nature biotechnology

© 2007 Nature Publishing Group http://www.nature.com/naturebiotechnology

High-frequency generation of viable mice from engineered bi-maternal embryos

Manabu Kawahara[1,2], Qiong Wu[1,2,4], Nozomi Takahashi[1], Shinnosuke Morita[1], Kaori Yamada[1], Mitsuteru Ito[3], Anne C Ferguson-Smith[3] & Tomohiro Kono[1,2]

Mammalian development to adulthood typically requires both maternal and paternal genomes, because genomic imprinting places stringent limitations on mammalian development, strictly precluding parthenogenesis[1,2]. Here we report the generation of bi-maternal embryos that develop at a high success rate equivalent to the rate obtained with *in vitro* fertilization of normal embryos. These bi-maternal mice developed into viable and fertile female adults. The bi-maternal embryos, distinct from parthenogenetic or gynogenetic conceptuses, were produced by the construction of oocytes from fully grown oocytes and nongrowing oocytes that contain double deletions in the *H19* differentially methylated region (DMR) and the *Dlk1-Dio3* intergenic germline–derived DMR. The results provide conclusive evidence that imprinted genes regulated by these two paternally methylated imprinting-control regions are the only paternal barrier that prevents the normal development of bi-maternal mouse fetuses to term.

Construction of oocytes containing two sets of haploid genomes, one from a nongrowing (ng) oocyte and the other from a fully grown (fg) oocyte, allows manipulation of the expression of imprinted genes that are regulated by maternal methylation imprinting[3–5]. Whereas the ng oocyte genome can be considered 'imprint free', the fg oocyte genome has established its maternal methylation imprints. The paternal contribution to mammalian development can be delineated by studying the development of these ng/fg bi-maternal conceptuses. We previously constructed ng$^{\Delta ch7}$/fg bi-maternal embryos using ng oocytes derived from mutant mice harboring a 13-kb deletion that includes the *H19* transcription unit and its differentially methylated region (DMR) on chromosome 7 (Δch7)[6]. However, the efficiency of this reconstruction was extremely low, as we recovered only two normal, live bi-maternal mice from a total of 371 oocyte *H19*-deletion manipulations (0.5% recovery).

To date, three DMRs that are methylated during spermatogenesis, located on chromosomes 7, 9 and 12, have been identified as imprinting-control regions[7–9]. We first constructed bi-maternal embryos using ng oocytes derived from double-knockout mice (ΔDouble) that harbored deletions in both the *H19*-DMR (Δch7) and the *Dlk1-Dio3* intergenic germline-derived (IG)-DMR (Δch12)[9,10]. Females homozygous for the Δch7 deletion were mated with males heterozygous for the Δch12 deletion, resulting in the production of double-mutant females with heterozygous Δch7$^{-/+}$ and heterozygous Δch12$^{+/-}$ deletions (**Fig. 1a**). Furthermore, males with heterozygous Δch7$^{-/+}$ and heterozygous Δch12$^{+/-}$ deletions were mated with homozygous Δch7$^{-/-}$ females, resulting in the production of double-mutant females with homozygous Δch7$^{-/-}$ and heterozygous Δch12$^{+/-}$ deletions. The newborn females harboring double mutations were selected by detecting the deletion of both *H19*-DMR and IG-DMR by PCR. The ng$^{\Delta Double}$ oocytes with double mutations at the diplotene stage of the first meiotic division were collected from each female of genotype Δch7$^{-/+}$ and Δch12$^{+/-}$ or Δch7$^{-/-}$ and Δch12$^{+/-}$. The ng$^{\Delta Double}$/fg bi-maternal embryos were reconstructed by serial nuclear transfer (**Supplementary Fig. 1** online).

