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The Influence of Polyploidy and Genome Composition on Genomic Imprinting in Mice*

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Genomic imprinting is an epigenetic mechanism that switches the expression of imprinted genes involved in normal embryonic growth and development in a parent-of-origin-specific manner. Changes in DNA methylation statuses from polyploidization are a well characterized epigenetic modification in plants. However, how changes in ploidy affect both imprinted gene expression and methylation status in mammals remains unclear. To address this, we used quantitative real time PCR to analyze expression levels of imprinted genes in mouse tetraploid fetuses. We used bisulfite sequencing to assess the methylation statuses of differentially methylated regions (DMRs) that regulate imprinted gene expression in triploid and tetraploid fetuses. The nine imprinted genes *H19*, *Gtl2*, *Dlk1*, *Igf2r*, *Grb10*, *Zim1*, *Peg3*, *Ndn*, and *Ipw* were all unregulated; in particular, the expression of *Zim1* was more than 10-fold higher, and the expression of *Ipw* was repressed in tetraploid fetuses. The methylation statuses of four DMRs *H19*, intergenic (IG), *Igf2r*, and *Snrpn* in tetraploid and triploid fetuses were similar to those in diploid fetuses. We also performed allele-specific RT-PCR sequencing to determine the alleles expressing the three imprinted genes *Igf2*, *Gtl2*, and *Dlk1* in tetraploid fetuses. These three imprinted genes showed monoallelic expression in a parent-of-origin-specific manner. Expression of non-imprinted genes regulating neural cell development significantly decreased in tetraploid fetuses, which might have been associated with unregulated imprinted gene expression. This study provides the first detailed analysis of genomic imprinting in tetraploid fetuses, suggesting that imprinted gene expression is disrupted, but DNA methylation statuses of DMRs are stable following changes in ploidy in mammals.

In mammals, imprinted genes are monoallelically expressed from a single parental allele that is regulated by epigenetic mechanisms, including DNA methylation (1). Acquisition of cytosine guanine (CpG)[2] dinucleotide methylation in differentially methylated regions (DMRs) that are methylated on one of the two parental chromosomes occurs during gametogenesis. Imprinted genes are usually clustered with *cis*-acting DMRs carrying allele-specific methylation markers (2, 3). Moreover, major imprinted genes play critical roles in normal postimplantation development and behavior (4). Mouse uniparental fetuses (androgenetic, parthenogenetic, and gynogenetic) exhibit severe developmental anomalies, resulting in lethality by embryonic day 9.5 (E9.5) (5, 6). Uniparental fetuses have two sets of either maternal or paternal genomes, in which imprinted gene expression is extremely unregulated (7–9). Additionally, the methylation status at *H19*-DMR required for paternal-specific silencing is partially unmethylated in the androgenetic fetus (8). This raises the possibility that uniparental genomes result in disruption of not only imprinted gene expression but also the methylation status of DMRs. In other words, the abundance ratio of maternal and paternal genomes could influence imprinted gene transcription from the expressed allele and the methylation status of the DMR regulating the parent-of-origin-specific expression.

Polyploidy is a state that the parental genome dosage alters dramatically, and it occurs relatively frequently among plants and some animal groups (10–12). In mammals, polyploidy is typically fatal, with embryos dying early in development, although a few mammals, such as the red viscacha rat *Tympanoctomys barrerae*, have tetraploid lineages (13). In plants, polyploidy is much more accommodated and is not typically fatal (14). Furthermore, polyploidization in plants leads to epigenetic alterations, including DNA methylation and histone modification (15–17). Thus, it is possible that the change in genome composition from polyploidy impacts the epigenetic statuses of eukaryotic organisms. However, the interaction between polyploidy and epigenetic modification has not been fully examined in mammals.

Tetraploid genotypes have two sets of maternal and paternal chromosomes, rather than the usual bi-parental diploid set. Therefore, the tetraploid fetus is as "genetically balanced" as the diploid fetus in terms of parental genome ratio (18, 19). However, triploid genotypes, which have only one set of additional maternal or paternal chromosomes to the bi-parental diploid set, are genetically unbalanced. We previously confirmed

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This article contains supplemental Tables S1–S3.

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[2] The abbreviations used are: CpG, cytosine guanine; DMR, differentially methylated region; HTF, human tubal fluid; IVF, *in vitro* fertilization; IG, intergenic.

extremely unregulated expression of imprinted genes, including *H19*, *Gtl2*, *Igf2r*, *Grb10*, *Igf2*, *Dlk1*, *Ndn*, and *Peg3* in triploid fetuses (20), and we suggest investigation of both imprinted gene expression and DNA methylation status in tetraploid and triploid fetuses is needed to fully explore the relationship among ploidy, genome composition, and epigenetic status. Here, we investigate the gene expression levels of five maternally expressed and six paternally expressed imprinted genes in tetraploid fetuses, and we assess the methylation statuses of four DMRs in both tetraploid and triploid fetuses. We also identify the alleles expressing three imprinted genes in tetraploid fetuses.

Results

Developmental Characteristics of Embryos—Chromosome analysis showed successful production of tetraploid and triploid embryos (Fig. 1, *A* and *B*). The blastocyst formation rate of both the tetraploid and triploid embryos was equivalent to that of the diploid embryos (Table 1). However, the developmental rate up to E10.5 was significantly lower in the tetraploid and triploid than the diploid embryos (tetraploid, 8.9%; diandric triploid, 2.2%; digynic triploid, 4.9%; and diploid, 51.9%) (Table 1), which concurs with previous studies (18, 20). Physical appearances of the tetraploid fetuses at E10.5 were craniofacial abnormal, as sizes were much smaller than diploid fetuses, as reported in an earlier study (21). Phenotypes of the triploid fetuses were similar to those observed in earlier studies (20, 22). Furthermore, we examined the expression of the *p53* gene, a central regulatory molecule of apoptosis, in both diploid and tetraploid fetuses using quantitative expression analysis (Fig. 1*D*). The expression of *p53* mRNA was the same in both tetraploid and diploid fetuses at E10.5.

