suggests that the absence and/or overexpression of chromosome 12 imprinted genes make a major contribution to the dysplasia in ngWT/fg placentae, but cannot rescue the size defect or trophoblast giant cell phenotype that are no longer evident when normal H19 and Igf2 levels are restored. Thus, we provide the first demonstration that the genes regulated by the two paternally imprinted methylated regions on chromosomes 7 and 12 contribute distinct and complementary functions to mouse placenta.

RESULTS

The developmental ability of the ngWT/fg and ngCh7/fg bi-maternal conceptuses had previously been determined; however, the developmental ability of the ngCh12/fg bi-maternal conceptuses remained to be elucidated (17,20). We first examined the ngCh12/fg bi-maternal conceptuses carrying the ng-oocyte genome that contained a deletion of the IG-DMR. The results clearly showed that the ngCh12/fg bi-maternal conceptuses could develop into at least E18.5 (Kawahara et al., manuscript in preparation). Next, we examined the masses of all the four placental types. The ngWT/fg placenta showed severe growth retardation at E12.5 (Fig. 1A and B). Until E18.5, the ngCh12/fg placenta consistently showed the highest masses among the placental types derived from bi-maternal conceptuses, but nonetheless, they were significantly lower than those of the WT placentae (Fig. 1B). Among the ngCh7/fg, ngCh12/fg and WT placentae,
the ngΔch12/fg placentae maintained the lowest weight until E18.5 (Fig. 1B). To understand the feature of each placenta in detail, we carried out the following analyses: quantitative analysis of gene expression, in situ hybridization, histological analysis and scanning electron microscopic (SEM) studies to observe the placental circulatory system.

Figure 1. (A) Four types of placentae, namely the WT, ngWT/fg, ngΔch7/fg and ngΔch12/fg at E12.5, E15.5 and E18.5. (B) Graphical representation of placental masses (n = 5). Values are represented as means ± s.d. (indicated by error bars). (a and b) Values with different superscripts are significantly different within the same gestational age (P < 0.05).

Gene expression of the paternally methylated imprinted genes H19, Igf2, Gtl2 and Dlk1 in the placenta

We performed quantitative expression analysis of the H19, Igf2, Gtl2 and Dlk1 genes in individual WT, ngWT/fg, ngΔch7/fg and ngΔch12/fg placentae by using real-time PCR (Fig. 2A). As expected, the expression of the H19 and Igf2 genes at E12.5 was corrected in the ngΔch7/fg placenta, but not in the ngWT/fg and ngΔch12/fg placenta. As expected, in both ngWT/fg and ngΔch7/fg placenta, the Gtl2 RNA expression level was approximately twice that of the mean value in the WT placentae, whereas the Dlk1 RNA expression level remained reduced until E18.5. The ngΔch12/fg placentae showed appropriate expression patterns of the Gtl2 and Dlk1 genes, except significantly higher expression of the Gtl2 gene at E12.5. Interestingly, the H19 RNA expression level in the ngΔch7/fg placenta was corrected to that in the WT placentae at E18.5, but not at earlier stages. The Igf2 RNA expression level was repressed in the ngΔch12/fg placenta. Next, we analysed the localization signal of RNA expression by using in situ hybridization (Fig. 2B). The intensities of the expression signals reflected the results of the quantitative expression analysis. Although, in previous published studies, the signals of Gtl2 RNA expression had not been detected in giant cells (24), other studies have observed Gtl2 transcription in some though not all giant cells (da Rocha et al., manuscript in preparation); signals were detected in the giant cells of the ngWT/fg and ngΔch7/fg placentae. Dlk1 RNA expression signals were distinctly recognized in the endothelial cells of the fetal blood vessels within the labyrinth of the WT and ngΔch12/fg placentae; however, no expression signals could be detected in the ngWT/fg and ngΔch7/fg placentae. Thus, we confirmed that in the ngΔch7/fg and ngΔch12/fg placentae, the expression patterns of H19-Igf2 and Gtl2-Dlk1 RNAs were respectively corrected to approximately normal levels seen in the WT placentae.