The developmental ability of ng$^{\Delta Double}$/fg bi-maternal embryos was verified by a 3-d *in vitro* culture of the 323 reconstructed embryos and by transplantation of the resulting 286 blastocysts to 29 recipient females (**Table 1**). A total of 42 live pups were recovered by autopsy at 19.5 d of gestation and all were heterozygous for the double-mutation on chromosomes 7 and 12 (**Fig. 1** and **Supplementary Figs. 1** and **2** online), indicating that only one genotype—ng$^{\Delta Double}$/fg—was permissive for survival. Thirty-eight pups were successfully nursed by foster mothers. Twenty-seven out of 38 pups had body weights ≥1.1 g and grew to adulthood (**Fig. 1b–d**). The mean body weight of these 27 bi-maternal pups at birth, 1.19 ± 0.02 g, was similar to the mean body weight of the wild-type C57BL/6J × B6D2F1 control pups, 1.20 ± 0.02 g. The remaining 11 bi-maternal pups (mean birth weight, 0.90 ± 0.06 g) exhibited slight growth retardation at birth and died within 3 d. The mean placental weights for these groups showed differences similar to the ones observed for the mean body weights (**Fig. 1d,e**).

After oocyte reconstruction and meiotic division *in vitro*, the theoretical proportion of ng$^{\Delta Double}$ oocytes is one-fourth when heterozygous Δch7$^{+/-}$ ng oocytes are used, and one-half when homozygous Δch7$^{-/-}$ ng oocytes are used (**Supplementary Fig. 1**). This theoretical proportion was confirmed by genotyping blastocysts derived from the reconstructed bi-maternal embryos. For

[1]Department of BioScience Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan. [2]Bio-oriented Technology Research Advancement Institution (BRAIN), Minato-ku, Tokyo 105-0001, Japan. [3]Department of Physiology, Development & Neuroscience, University of Cambridge, Downing St., Cambridge CB2 3DY, UK. [4]Present address: Department of Life Science and Engineering Harbin Institute of Technology, No.92, West Da-Zhi Street, Harbin, Heilongjiang. Correspondence should be addressed to (tomohiro@nodai.ac.jp).

Received 1 March; accepted 24 July; published online 19 August 2007; doi:10.1038/nbt1331

