Defining Contributions of Paternally Methylated Imprinted Genes at the Igf2-H19 and Dlk1-Gtl2 Domains to Mouse Placentation by Transcriptomic Analysis*¶§

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Parental genome functions in ontogeny are determined by interactions among transcripts from the maternal and paternal genomes, which contain many genes whose expression is strictly dependent on their parental origin as a result of genomic imprinting. Comprehensive recognition of the interactions between parental genomes is important for understanding genomic imprinting in mammalian development. The placenta is a key organ for exploring the biological significance of genomic imprinting. To decipher the unknown roles of paternally methylated imprinted genes on chromosomes 7 and 12 in mouse placentation, we performed a transcriptomic analysis on placentae in three types of bimaternal conceptuses that contained genomes derived from both non-growing and fully grown oocytes. Furthermore, we used the Ingenuity pathway analysis software to predict key networks and identify functions specific to paternally methylated imprinted genes regulated by the Igf2-H19 imprinting control region and Dlk1-Dio3 imprinting control region. The data suggested that dynamic conversion of the gene expression profile by restoring the expression of paternally methylated imprinted genes resulted in phenotypic improvements in bimaternal placentae. These results provide a framework to further explore the role of epigenetic modifications in paternal genome during mouse placentation.

In mammals, embryonic development and other phenotypic characteristics are influenced by the genetic constitution of zygotes. Maternal and paternal genetic contributions are usually distinctive. However, certain differences are present in the epigenetic modifications of some parental alleles, which lead to functional differences between certain homologous chromosomal regions. Genomic imprinting results in functional non-equivalence of the maternal and paternal genomes, thereby preventing the development of viable parthenotes in eutherian mammals. Usually, parthenotes die before embryonic day 9.5 (E9.5)² and have poorly developed extraembryonic tissues (1, 2). Therefore, genomic imprinting has been regarded as an obligatory step in mouse placentation.

In mice, an extremely small number of primordial germ cells, i.e. cells from which germ cells originate, first differentiate from pluripotent epiblast cells at around day 6.5 of gestation (3, 4). The primordial germ cells acquire epigenetic modifications that are differentially imposed during spermatogenesis and oogenesis (5, 6). The paternal- or maternal-specific DNA methylation at differentially methylated regions associated with imprinted genes regulates their allele-specific expression. This leads to decisive functional differences between the maternal and paternal genomes; thus, both parental genomes are required for normal development to term in mammals. It is known that Dnm3L cooperates with Dnmt3a and Dnmt3b to methylate DNA de novo (7). Studies on the extraembryonic tissues of Dnmt3L mat−/− embryos have revealed that appropriate paternal methylation of imprinted genes is absolutely essential for mouse placentation (8). Additionally, in our previous study, we showed that when non-growing oocytes of wild-type mice (WT) (ngWT), in which imprints are not established, were combined with fully grown (fg) oocytes, the resulting ngWT/fg bimaternal conceptuses could have placentae comprising three basic layers: the trophoblastic giant cells, spongiosplrophoblast layer, and labyrinthine layer (9). However, the ngWT/fg placenta showed diagnostic defects such as severe growth retardation, disproportionate expansion of the spongiosplrophoblast layer, distorted and ambiguous boundary between the spongiosplrophoblast and labyrinthine layers, and the presence of abnormally enlarged giant cells (10). This probably occurs because the maternal epigenotype on chromosomes 7 and 12, the Igf2-H19 imprinting control region (ICR) and Dlk1-Dio3 ICR, causes inappropriate expression of the imprinted genes.

To further understand the roles of paternally methylated imprinted genes regulated by Igf2-H19 ICR and Dlk1-Dio3 ICR in mouse placentation, we have analyzed the ng/fg bimaternal placentae that were switched from the maternal to the paternal epigenotype in the imprinted domain at the distal regions on chromosomes 7 and 12 (10). The genetic backgrounds of these ng oocytes were as follows. One was derived from mutant mice carrying a 13-kb deletion in the H19 transcription unit including the germline-derived differentially methylated region on chromosome 7 (ngΔH19). Another set of oocytes was derived

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2 The abbreviations used are: E, embryonic day; WT, wild type; fg, fully grown; ng, non-growing; ICR, imprinting control region; IPA, Ingenuity pathway analysis; GCOS, GeneChip operating software; PCA, principal component analysis; RT-PCR, real-time PCR.
from mutant mice carrying a 4.15-kb deletion in the intergenic germline-derived differentially methylated region on chromosome 12 (ng\textsuperscript{ch12}) (11, 12). Interestingly, ng\textsuperscript{ch/fg} and ng\textsuperscript{ch12/fg} placentae showed reciprocal phenotypes, i.e. the ng\textsuperscript{ch/fg} placenta exhibited severe dysplasia, such as an expanded spongiotrophoblast layer and a malformed labyrinthine zone, but exhibited normal-sized giant cells. In contrast, the cell layers in the ng\textsuperscript{ch12/fg} placenta retained their normal structure with a normal circulatory system, but its total mass was extremely low and expanded giant cells were present. These findings strongly suggested that investigating the ng/fg bimaternal placenta was a powerful means of defining paternal genetic contribution to mouse placentation and for understanding how paternally methylated imprinted genes controlled by IGF2-H19 ICR and Dlk1-Dio3 ICR complementarily organize placentation. The manner in which the appropriate expression of imprinted genes regulated by these two ICRs leads to definitive placentation in bimaternal conceptuses is unclear, and the global gene expression dynamics underlying bimaternal placentation also remain to be elucidated.

