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Roles of Genes Regulated by Two Paternally Methylated Imprinted Regions on Chromosomes 7 and 12 in Mouse Ontogeny

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Abstract. We studied the longevity of mice produced without sperm using the genomes of oocytes that are already committed to a germline cell lineage. The first sperm-free mouse “KAGUYA”, which we term ‘bi-maternal mouse’, was born on 3 February, 2003. Bi-maternal embryos were generated using 2 sets of female genomes—one derived from fully grown oocytes from normal adults and the other from non-growing oocytes from newborn pups. These genomes were combined by nuclear transfer. We refined the technique for generating bi-maternal mice and found that genetic manipulations in only 2 regions—the imprinting centres of *Igf2-H19* and *Dkl1-Gtl2*—on chromosomes 7 and 12 of the newborn pups allowed us to generate bi-maternal mice at a high rate. Studying bi-maternal conceptuses and mice provides further insight into the mechanisms by which paternally methylated imprinted genes regulate mammalian ontogenesis.

Key words: Development, *Dkl1*, Genomic imprinting, *Gtl2*, *H19*, *Igf2*, Mouse

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Androgenetic and parthenogenetic embryos fail to develop past early post-implantation and mid-gestation respectively, and disruption of imprinting is implicated in serious human diseases such as Angelman syndrome, Prader-Willi syndrome, Beckwith-Wiedemann syndrome, and Silver-Russell syndrome [1–3]. However, parthenogenesis is not unusual for non-mammals. For instance, parthenogenesis in turkey was studied for many years, and in fact, as many as 1 to 30% of unfertilized turkey eggs develop embryos [4–9]. In mammals, full development typically requires genomes from both the oocyte and spermatozoon. Previous studies have revealed that embryos possessing two paternally derived pronuclei (androgenones) or two maternally derived pronuclei (gynogenones and parthenogenones) fail to develop to term [10–12]. In mammals, imprinted genes, wherein only one of the two parental chromosome copies is expressed, are regulated by epigenetic modifications, including DNA methylation [13]. Genomic imprinting regulates the expression of developmentally crucial mammalian genes. How can the function of the germ line-specific methylation imprints during embryo development be elucidated? It is impossible to directly assess the genome competence of non-growing (ng) and growing-stage oocytes to support development. This is because at these stages, the oocytes are entirely incompetent and are unable to mature to the metaphase of the second meiosis (MII), undergo fertilization and develop.

We established serial nuclear transfer as a reliable procedure for reconstructing oocytes containing two haploid sets of maternal genomes by using oocytes obtained from different sources [14–17].

In the first nuclear transfer, an ng oocyte from growing stage oocytes was fused with a fully grown germinal vesicle (GV)-stage (GV-fg) oocyte from which the GV was previously removed. In the second nuclear transfer, the resultant MII spindle derived from the ng oocyte was transferred into an intact MII oocyte. Following artificial activation, the reconstructed embryos became diploid, forming two pronuclei and polar bodies, and began to develop. The embryos that were derived solely from maternal genomes were designated as bi-maternal embryos (BMEs) in order to clearly distinguish them from parthenogenetic/gynogenetic embryos. This method enables the assessment of the nuclear properties of ng and growing-stage oocytes. Furthermore, by using this serial nuclear transfer system, we have demonstrated several lines of direct evidence that the epigenetic modifications occurring during oocyte growth or spermatogenesis exert a decisive effect on mammalian development.

Riddle Arising from Birth of “Kaguya”

So far three differentially methylated regions (DMRs) that are methylated during spermatogenesis and located on chromosomes 7, 9 and 12 have been identified as imprinting control regions [18–20]. We constructed three types of BMEs; that is, using ng oocytes derived from mutant mice harbouring a 13-kb deletion including the *H19* transcription unit and its DMR on chromosome 7 (Δ ch7), mutant mice carrying a 4.15-kb deletion in the intergenic germline-derived DMR (IG-DMR) on chromosome 12 (Δ ch12), and double-knockout mice (Δ Double) that harboured deletions in both the *H19*-DMR and the IG-DMR.