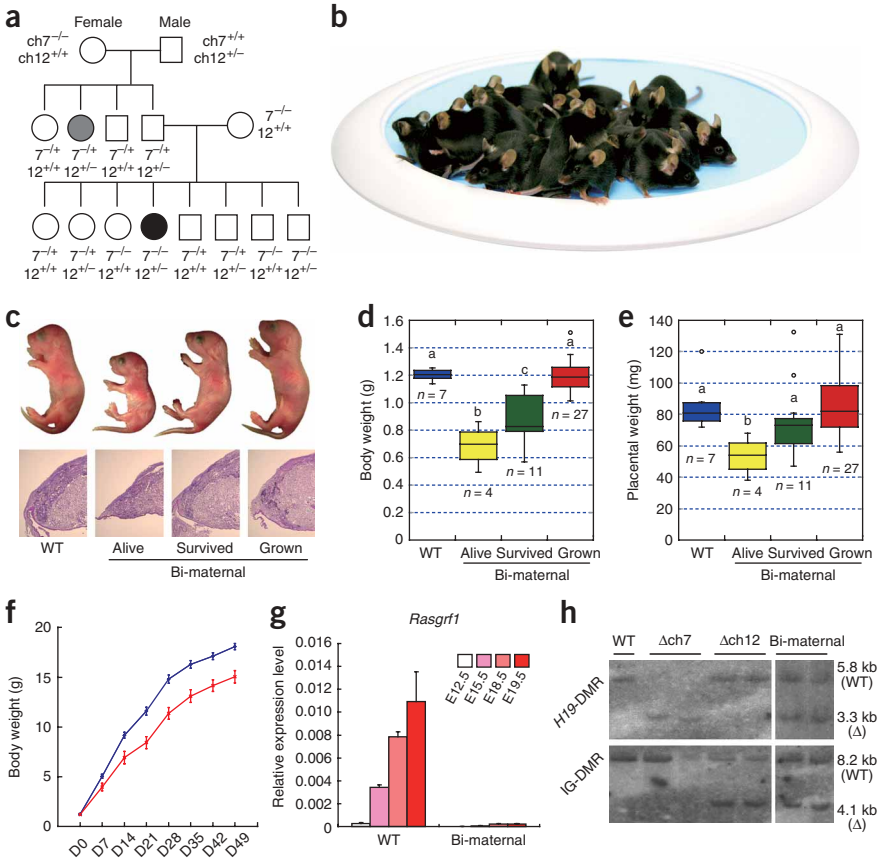

Figure 1 The bi-maternal mice derived from the ng$^{\Delta Double}$/fg embryos. (**a**) Production of a double-knockout mouse in which the *H19*-DMR and the IG-DMR are deleted. Double-knockout mice were obtained from the mating of each mutant mouse—Δch7 female (ch7$^{-/-}$) and Δch12 male (ch12$^{+/-}$). Nongrowing oocytes in ch7$^{-/+}$, ch12$^{+/-}$ (gray) or ch7$^{-/-}$, ch12$^{+/-}$ (black) female mice were used for oocyte reconstruction. (**b**) The bi-maternal mice that grew to normal adults are shown ($n = 27$). Of these mice, five were tested for reproductive performance and delivered pups with an intact IG-DMR. Maternal transmission of the IG-DMR deletion is lethal prenatally[9]. (**c**) ng$^{\Delta Double}$/fg bi-maternal pups at birth and midline placental sections (PAS) at E19.5. Of the bi-maternal mice, the left pup was alive at birth, but did not show evidence of subsequent respiration (Alive). The center pup exhibited normal respiration, but did not grow beyond 3 d after birth (Survived). The right pup exhibited normal respiration and grew to adulthood (Grown). (**d**,**e**) The boxplot graphs represent body (**d**) and placental (**e**) weights of the bi-maternal mice at birth (WT (wild-type), $n = 7$; Alive, $n = 4$; Survive, $n = 11$; Grown, $n = 27$): the two quartiles, the 25th and 75th percentile 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. The circles denote the outliers outside the acceptable range. Different letters denote significant differences ($P < 0.05$; one-way ANOVA with Fisher's post-hoc test). (**f**) Body weights of the wild-type and the bi-maternal mice from Day 0 to Day 49 after birth (wild-type, red, $n = 11$; bi-maternal mice, blue, $n = 9$). The values represent the means ± s.e.m. (**g**) The expression of the *Rasgrf1* gene in the brains of the bi-maternal mice at E12.5, E15.5 and E18.5 was analyzed by quantitative real-time PCR ($n = 3$). The values (means ± s.e.m.) represent the levels of expression relative to that of the internal control gene (*Gapdh*). (**h**) Southern analyses of *H19*-DMR and IG-DMR in wild-type, Δch7(–/–), Δch12(+/–) and bi-maternal offspring. The differences in band size were used to distinguish between the mutant and the wild-type allele; wild-type allele (WT): 5.8 and 8.2 kb, mutant allele (Δ): 3.3 (Δch7) and 4.1 (Δch12) kb.

© 2007 Nature Publishing Group http://www.nature.com/naturebiotechnology

heterozygous Δch7$^{-/+}$ and Δch12$^{+/-}$ ng oocytes as source material, the genotypes and proportions of 51 bi-maternal blastocysts were: ng^{WT}/fg, 15/51 (29.4%); ng$^{\Delta ch7}$/fg, 12/51 (23.5%); ng$^{\Delta ch12}$/fg, 14/51 (27.5%); and ng$^{\Delta Double}$/fg, 10/51 (19.6%), respectively. Although this segregation ratio approximated the theoretical ratio—ng^{WT}/fg: ng$^{\Delta ch7}$/fg: ng$^{\Delta ch12}$/fg: ng$^{\Delta Double}$/fg = 1: 1: 1: 1—a significant difference was observed (**Supplementary Fig. 1a**; χ^2-test (χ^2-statistic = 4.5), significance level of 1%). For Δch7$^{+/-}$ and Δch7$^{-/-}$ ng oocytes as source material, the production rate of bi-maternal mice was calculated as 27.5% and 32.5%, respectively (**Table 1**). Overall, the survival rate to adulthood of bi-maternal mice was similar to that of *in vitro*-fertilized embryos (39.4%)[3]. The bi-maternal mice that survived beyond 3 d after birth grew successfully to adulthood, although they did exhibit postnatal growth retardation (>20% compared to wild-type mice). The most likely reason for growth retardation is the repression of *Rasgrf1* transcription. *Rasgrf1* is regulated by paternal methylation imprinting and induces growth hormone secretion from the pituitary. Future studies are required to determine whether *Rasgrf1* transcription is repressed in bi-maternal mice (**Fig. 1f**,**g**)[11].