Expression Patterns of Imprinted Genes in Tetraploid Fetuses—To investigate the expression levels of imprinted genes in tetraploid fetuses, we performed quantitative expression analysis for 11 imprinted genes (*H19*, *Igf2*, *Gtl2*, *Dlk1*, *Igf2r*, *Grb10*, *Peg3*, *Snrpn*, *Ndn*, *Ipw*, and *Zim1*) using quantitative real time PCR. Of the 11 imprinted genes analyzed, seven (*H19*, *Gtl2*, *Dlk1*, *Igf2r*, *Grb10*, *Peg3*, and *Zim1*) showed significantly increased mRNA expression levels, and two (*Ndn* and *Ipw*) were significantly decreased ($p < 0.05$, Fig. 2). In particular, mRNA expression of *Zim1* was extremely elevated (10-fold of the diploid control value). Conversely, *Ipw* mRNA expression was repressed (35-fold down-regulation). These results suggested that imprinted gene expression patterns were disrupted in tetraploid fetuses.

Methylation Statuses of Paternally and Maternally Methylated DMRs in Tetraploid and Triploid Fetuses—To more fully understand the disrupted expression levels of imprinted genes in polyploid fetuses, we investigated the methylation statuses of both tetraploid and triploid fetuses in two paternally methylated DMRs (*H19* and IG) and two maternally methylated DMRs (*Igf2r* and *Snrpn*) (Fig. 3). Both *H19* and *Igf2* genes are regulated by *H19*-DMR (23). In addition, both *Gtl2* and *Dlk1* genes are regulated by IG-DMR. The *Igf2r* gene is regulated by *Igf2r*-DMR2, and the *Snrpn*, *Ndnn*, and *Ipw* genes are regulated by *Snrpn*-DMR. Parental alleles of DMRs were distinguished by differences in SNPs between subspecies, *i.e.* B6D2F1 (C57BL/6N and DBA/2) and JF1/Ms strains. As we did not detect any SNPs between these strains regarding *Snrpn*-DMR, we compared the percentages of methylated cytosines in all detected cytosines between diploid controls and polyploid fetuses.

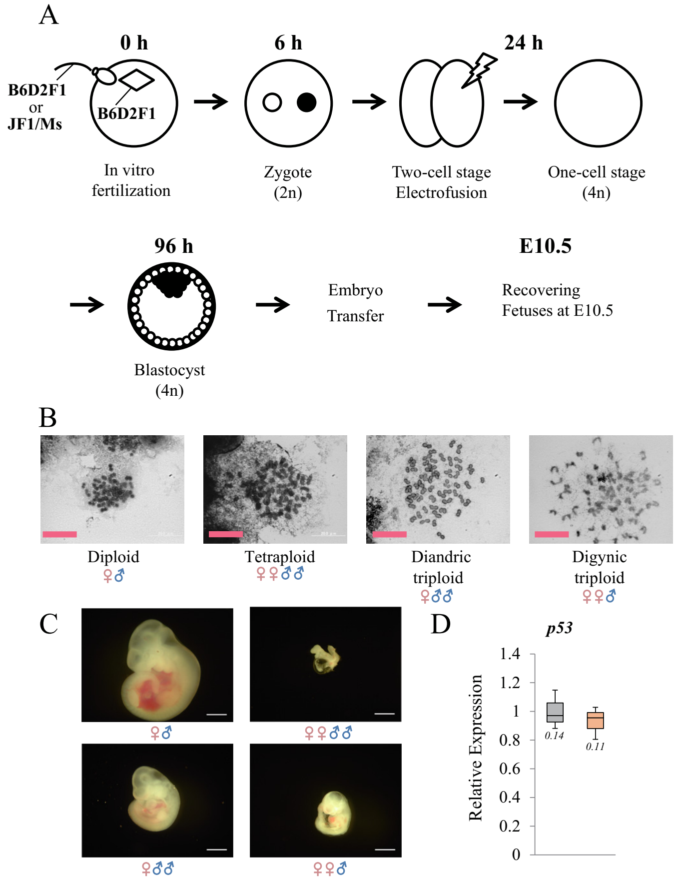

FIGURE 1. **Production of mouse tetraploid fetuses.** *A*, schematic diagram showing the production of tetraploid embryos produced by electrofusion of embryo blastomeres at the two-cell stage. Oocytes donated from B6D2F1 females were used for IVF. Two types of sperm from B6D2F1 and JF1/Ms were used for IVF. The time (0 h) indicates the start of insemination for IVF. After embryo transfer, tetraploid fetuses with heartbeats were retrieved from uteruses at E10.5. *B*, karyotypes at the blastocyst stage: *left*, diploid; *middle left*, tetraploid; *middle right*, diandric triploid; *right*, digynic triploid. Chromosome numbers are 40 in diploid, 80 in tetraploid, and 60 in both diandric and digynic triploid embryos. Analyses were performed on more than 10 blastomeres. The *scale bar*, 20 μm. *C*, representative appearance of fetuses at E10.5: *upper left*, diploid; *upper right*, tetraploid; *lower left*, diandric triploid; *lower right*, digynic triploid. The *scale bar*, 1 mm. *D*, relative expression levels of the *p53* gene, a central regulatory molecule of apoptosis, in both diploid and tetraploid fetuses were analyzed using quantitative real time PCR: *gray*, diploid; *orange*, tetraploid. Values represent the levels of expression relative to an internal control gene (*Gapdh*). The 25th and 75th percentiles form the *box*, with the median marked as a *line*, nd the maximum and minimum values form the *whiskers* within the acceptable range that is defined by two quartiles. Standard deviation (*S.D.*) values are represented at bottom of boxplots ($n = 3$).