We first reported that ng ^{Δ ch7}/fg BMEs developed to adulthood with the ability to reproduce offspring; however, the efficiency of this technique was extremely low [15]. It took 371 oocyte *H19*-

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deletion manipulations to produce only two normal live bi-maternal mice (0.5%). Those survived ng^{Ach7}/fg BMEs exhibited the normal level of paternally expressed *Dlk1*, which grew up to adulthood with normal reproductive ability. We carried out further methylation analysis of the IG-DMR of *Dlk1-Gtl2* in survivor ng^{Ach7}/fg BMEs. Interestingly, we observed a higher frequency of hypermethylated patterns in the ng^{Ach7} allele and partial methylation in the *fg* allele [21]. The reason for the different IG-DMR methylation patterns of *Dlk1* in the ng^{Ach7}/fg BMEs is unknown. However, we considered that the normal expression of *Dlk1* together with the neighbouring imprinted genes was an important factor in the normal development and survival of ng^{Ach7}/fg BMEs.

Paternally Methylated Imprinted Genes on Mouse Chromosome 12

Paternally imprinted *Dlk1* and *Gtl2* are also important with regard to foetal lethality and growth retardation in maternal disomic mice [20, 22, 23]. *Dlk1*-null mice displayed growth retardation, obesity, blepharophimosis, skeletal malformation, and increased serum lipid metabolites [24]. The deletion of *Gtl2* exons 1–5 also affected the expression of the neighboring imprinted genes via the *cis*-regulatory elements and mice carrying the paternal deletion (*Gtl2*^{+m/-p}) showed severe growth retardation and perinatal lethality [25]. They are another set of coordinately regulated but oppositely expressed genes, located on the distal region of chromosome 12. It is known that the deletion of the IG-DMR from maternally inherited chromosome 12 leads to loss of imprinting of all genes in the 1-Mb cluster that carries the maternally repressed genes *Dlk1*, *Dio3* and *Rtl1*; and the maternally expressed non-coding RNAs, including *Gtl2*, and microRNAs. Hence the IG-DMR is the imprinting control region for this cluster of imprinted genes. This IG-DMR deletion from the maternally inherited chromosome results in lethality in mutant mice [20]. In contrast, the paternally methylated imprinted genes *Igf2* and *H19* present on chromosome 7 do not appear to be essential for foetal viability, since loss of paternal methylation imprinting by *H19*-DMR deletion did not result in lethality in mice [26, 27]. These results appear to indicate that for foetal viability, paternal methylation imprinting on chromosome 12 is more important than that on chromosome 7. To address this issue, we investigated the developmental ability of ng^{Ach12}/fg BMEs in which the maternal unmethylated status of *H19*-DMR of the *Igf2-H19* locus on both maternal chromosomes would likely be maintained. By restoration of the expression of paternally methylated imprinted genes on chromosome 12, we found that the ng^{Ach12}/fg BMEs could develop to E19.5 but showed severe growth retardation as compared to the wild type (wt) foetuses and the ng^{Ach7}/fg BMEs (Fig. 1). At E19.5, 5 live foetuses could be recovered from 253 transferred embryos. However, during gestation the weight of ng^{Ach12}/fg BMEs was significantly less than that of wt foetuses [28]. We found that ng^{Ach12}/fg BMEs were alive at E19.5, but their body weight was less than that of wt foetuses (<55%). Furthermore, ng^{Ach12}/fg BMEs could not survive further due to failure of normal respiration, suggesting difficulty in full-term development. Histological evaluation of the lungs of ng^{Ach12}/fg BMEs revealed thick alveolar septa and poorly organized alveoli [28]. The lungs of both ng^{Ach7}/fg and ng^{Ach12}/fg