Recently, we used ng oocytes of double mutant mice (ng\textsuperscript{Double}) that harbored deletions in both the IGF2-H19 ICR and the Dlk1-Dio3 ICR to demonstrate the generation of bimater- nal embryos that develop as normal individuals at a high success rate equivalent to that obtained by in vitro fertilization of normal embryos (13). This result was obtained by the expression of imprinted genes regulated by IGF2-H19 ICR and Dlk1-Dio3 ICR, which was appropriately corrected in the ng\textsuperscript{Double/fg} bimaternal embryo. In this study, we determined the placenta-forming ability of the ng\textsuperscript{Double/fg} bimaternal embryo and further investigated the genes that are dominated by the paternally methylated imprinted genes controlled by IGF2-H19 ICR and Dlk1-Dio3 ICR. First, we used the same methods as those described in our previous report to evaluate the phenotypic characteristics of the ng\textsuperscript{Double/fg} placenta (10). Next, we compared the global gene expression profiles of four placenta types, namely, the ng\textsuperscript{ch7/fg}, ng\textsuperscript{ch12/fg}, ng\textsuperscript{Double/fg}, and WT placentae (Fig. 1). Finally, for in-depth investigation into the roles of the corrected expression level of paternally methylated imprinted genes regulated by IGF2-H19 ICR and Dlk1-Dio3 ICR in the mouse placenta, we conducted the Ingenuity Pathway Analysis (IPA) to predict the key networks of genes that function during mouse placentation.

In this study, we observed that the ng\textsuperscript{Double/fg} placenta showed remarkably improved phenotypes in comparison with the ng\textsuperscript{ch7/fg} and ng\textsuperscript{ch12/fg} placentae. Additionally, comparative global gene expression analyses of the ng\textsuperscript{Double/fg} placenta and the other bimater- nal placentae, i.e. the ng\textsuperscript{ch7/fg} and ng\textsuperscript{ch12/fg} placentae, enabled us to predict the functions of paternally methylated imprinted genes regulated by IGF2-H19 ICR and Dlk1-Dio3 ICR in the mouse placenta. They demonstrated that the restored gene expression of paternally methylated imprinted genes on chromosomes 7 and 12 led to widespread alteration of the expression of other genes and remarkable improvements in the placental phenotypes. Thus, these global gene expression profiles would be useful for defining paternal genetic contribution regulated by IGF2-H19 ICR and Dlk1-Dio3 ICR to mouse placentation.

Oocyte Manipulations—Fully grown germlinal vesicle oocytes from the ovarian follicles of B6D2F1 (C57BL/6N × DBA2) female mice were collected in the M2 medium 44–48 h after the mice were injected with equine chorionic gonadotropin. Ovulated MII oocytes were also collected from superovulated B6D2F1 mice 16 h after they were injected with human chronic gonadotropin. 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 performed using a previously described method (14). The ng oocytes derived from double mutant females with heterozygous \( \Delta \text{ch7} (-/+) \) and heterozygous \( \text{Ach12} (+/-) \) were fused with enucleated germlinal vesicle oocytes. After fusion with inactivated Sendai virus, the reconstructed oocytes were cultured for 14 h in α-minimum essential medium (Invitrogen). A spindle from the reconstructed oocytes was again transferred into the ovulated MII oocytes and treated with 10 mM SrCl\(_2\) in Ca\(^{2+}\)-free M16 medium for 2 h. Bimaternal embryos of four different genotypes were obtained: the ng\textsuperscript{WT/fg}, ng\textsuperscript{ch7/fg}, ng\textsuperscript{ch12/fg}, and ng\textsuperscript{Double/fg} embryos (13). These embryos were cultured in the M16 medium at 37 °C for 3.5 days in an atmosphere containing 5% CO\(_2\), 5% O\(_2\), and 90% N\(_2\). 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 and used in subsequent microarray and morphometric analyses. All experiments were carried out on three individual placentae of each class: the ng\textsuperscript{ch7/fg}, ng\textsuperscript{ch12/fg}, ng\textsuperscript{Double/fg} bimaternal, and WT placentae. Genotyping of the bimaternal conceptuses was performed with the use of their yolk sacs, as reported previously (13).

Histological and Morphometric Analyses—We measured the areas of the labyrinthine and spongiotrophoblast layers using our previously described method (10). Briefly, we captured digitized images of the midline paraffin sections stained with hematoxylin/eosin. Placental entire images were saved as high quality TIF files and analyzed by the MetaMorph software (Universal Imaging Co., Downingtown, PA). The total number of pixels in each layer was calculated with the measurement tool of this software. Additionally, we analyzed the average areas of giant cells and the number of blood vessels within the labyrinth using the PALM Robo software 2.2.0103 (P.L.M. Microlaser Technologies AG). We calculated the number of blood vessels by tracing the area surrounding each blood vessel, i.e. within 114,550 \( \mu \text{m}^2 \) of the labyrinth. In the case of giant cells, the average areas of five giant cells per hematoxylin/eosin section from each placental type were calculated. Morphological analyses were carried out at the end point of mouse gestation, namely E19.5, because the phenotypical gaps between the WT and the bimaternal placenta trended to expand with the advance of gestation (10).

Microarray Analysis—We used a commercially available acid-phenol reagent (TRIzol; Invitrogen) for total RNA extraction from four types of whole placentae, ng\textsuperscript{ch7/fg}, ng\textsuperscript{ch12/fg}, ng\textsuperscript{Double/fg} bimaternal, and WT, at E12.5. Transcriptomic analysis was performed at E12.5, when all types of the bimaternal-
Contributions of Imprinted Genes to Mouse Placentation

FIGURE 1. Experimental design. First of all, we precisely investigated that the ng<sub>ADouble</sub>/fg placenta is phenotypically identical to the WT placenta followed by transcriptomic analysis. a, investigation of differentially expressed genes in the ng<sub>ADouble</sub>/fg placenta relative to those in the WT placenta may lead to normality verification of the ng<sub>ADouble</sub>/fg placenta (Table 1; Fig. 3). b, differentially expressed genes in the ng<sub>ADouble</sub>/fg placenta relative to those in the ng<sub>ADouble</sub>/fg placenta may be associated with peculiar disorders to the ng<sub>ADouble</sub>/fg placenta, suggesting biological functions of paternally methylated imprinted genes on chromosome 12 in placentation (Tables 2 and 4; Figs. 3, 4, 6, and 7). c, similarly, differentially expressed genes in the ng<sub>ADouble</sub>/fg placenta relative to those in the ng<sub>ADouble</sub>/fg placenta may be associated with peculiar disorders to the ng<sub>ADouble</sub>/fg placenta, suggesting biological functions of paternally methylated imprinted genes on chromosome 7 in placentation (Tables 3 and 5; Figs. 3, 5, 6, and 8).