All DMR methylation statuses of diploid controls showed parent-of-origin-specific patterns (Fig. 3*A* and Table 2). In both the *H19*-DMR and IG-DMR of tetraploid fetuses, paternal alleles were hypermethylated, whereas maternal alleles were hypomethylated (Fig. 3*B* and Table 2). In contrast, paternal alleles of *Igf2r*-DMR2 were hypomethylated and maternal alleles were hypermethylated (Fig. 3*B* and Table 2). The *Snrpn*-DMR of tetraploid fetuses showed the same methylation level

TABLE 1

Pre- and postimplantation development of mouse tetraploid and triploid embryos up to E10.5

	No. of embryos cultured	No. of blastocysts	No. of recipients	No. of blastocysts transferred	No. of implantations	No. of viable fetuses[a]
		%			%	%
Diploid	121	116 (95.9)	3	27	25 (92.6)	14 (51.9)
Tetraploid	191	190 (99.5)	9	105	74 (70.5)	9 (8.6)
Diandric triploid	301	256 (85.0)	18	178	108 (60.7)	4 (2.2)
Digynic triploid	91	81 (89.0)	12	81	53 (65.4)	4 (4.9)

[a] Viable fetuses exhibited obvious heartbeats when recovered from uterine horns of recipients.

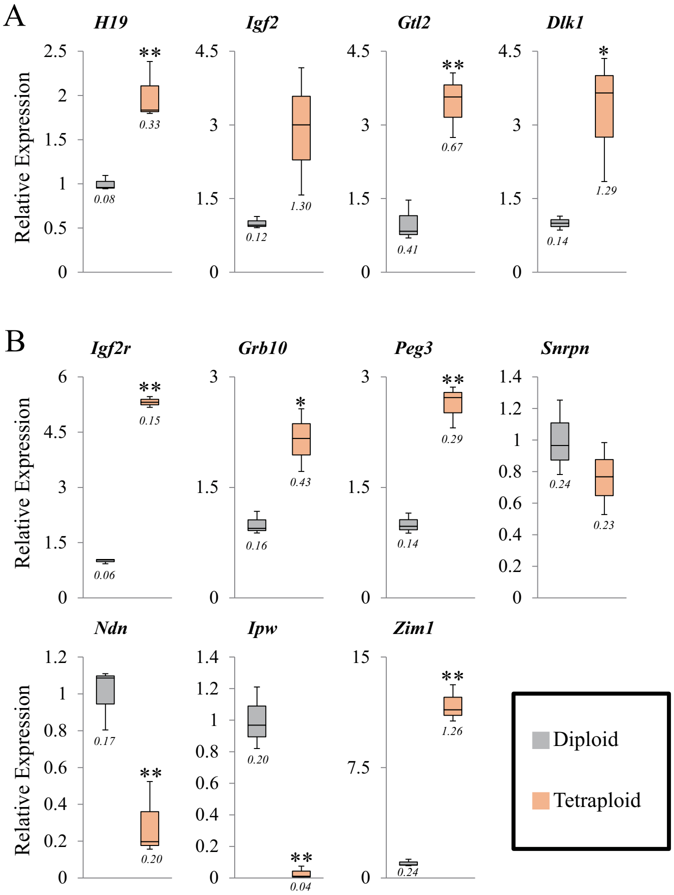

FIGURE 2. **Boxplot representations of the expression of paternally methylated and maternally methylated imprinted genes in tetraploid fetuses at E10.5.** Relative expression levels of imprinted genes in both diploid and tetraploid fetuses were analyzed using quantitative real time PCR. *A*, paternally methylated imprinted genes (maternally expressed, *H19* and *Gtl2*; paternally expressed, *Igf2* and *Dlk1*). *B*, maternally methylated imprinted genes (maternally expressed, *Igf2r*, *Grb10*, and *Zim1*; paternally expressed, *Peg3*, *Snrpn*, *Ndn*, and *Ipw*). Values represent the levels of expression relative to an internal control gene (*Gapdh*). The 25th and 75th percentiles form the *box*, with the median marked as a *line*, the maximum and minimum values form the *whiskers* within the acceptable range that is defined by the two quartiles. Standard deviation (*S.D.*) values are represented at *bottom* of boxplots ($n = 3$). *, $p < 0.05$; **, $p < 0.01$.