We conducted a series of phenotypic analyses—anatomical comparison, histological examination, blood property tests, serum biochemical tests, pulse rate and blood pressure measurements—in these bi-maternal mice (**Supplementary Tables 1–4** online). The results indicate that the bi-maternal mice are phenotypically similar to the wild-type controls. A rotarod test at 5 and 8 weeks after birth and a Water maze–learning test at 6 weeks after birth were also carried out (**Supplementary Tables 5** and **6** online). These experiments suggested that the bi-maternal animals' exercise and learning performance were normal. However, additional long-term memory studies are required in bi-maternal mice because *Rasgrf1* null mutants showed long-term memory defects[12]. In conclusion, these results demonstrate that bi-maternal mice are phenotypically equivalent to wild-type mice, with the exception of the low body weight after birth and the smaller litter size when the IG-DMR deletion is transmitted from bi-maternal double-mutant mothers[9].

To obtain further insight into the development of ng$^{\Delta Double}$/fg bi-maternal embryos to term, we conducted quantitative gene expression analysis by using real-time RT-PCR at E15.5 for *Igf2*, *H19* and for genes in the *Dlk1* cluster—*Dlk1*, *Gtl2*, *Mirg* and *Dio3*—and by northern blotting for *Rtl1* (**Fig. 2a–g**). In the major organs of the bi-maternal double-mutant fetuses, the genes were expressed at levels similar to those of the wild-type control. The noncoding RNA *Gtl2*, however, shows greater variability and an unexplained increase in expression in the liver. The results suggest that the favorable imprinting status generated in the ng$^{\Delta Double}$/fg bi-maternal embryos enables development to term and subsequent growth to adulthood. As expected, the *Igf2* expression level was reduced in the ng$^{\Delta ch12}$/fg bi-maternal fetuses, and the *Dlk1* and *Rtl1* expression levels were

Table 1 Development of ng$^{\Delta Double}$/fg bi-maternal embryos

Genotypes of ng oocytes	Developmental progress	Number
Control	Embryos transferred	66 (86.8% of reconstructed and fertilized eggs)
	Pregnant recipients	7
	Total pups	26 (39.4% of embryos transferred to pregnant recipients)
Δch7$^{-/+}$ Δch12$^{+/-}$ Heterozygous on chromosome 7	Reconstructed eggs	238
	Embryos transferred	206 (86.6% of reconstructed eggs)
	Pregnant recipients	21
	Total pups	26 (10.9% of reconstructed eggs)
	Alive	4 (1.9% of embryos transferred to pregnant recipients)
	Survived	8 (3.9% of embryos transferred to pregnant recipients)
	Grown	14 (6.8% of embryos transferred to pregnant recipients) (27.2%)[a]
Δch7$^{-/-}$ Δch12$^{+/-}$ Homozygous on chromosome 7	Reconstructed eggs	85
	Embryos transferred	80 (94.1% of reconstructed eggs)
	Pregnant recipients	8
	Total pups	16 (18.8% of reconstructed eggs)
	Alive	0
	Survived	3 (3.8% of embryos transferred to pregnant recipients)
	Grown	13 (16.3% of embryos transferred to pregnant recipients) (32.6%)[a]

[a]Expected percentages (%) estimated from the transmission rates of two deletions on chromosomes 7 and 12. When ng oocytes with Δch7$^{-/+}$, Δch12$^{+/-}$ or Δch7$^{-/-}$, Δch12$^{+/-}$ genotypes are used to produce ng/fg embryos, the transmission rate of double deletions on chromosomes 7 and 12 to ng/fg embryos is estimated to be 25% or 50%, respectively. To confirm the validity of the estimated rate, we have examined the transmission rate of double deletions to ng/fg embryos that developed to the blastocyst stage by using polymerase chain reaction (PCR) for the neo cassette sequence and the deleted sequence of IG-DMR (data not shown). Alive, Animals were alive at birth but did not exhibit respiration; Survived, Animals exhibited normal respiration, but did not grow beyond 3 d after birth; Grown, Animals exhibited normal respiration and grew to adulthood. As the positive controls for bi-maternal embryos, reconstructed MII oocytes produced by serial nuclear transfer were applied for *in vitro* fertilization (IVF), as we previously reported[3]. To produce controls with genetic background identical to that of bi-maternal embryos, B6D2F1 females and C57BL/6N males were used as oocyte and sperm donors, respectively.