Results and Discussion

Total RNA preparation was carried out as described in microarray analysis. The cDNAs were then synthesized using the SuperScript<sup>TM</sup> III RNase H reverse transcriptase kit (Invitrogen) in a reaction solution (20 μl) containing the total RNA (1 μg) prepared from each placenta. Finally, we performed a quantitative analysis of the gene expression by using real-time PCR (7500 real-time PCR system, Applied Biosystems) after preparing a reaction mixture (SYBR<sup>®</sup> GREEN PCR master mix, Applied Biosystems). The primers used for the analysis were as described in supplemental Table 1. Ten transcripts were selected from the results of the array expression analysis (see Figs. 7 and 8) for verification by real-time RT-PCR; however, the expression level of the Pla2g2c gene was below the detection limit of this method of analysis.

Statistical Analyses—Data on the mRNA expression level by real-time RT-PCR were examined using analysis of one-way analysis of variance and the Fisher’s protected least significance difference test by using the statistical analysis software Statview (Abacus Concepts, Inc., Berkeley, CA). A p value of <0.05 was considered significant.

RESULTS AND DISCUSSION

Definitive Placentation of ng<sub>ADouble</sub>/fg Bimetalar Conceptuses—As stated previously, we observed reciprocal defects in the ng<sub>Δch7</sub>/fg and ng<sub>Δch12</sub>/fg placenta (10). Furthermore, we found that the weight of the ng<sub>ADouble</sub>/fg placenta, which could
Contributions of Imprinted Genes to Mouse Placentation

a) Labyrinthine layer (Lab) vs Spongiotrophoblastic layer (Spo)

b) Ratio (Spo/Lab)

c) Size of giant cells

d) Average area of vascular

wt

bi-maternal
support term development of bimaternal fetuses, was equivalent to that of the WT placenta (13); however, in-depth phenotypic studies are required to confirm this. In this study, we observed for the first time that the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta showed proportionate ratios of the spongiotrophoblast and labyrinthine layers (Fig. 2, a and b) with normal-sized giant cells (Fig. 2, c and e). Furthermore, the average area of the vasculature in the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta was similar to that of the WT placenta (Fig. 2d), indicating that the circulatory system of the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta was normal. In the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta, malformations specific to the \( \text{ng}^{\text{ch7}}/\text{fg} \) and \( \text{ng}^{\text{ch12}}/\text{fg} \) placentae were entirely restored, as typified by the distinct boundary between the spongiotrophoblast and labyrinthine layers (Fig. 2e). These results demonstrate that the \( \text{ng}^{\text{Double}}/\text{fg} \) bimaternal conceptuses have a definitive placenta with regard to the competency to support both term development and morphogenesis. Therefore, comparison of the gene expression profiles of the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta and other bimaternal placentae, namely, the \( \text{ng}^{\text{ch7}}/\text{fg} \) and \( \text{ng}^{\text{ch12}}/\text{fg} \) placentae that showed reciprocal defects (10), may provide further insight into the roles of paternally methylated imprinted genes on chromosomes 7 and 12 in mouse placenta.

Global Gene Expression Analysis of the Bimaternal Placentae—Global gene expression analysis was performed using a microarray method to investigate the underlying alterations in gene expression from defective placenta in the \( \text{ng}^{\text{ch7}}/\text{fg} \) and \( \text{ng}^{\text{ch12}}/\text{fg} \) conceptuses to normalized placenta in the \( \text{ng}^{\text{Double}}/\text{fg} \) conceptuses. We constructed hierarchical clustering with all the transcripts on the Genome 430 2.0 chip set using microarray data from the WT, \( \text{ng}^{\text{ch7}}/\text{fg} \), \( \text{ng}^{\text{ch12}}/\text{fg} \), and \( \text{ng}^{\text{Double}}/\text{fg} \) placentae (\( n = 3 \)) (Fig. 3a). As expected, each placental type was clustered depending on the genetic background, suggesting that the results reflected the gene expression pattern specific to each ng/fg bimaternal placental type. The validity of this clustering was supported by the principal component analysis (PCA) (x axis, PCA component 1, 83.83% variance; y axis, PCA component 2, 4.575% variance; z axis, PCA component 3, 3.984% variance). The results indicated that among the three variances, the x axis showed the most significant variance (Fig. 3b). In the x-y plane, the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta was relatively closer to the WT placenta, whereas the \( \text{ng}^{\text{ch7}}/\text{fg} \) and \( \text{ng}^{\text{ch12}}/\text{fg} \) placentae almost coincided with each other. Both of them were distant from the WT placenta, indicating that the expression pattern of the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta was comparatively closer to that of the WT placenta than to those of the \( \text{ng}^{\text{ch7}}/\text{fg} \) and \( \text{ng}^{\text{ch12}}/\text{fg} \) placentae. Thus, the concordance between the results from the hierarchical cluster analysis and PCA indicated that the global gene expression pattern of the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta was closer to that of the WT placenta than to those of the other bimaternal placentae, i.e. the \( \text{ng}^{\text{ch7}}/\text{fg} \) and \( \text{ng}^{\text{ch12}}/\text{fg} \) placenta.

