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Defining Contributions of Paternally Methylated Imprinted Genes at the *Igf2-H19* and *Dlk1-Gtl2* Domains to Mouse Placentation by Transcriptomic Analysis^{*[5]}♦

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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 *Dnmt3L* cooperates with *Dnmt3a* and *Dnmt3b* to methylate DNA *de novo* (7). Studies on the extraembryonic tissues of *Dnmt3L*^{mat-/-} embryos have revealed that appropriate maternal 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) (ng^{WT}), in which imprints are not established, were combined with fully grown (fg) oocytes, the resulting ng^{WT}/fg bimaternal conceptuses could have placentae comprising three basic layers: the trophoblastic giant cells, spongiotrophoblast layer, and labyrinthine layer (9). However, the ng^{WT}/fg placenta showed diagnostic defects such as severe growth retardation, disproportionate expansion of the spongiotrophoblast layer, distorted and ambiguous boundary between the spongiotrophoblast 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 (ng^{Δch7}). Another set of oocytes was derived

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains two supplemental figures and a supplemental table.

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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 ($ng^{\Delta ch12}$) (11, 12). Interestingly, $ng^{\Delta ch7}/fg$ and $ng^{\Delta ch12}/fg$ placentae showed reciprocal phenotypes, *i.e.* the $ng^{\Delta ch7}/fg$ 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 $ng^{\Delta ch12}/fg$ 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 ($ng^{\Delta Double}$) that harbored deletions in both the *Igf2-H19* ICR and the *Dlk1-Dio3* ICR to demonstrate the generation of bimaternal 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 $ng^{\Delta Double}/fg$ bimaternal embryo. In this study, we determined the placenta-forming ability of the $ng^{\Delta Double}/fg$ 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 $ng^{\Delta Double}/fg$ placenta (10). Next, we compared the global gene expression profiles of four placenta types, namely, the $ng^{\Delta ch7}/fg$, $ng^{\Delta ch12}/fg$, $ng^{\Delta Double}/fg$, and WT placentae (Fig. 1). Finally, for in-depth investigation into the roles of the corrected expression level of paternally methylated imprinted genes on chromosome(s) 7 and/or 12 in mouse placentation, 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 $ng^{\Delta Double}/fg$ placenta showed remarkably improved phenotypes in comparison with the $ng^{\Delta ch7}/fg$ and $ng^{\Delta ch12}/fg$ placentae. Additionally, comparative global gene expression analyses of the $ng^{\Delta Double}/fg$ placenta and the other bimaternal placentae, *i.e.* the $ng^{\Delta ch7}/fg$ and $ng^{\Delta ch12}/fg$ 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.

EXPERIMENTAL PROCEDURES

Oocyte Manipulations—Fully grown germinal vesicle oocytes from the ovarian follicles of B6D2F1 (C57BL/6N $\times$ 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 chorionic 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 $\Delta ch7$ ($-/+$) and heterozygous $\Delta ch12$ ($+/-$) were fused with enucleated germinal 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^{WT}/fg , $ng^{\Delta ch7}/fg$, $ng^{\Delta ch12}/fg$, and $ng^{\Delta Double}/fg$ 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 $ng^{\Delta ch7}/fg$, $ng^{\Delta ch12}/fg$, $ng^{\Delta Double}/fg$ 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.A.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 μm^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, $ng^{\Delta ch7}/fg$, $ng^{\Delta ch12}/fg$, $ng^{\Delta Double}/fg$ bimaternal, and WT, at E12.5. Transcriptomic analysis was performed at E12.5, when all types of the bimaternal-

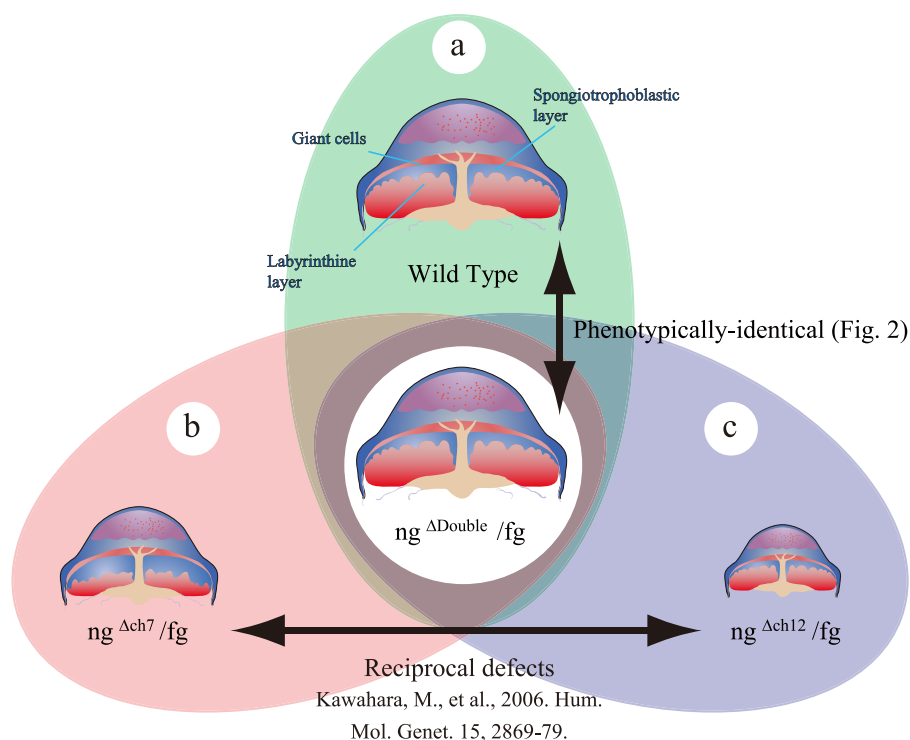


FIGURE 1. Experimental design. First of all, we precisely investigated that the $ng^{\Delta Double}/fg$ placenta is phenotypically identical to the WT placenta followed by transcriptomic analysis. *a*, investigation of differentially expressed genes in the $ng^{\Delta Double}/fg$ placenta relative to those in the WT placenta may lead to normality verification of the $ng^{\Delta Double}/fg$ placenta (Table 1; Fig. 3). *b*, differentially expressed genes in the $ng^{\Delta Double}/fg$ placenta relative to those in the $ng^{\Delta ch7}/fg$ placenta may be associated with peculiar disorders to the $ng^{\Delta ch7}/fg$ placenta, suggesting biological functions of paternally methylated imprinted genes on chromosome 12 in placenta (Tables 2 and 4; Figs. 3, 4, 6, and 7). *c*, similarly, differentially expressed genes in the $ng^{\Delta Double}/fg$ placenta relative to those in the $ng^{\Delta ch12}/fg$ placenta may be associated with peculiar disorders to the $ng^{\Delta ch12}/fg$ placenta, suggesting biological functions of paternally methylated imprinted genes on chromosome 7 in placenta (Tables 3 and 5; Figs. 3, 5, 6, and 8).

nal conceptuses showed relatively high survivability as compared with the later embryonic days, and their placentae appeared to be appropriate for reliable evaluation (15). The total RNA from three replicates of each placenta was used for the GeneChip® one-cycle eukaryotic target labeling assay (Affymetrix, Santa Clara, CA). Fifteen micrograms per replicate of fragmented cRNA samples was serially hybridized to the GeneChip mouse genome 430 2.0 array (Affymetrix) that contained 45,101 probe sets, and these were then processed according to the manufacturer's instructions (GeneChip Expression Analysis Technical Manual, Affymetrix (34)).

Briefly, the GeneChip operating software 1.3 (GCOS; Affymetrix) was first used to quantify microarray signals with default analysis parameters. The GCOS output files were then loaded into GeneSpring v7.3 (Agilent Technologies, Santa Clara, CA) with per chip normalization to the 50th percentile and per gene normalization to the average expression level of the WT placenta. The first operation simply identified transcripts with a raw signal of at least 50 in terms of the expression level of the WT placenta. A filtered list was created of all the genes detected (GCOS "P" and "M" calls) in all replicates. The GCOS output files of all the genes after GeneSpring normalization were also used in a one-way analysis of variance analysis with post hoc testing using Tukey's true test for significance ($p < 0.01$ at E12.5). These lists were referred to as the chr12 and chr7 gene lists. All genes in the chr12 and chr7 gene lists were

integrated into the IPA pathways on the basis of the biological relationship described in previous studies.