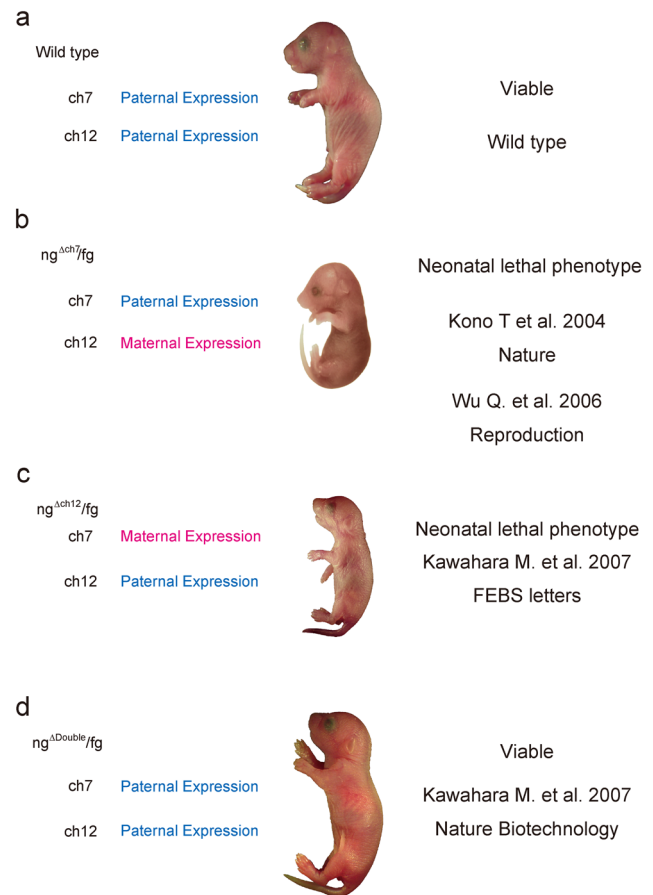


Fig. 1. Developmental outcome of reconstructed bi-maternal embryos. a: wild type foetus at E19.5, b: ng^{Ach7}/fg bi-maternal foetus at E19.5, c: ng^{Ach12}/fg bi-maternal foetus at E19.5, and d: $ng^{ADouble}/fg$ bi-maternal foetus at E19.5. a and d showed evidence of subsequent normal respiration and grew to adulthood. Note that the body dimension of the ng^{Ach7}/fg bi-maternal foetus is extremely disproportionate in comparison to those of other bi-maternal foetuses.

BMEs were atrophic and had severe anomalies. When compared with wt foetuses, ng^{Ach7}/fg and ng^{Ach12}/fg BMEs showed thicker alveolar septae and poorly organized alveoli [28]. However, the severity of lung defects in ng^{Ach12}/fg BMEs was greater than that in ng^{Ach7}/fg BMEs, and is most likely due to the absence of *Igf2* mRNA expression. Anomalies in the lungs of *Igf2* null foetuses have been described previously [29]. Next, we compared the diaphragms of the wt foetuses with those of ng^{Ach7}/fg and ng^{Ach12}/fg BMEs, because studies on mice with uniparental disomy for chromosome 12 suggested that the imprinted genes on chromosome 12 were associated with abnormalities in muscle development including in the diaphragm. Diaphragms of ng^{Ach7}/fg BMEs ($81.4 \pm 8.2 \mu m$) had significantly ($P < 0.05$) reduced thickness in the cross-sectional area compared to wild-type ($109.8 \pm 6.9 \mu m$), but not those of ng^{Ach12}/fg BMEs ($103.2 \pm 11.0 \mu m$).