Next, one-way analysis of variance post hoc testing results after GeneSpring normalization revealed that in terms of the number of genes that were significantly expressed, there was a smaller difference between the WT and \( \text{ng}^{\text{Double}}/\text{fg} \) placentae than between the WT and other bimaternal placentae (Fig. 3c). We also prepared a list of genes that were differentially expressed in the \( \text{ng}^{\text{Double}}/\text{fg} \) and WT placentae. Of the transcripts that were significantly altered in the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta, 2 and 13 genes were up- and down-regulated by at least 2-fold each (Table 1). However, the biological function of most of these genes in the placenta is unknown; for example, Serpinb1 was up-regulated by more than 6-fold in the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta. This gene encodes an efficient inhibitor of neutrophil serine proteases and is required to protect the neutrophil and collectin surfactant protein-D from excess neutrophil serine proteases in the lung (16). However, the function of this gene in the placenta is unknown. Furthermore, Dnmt3a was down-regulated by more than 2-fold in the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta; its gene product functions as a de novo methyltransferase that is essential for normal development (17). Down-regulation of Dnmt3a by \( \approx 2 \)-fold is likely to cause little change in early mouse placentation, and this observation was supported by the viability of mutants carrying heterozygously deleted Dnmt3a from E8.5 to E15.5 (17). However, at present, the reason for Dnmt3a down-regulation is unclear. Nevertheless, by analogy with the phenotypic similarity between the WT and \( \text{ng}^{\text{Double}}/\text{fg} \) placentae, genes that were altered in the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta including Serpinb1 and Dnmt3a do not appear to be involved in placental morphogenesis, at least, with regard to the phenotypes examined in Fig. 2.

Furthermore, 138 and 159 genes in the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta were differentially expressed as compared with those in the \( \text{ng}^{\text{ch7}}/\text{fg} \) and \( \text{ng}^{\text{ch12}}/\text{fg} \) placentae, respectively. Using these genes, we constructed a Venn diagram with the Gene Spring software, and gene lists that represented differentially and specifically expressed genes in the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta relative to the \( \text{ng}^{\text{ch7}}/\text{fg} \) and \( \text{ng}^{\text{ch12}}/\text{fg} \) placentae were prepared (Fig. 3d). Identifying differentially expressed genes in the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta relative to those in the \( \text{ng}^{\text{ch7}}/\text{fg} \) and \( \text{ng}^{\text{ch12}}/\text{fg} \) placentae may assist in elucidating the peculiar roles played by paternally methylated imprinted genes on chromosomes 7 and 12 in mouse placentation. The results indicated that between the \( \text{ng}^{\text{Double}}/\text{fg} \) and the \( \text{ng}^{\text{ch7}}/\text{fg} \) placenta (ch12 gene list), 96 differentially expressed genes were present, whereas between the \( \text{ng}^{\text{Double}}/\text{fg} \) and the \( \text{ng}^{\text{ch12}}/\text{fg} \) placenta, 117 differentially and specifically expressed genes were present (ch7 gene list) (Fig. 3d).

Complementary Functions of Paternally Methylated Imprinted Genes on Chromosomes 7 and 12 in Mouse Placentation—To understand the biological and molecular processes represented in the ch12 and ch7 gene lists, we per-

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**FIGURE 2. Morphometric analyses of the WT and \( \text{ng}^{\text{Double}}/\text{fg} \) placentae (\( n = 3 \)).** a and b, spongiotrophoblast layer (Spo), labyrinthine layer (Lab) (a), and spongiotrophoblast layer/labyrinthine layer (Spo/Lab) ratios (b) in the WT and \( \text{ng}^{\text{Double}}/\text{fg} \) placentae. Error bars represent mean ± S.E. c, the average areas of giant cells were also calculated. d, the average areas of the vasculature in the WT and \( \text{ng}^{\text{Double}}/\text{fg} \) placentae were investigated by tracing the surrounding area of both maternal and fetal blood vessels within 114,550 μm² of the labyrinth. Error bars indicate means ± S.E. e, midline placental sections (hematoxylin/eosin) at E19.5. The left panels present an overview of the WT and \( \text{ng}^{\text{Double}}/\text{fg} \) placentae, and their magnified images are shown in the center. Note that the boundary in the \( \text{ng}^{\text{Double}}/\text{fg} \) placenta is entirely restored. The right panels show high magnification views of giant cells (yellow arrowsheads) adjacent to the spongiotrophoblast layer in the WT and \( \text{ng}^{\text{Double}}/\text{fg} \) placentae.
formed IPA. On the basis of their known biological relationships, which have been described previously (13, 18–20), the genes in these two lists were integrated into the IPA pathways. The result of the integration showed that six networks were produced from the ch12 and ch7 gene lists (Tables 2 and 3; Figs. 4 and 5). In the ch12 gene list, we investigated the biological functions of six networks that resulted from globally altered gene expression due to paternalization of the gene expression pattern in the Dlk1-Dio3 region in the ng\textsuperscript{\text{Double/fg}} placenta; this expression pattern differed from that in the ng\textsuperscript{\text{ch7/fg}} placenta (Table 2; Fig. 4). This list of genes includes members of gene families that have been identified in previous studies and genes involved in the cell cycle, organ morphology, cellular development, etc. In the ch7 gene list, the biological functions of the six constructed networks largely showed genes involved in the cell cycle, carbohydrate metabolism, and cellular growth.

**FIGURE 3.** Global gene expression analyses of four types of placentae: WT, ng\textsuperscript{-h7/fg}, ng\textsuperscript{-hch12/fg}, and ng\textsuperscript{\text{Double/fg}} bimaternal placentae (n = 3). a, hierarchical clustering of all the samples from different genetic backgrounds. Colors correspond to the relative RNA abundance for more than 39,000 transcripts. b, principal component analysis of gene expression in all the groups subjected to the hierarchical clustering analysis. c, one-way analysis of variance post hoc testing analysis of the following four placenta types: WT, ng\textsuperscript{-h7/fg}, ng\textsuperscript{-hch12/fg}, and ng\textsuperscript{\text{Double/fg}} placenta. Each box shows the number of genes that are statistically similar (green) or different (red) in a group-to-group comparison. The intensity of the green and red colors indicates whether the value is more or less than the sum of the gene number. d, Venn diagrams constructed with the GeneSpring software show genes that were differentially and specifically expressed in the ng\textsuperscript{\text{Double/fg}} placenta in comparison with the ng\textsuperscript{\text{h7/fg}} (red, ch12 gene list) and the ng\textsuperscript{\text{hch12/fg}} (blue, ch7 gene list) placenta individually. These gene lists were integrated into the IPA pathways (Figs. 4 and 5).
Contributions of Imprinted Genes to Mouse Placentation