Ingenuity Pathway Analysis—IPA version 3.0 was used to determine the possible biological pathways and the inter-relationships between network genes in subsets of candidate genes that had particularly interesting patterns. A detailed description of IPA can be found at the Ingenuity Systems site. Data sets containing the Affymetrix gene identifiers and their corresponding expression-fold change values were uploaded as Excel files. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. Network building was initiated with the program for querying the Ingenuity Pathways Knowledge Base for interactions between focus genes and all the other gene objects stored in the Knowledge Base. A set of networks was then generated, and the IPA computed a score for each network according to the fit of the network to the set of focus genes. Biological functions were calculated and assigned to each network.

Quantitative RT-PCR Analysis

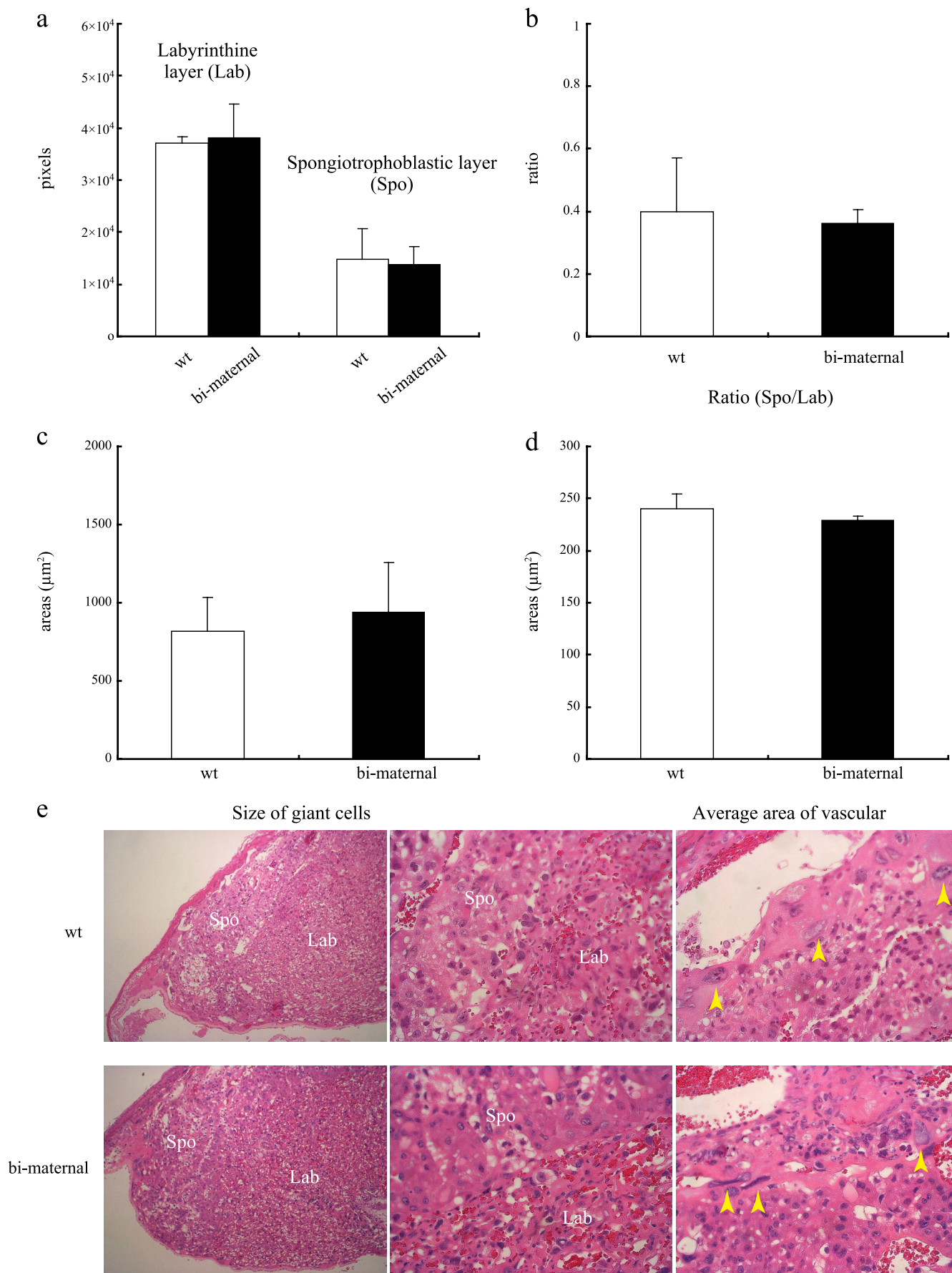
Total RNA preparation was carried out as described in microarray analysis. The cDNAs were then synthesized using the SuperScript™ III RNase H reverse transcriptase kit (Invitrogen) in a reaction solution (20 μ l) containing the total RNA (1 μ g) prepared from each placenta. Finally, we performed a quantitative analysis of the gene expression by using real-time PCR (7500 real-time PCR system, Applied Biosystems) after preparing a reaction mixture (SYBR® 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 $ng^{\Delta Double}/fg$ Bimaternal Conceptuses—As stated previously, we observed reciprocal defects in the $ng^{\Delta ch7}/fg$ and $ng^{\Delta ch12}/fg$ placentae (10). Furthermore, we found that the weight of the $ng^{\Delta Double}/fg$ placenta, which could

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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 $ng^{\Delta Double}/fg$ 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 $ng^{\Delta Double}/fg$ placenta was similar to that of the WT placenta (Fig. 2*d*), indicating that the circulatory system of the $ng^{\Delta Double}/fg$ placenta was normal. In the $ng^{\Delta Double}/fg$ placenta, malformations specific to the $ng^{\Delta ch7}/fg$ and $ng^{\Delta ch12}/fg$ placentae were entirely restored, as typified by the distinct boundary between the spongiotrophoblast and labyrinthine layers (Fig. 2*e*). These results demonstrate that the $ng^{\Delta Double}/fg$ 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 $ng^{\Delta Double}/fg$ placenta and other bimaternal placentae, namely, the $ng^{\Delta ch7}/fg$ and $ng^{\Delta ch12}/fg$ 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 placentation.

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 placentation in the $ng^{\Delta ch7}/fg$ and $ng^{\Delta ch12}/fg$ conceptuses to normalized placentation in the $ng^{\Delta Double}/fg$ conceptuses. We constructed hierarchical clustering with all the transcripts on the Genome 430 2.0 chip set using microarray data from the WT, $ng^{\Delta ch7}/fg$, $ng^{\Delta ch12}/fg$, and $ng^{\Delta Double}/fg$ placentae ($n = 3$) (Fig. 3*a*). 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. 3*b*). In the x - y plane, the $ng^{\Delta Double}/fg$ placenta was relatively closer to the WT placenta, whereas the $ng^{\Delta ch7}/fg$ and $ng^{\Delta ch12}/fg$ placentae almost coincided with each other. Both of them were distant from the WT placenta, indicating that the expression pattern of the $ng^{\Delta Double}/fg$ placenta was comparatively closer to that of the WT placenta than to those of the $ng^{\Delta ch7}/fg$ and $ng^{\Delta ch12}/fg$ placentae. Thus, the concordance between the results from the hierarchical cluster analysis and PCA indicated that the global gene expression pattern of the $ng^{\Delta Double}/fg$ placenta was closer to that of the WT placenta than to those of the other bimaternal placentae, *i.e.* the $ng^{\Delta ch7}/fg$ and $ng^{\Delta ch12}/fg$ placentae.

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 $ng^{\Delta Double}/fg$ placentae than between the WT and other bimaternal placentae (Fig. 3*c*). We also prepared a list of genes that were differentially expressed in the $ng^{\Delta Double}/fg$ and WT placentae. Of the transcripts that were significantly altered in the $ng^{\Delta Double}/fg$ 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 $ng^{\Delta Double}/fg$ 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 $ng^{\Delta Double}/fg$ placenta; its gene product functions as a *de novo* methyltransferase that is essential for normal development (17). Down-regulation of *Dnmt3a* by ~ 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 $ng^{\Delta Double}/fg$ placentae, genes that were altered in the $ng^{\Delta Double}/fg$ 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 $ng^{\Delta Double}/fg$ placenta were differentially expressed as compared with those in the $ng^{\Delta ch7}/fg$ and $ng^{\Delta ch12}/fg$ 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 $ng^{\Delta Double}/fg$ placenta relative to the $ng^{\Delta ch7}/fg$ and $ng^{\Delta ch12}/fg$ placentae were prepared (Fig. 3*d*). Identifying differentially expressed genes in the $ng^{\Delta Double}/fg$ placenta relative to those in the $ng^{\Delta ch7}/fg$ and $ng^{\Delta ch12}/fg$ 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 $ng^{\Delta Double}/fg$ and the $ng^{\Delta ch7}/fg$ placentae (ch12 gene list), 96 differentially expressed genes were present, whereas between the $ng^{\Delta Double}/fg$ and the $ng^{\Delta ch12}/fg$ placentae, 117 differentially and specifically expressed genes were present (ch7 gene list) (Fig. 3*d*).