Bi-maternal Embryos that Develop as Normal Individuals at a High Success Rate

Finally, we aimed to construct bi-maternal embryos using ng oocytes derived from double-knockout mice (ΔDouble) that harboured deletions in both the *H19*-DMR and the IG-DMR [16]. Females with Δch7 ($-/-$) were mated with males transmitting Δch12 ($+/-$), resulting in the production of double-mutant females with heterozygous Δch7 ($-/+$) and heterozygous Δch12 ($+/-$). Furthermore, males with heterozygous Δch7 ($-/+$) and heterozygous Δch12 ($+/-$) were mated with females with Δch7 ($-/-$), resulting in the production of double-mutant females with homozygous Δch7 ($-/-$) and heterozygous Δch12 ($+/-$). The newborn females harbouring double mutants were selected by detecting the deletion of both *H19*-DMR and IG-DMR by polymerase chain reaction (PCR). The ng ΔDouble oocytes with double mutations at the diplo-tenne stage of the first meiotic division were collected from each female of genotype Δch7 ($-/+$) and Δch12 ($+/-$) or Δch7 ($-/-$) and Δch12 ($+/-$). After transplantation of the resultant 286 blastocysts to 29 recipient females, a total of 42 live pups were recovered by autopsy at 19.5 days of gestation and all were heterozygous for the double-mutation on chromosomes 7 and 12 indicating that only one genotype was permissive for survival (Fig. 1) [16]. The mean body weight of the 27 bi-maternal pups at birth was 1.19 ± 0.02 g; this was similar to the weights of the wild type C57BL/6J $\times$ B6D2F1 pups, 1.20 ± 0.02 g. Phenotypic analyses revealed no significant differences between the 2 mice groups with regard to learning performance, fertility, athletic ability, and the histological features of various tissues, including the liver [16]. However, only two phenotypes different were postnatal growth retardation ($>20\%$ compared to wild-type mice) and longer lifespan. Of the latter, we compared the longevity of naturally mating control mice ($n = 13$) and BM ($n = 13$) born between October 2005 and March 2006 [30]. The average lifespan was found to be 185.9 days longer in bi-maternal mice (BM) than in the controls. The bi-maternal genotype clearly shifted the entire survival curve to the right, indicating the delay in the expression of all causes of mortality. The cumulative survival rate of the BM significantly differed from those of the controls ($P < 0.01$, Kaplan-Meier analysis). Meanwhile, the maximal longevity were 996 and 1045 days in the control mice and BM, respectively. The control mice were also female and genetically identical (B6D2F1 $\times$ C57BL/6) to the BM. Although all the animals were nonpregnant and were housed together under free-feeding and specific pathogen-free conditions, the BM lived approximately 30% longer than the controls.

We demonstrated that fecund, bi-maternal mice can be consistently produced at a high rate equivalent to those produced by bi-parental embryos. So far, it has been argued that lacking of male-derived factors like spermatozoan RNAs may be a crucial cause for the low success rate of somatic cell nuclear transfer or previous low frequency attempts to generate bi-maternal mice resulting in 'Kaguya', the sole adult mouse produced from the ng Δch7 /fg bi-maternal embryos [31]. However, the data clearly demonstrate that transcripts that are specific to male germ cells are unnecessary for mammalian development after gamete fusion. We considered that this bi-maternal mouse production system opens a

new frontier in possibilities for investigating the biological roles of the paternal genome.

Investigating the Biological Roles of the Paternal Genome in Placentation

Investigating placentation in ng/fg BMEs may help in elucidating the roles that paternally methylated imprinted genes play in mouse placentation in three ways. First, our system provides the only way of manipulating the expression of multiple imprinted genes, that can be compared with the extremes of full parthenogenesis and androgenesis. Second, we can monitor the development of placentae that exclusively possessed the maternal genomes during gestation; that is, we can investigate the phenotypes of the placentae in which all paternally methylated imprinted genes are absent by default. In the case of the ng WT /fg placenta, maternal methylation imprinting was globally modified, differing from the imprinting that is observed in case of normal parthenotes because one set of maternal imprints are erased and neither paternal methylation nor a second set of maternal methylation imprints are imposed. Third, the evaluation of the ng Δch7 /fg, ng Δch12 /fg, and ng ΔDouble /fg placentae enable an understanding of the relative contributions of paternally methylated imprinted genes on chromosomes 7 and 12 to the development of the placenta.