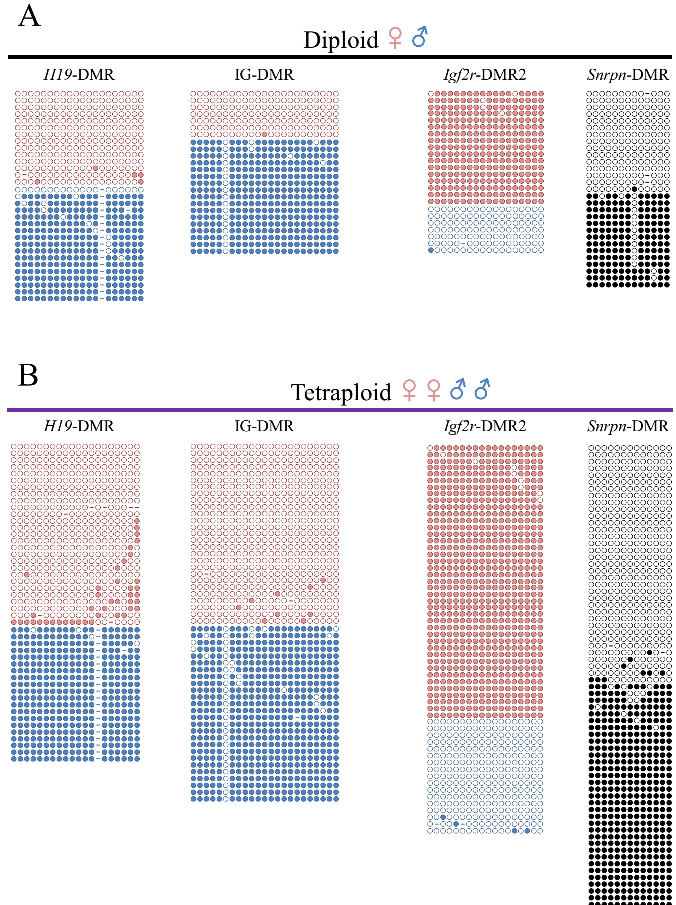

FIGURE 3. **Bisulfite sequencing analysis of DNA methylation statuses at two paternally and two maternally methylated DMRs in diploid and tetraploid fetuses at E10.5.** *A*, bisulfite sequencing results in diploid fetuses ($n = 3$) showing methylated (*filled circles*) and unmethylated (*open circles*) CpG sites of two paternally methylated DMRs, *i.e. H19* and IG, and two maternally methylated DMRs, *i.e. Igf2r* and *Snrpn*. In the bisulfite sequencing profiles, *hyphens* represent missing or undetermined CpG sites due to SNPs or sequencing failures. Paternal and maternal alleles were distinguished from SNPs where available. *Red*, female allele sequences from B6D2F1 (C57BL/6N × DBA/2); *blue*, male allele sequences from JF1/Ms. We did not detect any differences in SNPs between these strains regarding *Snrpn*-DMR. Therefore, *Snrpn*-DMR did not distinguish paternal and maternal alleles. *Black*, female and male allele sequences. *B*, CpG methylation profiles of four DMRs are presented as described above in tetraploid fetuses ($n = 3$).

as diploid fetuses (48.3% *versus* 44.9%) (Fig. 3*B* and Table 2). Furthermore, the parent-of-origin-specific methylation statuses in the above-mentioned DMRs were maintained in triploid (diandric and digynic) fetuses (Fig. 4 and Table 3). Together, these results demonstrated that the parent-of-origin-specific methylation statuses were maintained in examined DMRs, irrespective of ploidy.

Allele-specific RT-PCR Sequencing Analysis in Tetraploid Fetuses—To determine the allele expressing the imprinted genes in tetraploid fetuses, we tracked the three imprinted genes *Igf2*, *Gtl2*, and *Dlk1* and analyzed the RNA products of cDNAs from B6D2F1 × JF1/Ms (named BDJF) tetraploid fetuses by direct sequencing. As a control, we repeated the procedure for cDNAs of BDJF diploid fetuses. All three of the imprinted genes *Igf2*, *Gtl2*, and *Dlk1* were expressed from a single parental origin-specific allele; *Igf2* from the paternal allele, *Gtl2* from the maternal allele, and *Dlk1* from the paternal allele (Fig. 5). Therefore, the parent-of-origin-specific expres-

TABLE 2
Number of methylated CpGs in female and male alleles in DMRs in diploid and tetraploid fetuses

DMR	Allele	No. of methylated CpG/no. of all CpG (% ± S.E.) Diploid	Tetraploid
H19	Female	5/279 (1.8 ± 1.0)	42/533 (7.9 ± 2.7)
	Male	291/322 (90.4 ± 5.7)	376/ 380 (98.9 ± 0.62)
IG	Female	1/161 (0.62 ± 0.62)	9/619 (1.5 ± 0.52)
	Male	369/374 (98.7 ± 0.65)	555/571 (97.2 ± 0.67)
Igf2r	Female	301/306 (98.4 ± 0.79)	729/739 (98.8 ± 0.36)
	Male	1/125 (0.80 ± 0.79)	4/304 (1.3 ± 0.77)
Snrpn	Not determined	168/374 (44.9 ± 8.6)	426/882 (48.3 ± 5.9)

TABLE 3
Number of methylated CpGs in female and male alleles in DMRs in diandric and digynic triploid fetuses

DMR	Allele	No. of methylated CpG/no. of all CpG (% ± S.E.) Diandric triploid	Digynic triploid
H19	Female	44/655 (6.7 ± 2.1)	29/908 (3.2 ± 0.7)
	Male	422/436 (96.8 ± 0.72)	435/494 (88.1 ± 5.17)
IG	Female	12/482 (2.5 ± 0.64)	25/1102 (2.3 ± 0.52)
	Male	537/572 (93.9 ± 3.7)	646/659 (98.0 ± 0.64)
Igf2r	Female	690/699 (98.7 ± 0.43)	1519/1545 (98.3 ± 0.37)
	Male	20/288 (6.9 ± 6.2)	2/88 (2.3 ± 1.4)
Snrpn	Not determined	431/818 (52.7 ± 5.9)	656/1155 (56.8 ± 4.7)