TABLE 1
Genes showing a 2-fold or higher expression difference between the WT and ngADouble/fg placenta

| Gene      | Description                                      | Fold change | Networks | Location           |
|-----------|--------------------------------------------------|-------------|----------|--------------------|
| Serpinb1  | Serpin peptidase inhibitor, clade B (ovalbumin), member 1 | 6.342       | 6        | Cytoplasm          |
| Il18      | Interleukin 18 (interferon-γ-inducing factor)     | 2.113       | 5        | Extracellular space|
| Dhar24    | 24-Dehydrocholesterol reductase                  | -2.008      | 4        | Cytoplasm          |
| Dmnt3a    | DNA (cytosine-5-)methyltransferase 3-α           | -2.062      | 2        | Nucleus            |
| Rcrel     | Rq protein-like (DNA helicase Q1-like)            | -2.09       | 1        | Nucleus            |
| Zbtb7a    | Zinc finger and BTR domain containing 7A          | -2.099      | 1        | Nucleus            |
| Fblm1     | Filamin-binding LIM protein 1                     | -2.12       | 8        | Plasma membrane    |
| Jarid1a    | Jumonji, AT-Rich interactive domain 1A            | -2.156      | 4        | Nucleus            |
| Tjap2     | Tjap2 (TCDD-inducible poly(ADP-ribose) polymerase) | -2.21       | 9        | Unknown            |
| Uhf1      | Ubiquitin-like, containing PHD and RING finger domains, 1 | -2.234      | 2        | Nucleus            |
| Rgs5      | Regulator of G-protein signaling 5                | -2.337      | 6        | Plasma membrane    |
| Polr1a    | Polr1a (RNA 1) polypeptide A, 194 kDa            | -2.355      | 1        | Nucleus            |
| Rtn1      | Rtn1                                              | -2.39       | 9        | Cytoplasm          |
| Cdc25a    | Cell division cycle 25 homolog A (Schizosaccharomyces pombe) | -2.606      | 1, 8, 11 | Nucleus            |
| Lrrc4     | Leucine-rich-repeat-containing 4                  | -2.98       | 16       | Unknown            |

and proliferation (Table 3). In addition, as expected, Dlk1 and Igf2 were incorporated into network 3 in Table 2 (Fig. 4) and networks 1 and 4 in Table 3 (Fig. 5). The biological functions of network 3 in Table 2 were cellular development, connective tissue development, and functional and developmental disorders; this result is consistent with our previous observations of severe dysplasia in the ngAch7/fg placenta (10). Genes in networks 1 and 4 in Table 3 are involved in the cell cycle (10), cancer, reproductive system diseases, cellular movement, and cell death. Cell cycle and cell death may be responsible for the phenotypes of the ngAch7/fg placenta, namely, extremely low placental mass and the presence of expanded giant cells. Furthermore, the constructed networks were bound in the commonly appearing genes as shown in Fig. 6. Thus, the present transcriptomic study suggests that Dlk1 and Igf2 play pivotal roles in improving the placentation of bimaturel conceptuses. Both genes appear to have propagating effects on other multiple genes involved in mouse placentation.

Further Investigations into the ch12 and ch7 Gene Lists—We also screened the genes in the ngADouble/fg placenta whose differential expression was more than 2-fold that of the genes in the ngAch7/fg and ngAch12/fg placenta (Tables 4 and 5). Furthermore, to evaluate the definite effects of paternalization in the Dlk1-Dio3 and Igf2-H19 regions, we noted the experimental variability after determining the expression levels in three microarray trials (Figs. 7 and 8). If the expression level of a given gene remains within the range between the levels of the WT and ngADouble/fg placenta, the gene should be regarded as being within the range of normality. This is because genes that are differentially expressed between the WT and ngADouble/fg placenta are apparently not directly/indirectly associated with mouse placentation, at least in placental morphogenesis analyzed in this study (Fig. 2).

In comparison with the ngAch7/fg placenta, the most altered gene in the ngADouble/fg placenta was Dlk1 (Table 4; Fig. 7). This gene is paternally expressed in the endothelial cells of fetal
**Conclusions of Imprinted Genes to Mouse Placentation**

**TABLE 3**

 Networks generated by IPA for transcripts that were specifically and differentially expressed in the ng^{Double}/fg placenta in comparison with the ng^{ch7}/fg placenta.

Statistically significant genes were specifically and differentially expressed in the ng^{Double}/fg placenta in comparison with the ng^{ch7}/fg placenta (ng^{Double}/fg versus ng^{ch7}/fg). These were used as the input in the IPA analysis.