Histological analyses of the placenta

Histological analysis revealed that the ngWT/fg placentae showed disproportionate expansion of the sponggiotrophoblast layer along with a distorted and ambiguous boundary between the sponggiotrophoblast layer and the labyrinth; they also showed enlarged giant cells (Fig. 3A and B). However, in the ngΔch7/fg placentae, enlargement of the trophoblast giant cells was not detected, in contrast to the morphology of the boundary that was not restored. In contrast, the boundary in the ngΔch12/fg placenta was entirely restored, but enlargement of giant cells seen in the ngWT/fg placenta was not entirely corrected (Fig. 3A and B). Further, morphometric analysis revealed that the labyrinth of the ngWT/fg placenta was reduced in size, and the ratio of the sponggiotrophoblast layer to the labyrinth showed an increase of greater than 4-fold (Fig. 3B). This disproportionate appeared to be corrected in the ngΔch7/fg placentae at E12.5. However, the ratio of the sponggiotrophoblast layer to the labyrinth in the ngΔch7/fg placenta was 1.5-fold higher than that in the WT placentae. The ngΔch7/fg placenta consistently showed disproportionate expansion of the sponggiotrophoblast layer and a reduction in the labyrinthine layer. In contrast, the ngΔch12/fg placentae never showed this type of disproportionate expansion of the sponggiotrophoblast layer, and the ratio of the sponggiotrophoblast layer to the labyrinth was identical to that in the WT placenta up to E18.5 (Fig. 3B). To gain further insight into the disproportionate expansion of the sponggiotrophoblast layer in the ngWT/fg and ngΔch7/fg placentae, we examined the expression of the Phlda2 gene by real-time PCR and in situ hybridization in the four placental types (Fig. 4A...
and B). This gene is located on chromosome 7, is exclusively expressed from the maternal allele and encodes a cytoplasmic protein with a pleckstrin-homology domain. It controls placental size via a mechanism that is independent of Igf2 signalling, and its knockout results in global hyperplasia of placental tissues with disproportionate expansion of the spongiotrophoblast layer (11,25). The expression of the Phlda2 gene was repressed in all placental types derived from bi-maternal conceptuses, namely...
Figure 3. Histological and morphometric analyses of the WT, ngWT/fg, ngΔch7/fg and ngΔch12/fg placentae. (A) Midline placental sections (H&E) at E12.5, E15.5 and E18.5. The blue arrowheads indicate the border between the spongiotrophoblast layer and the labyrinth. High-magnification views of giant cells adjacent to the spongiotrophoblast layer at E12.5 are shown in the bottom row. (B) Spongiotrophoblastic layer (Spo), labyrinthine layer (Lab) and Spo/Lab ratios in the WT, ngWT/fg, ngΔch7/fg and ngΔch12/fg placentae (n = 4). The average areas of giant cells at E12.5 was also calculated (n = 4). (a and b) Values with different superscripts are significantly different within the same gestational age (P < 0.05).
ngWT/fg, ngΔch7/fg and ngΔch12/fg placenta (11, 14 and 22%, respectively). This was in accordance with the results of the in situ hybridization (Fig. 4A and B). Hence, these results indicated that the ngWT/fg and ngΔch7/fg placentae show disproportionate expansion of the spongiotrophoblast layer, independently of the repression of the Phlda2 gene. However, the ngΔch7/fg placentae showed normal giant cells. Furthermore, the ngΔch12/fg placentae showed no histological defects except the enlarged giant cells. These results indicate that the spongiotrophoblast expansion and its defective interface with the labyrinthine zone in ngWT/fg is rescued by corrected expression of imprinted genes on chromosome 12 and the trophoblast giant cell expansion by corrected Igf2-H19 expression. More precise gene expression analysis using each placenta tissue might provide further insight into a various placental abnormalities in the bi-maternal conceptuses.