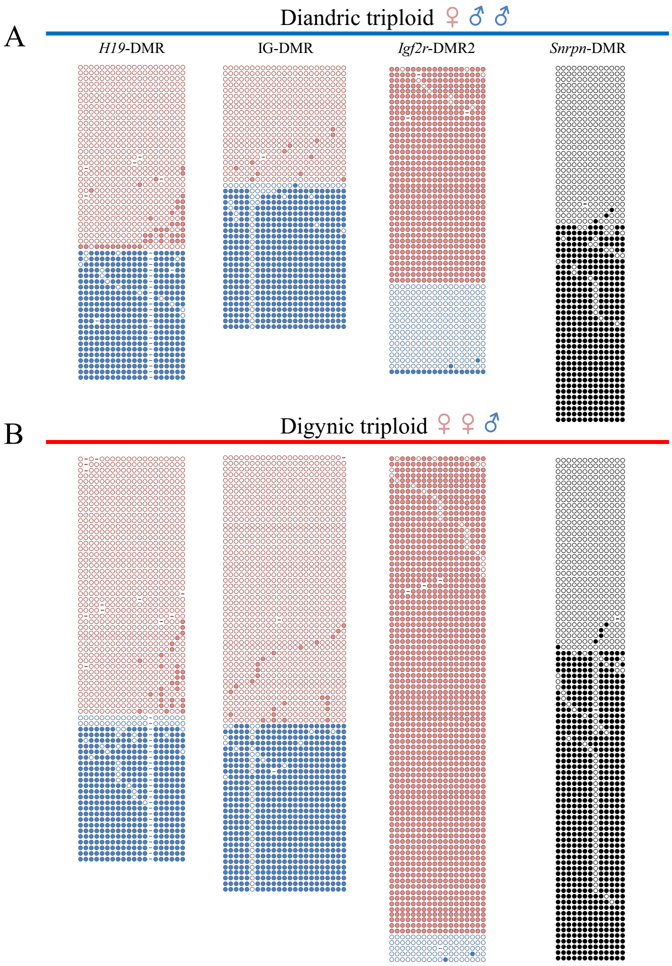

FIGURE 4. **Bisulfite sequencing analysis of DNA methylation statuses of two paternally and two maternally methylated DMRs in triploid fetuses at E10.5.** CpG methylation profile of two paternally methylated DMRs, *i.e. H19* and IG, and two maternally methylated DMRs, *i.e. Igf2r* and *Snrpn*, in the diandric ($n = 3$) (*A*) and digynic ($n = 4$) (*B*) triploid fetuses. As described in Fig. 3, methylated and unmethylated CpG sites are represented by *filled circles* and *open circles*, respectively. In the bisulfite sequencing profiles, *hyphens* represent missing or undetermined CpG sites due to SNPs or sequencing failures. Paternal and maternal alleles were distinguished from SNPs where available. *Red,* female allele sequences from B6D2F1 (C57BL/6N × DBA/2); *blue,* male allele sequences from JF1/Ms. We did not detect any differences in SNPs between these strains regarding *Snrpn*-DMR. Therefore, *Snrpn*-DMR did not distinguish paternal and maternal alleles. *Black,* female and male allele sequences.

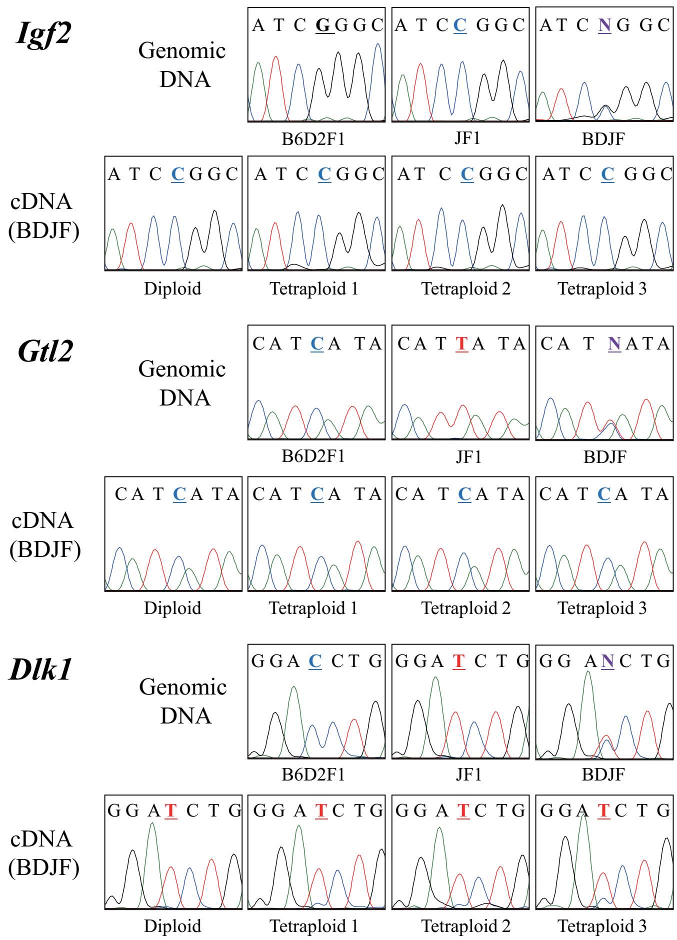

FIGURE 5. **Identification of the allele expressing imprinted genes in tetraploid fetuses.** We performed allele-specific RT-PCR sequencing analysis of *Igf2*, *Gtl2*, and *Dlk1* in both the diploid ($n = 3$) and tetraploid ($n = 3$) fetuses at E10.5. *Upper three panels* for each imprinted gene show the polymorphism detected with genomic DNA obtained from B6D2F1 (C57BL/6N × DBA/2) liver, JF1/Ms liver, and BDJF (B6D2F1 × JF1/Ms) fetus, respectively. *Lower panels* represent the direct sequencing of cDNAs from BDJF diploid and tetraploid fetuses at E10.5. The SNP of each imprinted gene is highlighted in *boldface* and *underlined characters.*

sion patterns of *Igf2*, *Gtl2*, and *Dlk1* were maintained in tetraploid fetuses.

Expression Patterns of Non-imprinted Genes in Tetraploid Fetuses—We observed down-regulation of *Ndn* and *Ipw*, which are associated with the neurogenetic disorder Prader-Willi syndrome (24–26) in tetraploid fetuses (Fig. 2). Therefore, we examined the expression levels of the non-imprinted *Map1b*, *Pax6*, and *Nestin* genes that play critical roles in nervous system development using quantitative real time PCR (Fig. 6) (27–30). The expression levels of all three genes significantly decreased in tetraploid fetuses ($p < 0.01$).