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-

FIGURE 2. **Morphometric analyses of the WT and $ng^{\Delta Double}/fg$ 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 $ng^{\Delta Double}/fg$ placentae. Error bars represent means $\pm$ S.E. *c*, the average areas of giant cells were also calculated. *d*, the average areas of the vasculature in the WT and $ng^{\Delta Double}/fg$ placentae were investigated by tracing the surrounding area of both maternal and fetal blood vessels within 114,550 μm^2 of the labyrinth. Error bars indicate means $\pm$ S.E. *e*, midline placental sections (hematoxylin/eosin) at E19.5. The left panels present an overview of the WT and $ng^{\Delta Double}/fg$ placentae, and their magnified images are shown in the center. Note that the boundary in the $ng^{\Delta Double}/fg$ placenta is entirely restored. The right panels show high magnification views of giant cells (yellow arrowheads) adjacent to the spongiotrophoblast layer in the WT and $ng^{\Delta Double}/fg$ placentae.

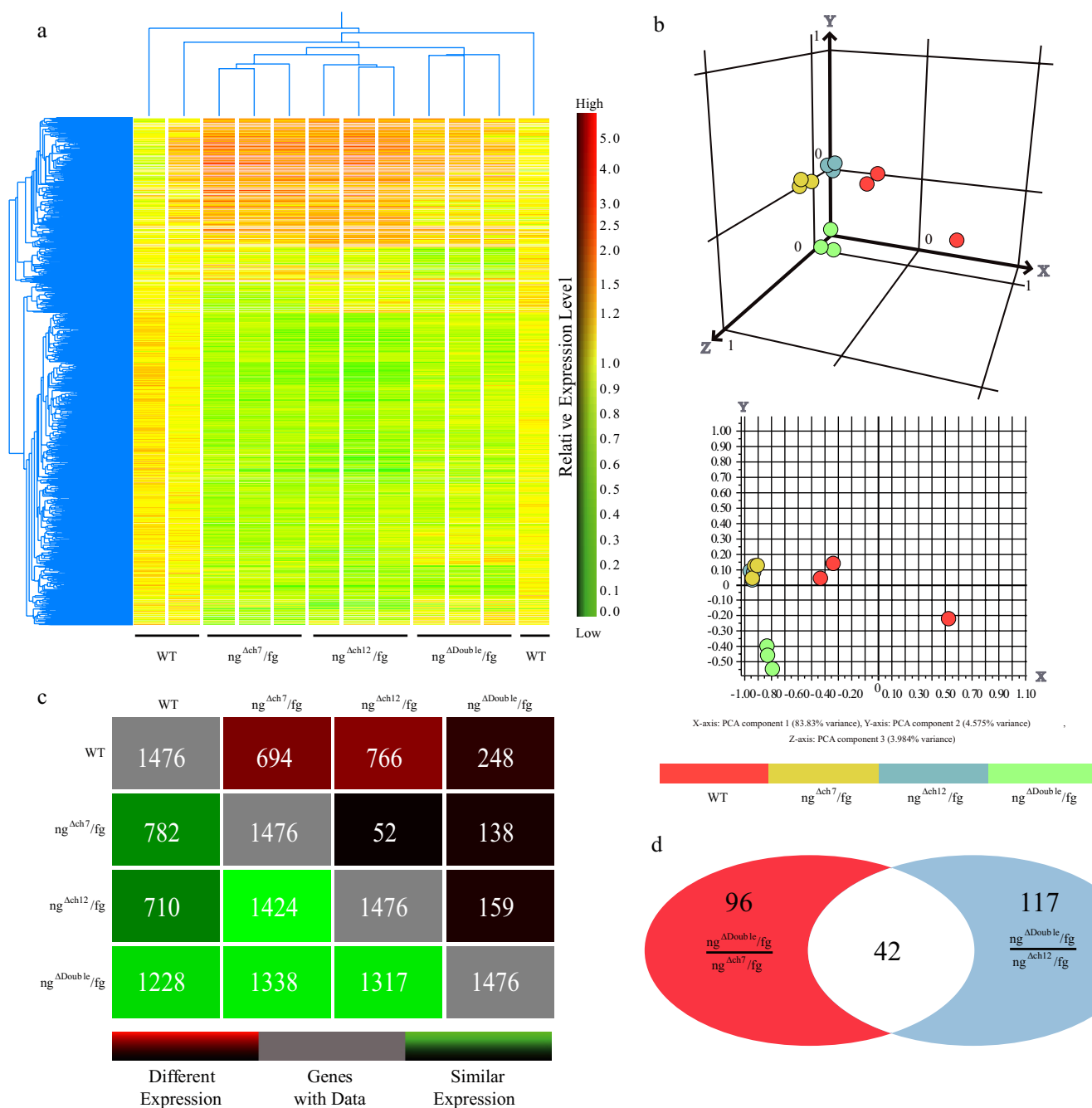


FIGURE 3. Global gene expression analyses of four types of placentae: WT, $ng^{\Delta ch7}/fg$, $ng^{\Delta ch12}/fg$, and $ng^{\Delta Double}/fg$ bimaternal placentae ($n = 3$). *a*, hierarchical clustering of all the samples from different genetic backgrounds. Colors correspond to the relative RNA 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, $ng^{\Delta ch7}/fg$, $ng^{\Delta ch12}/fg$, and $ng^{\Delta Double}/fg$ placentae. 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 $ng^{\Delta Double}/fg$ placenta in comparison with the $ng^{\Delta ch7}/fg$ (red, ch12 gene list) and the $ng^{\Delta ch12}/fg$ (blue, ch7 gene list) placentae individually. These gene lists were integrated into the IPA pathways (Figs. 4 and 5).

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 $ng^{\Delta Double}/fg$ placenta; this expression pattern differed from that in the $ng^{\Delta ch7}/fg$ 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

TABLE 1

Genes showing a 2-fold or higher expression difference between the WT and $ng^{\Delta Double}/fg$ placentaeFifteen representative genes in the $ng^{\Delta Double}/fg$ placenta were expressed at a level that was 2-fold higher than that in the WT placenta (bold, up-regulated; italic, down-regulated). BTB, Bric-a-brac, Tramtrack, Broad complex; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PHD, plant homeodomain.

Gene	Description	-Fold change	Networks	Location
<i>Serpinb1</i>	Serpin peptidase inhibitor, clade B (ovalbumin), member 1	6.342	6	Cytoplasm
<i>Il18</i>	Interleukin 18 (interferon- γ -inducing factor)	2.113	5	Extracellular space
<i>Dhcr24</i>	24-Dehydrocholesterol reductase	-2.008	4	Cytoplasm
<i>Dnmt3a</i>	DNA (cytosine-5)-methyltransferase 3- α	-2.062	2	Nucleus
<i>Recql</i>	RecQ protein-like (DNA helicase Q1-like)	-2.09	1	Nucleus
<i>Zbtb7a</i>	Zinc finger and BTB domain containing 7A	-2.099	1	Nucleus
<i>Fblim1</i>	Filamin-binding LIM protein 1	-2.12	8	Plasma membrane
<i>Jarid1a</i>	Jumonji, AT-rich interactive domain 1A	-2.156	4	Nucleus
<i>Tiparp</i>	TCDD-inducible poly(ADP-ribose) polymerase	-2.21	9	Unknown
<i>Uhrf1</i>	Ubiquitin-like, containing PHD and RING finger domains, 1	-2.234	2	Nucleus
<i>Rgs5</i>	Regulator of G-protein signaling 5	-2.337	6	Plasma membrane
<i>Polr1a</i>	Polymerase (RNA) I polypeptide A, 194 kDa	-2.355	1	Nucleus
<i>Rtn1</i>	Reticulon 1	-2.39	9	Cytoplasm
<i>Cdc25a</i>	Cell division cycle 25 homolog A (<i>Schizosaccharomyces pombe</i>)	-2.606	1, 8, 11	Nucleus
<i>Lrrc4</i>	Leucine-rich repeat-containing 4	-2.98	16	Unknown

TABLE 2

Networks generated by IPA for transcripts that were specifically and differentially expressed in the $ng^{\Delta Double}/fg$ placenta in comparison with the $ng^{\Delta ch7}/fg$ placentaStatistically significant genes were specifically and differentially expressed in the $ng^{\Delta Double}/fg$ placenta in comparison with the $ng^{\Delta ch12}/fg$ placenta ($ng^{\Delta Double}/fg$ versus $ng^{\Delta ch12}/fg$). These were used as the input in the IPA.