| ID | Molecules in network | Score | Focus molecules | Top functions |
|----|----------------------|-------|-----------------|--------------|
| 1  | Aldh1a3, Aurka, Bir2c, Ccbl1, Cen1, Cdes2, Cda8, Cepha, Chafl, Cyclin A, Cyclin B, Cyclin E, E2F, Elaf1, Fosm1, Ghr, Histone H3, Id2, Igf2, Igfbp7, Ikb, Igbkd1, Lamp2, Mcm10, M6hd11, Myh2, Njfb, Pde4b, Pdgf, Palinin1, Prm1, R19b, Satb1, Tbx1 | 55   | 26              | Cell cycle, cancer, reproductive system disease |
| 2  | Afpap1, Akt, Calpain, Caspase, Cd47, Cd200, Cc11, Ccxl10, Dusp1, F Actin, Gas2, H2a2, Ier3, Il11, Insulin, Jnk, Lepnp, Mapk, Mmp11, P38, MAPK, Pkk1, Pkap1, Pof6b, Ppek, Ppina1, Psmsc1p, Rac, Racs, Rcs16, RhoB, Sh2b, Sh3gb1, Tgfb, Tbx1, Vegf | 24   | 14              | Carbohydrate metabolism, immune response, small molecule biochemistry |
| 3  | Ap1m2, Cts2, Dccl, Egr, Erbb2, Ereg, Feni, Gas1, Hmnr, Iid1, Ier2, Ier3, Myc, Ndry, Njfb, Nofa1, Nrg1, Pena, Polg, Pou3f1, Prim1, Piptr, Purb, Rod5s1, Rre4, Rgs6, Rrm2, Sglh, Shnt1, Smn1, Srv, Synfl5, Tgf4, Tps53, Zfp4 | 24   | 14              | Cellular growth and proliferation, DNA replication, recombination, and repair, cancer |
| 4  | Areg, Cdt2, Dihydrotestosterone, Dtx3, Ephi4b, Eli, Ghr, Gw4d1, Hhegf, Hgf, Ifng, Igf2, Ighp4, Il6r, Kif22, Mnp1, Mybl2, Ncor1, Nr6a1, P70 S6k, Pim1, Epa1, progestosterone, Pigs2, Rapp5, RhoB, Sepp1, Sc1a5, Sc32a1, Sles4a1, Tmp1, Tgipar, Tmp13, Uimc | 22   | 13              | Cancer, cellular movement, cell death |
| 5  | Akt1, Bcl, Cisorf3, Cdc2, Cdc6, Cdc8, Cdx1a, Chafl, Cts2, E2f1, Fen1, Gadd45a, Gsk3b, Il11, Il13, Il13ar1, Kif5b, Kitlg (includes Egr2-544), Kbb2, Mamm3, Mcm2, Monc, Mmm1, Mybl2, Nin, Paccs2, Pim1, Rad51, Rad54l, RhoB, Rps6kl1, Rrm1, Sirt2, Thra | 20   | 12              | Cancer, cell death, cell cycle |
| 6  | Aqp3, Cdk2a1, Dusp1, Ep300, Feni, Gart, Ghr, Grn, Ie, Idh1, Ier1, Ier2, Ier3, Jf47, Lgfas, Lgfbp1, Lgfbp4, Lh, Ins, Ins7, Lep, Lmpc1, Mmp1, Myog, Njfb, Nrc3c1, P70 S6k, Prkab2, Rrm2, Sgp1, Tacc2, Tbp, Thra, Tk, Trim50, Ulf1 | 16   | 10              | Gene expression, organismal development, DNA replication, recombination, and repair |

blood vessels and is present on chromosome 12. It encodes a transmembrane protein containing epidermal growth factor repeats and is a member of the Notch/Delta/Serrate family of developmental signaling molecules. It 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 (21–26).

After Dlk1, the gene that was most up-regulated and showed more than a 9-fold change in the ng^{Double}/fg placenta was *Serpin6* (Fig. 7). This gene encodes an α-globulin protein with corticosteroid binding properties and is the major transport protein for glucocorticoids and progestins in the blood of most vertebrates. The function of *Serpin6* in the placenta remains unknown; however, it is known that its corresponding protein localizes to the placenta, particularly to the maternal and fetal blood vessels (27). A low level of *Serpin6* expression may be a possible cause of vascular defects in the ng^{ch7}/fg placenta. In contrast, the *Synec2* mRNA was overexpressed in the ng^{ch7}/fg placenta. In humans, the *SYNE2* mRNA is expressed in vascular smooth muscle cells and skeletal muscle cells, which are associated with the sarcoplasmic reticulum, and it may provide a network of scaffolds that spatially orient the myofibrils, sarcoplasmic reticulum, and cytoskeleton (28). However, there are no further details on the specific functions of *SYNE2*. *SYNE2* transcription is positive in the mouse placenta (29), but further studies are required to determine whether *SYNE2* overexpression in the ng^{ch7}/fg placenta is involved in a spectrum of placental vascular and angiogenic defects.

In the ch7 gene list, *Igf2* was the most altered gene in the ng^{Double}/fg placenta in comparison with the ng^{ch12}/fg placenta. This gene encodes a member of the insulin family of polypeptide growth factors, which is involved in placental development and growth (30) (Table 5; Fig. 8). It is an imprinted gene on chromosome 7 and is expressed only from the paternally inherited allele in the placenta and fetal tissues, excluding the brain (31). *Igf2* mRNA expression level in the ng^{Double}/fg placenta was equivalent to that in the WT placenta (Fig. 8) and, therefore, the normal-sized ng^{Double}/fg placenta was attributed to restored *Igf2* mRNA expression. In our previous study, the ng^{ch7}/fg placenta consistently showed a greater mass than the ng^{ch12}/fg placenta; however, these masses were significantly lesser than that of the WT placenta (10). In the ng^{ch7}/fg placenta, the *Igf2* mRNA expression level did not reach the range between the levels of the WT and ng^{Double}/fg placenta, although it was much higher than the levels of the ng^{ch12}/fg placenta. This suggests that appropriate *Igf2* expression requires proper expression of paternally methylated imprinted genes on chromosome 12. The appropriate expression pattern of paternally methylated imprinted genes on chromosome 12 leads to normal differentiation of the trophoblast, resulting in orderly histogenesis (10). This histological normality may be one of the conditions required for appropriate *Igf2* mRNA expression in the placenta. The slight hypoplasia of the ng^{ch7}/fg placenta with a disproportionally layered structure suggests that the mass of the ng^{ch7}/fg placenta was equivalent to that in the WT placenta (Fig. 8) and, therefore, the normal-sized ng^{Double}/fg placenta was attributed to restored *Igf2* mRNA expression. In our previous study, the ng^{Double}/fg placenta in comparison with the ng^{ch7}/fg placenta was equivalent to that in the WT placenta (Fig. 8) and, therefore, the normal-sized ng^{Double}/fg placenta was attributed to restored *Igf2* mRNA expression. In our previous study, the ng^{Double}/fg placenta in comparison with the ng^{ch7}/fg placenta was equivalent to that in the WT placenta (Fig. 8) and, therefore, the normal-sized ng^{Double}/fg placenta was attributed to restored *Igf2* mRNA expression.