Additionally, to explore the maintenance of parent-of-origin-specific methylation statuses in tetraploid fetuses, we

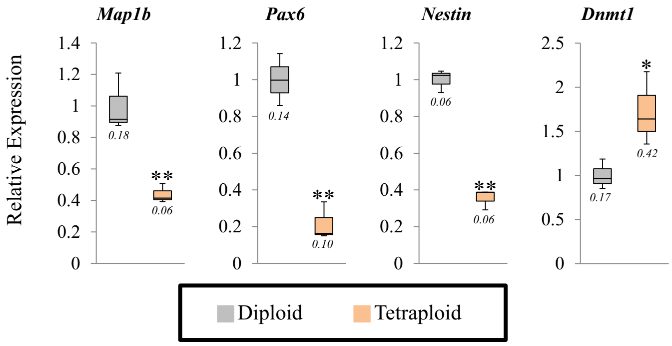

FIGURE 6. **Boxplot representations of non-imprinted genes in tetraploid fetuses at E10.5.** Relative expression levels of non-imprinted genes, *Map1b*, *Pax6*, *Nestin,* and *Dnmt1*, in both diploid and tetraploid fetuses were analyzed using quantitative real time PCR. Values represent the levels of expression relative to an internal control gene (*Gapdh*). The 25th and 75th percentiles form the *box*, with the median marked as a *line*, the maximum and minimum values form the *whiskers* within the acceptable range that is defined by the two quartiles. Standard deviation (*S.D.*) values are represented at bottom of boxplots ($n = 3$). *, $p < 0.05$; **, $p < 0.01$.

examined the expression levels of DNA methyltransferase 1, *Dnmt1,* using quantitative real time PCR (Fig. 6). The *Dnmt1* expression level in tetraploid fetuses significantly increased ($p < 0.05$, Fig. 6).

Discussion

Generally, the main regulator of imprinted gene expression is the *cis*-acting mechanism that acts only on one-sided chromosomes (2). Imprinted gene expression is controlled by epigenetic modifications, including DNA methylation of DMRs. However, the expression levels of imprinted genes in tetraploid fetuses, with equal ratios of maternal and paternal chromosomes, were aberrant, despite the methylation statuses of DMRs remaining parent-of-origin-specific. These results could not be explained by conserved *cis*-acting regulation alone. Recently, it was revealed that imprinted gene expression levels could be regulated by cell cycle, proliferation, and differentiation, independently of changes in the methylation patterns of DMRs (31). Mouse embryonic fibroblasts treated with serum/primary growth factors showed that imprinted genes that were in a quiescent state were up-regulated, whereas those that were in a proliferative state were down-regulated. Both the methylation statuses and monoallelic expression patterns remained parent-of-specific (31). Our observations of tetraploid fetuses were consistent with DMR methylation-independent alteration of imprinted gene expression. In general, tetraploidy induces G_1 arrest in cells and reduces cell proliferation in *in vitro* culture (32, 33). Therefore, up-regulation of imprinted gene expression in tetraploid fetuses might occur through suppressed cell proliferation and arrested cycles of tetraploid cells. In addition, altering the expression of restricted numbers of imprinted genes may have further effects on numerous non-imprinted genes, from the indirect effects of improved growth by restored imprinted gene expression (34–36). Although we confirmed that the expression of *p53* was equivalent in tetraploid and diploid fetuses, cell proliferation in tetraploid fetuses was suppressed. Thus, the intrinsic characteristics of tetraploid cells might influence imprinted gene expression levels. Precisely how cell differentiation, proliferation, and cell cycle contribute to imprinted gene expression remains unknown, particularly *in vivo*.

The influence of *trans*-acting interactions on regulation of imprinted genes has been observed at several imprinted loci, for instance, between *Ipw* and maternally expressed genes, including *Gtl2* within the *Dlk1-Dio3* region, and between *Peg3* and *Zim1* (37, 38). We found that *Ipw* expression was repressed and that *Gtl2* expression was increased in tetraploid fetuses. In human-induced pluripotent stem cells, *IPW* is a noncoding RNA located in the *SNRPN* imprinted cluster on human chromosome 15 that regulates maternally expressed genes in the *DLK1-DIO3* region on human chromosome 14 (37). The absent expression of *IPW* results in up-regulation of maternally expressed genes in the *DLK1-DIO3* region. In contrast, overexpressed *IPW* in the *IPW*-lacking induced pluripotent stem cells leads to down-regulation of maternally expressed genes in the *DLK1-DIO3* region. Thus, maternally expressed gene expression in the *DLK1-DIO3* region could be regulated by *cis*-acting IG-DMR methylation and the noncoding RNA *IPW* in *trans*. Although it is unknown whether the same regulatory mechanism is conserved in mice, the up-regulation of maternally expressed genes in the *Dlk-Dio3* region might be due to repression of *Ipw* in the tetraploid fetus. Furthermore, the mechanism of *Ipw* down-regulation in tetraploid fetuses suggests that tetraploidy is associated with the upstream regulation of *Ipw* expression. Meanwhile, PEG3 protein binds to the zinc finger exon of *Zim1*, concomitant with histone modification in mouse embryonic fibroblasts. Lack of PEG3 protein causes up-regulation of *Zim1* at transcription in mice (38). Additionally, the restoration of *Peg3* transcription in the *Peg3*-deficient cells results in *Zim1* down-regulation. Hence, the interactive relationship between *Peg3* and *Zim1* is also regarded as a *trans*-acting mechanism as well as regulation by *Ipw*. However, we found that expression of *Peg3* was significantly increased, and expression of *Zim1* was extremely elevated in tetraploid fetuses. This clearly demonstrated that regulation of *Zim1* expression was not only due to the PEG3 protein binding to the zinc finger exon of *Zim1* in tetraploids.