ID	Molecules in network	Score	Focus molecules	Top functions
1	<i>Aldosterone</i> , <i>Apobec1</i> , <i>Brs3</i> , <i>Ca²⁺</i> , <i>Clk2</i> , <i>Col1a1</i> , <i>Col4a1</i> , <i>Dmp1</i> , <i>Egfr</i> , <i>F2</i> , <i>F11r</i> , <i>Fxyd3</i> , <i>Hsph1</i> , <i>Ibsp</i> , <i>Klf6</i> , <i>Kras</i> , <i>Mad11l</i> , <i>Mtss1</i> , <i>Nagk</i> , <i>Ndc80</i> , <i>Nde1</i> , <i>Nek2</i> , <i>Ppil1</i> , <i>Ppp2r3a</i> (includes EG:5523), <i>Ptpn1</i> , <i>Sfrs3</i> , <i>Slc19a2</i> , <i>Smad1</i> , <i>Stt4</i> , <i>Tcp1</i> , <i>Tob1</i> , <i>Tp53</i> , <i>Tram2</i> , <i>Tik</i> , <i>Vhl</i>	27	14	Cell cycle, cancer, cellular growth and proliferation
2	<i>Ager</i> , <i>Ankrd11</i> , <i>Brd2</i> , <i>Cbl</i> , <i>Cldnd1</i> , <i>Ecm1</i> , <i>Ehmt2</i> , <i>Epha4</i> , <i>Ephb2</i> , <i>Fshb</i> , <i>Histone H3</i> , <i>Hmgb1</i> (includes EG:3146), <i>Ifna1</i> , <i>Ifnb1</i> , <i>Inha</i> , <i>L-Triiodothyronine</i> , <i>Lep</i> , <i>Mapk</i> , <i>Mia</i> , <i>Mst1</i> , <i>Mst1r</i> , <i>Mt1e</i> , <i>Mtpn</i> , <i>Ncoa1</i> , <i>Phosphate</i> , <i>Prpf31</i> , <i>Pum1</i> , <i>Rgs20</i> , <i>Stmn2</i> , <i>Syne2</i> , <i>Thbd</i> , <i>Tlr9</i> , <i>Tubb3</i> , <i>Ube2l3</i> , <i>Xrn2</i>	24	13	Organ morphology, reproductive system development and function, cellular growth and proliferation
3	<i>17-Hydroxyprogesterone</i> , <i>aldosterone</i> , <i>Cdcp1</i> , <i>Corticosterone</i> , <i>Ctsb</i> , <i>Ctsc</i> , <i>Ctsd</i> , <i>Cul3</i> , <i>dehydroisoandrosterone</i> , <i>Dlk1</i> , <i>Eea1</i> , <i>Egf</i> , <i>Ethe1</i> , <i>F11r</i> , <i>Foxa2</i> , <i>Gdf15</i> , <i>Il11</i> , <i>Insig1</i> , <i>Itgal</i> , <i>Klf6</i> , <i>Meis2</i> , <i>Mmp1</i> , <i>Mt1e</i> , <i>Pdx1</i> (includes EG:29535), <i>progesterone</i> , <i>Rab22a</i> , <i>retinoic acid</i> , <i>Srsf5</i> , <i>Serpina6</i> , <i>Spop</i> , <i>Src</i> , <i>Srebf1</i> , <i>Sucg1</i> , <i>Tnf</i> , <i>TP53i3</i>	20	11	Cellular development, connective tissue development and function, developmental disorder
4	<i>Acvr1</i> , <i>amino acids</i> , <i>Ap3s1</i> , <i>C12orf11</i> , <i>Ctdp1</i> , <i>Daxx</i> , <i>Dynll1</i> , <i>Foxg1</i> , <i>Frap1</i> , <i>Gatad2b</i> , <i>Gggs1</i> , <i>Gphn</i> , <i>Hdac1</i> (includes EG:3065), <i>Irs1</i> , <i>Lep</i> , <i>Mad11l</i> , <i>Mast2</i> , <i>Mbd2</i> , <i>Mbd3</i> (includes EG:53615), <i>Pkn1</i> , <i>Ppp2r2a</i> , <i>Prmt5</i> , <i>Runx3</i> , <i>Ski</i> , <i>Smad5</i> , <i>Smad6</i> , <i>Snrp70</i> , <i>Snrpd3</i> , <i>Sntb2</i> , <i>St6galnac3</i> , <i>Stk16</i> , <i>Tgfb1</i> , <i>Tiparp</i> , <i>Vasp</i> , <i>Wdr77</i>	20	11	Cancer, amino acid metabolism, post-translational modification
5	<i>Atf3</i> , <i>C1orf2</i> , <i>Ccl2</i> , <i>Ccna2</i> , <i>Ccne1</i> , <i>Cdkn1a</i> , <i>Ctsb</i> , <i>Dnmt1</i> , <i>Eftud2</i> , <i>Elavl1</i> , <i>Fos</i> , <i>Heparin</i> , <i>Hgs</i> , <i>Hnrpd</i> , <i>Inhba</i> , <i>Itga5</i> , <i>Lif</i> , <i>Mmp1</i> , <i>Myog</i> , <i>Pcbp1</i> (includes EG:5093), <i>Pdgr</i> , <i>Pdgfrb</i> , <i>Ren</i> , <i>Rhob</i> , <i>Rps24</i> (includes EG:20088), <i>Runx2</i> , <i>Serpine1</i> , <i>Set</i> , <i>Sfrs12</i> , <i>Spag5</i> , <i>Spry2</i> , <i>Srebf1</i> , <i>Stat5b</i> , <i>Vav1</i> , <i>Vav2</i>	7	5	Gene expression, cellular growth and proliferation, cell cycle
6	<i>Brp44</i> , <i>Stat5b</i>	2	1	Cancer, developmental disorder, genetic disorder

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 $ng^{\Delta ch7}/fg$ placenta (10). Genes in networks 1 and 4 in Table 3 are involved in the cell cycle, cancer, reproductive system diseases, cellular movement, and cell death. Cell cycle and cell death may be responsible for the phenotypes of the $ng^{\Delta ch12}/fg$ 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 bimaternal 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 $ng^{\Delta Double}/fg$ placenta whose differential expression was more than 2-fold that of the genes in the $ng^{\Delta ch7}/fg$ and $ng^{\Delta ch12}/fg$ placentae (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 $ng^{\Delta Double}/fg$ placentae, the gene should be regarded as being within the range of normality. This is because genes that are differentially expressed between the WT and $ng^{\Delta Double}/fg$ placentae are apparently not directly/indirectly associated with mouse placentation, at least in placental morphogenesis analyzed in this study (Fig. 2).

In comparison with the $ng^{\Delta ch7}/fg$ placenta, the most altered gene in the $ng^{\Delta Double}/fg$ placenta was *Dlk1* (Table 4; Fig. 7). This gene is paternally expressed in the endothelial cells of fetal

TABLE 3

Networks generated by IPA for transcripts that were specifically and differentially expressed in the $ng^{ADouble}/fg$ placenta in comparison with the ng^{Ach12}/fg placenta

Statistically significant genes were specifically and differentially expressed in the $ng^{ADouble}/fg$ placenta in comparison with the ng^{Ach7}/fg placenta ($ng^{ADouble}/fg$ versus ng^{Ach7}/fg). These were used as the input in the IPA analysis.