Aberrant vasculature of the labyrinth of placenta derived from bi-maternal conceptuses

The labyrinth plays a crucial role in the exchange of nutrients, gases and waste between maternal and fetal blood. The number of blood vessels was counted in the WT and ngΔch12/fg placentae, and this value was found to increase with gestational age (Fig. 5B), SEM studies of casts additionally revealed that in the ngΔch7/fg placenta, both the maternal sinusoids and the fetal blood vessels within the labyrinth were defective (Fig. 5A, B and D). The ngWT/fg and the ngΔch7/fg placenta showed irregular dilation of the maternal sinusoids (Fig. 5Bb). At E12.5 and later, on examining the vascular casts of the maternal sinusoids in the WT placentae, we observed that the maternal sinusoids become smaller and more intricate, and the ring-like space at the base of the labyrinth (embryonic side) becomes enlarged as gestation proceeds (Fig. 5Ba, e and h and C). However, in the ngΔch7/fg placentae, the sinusoidal pattern was disordered and the degree of arborization was reduced, and the development of the ring-like space of the labyrinth was inadequate (Fig. 5Bc, f and i and C). In contrast, the maternal sinusoids of the ngΔch12/fg placentae showed none of the serious defects that were shown by the ngΔch7/fg placentae, except that their entire structure was very small (Fig. 5Bd, g and j and C). To visualize the feto-placental circulation, we prepared casts of the fetal circulation by injecting the casting compound into the umbilicals cords (Fig. 5D). The umbilical cord of the ngΔch12/fg placenta was so narrow and thin that the casting compound could not be injected into them and we obtained casts only for the WT and ngΔch7/fg placentae. We found that in the ngΔch7/fg placenta, the fetal blood vessels within the labyrinth were strikingly disordered, particularly near the spongiotrophoblast border. Moreover, the blood vessels were remarkably dilated (centre of Fig. 5D). In the middle of the labyrinth, we observed that the fetal blood vessels were abnormally aggregated (right side of Fig. 5D). These results indicate that both the maternal sinusoids and fetal blood vessels in the ngΔch7/fg placentae are larger and have lower density than those in the WT placentae, whereas the ngΔch12/fg placentae retain the intact vascular architecture.

DISCUSSION

The various analyses reported here of the placental defects caused by the disturbance of paternally methylated imprinted genes located on chromosomes 7 and 12 facilitate the understanding of the manner in which the genes in these regions contribute to mouse placentation. We found that many phenotypes displayed by the ngΔch7/fg and ngΔch12/fg placentae were heterogeneous and complementary. This suggests that the two imprinted regions complementarily contribute to mouse placentation and the respective regions have multiple functions in mouse placentation (Fig. 6). Appropriate expression of the two imprinted genes on chromosome 7 results in some corrections to the ngWT/fg placental phenotypes, namely some increase in placental size and normalization of the expanded giant cells arising in the ngWT/fg placentae. Furthermore, when the imprinted genes on chromosome 12 were appropriately expressed in the ngΔch12/fg placentae, these placentae had the three layers in order and a labyrinthine zone with intricate vasculature.
Roles of the paternally methylated imprinted genes on chromosome 7

The masses of the ng<sup>Δch7</sup>/fg placentae were higher than those of the ng<sup>WT</sup>/fg and ng<sup>Δch12</sup>/fg placentae. Previous studies reported that the placental phenotype that was associated with the lack of Igf2 exhibits a reduced mass (8,26,27). On the basis of this, we surmise that these corrections are due to the retrieval of Igf2 expression in the ng<sup>Δch7</sup>/fg placentae. However, the masses of the ng<sup>Δch7</sup>/fg placentae were never equal to those of the WT placentae. This might be due to multiple histological defects in the ng<sup>Δch7</sup>/fg placentae (Fig. 3). Indeed, further correction of the disproportionate growth of both the spongiotrophoblastic layer and labyrinthine zone by the appropriate transcription of the imprinted
genes on chromosome 7 and 12 may be necessary. This could also ensure that the placental masses of the ng WT/fg placentae equal those of the WT placentae. Analysis of the placental phenotypes of double mutant ng/fg oocytes will provide further insight into this.