After *Igf2*, the genes that were up-regulated by more than 2-fold in the ng^{Double}/fg placenta were *Pla2g2c*, *Tacc2*, and *Tnn1* (Fig. 8), although the biological functions of these genes in the placenta have not yet been established. Meanwhile, *Aldh1a3* and *Cxc10* were down-regulated by more than 2-fold in the ng^{Double}/fg placenta. The function of *Aldh1a3* in the placenta is also unknown, but it is known that *Cxc10* encodes a chemokine of the CXC subfamily, and the binding of this pro-
Contributions of Imprinted Genes to Mouse Placentation

Network 1

Network 2

Network 3

Network 4

Network 5

Network 6

FIGURE 4. Functional networks of genes that are differentially and specifically expressed in the ng^ΔDouble/fg placenta in comparison with the ng^ΔWT/fg placenta. Genes marked in red and green are up-regulated and down-regulated, respectively. The network is displayed graphically as nodes (gene or gene product) and edges (the biological relationships between nodes, including the functional or physical interactions. For example, A, activation/deactivation; RB, regulation of binding; PR, protein-mRNA binding; PP, protein-protein binding; PD, protein-DNA binding; B, binding; E, expression; I, inhibition; L, proteolysis; M, biochemical modification; O, other; P, phosphorylation/dephosphorylation; T, transcription; Lo, localization). The numbers under a node represent the -fold change. The shape of the objects (e.g. circle and diamond) indicates whether the protein is a structural protein, transcription factor, etc. (refer to supplemental Fig. 1).
Contributions of Imprinted Genes to Mouse Placentation

FIGURE 5. Functional networks of genes that are differentially and specifically expressed in the ng<sup>Double</sup>/fg placenta in comparison with the ng<sup>Double</sup>/fg placenta (refer to the legends for Fig. 4 and supplemental Fig. 1).
tein to CXCR3 results in pleiotropic effects. Additionally, Cxcl10 overexpression stimulates cell proliferation (32, 33), which may be associated with the gain of placental mass. In fact, we had observed that the placental mass in the ngMash1/fg conceptus increased as compared with that in the ngWT/fg conceptus. Therefore, this result indicates that up-regulation of Cxcl10 compensates for the gain in placental mass in the ngMash1/fg conceptus lacking Igf2 expression. However, in the ngMash Double/fg placenta, Cxcl10 overexpression was entirely restored. This is attributed to the fact that Igf2 transcription level in the ngMash Double/fg placenta is normalized.

**Validation of the Microarray Data**—Ten transcripts were selected from the array expression profile to validate the microarray data by quantitative RT-PCR (Fig. 9). Among them, nine transcripts were detectable as follows: Dlk1, Serpin6a, Tram2, Syn2, Igf2, Tacc2, Tnx, Aldh1a3, and Cxcl10. The expression level of the Pla2g2c gene was undetectable with little expression; therefore, we could not verify the further analysis by quantitative RT-PCR. The expression patterns of Dlk1 and Igf2 genes were identical to those by the microarray expression analysis (Fig. 9, a and b). Furthermore, in the ch12 gene list, the Serpin6a and the Syn2 genes were significantly down- and up-regulated in the ngMash/fg placenta as expected (p values; 0.0061 and 0.0258, respectively) (Fig. 9a). In the ch7 gene list, the abnormal expression patterns of the Tacc2, Aldh1a3, and Cxcl10 RNAs in the

**FIGURE 6. Overlapping networks constructed from the gene lists.** a, ch12 gene list; b, ch7 gene list. Each constructed network is bound in commonly appearing genes. The number of each network corresponds to the network IDs shown in Tables 4 and 5.

**TABLE 4**

Genes showing a 2-fold or higher expression difference between the ngMash Double/fg and ngMash7/fg placentae

| Gene     | Description | Fold change | Networks | Location          |
|----------|-------------|-------------|----------|------------------|
| Dlk1     | δ-Like 1 homolog (Drosophila) | 15.993 | 3 | Extracellular space |
| Serpin6a | Serpin peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 6 | 9.921 | 3 | Extracellular space |
| Bmx3     | Bombesin-like receptor 3 | 2.427 | 1 | Plasma membrane |
| Tram2    | Translocation-associated membrane protein 2 | 2.347 | 1 | Unknown |
| Stmn2    | Stathmin-like 2 | 2.154 | 2 | Cytoplasm |
| Nagk     | N-Acetylglucosamine kinase | 2.052 | 1 | Cytoplasm |
| Aproec1  | Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 | −2.001 | 1 | Cytoplasm |
| St6galnac3 | ST6 (α-N-acetyl-neuraminyl-2,3-β-galactosyl-1,3,4-N-acetylglactosaminide α-2,6-sialyltransferase 3 | −2.121 | 4 | Cytoplasm |
| Tiparp   | TCDD-inducible poly(ADP-ribose) polymerase | −2.471 | 4 | Unknown |
| Syn2     | Spectrin repeat-containing, nuclear envelope 2 | −2.629 | 2 | Nucleus |
| Spop     | Speckle-type POZ protein | −2.641 | 3 | Extracellular space |
| Crem1    | Cysteine-rich transmembrane BMP regulator 1 (chordin-like) | −2.698 | 7 | Extracellular space |
| Mt1e     | Metallothionein 1E | −3.43 | 2,3 | Cytoplasm |