ID	Molecules in network	Score	Focus molecules	Top functions
1	<i>Aldh1a3, Aurka, Birc5, Ccnb1, Ccne1, Cdc20, Cdca8, Cenpa, Chaf1b, Cyclin A, Cyclin B, Cyclin E, E2f, Elac2, Foxm1, Ghr, Histone H3, Id2, Igf2, Igfbp7, Ikb, Ikbkb, Lamp2, Mcm10, Mthfd1, Mybl2, Nfkb, Pde4b, Pdgf, Pdlim5, Prim1, Rnf19b, Satb1, Tcr, Txn</i>	55	26	Cell cycle, cancer, reproductive system disease
2	<i>Afap1, Akt, Calpain, Caspase, Cd47, Cd200, Cotl1, Cxcl10, Dusp1, F Actin, Gas2, Hba2, Ier3, Il11, Insulin, Jnk, Lnpep, Mapk, MMP11, P38 MAPK, P70 S6k, Pak1, Pdgf BB, PI3K, PPM1A, PSMC3IP, Rac, RAC3, RGS16, RHOB, Sh2b2, Sh3glb1, Tgfb, Thbs1, Vegf</i>	24	14	Carbohydrate metabolism, immune response, small molecule biochemistry
3	<i>Ap1m2, Ctsl2, Dcc1, Egfr, Erbb2, Ereg, Fen1, Gas1, Hmnr, Id1, Ier2, Ier3, Myc, Ndrgr1, Nfib, Nola1, Nrg1, Pcnal, Polg, Pou3f1, Prim1, Ptprd, Purb, Rad51ap1, Rfc4, Rgs16, Rrm2, Sgsh, Shmt1, Smn1, Src, Sumf1, Tcf4, Tp53, Zrf1</i>	24	14	Cellular growth and proliferation, DNA replication, recombination, and repair, cancer
4	<i>Areg, Ctsd, Ctsl2, dihydrotestosterone, Dtx3l, Eif4ebp1, Elk1, Ghr, Grw1, Hbegf, Hgf, Ifng, Igf2, Igfbp4, Il6r, Kif22, Mmp1, Mybl2, Ncor1, Nr6a1, P70 S6k, Pim1, Ppa1, progesterone, Ptgs2, Rapgef5, Rhob, Sepp1, Slc1a5, Slc32a1, Slco3a1, Timp1, Tiparp, Trip13, Uimc1</i>	22	13	Cancer, cellular movement, cell death
5	<i>Akt1, Bid, C15orf23, Cdc2, Cdc6, Cdca3, Cdkn1a, Chaf1b, Ctsl2, E2f1, Fen1, Gadd45a, Gsk3b, Il11, Il13, Il13ra1, Kif20a, Kitlg (includes EG:4254), Khlh24, Matr3, Mcm2, Mcm4, Mcm7, Mmp1, Mybl2, Nin, Pacs2, Pim1, Rad51, Rad54l, Rhob, Rps6kb1, Rrm1, Sirt2, Thra</i>	20	12	Cancer, cell death, cell cycle
6	<i>Aqp3, Cdkn2a, Dusp1, Ep300, Fen1, Gart, Ghr, Grn, Ide, Idh1, Ier2, Ier3, Ifi47, Igfbp1, Igfbp4, Il1b, Ins, Ints7, Lep, Melk, Mmp1, Myog, Nfyb, Nr3c1, P70 S6k, Prkab2, Rrm2, Sepp1, Tacc2, Tbp, Thra, Tk, Trim30, Utf1</i>	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 $ng^{ADouble}/fg$ placenta was *Serpina6* (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 *Serpina6* 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 *Serpina6* expression may be a possible cause of vascular defects in the ng^{Ach7}/fg placenta. In contrast, the *Syne2* mRNA was overexpressed in the ng^{Ach7}/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 orients 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^{Ach7}/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 $ng^{ADouble}/fg$ placenta in comparison with the ng^{Ach12}/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 $ng^{ADouble}/fg$ placenta was equivalent to that in the WT placenta (Fig. 8) and, therefore, the normal-sized $ng^{ADouble}/fg$ placenta was attributed to restored *Igf2* mRNA expression. In our previous study, the ng^{Ach7}/fg placenta consistently showed a greater mass than the ng^{Ach12}/fg placenta; however, these masses were significantly lesser than that of the WT placenta (10). In the ng^{Ach7}/fg placenta, the *Igf2* mRNA expression level did not reach the range between the levels of the WT and $ng^{ADouble}/fg$ placentae, although it was much higher than the levels of the ng^{Ach12}/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^{Ach7}/fg placenta with a disproportionally layered structure may be due to the improper expression pattern of paternally methylated imprinted genes on chromosome 12. The observation that the mass of the $ng^{ADouble}/fg$ placenta was completely normalized provided further evidence for this (Fig. 2).

After *Igf2*, the genes that were up-regulated by more than 2-fold in the $ng^{ADouble}/fg$ placenta were *Pla2G2C*, *Tacc2*, and *Txn* (Fig. 8), although the biological functions of these genes in the placenta have not yet been established. Meanwhile, *Aldh1a3* and *Cxcl10* were down-regulated by more than 2-fold in the $ng^{ADouble}/fg$ placenta. The function of *Aldh1a3* in the placenta is also unknown, but it is known that *Cxcl10* encodes a chemokine of the CXC subfamily, and the binding of this pro-

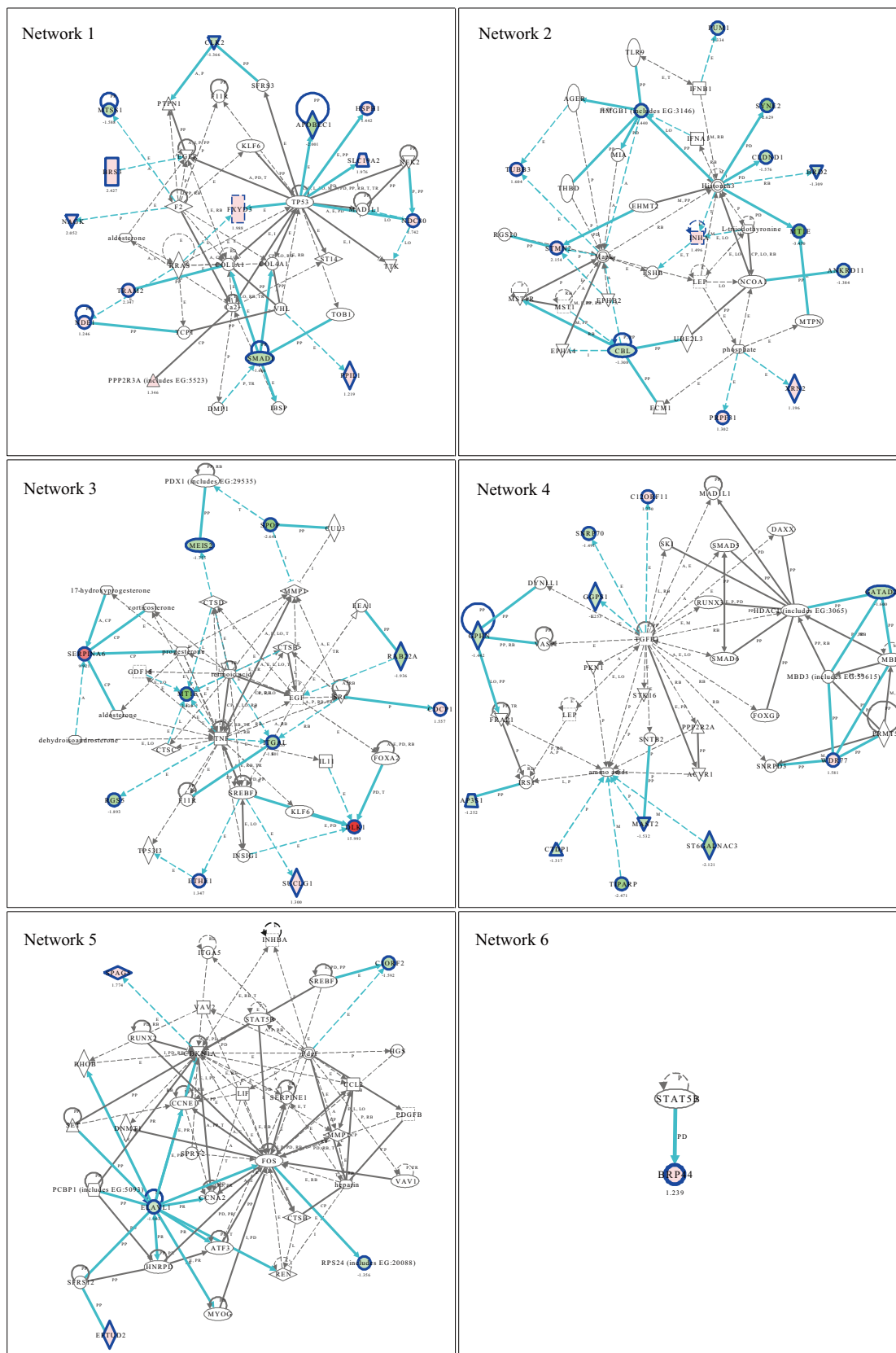


FIGURE 4. Functional networks of genes that are differentially and specifically expressed in the $ng^{\Delta Double}/fg$ placenta in comparison with the $ng^{\Delta ch7}/fg$ placenta. Genes marked in red and green are up-regulated and down-regulated, respectively. The network is displayed graphically 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).