We additionally found that among the three placental types of bi-maternal placentae, only the ng D ch7/fg placentae had normal giant cells. Middleton et al. (28) also reported the possibility that Igf2 plays an important role in the growth and development of the giant cell tumours of the bone. Our results are consistent with the idea that the level of Igf2 RNA expression is important for the normal formation of trophoblastic giant cells in mouse placentae.

Giant cells contribute to invasiveness and the process of pregnancy by expressing the placental lactogen 1 and proliferin genes (29,30). In addition, oligonucleotide microarray analysis revealed that, at E12.5, the expression of both these genes in the ng WT/fg placentae was greater than twice that in WT placentae (Kawahara et al., unpublished data). In contrast, in the ng D ch7/fg placentae, the expression of these genes was normal. Taken together, these results and the present findings indicate that the expression of Igf2 and H19 on chromosome 7 contributes to the differentiation of functional giant cells.

Roles of the imprinted genes on chromosome 12
The ng D ch7/fg placentae showed abnormal expression levels of the paternally methylated imprinted genes on chromosome 12; these placentae evidently had many gross abnormalities. These anomalous phenotypes were entirely restored in the ng D ch12/fg placentae. The ng D ch12/fg placentae had a restored boundary between the spongiotrophoblast and labyrinthine zone and the ratio of the spongiotrophoblast layer to the labyrinth totally correlated with that of the WT placentae. The trophoblastic giant cells of the ng D ch12/fg placentae, however, remained abnormally expanded. Consequently, these findings suggest that the imprinted genes on distal mouse chromosome 12 regulate a balance between spongiosotropic growth and labyrinthine growth.

Dlk1 is a paternally expressed, protein-coding gene on chromosome 12; this gene encodes a transmembrane protein containing epidermal growth factor repeats and is a member of the Notch/Delta/Serrate family of developmental signalling molecules. This gene is involved in several differentiation processes and is expressed in the fetal endothelial cells of the murine placenta; however, its precise function in the placenta is yet to be determined (5,31–35). We examined the subcellular localization of Dlk1 RNA in the mouse placenta. The Dlk1 RNA was specifically transcribed in the endothelial cells of the fetal blood vessels (Fig. 2B). Therefore, the disrupted fetal blood vessels observed in the ng D ch7/fg placentae might be due to the absence of Dlk1 RNA. In fact, we observed that the ng D ch12/fg placentae was comparable to the WT placental vasculature. However, we cannot rule out the possibility that other genes located on chromosome 12, such as Rtl1 or Dio3, might be involved in placental vasculogenesis. Although placental defects in Dlk1 knockout animals were not described, conceptuses with paternal uniparental disomy for chromosome 12 have defects in the fetal capillary component of the labyrinthine zone (6,36). It is known that Rtl1 is a retrotransposon-like gene with an open-reading frame of unknown function and that Dio3 is a negative regulator of thyroid hormone metabolism; however, the particular roles of both these genes in placentation has not been reported thus far.

Low level of Phlda2 RNA expression in both the ng D ch7/fg and ng D ch12/fg placentae
To understand the reason behind the anomalous expansion of the spongiosotropic layer in the ng WT/fg and ng D ch7/fg
placentae, we investigated the expression pattern of the Phlda2 gene in the four placental types (Fig. 4). It is known that overgrowth along with the expansion of the spongiotrophoblast layer is detected in the Phlda2-null placentae, independent of Igf2 signalling (11). However, all the plental types exhibited low Phlda2 activity, including the ng<sup>ΔCH12/fg</sup> placentae, which did not show the expansion of the spongiotrophoblastic layer.

Phlda2 is a maternally expressed imprinted gene regulated by the Kvdmr1 located in the 10th intron of the Kcnq1 gene; we have confirmed that this methylation begins postnatally in oocytes that have attained a diameter greater than 40 μm (19). Hence, we had expected the level of Phlda2 transcription in the all plental types to be appropriately modified because the oocytes with a diameter less than 20 μm were selected as ng oocytes for nuclear transfer. We have confirmed the monoallelic expression of the Phlda2 gene from the fg-oocyte genome in the ng<sup>WT/fg</sup> placentae at E12.5 (Ogawa et al., submitted for publication). Therefore, the disproportionate expansion of the spongiotrophoblastic layer could not be explained based on the low level of Phlda2 transcription. The reasons for the repression of the Phlda2 gene in ng/fg bi-maternal placentae remain to be elucidated.