**TABLE 5**

Genes exhibiting a 2-fold or higher expression difference between the ngMash Double/fg and ngMash12/fg placentae

| Gene    | Description | Fold change | Networks | Location          |
|---------|-------------|-------------|----------|------------------|
| Igf2    | Insulin-like growth factor 2 (somatomedin A) | 26.566 | 1, 4 | Extracellular space |
| Pla2g2c | Phospholipase A2, group IIC | 3.621 | 8 | Extracellular space |
| Tacc2   | Transforming, acidic coiled-coil containing protein 2 | 3.014 | 6 | Nucleus |
| Fancb   | Fanconi anemia, complementation group B | 2.336 | 6 | Nucleus |
| Ptprd   | Protein tyrosine phosphatase, receptor type, D | 2.295 | 3 | Plasma membrane |
| Prim1   | Primase, DNA, polypeptide 1 (49kDa) | 2.215 | 1, 3 | Nucleus |
| Pou3f1  | POU class 3 homeobox 1 | 2.155 | 3 | Nucleus |
| N6ra1   | Nuclear receptor subfamily 6, group A, member 1 | 2.103 | 4 | Nucleus |
| Tnx     | Thioredoxin | 2.036 | 1 | Cytoplasm |
| Rad54l  | RAD54-like (Saccharomyces cerevisiae) | 2.025 | 5 | Nucleus |
| Dcc1    | Defective in sister chromatid cohesion homolog 1 (S. cerevisiae) | 2.005 | 3 | Unknown |
| Aldh1a3 | Aldehyde dehydrogenase 1 family, member A3 | −2.194 | 1 | Cytoplasm |
| Cxcl10  | Chemokine (CXC motif) ligand 10 | −2.501 | 2 | Extracellular space |
| Lrrc4   | Leucine-rich repeat-containing 4 | −2.837 | 11 | Unknown |
FIGURE 7. This figure shows the expression level of the genes in the ng^{Double}/fg placenta that are differentially expressed at a level higher than 2-fold that of the ng^{Shh}/fg placenta for each placenta (n = 3). Refer to Table 4. The y axes show the raw expression values from microarray analysis. If the expression level of a focused gene in only the ng^{Shh}/fg placenta is afield from the range between the levels of the WT and ng^{Double}/fg placentae (the realm between the two broken lines), the gene expression is considered to be altered in the ng^{Double}/fg placenta due to the restored expression of paternally methylated imprinted genes on chromosome 12 (colored in yellow).
FIGURE 8. This figure shows the expression level of genes in the \( \text{ng}^{\Delta \text{Double}/\text{fg}} \) placenta that are differentially expressed at a level higher than 2-fold that of the \( \text{ng}^{\Delta \text{ch}12/\text{fg}} \) placenta for each placenta (n = 3). Refer to Table 5. If the expression level of a focused gene in only the \( \text{ng}^{\Delta \text{ch}12/\text{fg}} \) placenta is afield from the range between the levels of the WT and \( \text{ng}^{\Delta \text{Double}/\text{fg}} \) placentae (the area between the two broken lines), we considered the gene expression to be altered in the \( \text{ng}^{\Delta \text{Double}/\text{fg}} \) placenta due to the restored expression of paternally methylated imprinted genes on chromosome 7 (colored in yellow). However, \( \text{Igf2} \) was an exception because the \( \text{Igf2} \) expression level was remarkably higher than that of the other genes, although expression in the \( \text{ng}^{\Delta \text{ch}7/\text{fg}} \) placenta narrowly missed the range between the two broken lines (shown in light blue).
FIGURE 9. Quantitative RT-PCR analysis of transcripts selected from microarray expression profiles (n = 3). Nine transcripts were selected for quantitative RT-PCR analysis from the array expression profile of the ng/H9004/Double/fg placenta to the ng/H9004/ch7/fg or ng/H9004/ch12/fg placentae (Fig. 7 and 8). a, four transcripts that were selected from 13 transcripts shown in Fig. 7, namely, Dlk1, Serpina6, Tram2, and Syne2. b, five transcripts that were selected from 14 transcripts shown in Fig. 8, namely, Igf2, Tacc2, Txn, Aldh1a3, and Cxcl10. Values are represented as means ± S.E. (indicated by error bars).
ng\textsuperscript{ach12}/fg placenta were also similar to those in microarray analysis, namely, Tacc2 transcript was characteristically downregulated, and Aldh1a3 and Cxcl10 were overexpressed in the ng\textsuperscript{ach12}/fg placenta (p values; 0.0147, 0.0386, and 0.0138, respectively) (Fig. 9b). Although of the nine transcripts, the Tram2 and Ttn expression levels in the ng\textsuperscript{ach}/fg and ng\textsuperscript{ach12}/fg placentae showed no significant differences as compared with those in the ng\textsuperscript{Double}/fg placenta, the expression patterns of the other seven genes were consistent with the result of microarray analysis. We also confirmed that the expression patterns of the other seven genes were consistent with the conclusion of the previous microarray analysis. We also confirmed that the expression patterns of these genes in the ng\textsuperscript{WT}/fg placenta containing wild-type ng genomes did not denote the same tendency of those in the ng\textsuperscript{ach12}/fg placenta (supplemental Fig. 2). This might be because grossly inequable tissues in cell composition in the ng\textsuperscript{WT}/fg placenta caused inexplicable downstream effects on transcription levels of these genes. Consequently, judging by the results from quantitative RT-PCR by using the ng\textsuperscript{ach}/fg, ng\textsuperscript{ach12}/fg, and ng\textsuperscript{Double}/fg placentae, the concordance of results obtained from these two independent lines of experimentation strongly suggests that the expression profiles derived from the microarray analysis precisely represent the qualitative changes in gene expression that occur during improved placentaion in the ng\textsuperscript{Double}/fg conceptus.

Conclusions
Bimaternal mouse conceptuses in which the expression pattern of the paternally methylated imprinted genes in the Igf2-H19 and Dlk1-Dio3 regions are inappropriate resulted in lethality before E13.5, accompanied by acute placental disorders. Experiments involving paternalization of both the Igf2-H19 and the Dlk1-Dio3 regions with the use of mutant mice clearly led to definitive placentaion. The global gene expression data sets in these bimaternal placentae verified the rigorous complementary correlation of paternally methylated imprinted genes on chromosomes 7 and 12. The results indicated that the contributions of paternally methylated imprinted genes on chromosomes 7 and 12 to placentaion were distinctive; the former mainly controlled cell proliferation- and cell growth-related genes. In contrast, the latter controlled tissue differentiation-related genes. Furthermore, the networks could be used to identify the functions of imprinted genes regulated by either of the two paternally methylated imprinting-control regions that have been annotated as hypothetical or as function unknown. Analysis of our transcriptome with bimaternal placentae lacking the paternal genome can help in deciphering the unknown functions and pathways of paternally methylated imprinted genes during mouse placentaion.

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