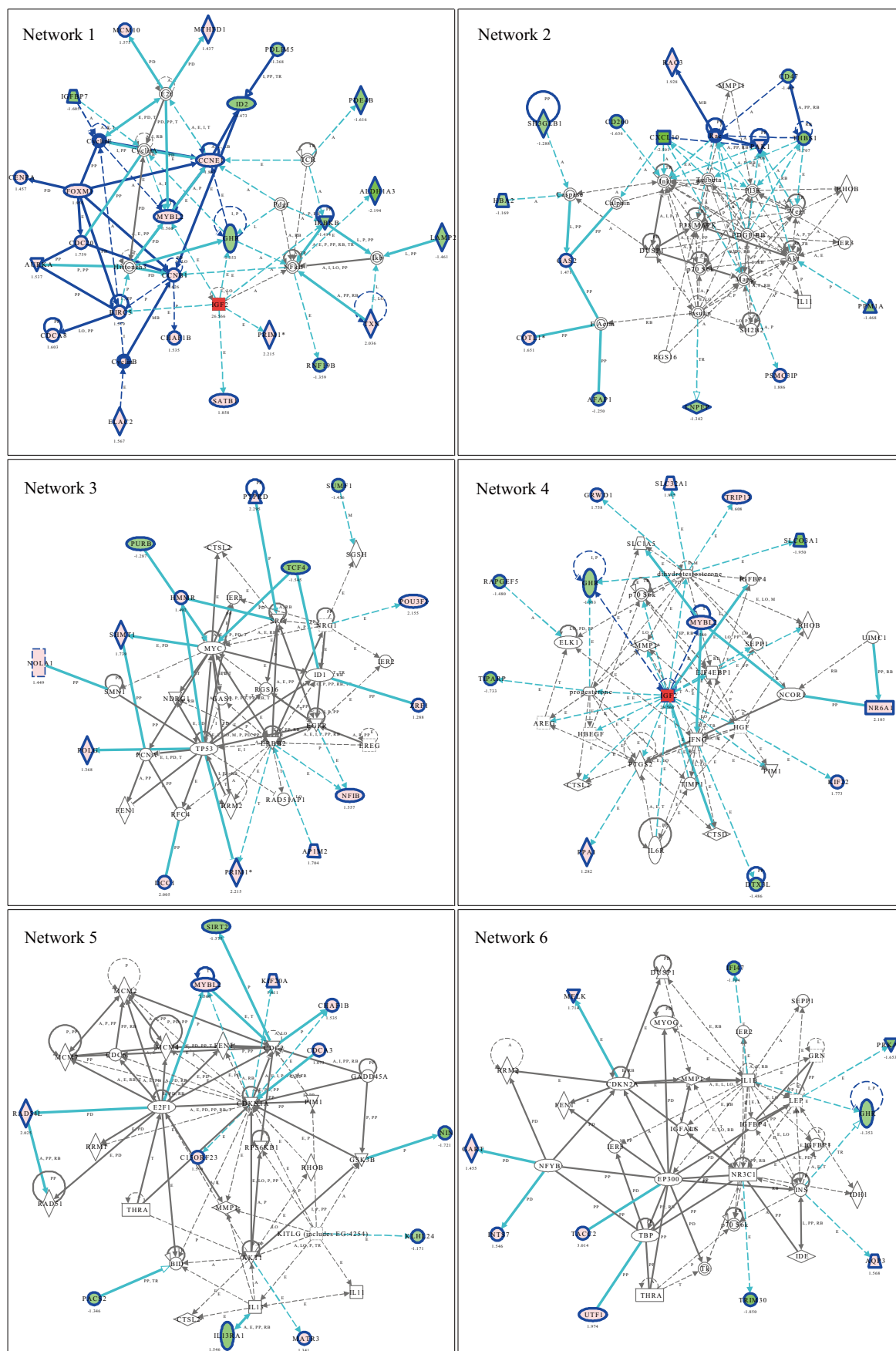


FIGURE 5. Functional networks of genes that are differentially and specifically expressed in the $ng^{\Delta Double}/fg$ placenta in comparison with the $ng^{\Delta ch12}/fg$ placenta (refer to the legends for Fig. 4 and supplemental Fig. 1).

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 $ng^{\Delta ch12}/fg$ conceptus increased as compared with that in the ng^{WT}/fg conceptus. Therefore, this result indicates that up-regulation of *Cxcl10* compensates for the gain in placental mass in the $ng^{\Delta ch12}/fg$

conceptus lacking *Igf2* expression. However, in the $ng^{\Delta Double}/fg$ placenta, *Cxcl10* overexpression was entirely restored. This is attributed to the fact that *Igf2* transcription level in the $ng^{\Delta Double}/fg$ 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*, *Serpina6*, *Tram2*, *Syne2*, *Igf2*, *Tacc2*, *Txn*, *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 *Serpina6* and the *Syne2* genes were significantly down- and up-regulated in the $ng^{\Delta ch7}/fg$ placenta as expected (*p* values; 0.0061 and 0.0258, respectively) (Fig. 9*a*). In the *ch7* gene list, the anomalous expression patterns of the *Tacc2*, *Aldh1a3*, and *Cxcl10* RNAs in the

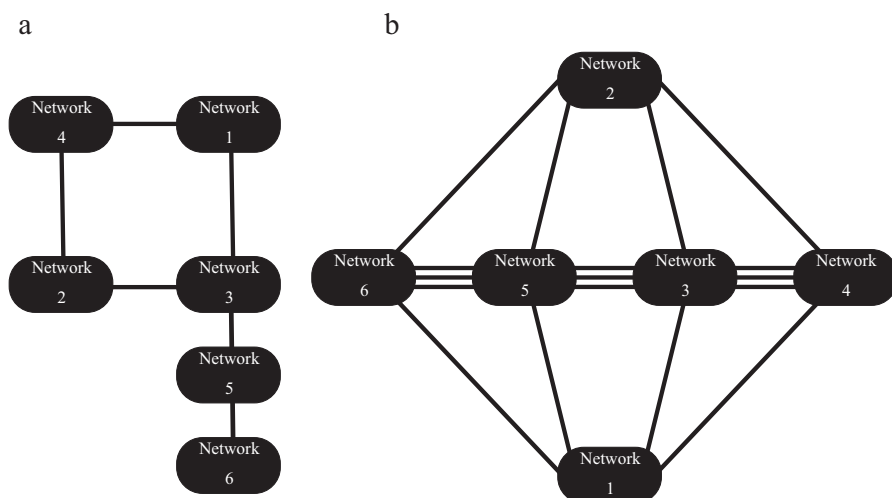


FIGURE 6. **Overlapping networks constructed from the gene lists.** *a*, *ch12* gene list; *b*, *ch7* gene list. Each constructed network is bound in commonly appearing genes. The number of each network corresponds to the network IDs shown in Tables 4 and 5.

TABLE 4

Genes showing a 2-fold or higher expression difference between the $ng^{\Delta Double}/fg$ and $ng^{\Delta ch7}/fg$ placentae

Thirteen representative genes in the $ng^{\Delta Double}/fg$ placenta were expressed at a level that was 2-fold higher than that in the $ng^{\Delta ch7}/fg$ placenta (bold, up-regulated; italic, down-regulated). TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; POZ, poxvirus and zinc finger; BMP, bone morphogenetic protein.

Gene	Description	-Fold change	Networks	Location
<i>Dlk1</i>	δ -Like 1 homolog (<i>Drosophila</i>)	15.993	3	Extracellular space
<i>Serpina6</i>	Serpin peptidase inhibitor, clade A (α -1 antiproteinase, antitrypsin), member 6	9.921	3	Extracellular space
<i>Brs3</i>	Bombesin-like receptor 3	2.427	1	Plasma membrane
<i>Tram2</i>	Translocation-associated membrane protein 2	2.347	1	Unknown
<i>Stmn2</i>	Stathmin-like 2	2.154	2	Cytoplasm
<i>Nagk</i>	<i>N</i> -Acetylglucosamine kinase	2.052	1	Cytoplasm
<i>Apobec1</i>	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1	<i>-2.001</i>	1	Cytoplasm
<i>St6galnac3</i>	ST6 (α - <i>N</i> -acetyl-neuraminyl-2,3- β -galactosyl-1,3)- <i>N</i> -acetylgalactosaminide α -2,6-sialyltransferase 3	<i>-2.121</i>	4	Cytoplasm
<i>Tiparp</i>	TCDD-inducible poly(ADP-ribose) polymerase	<i>-2.471</i>	4	Unknown
<i>Syne2</i>	Spectrin repeat-containing, nuclear envelope 2	<i>-2.629</i>	2	Nucleus
<i>Spop</i>	Speckle-type POZ protein	<i>-2.641</i>	3	Extracellular space
<i>Crim1</i>	Cysteine-rich transmembrane BMP regulator 1 (chordin-like)	<i>-2.698</i>	7	Extracellular space
<i>Mt1e</i>	Metallothionein 1E	<i>-3.43</i>	2,3	Cytoplasm

TABLE 5

Genes exhibiting a 2-fold or higher expression difference between the $ng^{\Delta Double}/fg$ and $ng^{\Delta ch12}/fg$ placentae

Fourteen representative genes in the $ng^{\Delta Double}/fg$ placenta were expressed at a level that was 2-fold higher than that in the $ng^{\Delta ch12}/fg$ placenta (bold, up-regulated; italic, down-regulated).