From the present study, we could derive the following conclusions (Fig. 6). We confirmed that the deletion of the IG-DMR on chromosome 12 which causes paternalization of the maternal chromosome alone could restore some imbalance imposed by two maternal genomes and facilitate the development of bi-maternal conceptuses to at least E18.5. A combination of our new system and our previous bi-maternal conceptuses production system facilitated a detailed understanding of the contribution of the paternally methylated imprinted genes on chromosomes 7 and 12 towards mouse placentation. The present study provides evidence that imprinted genes transcribed from both the regions, H19-Igf2 and Gtl2-Dlk1, complementarily contribute to mouse placentation. It is probable that the appropriate expression of these imprinted genes would enable ng/fg bi-maternal conceptuses to undergo definitive placentation.

**MATERIALS AND METHODS**

**Production of ng/fg bi-maternal conceptuses**

Fg germinal vesicle (GV) oocytes were collected into M2 medium from the ovarian follicles of B6D2F1 (C57BL/6N×DBA) female mice, 44–48 h after they were injected with equine chorionic gonadotrophin (37). Ovulated MI oocytes were also collected from superovulated B6D2F1 mice, 16 h after they were injected with human chronic gonadotrophin. We collected ng oocytes that were in the diplotene stage of the first meiosis from the ovaries of 1-day-old newborn mice. Serial nuclear transfer was then performed using a previously described method (17,18,22). Ng oocytes derived from WT B6D2F1 (ng<sup>WT</sup>) females, H19ΔA13 null mutants (ng<sup>ΔCH7</sup>) or IG-DMRΔ4.5 heterozygous mutants were reconstructed with enucleated GV oocytes. After fusion with inactivated Sendai virus, the reconstructed oocytes were cultured for 14 h in α-MEM medium (GIBCO, Grand Island, NY, USA). A spindle from the reconstructed oocytes was again transferred into ovulated MII oocytes, followed by treatment with 10 mM SrCl<sub>2</sub> in Ca<sup>2+</sup>-free M16 medium for 2 h. These embryos were cultured in M16 medium for 3.5 days in an atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> at 37°C (38). The embryos that developed to the blastocyst stage were transferred into the uterine horns of recipient female mice at 2.5 days of pseudopregnancy. The placentae were recovered from the pregnant mice at E12.5, E15.5 and E18.5 and used in the subsequent analyses. In order to distinguish the ng<sup>ΔCH12/fg</sup> bi-maternal conceptuses from the ng<sup>WT/fg</sup> bi-maternal conceptuses, we confirmed the deletion in IG-DMR by genotyping yolk sacs isolated from all recovered bi-maternal conceptuses.

**Quantitative gene expression analysis**

Total RNA was extracted using an RNAeasy Mini Kit (QIAGEN K.K., Tokyo, Japan) from the four types of whole placentae: WT placentae derived from fertilized embryos (B6D2F1×C57BL/6N), ng<sup>WT/fg</sup>, ng<sup>ΔCH7/fg</sup> and ng<sup>ΔCH12/fg</sup>. The cDNAs were then synthesized using the SuperScript<sup>™</sup> II RNaseH reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) in a reaction solution (20 μl) containing the total RNA (1 μg) prepared from each plenta. Finally, we performed a quantitative analysis of the gene expression by using real-time PCR (LightCyclerTM System, Roche Molecular Biochemicals, Mannheim, Germany) after preparing a reaction mixture (LightCycler FirstStart DNA Master SYBR Green I, Roche Molecular Biochemicals). The primers used for the analysis were as described in Wu et al. (22). The primers for Phlda2 gene expression analysis were used as follows, sense: 5’-CTT CGA AAA CCG TGA AGA CC-3’ and anti-sense primer: 5’-CCT TGT AAT AGT TGG TGA CGA TG-3’.