The parent-specific methylation statuses of DMRs were maintained in tetraploid fetuses. In bi-parental embryos, the parent-specific methylation statuses of DMRs are maintained until postimplantation development, involving several key enzymes and histone modifications. Throughout pre- and post-implantation development, maintenance methyltransferase, *Dnmt1*, and its cofactor, *Uhrf1*, are necessary for appropriate maintenance of methylation statuses of DMRs (1, 39–44). Overexpression of DNMT1 protein resulted in changes in *H19*-DMR methylation level in mouse embryonic stem cells (45). In this study, we observed that the *Dnmt1* gene mRNA expression significantly increased in tetraploid fetuses (Fig. 6), but this level of up-regulation might not lead to the changes of the DMRs methylation statuses because all the examined methylation statuses in DMRs, including *H19*-DMR, did not change in tetraploid fetuses (Fig. 3). Maintenance of methylation statuses in DMRs in a parent-of-origin-specific manner indicated that DNA methylation-regulating factors functioned appropriately in tetraploid fetuses. Our results suggested that polyploidy *per se* did not affect the mechanisms that maintain parent-specific

DNA methylation statuses of DMRs in embryos during development, at least until E10.5.

Increased expression in a part of imprinted genes was detected in tetraploid fetuses, which was also found in our previous investigation by using triploid fetuses (20). Monoallelic expression of imprinted genes was mainly controlled by alternate methylation patterns of DMRs between maternal and paternal alleles. The loss of DNA methylation within DMRs leads to a biallelic expression pattern, *i.e.* disruption of monoallelic expression, resulting in dysregulation of imprinted genes (39, 46, 47). However, our results demonstrated that parent-specific methylation statuses of DMRs and monoallelic expression patterns of imprinted genes were maintained in tetraploid fetuses. In other words, increased expression of imprinted genes in tetraploid fetuses did not result from disruption of DNA methylation maintenance in imprinted loci and biallelic expression of imprinted genes.

To further support our results, we focused on the expression levels of non-imprinted genes that are associated with well known aberrant neural development, including small forebrain vesicles and eyes in tetraploid fetuses (18, 48). Therefore, we investigated the expression levels of three non-imprinted genes that are essential for normal nervous system development, *Map1b*, *Pax6*, and *Nestin* (27–30). All the expression levels of these genes significantly decreased in tetraploid fetuses (Fig. 6). Interestingly, down-regulation of *Pax6* causes small eyes and severe craniofacial and forebrain defects in mice (28, 29), which clearly corresponds to the phenotypes of tetraploid fetuses. As described above, we observed down-regulation of *Ndn* and *Ipw* that are also associated with a neurogenetic disorder, Prader-Willi syndrome (24–26). Although the direct relationship between these imprinted genes and the three non-imprinted genes is unclear, the expression patterns of *Map1b*, *Pax6*, and *Nestin* might reflect phenotypes unique to tetraploid fetuses.

In conclusion, we demonstrated that expression of imprinted genes was disrupted, and parent-specific methylation statuses of DMRs and monoallelic expression patterns were maintained in tetraploid fetuses. Disrupted expression of imprinted genes might partially result from suppressed cell proliferation and arrested cell cycle of tetraploid cells. Our results clearly demonstrate that polyploidy of embryos did not affect maintenance of parent-specific methylation levels of DMRs and monoallelic expression patterns. This study contributes toward elucidating the effects of changes in ploidy on imprinted gene expression in mammals.

Experimental Procedures

All research and protocols were approved by the Regulatory Committee for the Care and Use of Laboratory Animals, Hokkaido University.

Production of Mouse Tetraploid Embryos—Diploid embryos were prepared by *in vitro* fertilization (IVF). IVF was performed by modifying the methods described in a previous study (20). The oocyte donors were B6D2F1 female mice. They were superovulated with administrations of 7.5 IU (international units) of equine chorionic gonadotropin (ASKA Pharmaceutical, Tokyo, Japan) and 7.5 IU of human chorionic gonadotropin (ASKA Pharmaceutical) given 48 h apart. At 16 h after human chorionic gonadotropin administration, oocytes at the metaphase of the second meiosis were collected from oviducts and used for IVF. Prior to IVF, spermatozoa were collected from the cauda epididymis of mature B6D2F1 for quantitative real time PCR and from JF1/Ms for bisulfite sequence and allelic expression analysis of male mice and preincubated in the droplets of the human tubal fluid medium (HTF) containing 0.4 mM methyl-β-cyclodextrin (Sigma) in a humidified atmosphere containing 5% CO_2 at 37 °C for 1.5 h (49, 50). Collected second meiosis oocytes were transferred into droplets of the HTF medium containing 1.25 mM glutathione (GSH; Sigma). Preincubated spermatozoa were added to the same HTF droplets. After 6 h of insemination, the presumptive zygotes were washed in M2 medium (51) and transferred into droplets of the M16 medium containing 0.1 mM EDTA (Dojindo Laboratories, Kumamoto, Japan) (52) for *in vitro* culture. The second polar body was removed from IVF embryos and cultured until the blastocyst stage in a humidified atmosphere containing 5% CO_2 at 37 °C.

To produce tetraploid embryos, we performed electrofusion using two-cell stage embryos at 24 h after insemination (Fig. 1*A*). Two-cell embryos were placed between two gold electrode fusion chambers filled with M2 medium and electroshocked with LF101 Electro Cell Fusion Generator (NEPAGENE, Chiba, Japan) set at 150 V, with a pulse duration of 50 μs. After electrostimulation, embryos were washed in M2 medium and incubated for fusion of cytoplasm in M16 for 1 h. After confirming blastomere fusion under a stereomicroscope, presumptive tetraploid embryos were cultured until the blastocyst stage at 96 h after insemination.

Triploid (diandric and digynic) embryos were produced by pronuclear transplantation, as described previously (20). The resulting diploid, tetraploid, and triploid blastocysts were transferred into the uterine horns of recipient ICR or B6D2F1 females after 2.5 days of pseudopregnancy. Fetuses for experiments were recovered from recipient uteruses at E10.5. Fetuses with a heartbeat were used for the experiments.