Gene	Description	-Fold change	Networks	Location
<i>Igf2</i>	Insulin-like growth factor 2 (somatomedin A)	26.566	1, 4	Extracellular space
<i>Pla2g2c</i>	Phospholipase A2, group IIC	3.621	8	Extracellular space
<i>Tacc2</i>	Transforming, acidic coiled-coil containing protein 2	3.014	6	Nucleus
<i>Fancb</i>	Fanconi anemia, complementation group B	2.336	3	Nucleus
<i>Ptpnrd</i>	Protein tyrosine phosphatase, receptor type, D	2.295	3	Plasma membrane
<i>Prim1</i>	Primase, DNA, polypeptide 1 (49kDa)	2.215	1, 3	Nucleus
<i>Pou3f1</i>	POU class 3 homeobox 1	2.155	3	Nucleus
<i>Nr6a1</i>	Nuclear receptor subfamily 6, group A, member 1	2.103	4	Nucleus
<i>Txn</i>	Thioredoxin	2.036	1	Cytoplasm
<i>Rad54l</i>	RAD54-like (<i>Saccharomyces cerevisiae</i>)	2.025	5	Nucleus
<i>Dcc1</i>	Defective in sister chromatid cohesion homolog 1 (<i>S. cerevisiae</i>)	2.005	3	Unknown
<i>Aldh1a3</i>	Aldehyde dehydrogenase 1 family, member A3	<i>-2.194</i>	1	Cytoplasm
<i>Cxcl10</i>	Chemokine (CXC motif) ligand 10	<i>-2.501</i>	2	Extracellular space
<i>Lrrc4</i>	Leucine-rich repeat-containing 4	<i>-2.837</i>	11	Unknown

Contributions of Imprinted Genes to Mouse Placentation

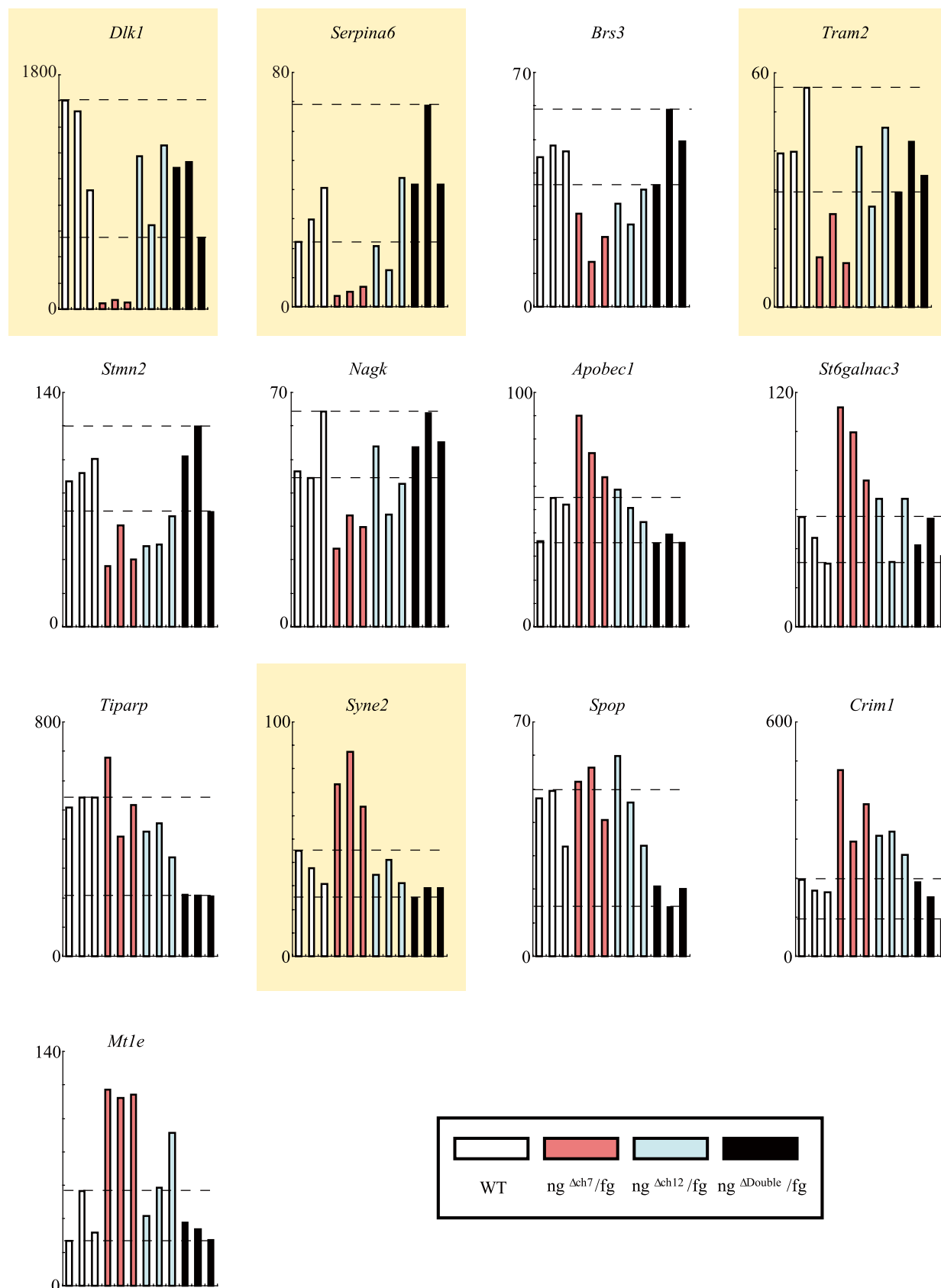


FIGURE 7. This figure shows the expression level of the genes in the $ng^{\Delta Double}/fg$ placenta that are differentially expressed at a level higher than 2-fold that of the $ng^{\Delta ch7}/fg$ placenta for each placenta ($n = 3$). Refer to Table 4. The y axes show the raw expression values from microarray analysis. If the expression level of a focused gene in only the $ng^{\Delta ch7}/fg$ placenta is afied from the range between the levels of the WT and $ng^{\Delta Double}/fg$ placentae (the realm between the two broken lines), the gene expression is considered to be altered in the $ng^{\Delta Double}/fg$ placenta due to the restored expression of paternally methylated imprinted genes on chromosome 12 (colored in yellow).