**mRNA in situ hybridization**

In order to prepare cryosections, we collected and fixed the four types of placentae at E12.5 with 4% paraformaldehyde, incubated them at 4°C overnight in 20% sucrose in PBS and subsequently embedded them in OCT compound for 10 min at room temperature. This was followed by freezing at −80°C until use. The sections were allowed to dry and were immediately processed for RNA in situ hybridization after slicing. For in situ hybridization, digoxigenin (DIG)-labelled RNA probes were prepared according to manufacturer’s instructions by using a DIG RNA labelling kit (Roche Diagnostics GmbH, Mannheim, Germany). To prepare RNA probes for each gene, a 27mer 14 kb mouse H19 cDNA clone (39) was used. For other probes, the following genes were amplified: Igf2 1346 bp (sense primer 5’-GCT GAC CTC ATT TTC CGA TAC-3’ and anti-sense primer 5’-AAA ATT TTG GTG CDT GCC CCT CT-3’), Git2 (sense primer 5’-AAC CCA CTA CCA TAC AGA GGA-3’ and anti-sense primer 5’-CGA GAG AAT GTG TGA GAC ACG A-3’), and Dlk1 (sense primer 5’-GTG GCG CGC TCG CTT GCT-3’ and anti-sense primer 5’-GAT GTG TGT TCG CCG CTG A-3’). Following amplification, these genes were inserted into a pGEM®-T Easy Vector (Promega). The Phlda2 probe
was prepared as described previously (25). None of these sense control probes produced significant signals.

**Histological and morphometric analyses**

In order to measure the areas of the labyrinthine and spongiosotrophoblast layers, we captured digitized images of the midline paraffin sections stained with haematoxylin/eosin (H&E). Placental entire images were saved as high-quality tif-files and analysed by using the MetaMorph software (Universal Imaging Co., Downingtown, PA, USA). The total number of pixels in each layer was calculated by using the ‘measurement’ tool of the MetaMorph software. Additionally, we analysed the average areas of giant cells at E12.5 and the number of blood vessels within the labyrinth by using PALM Robo Software 2.2-0103 (PALM Microlaser Technologies, AG). We calculated the number of blood vessels by tracing the area surrounding each blood vessel, that is, within 114.550 μm² of the labyrinth. In the case of giant cells, average areas of five giant cells per H&E section from each placental type were calculated.

**Scanning electron microscopy of vascular corrosion casts**

Vascular corrosion casts of the placentae were prepared based on methods described previously (29,40). To prepare casts of the maternal vasculature, pregnant mice at E12.5, E15.5 and E18.5 were anaesthetized and the left ventricle of the beating heart was injected with a heparinized saline solution. An incision that served as an exit point for perfusion was made in the right atrium. Subsequently, the placentae were perfused with Mercox solution (Okenshoji, Tokyo, Japan), a casting compound, by infusion via the left ventricle.

For preparing fetal side casts at E15.5, the pregnant mice were sacrificed by cervical dislocation, and the uterus was excised and immersed in PBS. An implantation site was prepared as described previously (25). None of these casts produced significant signals. For complete hardening, each cast was polymerized in hot water at 60°C for 2 hours, corrodend in 20% KOH, washed overnight in water and air-dried. The air-dried samples were frozen, cracked with a cooled razor blade to observe the internal structure of the labyrinth and then sputter-coated with platinum. Using a SEM (Hitachi S-4000, Tokyo) at low voltage, we examined five to 12 casts of the maternal vasculature and three to five casts of the fetal vasculature obtained from several pregnant mice.

**Statistical analyses**

Statistical analyses of all data for comparison were carried out using analysis of one-way analysis of variance and Fisher’s PLSD test by using the statistical analysis software Statview (Abacus Concepts, Inc., Berkeley, CA, USA). A P-value of <0.05 was considered significant. When depicting statistical significance in the figures, the use of a, b and c indicates that within a gestational stage, a is significantly different from b and c and b is significantly different from c. A value marked as ab is not significantly different from the values marked a or b.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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**REFERENCES**