Chromosome Analysis—At 96 h after insemination, diploid, tetraploid, and triploid blastocysts were incubated in M16 medium containing 1 μg/ml nocodazole (Sigma) in a humidified atmosphere containing 5% CO_2 at 37 °C for 6 h. After removing the zona pellucida with acetic Tyrode's solution (53), the embryos were placed in hypotonic solution (1% sodium citrate) at room temperature for 15 min and mildly fixed in methanol/acetic acid/water solution (5:1:4 v/v) for 5 min. Embryos were then transferred onto glass slides and fixed in methanol/acetic acid solution (3:1 v/v). Fixed embryos were dried using humidified warm air for 15 min. Chromosomes were stained with 2% (v/v) Giemsa solution for 10 min. Glass slides were washed with water and completely dried at room temperature before observing the chromosome number of embryos.

Genomic DNA and Total RNA Isolation—To isolate genomic DNA, one fetus at E10.5 from each of fetuses was lysed in 400 μl of TNE buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) containing 20 μl of 10% SDS solution and 8 μl of 10 mg/ml proteinase K solution, followed by incubation for 16 h at 37 °C. Incubated lysates were purified by phenol/chloroform extraction and ethanol precipitation. Resultant genomic DNAs were resuspended in distilled water. Additionally, total RNA from one tetraploid and diploid fetus at E10.5 was collected using ReliaPrep™ RNA cell miniprep system (Promega, Madison, WI) according to the manufacturer's instructions.

Genomic DNA and RNA concentrations were quantitated using Nanodrop (Thermo Fisher Scientific, Wilmington, DE). Quantities of extracted DNA and RNA were normalized to the concentrations of 1.0 μg of DNA for diploid and diandric triploid fetuses, 0.5 μg of DNA for tetraploid and digynic triploid fetuses, and 0.25 μg of RNA for tetraploid and diploid fetuses with one replicate for each experiment. After normalizing RNA quantities, cDNAs were synthesized using ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan) in a reaction mixture (10 μl) containing 0.25 μg of the total RNA extracted from each fetus.

Quantitative Real Time PCR—RNA samples from three tetraploid and diploid fetuses were prepared to analyze gene expression of imprinted and non-imprinted genes using quantitative real time PCR (LightCycler®; Roche Diagnostics, Basel, Switzerland). Reaction mixtures were prepared using Thunderbird SYBR® qPCR mix (Toyobo) in triplicate as described previously ($n = 3$) (20). The primer sets for qPCR are listed in supplemental Table S1. Transcript levels in each sample were calculated relative to transcription of the housekeeping gene *Gapdh*.

Bisulfite Sequencing—Genomic DNA was treated with bisulfite reagent using EZ DNA Methylation-Gold kit™ (Zymo Research, CA) according to the manufacturer's instructions. Bisulfite-treated genomic DNA was amplified by nested PCR using Takara Ex Taq Hot Start Version (TAKARA BIO, Shiga, Japan) for *H19*-DMR, IG-DMR, *Igf2r*-DMR2, and *Snrpn*-DMR. The primer sets for the bisulfite sequencing and nested PCR conditions are presented in supplemental Table S2 (54–58).

Amplified PCR products were purified and cloned into a pGEM®-T Easy Vector (Promega). Plasmid DNA was isolated using alkaline SDS method. Isolated plasmid DNA was sequenced with the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster, CA). At least 15 clones per 1 fetus of each polyploidy type were prepared, *i.e.* tetraploid ($n = 3$), diandric triploid ($n = 3$), and digynic triploid ($n = 4$). For the control diploid fetus, seven clones per 1 fetus were prepared, using three independent fetuses ($n = 3$).

Sequence alignments, methylation analysis, and visualization were conducted using the web-based tool QUMA (59). Maternal and paternal alleles were distinguished by differences in SNPs between subspecies.

Allele-specific RT-PCR Sequencing—To explore polymorphism, genomic DNAs were isolated from the liver of mature B6D2F1 and JF1/Ms male mice and BDJF diploid fetuses at E10.5. Isolation of genomic DNAs was performed as described above. The genomic DNAs were amplified by PCR using Go Taq Green Master Mix (Promega) for *H19*, *Igf2*, *Gtl2*, *Dlk1*, *Igf2r*, *Grb10*, *Peg3*, *Snrpn*, and *Ndn*. Amplified PCR products were purified and directly sequenced. Allelic expression analysis was performed on the basis of polymorphisms detected in *Igf2*, *Gtl2*, and *Dlk1* genes. Polymorphisms of *H19*, *Igf2r*, *Grb10*, *Peg3*, *Snrpn*, and *Ndn* genes were not detected in this study.

For allelic expression analysis, total RNAs were isolated from diploid and tetraploid fetuses at E10.5. Isolation of total RNAs and synthesis of cDNAs were performed as described above. The cDNA was amplified by PCR using Go Taq Green Master Mix (Promega) for *Igf2*, *Gtl2*, and *Dlk1*. Amplified PCR products were purified and directly sequenced using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The primer sets for allelic expression analysis are listed in supplemental Table S3.

Statistical Analysis—We compared the means using Student's t test for gene expression analysis. p values < 0.05 or < 0.01 were assumed as statistically significant. All calculations were performed using the software StatView (Abacus Concepts, Inc., Berkeley, CA).

Author Contributions—W. Y. conducted the experiments, analyzed the results, and discussed and wrote the draft manuscript. T. A. contributed to bisulfite sequencing analysis. H. B. and M. T. contributed to data analysis. M. K. contributed to project management, results analysis and discussion, writing the paper, and communication for publication.

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The Influence of Polyploidy and Genome Composition on Genomic Imprinting in Mice

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