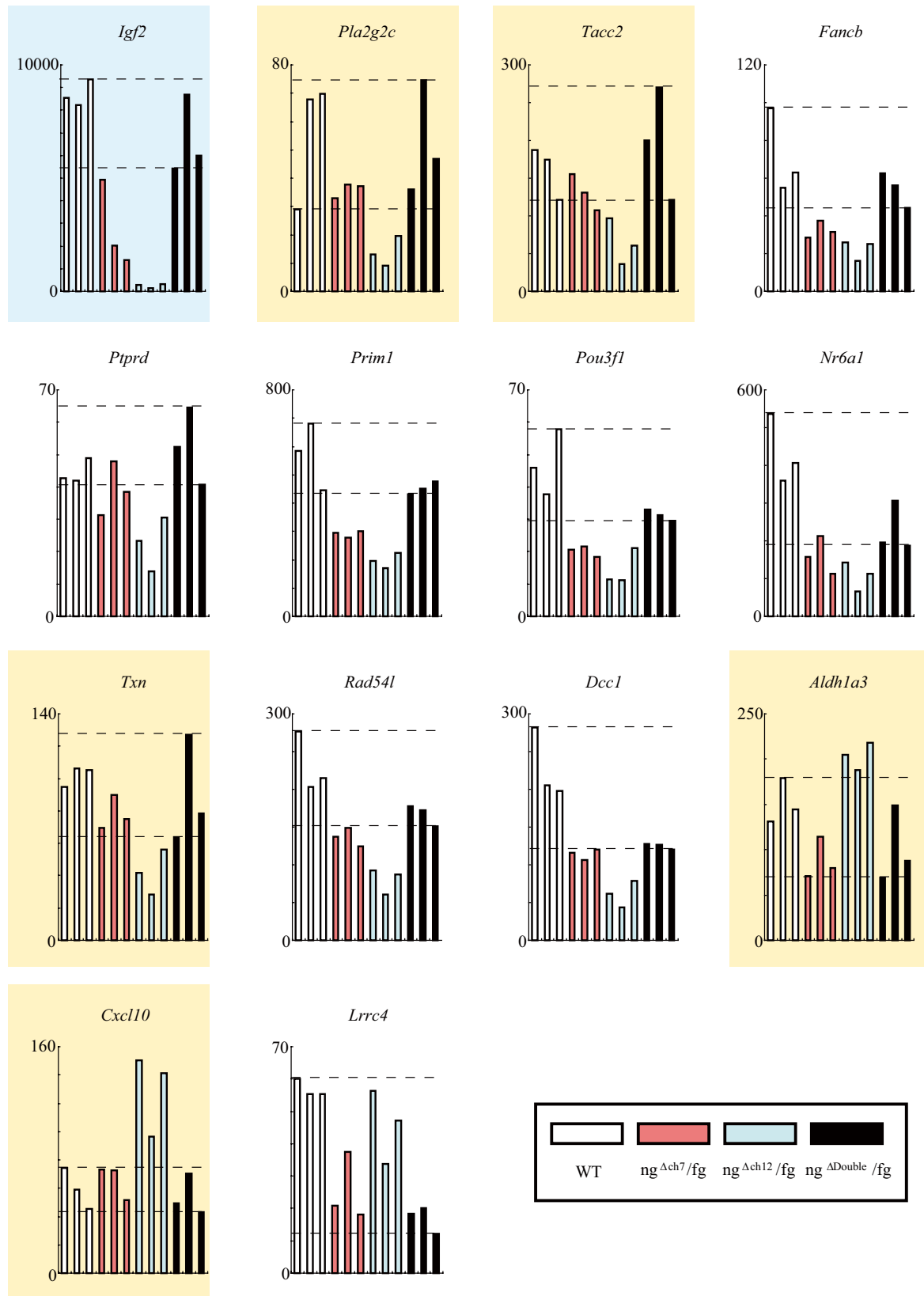


FIGURE 8. This figure shows the expression level of genes in the $ng^{\Delta Double}/fg$ placenta that are differentially expressed at a level higher than 2-fold that of the $ng^{\Delta ch12}/fg$ placenta for each placenta ($n = 3$). Refer to Table 5. If the expression level of a focused gene in only the $ng^{\Delta ch12}/fg$ placenta is afield from the range between the levels of the WT and $ng^{\Delta Double}/fg$ placentae (the area between the two broken lines), we considered the gene expression to be altered in the $ng^{\Delta Double}/fg$ placenta due to the restored expression of paternally methylated imprinted genes on chromosome 7 (colored in yellow). However, *Igf2* was an exception because the *Igf2* expression level was remarkably higher than that of the other genes, although expression in the $ng^{\Delta ch7}/fg$ placenta narrowly missed the range between the two broken lines (shown in light blue).

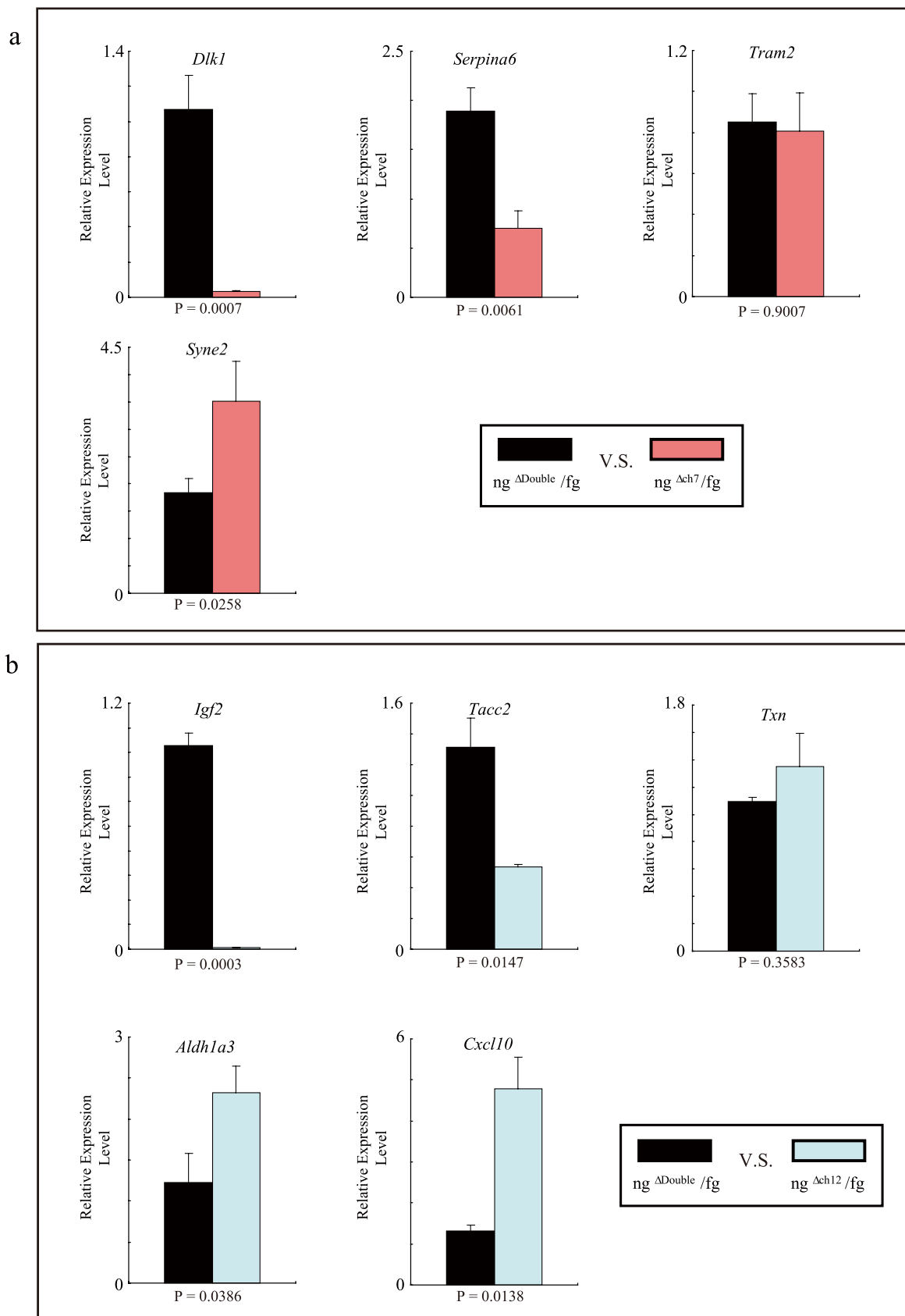


FIGURE 9. **Quantitative RT-PCR analysis of transcripts selected from microarray expression profiles ($n = 3$).** Nine transcripts were selected for quantitative RT-PCR analysis from the array expression profile of the ng Δ^{Double} /fg placenta to the ng Δ^{ch7} /fg or ng Δ^{ch12} /fg placentae (Fig. 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 $\pm$ S.E. (indicated by error bars).

ng^{Δch12}/fg placenta were also similar to those in microarray analysis, namely, *Tacc2* transcript was characteristically down-regulated, and *Aldh1a3* and *Cxcl10* were overexpressed in the ng^{Δch12}/fg placenta (*p* values; 0.0147, 0.0386, and 0.0138, respectively) (Fig. 9b). Although of the nine transcripts, the *Tram2* and *Txn* expression levels in the ng^{Δch7}/fg and ng^{Δch12}/fg placentae showed no significant differences as compared with those in the ng^{ΔDouble}/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 these genes in the ng^{WT}/fg placenta containing wild-type ng genomes did not denote the same tendency of those in ng^{Δch12}/fg placentae (supplemental Fig. 2). This might be because grossly inequable tissues in cell composition in the ng^{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^{Δch7}/fg, ng^{Δch12}/fg, and ng^{ΔDouble}/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 placentation in the ng^{ΔDouble}/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 placentation. 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 placentation 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 placentation.

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