Morphometric and histological analyses revealed that correction of the expression of paternally methylated imprinted genes on chromosome 7 affected not only the increase in placental mass but also the normal differentiation of giant cells (Fig. 2) [32]. However, ng Δch7 /fg BMEs formed placentae with severe dysplasia such as an anomalously expanded spongiotrophoblastic layer and a malformed labyrinthine layer with an anomalous circulatory system. This indicates that these phenotypes are caused by absence and/or overexpression of other imprinted genes in these placentae. We further observed that the circulatory system of the ng Δch12 /fg placenta was comparable to that of the wt placenta (Fig. 2) [32]. However, the placental weight barely increased, and the giant cells were abnormally expanded. This suggests that absence and/or overexpression of chromosome 12 imprinted genes make a major contribution to the dysplasia in ng Δch7 /fg placentae, but cannot rescue the size defect or trophoblast giant cell phenotype that are no longer evident when normal *H19* and *Igf2* levels are restored. Finally, we determined the placenta-forming ability of the ng ΔDouble /fg BME and further investigated the genes that were dominated by the paternally methylated imprinted genes controlled by *Igf2-H19* DMR and *Dlk1-Dio3* DMR. We observed that the ng ΔDouble /fg placenta showed remarkably improved phenotypes in comparison with the ng Δch7 /fg and ng Δch12 /fg placentae (Fig. 2) [33]. Additionally, comparative global gene expression analyses of the ng ΔDouble /fg placenta and the other bi-maternal placentae, i.e. the ng Δch7 /fg and ng Δch12 /fg placentae, enabled us to predict the functions of paternally methylated imprinted genes regulated by *Igf2-H19* DMR and *Dlk1-Dio3* DMR in the mouse placenta [33, 34]. 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, we

			Increase of placental mass	Orderly development of spongiotrophoblastic layer and labyrinthine layer	Size of giant cells	Arborization of vasculature	Reference
a Wild type	ch7 Paternal Expression		+	+	+	+	Wild type
	ch12 Paternal Expression						
b $ng^{Ach7/fg}$	ch7 Paternal Expression		±	-	+	-	Kawahara M et al. 2006 Human Molecular Genetics
	ch12 Maternal Expression						
c $ng^{Ach12/fg}$	ch7 Maternal Expression		-	+	-	+	Kawahara M et al. 2006 Human Molecular Genetics
	ch12 Paternal Expression						
d $ng^{\Delta Double/fg}$	ch7 Paternal Expression		+	+	+	Not Determined	Kawahara M. et al. 2009 The Journal of Biological Chemistry
	ch12 Paternal Expression						

+	Normal	-	Aberrant
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Fig. 2. Summary of the multiple roles played by the paternally methylated imprinted genes on chromosomes 7 and 12 in mouse placentation. Four types of placentae, namely, wild type, $ng^{Ach7/fg}$, $ng^{Ach12/fg}$, and $ng^{\Delta Double/fg}$. Midline placental sections (haematoxylin/eosin) revealed that the $ng^{Ach7/fg}$ placentae showed disproportionate expansion of the spongiotrophoblast layer along with a distorted and ambiguous boundary between the spongiotrophoblast layer and the labyrinth. The roles of the imprinted genes that were elucidated in our studies are listed in the table.

provided the first demonstration that the genes regulated by the two paternally imprinted methylated regions on chromosomes 7 and 12 contribute distinct and complementary functions to mouse placentation.

Conclusion

A series of our results suggests that a strict barrier to parthenogenesis is constituted independently by both maternal and paternal methylation imprinting; maternal methylation imprinting functions as the fundamental barrier to obstruct parthenogenetic development beyond the early implantation stage, and development beyond mid-gestation is severely inhibited by abnormal expression of genes normally regulated by elements that are paternally methylated/maternally unmethylated at the regions on chromosomes 7 and 12. The restored regulation of imprinted gene expression from these two regions is essential and sufficient for accomplishing normal term development. These results provide a framework for gaining further insight into the role of epigenetic modification of maternal and paternal genomes in mammalian development.

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