repressed in the ng$^{\Delta ch7}$/fg bi-maternal fetuses (**Fig. 2g** and data not shown). We have recently confirmed that the phenotypic characteristics of the ng$^{\Delta ch12}$/fg fetuses are different from the ng$^{\Delta ch7}$/fg fetuses; the ng$^{\Delta ch12}$/fg fetuses have a smaller body and placental size than the ng$^{\Delta ch7}$/fg fetuses (unpublished data)[13], reminiscent of what is observed in fg/sp$^{\Delta Igf2}$ animals. To understand why a minority of ng$^{\Delta Double}$/fg bi-maternal embryos did not grow to adulthood, we also examined imprinted gene expression at birth. In growth-retarded ng$^{\Delta Double}$/fg bi-maternal mice that were alive at term but did not exhibit respiration, the *Igf2* expression was reduced compared to wild-type and healthy bi-maternal pups (**Supplementary Fig. 3** online).

To explore the global change of gene expression caused by the correction of paternally methylated imprinted genes on chromosomes 7 and 12, we first characterized the expressed genes in the ng$^{\Delta Double}$/fg bi-maternal fetuses in comparison with the other three types—the ng$^{\Delta ch7}$/fg, ng$^{\Delta ch12}$/fg bi-maternal and wild-type fetuses—at embryonic day 12.5 (E12.5) and E15.5, using the Affymetrix Mouse Genome 430 2.0 Array (**Fig. 3a**). After constructing the Venn diagram with Gene Spring software, we established gene lists that represent differentially and specifically expressed genes in the ng$^{\Delta Double}$/fg bi-maternal fetus compared to each of the ng$^{\Delta ch7}$/fg and ng$^{\Delta ch12}$/fg bi-maternal fetuses at E12.5 and E15.5 (**Fig. 3b**). In the ng$^{\Delta ch7}$/fg bi-maternal fetus, 110 and 10 genes were specific at E12.5 and E15.5 (ch12 gene list), respectively, whereas in the ng$^{\Delta ch12}$/fg bi-maternal fetus, 111 and 282 genes were differentially and specifically expressed (ch7 gene list) at E12.5 and E15.5, respectively (**Fig. 3c**,**d**). We used Ingenuity Pathway Analysis (IPA)[14,15] to understand the biological and molecular processes represented by these genes. All the genes in the ch12 and ch7 gene lists were integrated into IPA pathways based on the biological relationships described in previous references (**Supplementary Tables 7–10** online). The result of the integration revealed that 8 and 2 networks were generated from the ch12 gene list at E12.5 and E15.5, respectively, whereas a corresponding 9 and 23 networks were produced from the ch7 gene list at E12.5 and E15.5, respectively. As expected, the *Igf2* and *Dlk1* genes were incorporated into the ch7 and ch12 gene lists, respectively (**Supplementary Figs. 4–7** online). The network containing the *Igf2* gene (**Supplementary Fig. 7**) revealed that the *Mmp13* mRNA exhibited a 13-fold change between the ng$^{\Delta Double}$/fg and ng$^{\Delta ch7}$/fg bi-maternal fetuses. *Mmp13* mRNA transcription is stimulated by *Igfbp3* whose mRNA expression is regulated by *Igf2* (refs. 16,17). It is known that the inactivation of *Mmp13* in mice through targeted mutagenesis leads to abnormal skeletal growth plate development[18]. Thus, this network suggests that the correction of *Igf2* mRNA expression in the ng$^{\Delta Double}$/fg fetuses might rescue the abnormal skeletal development via the correction of *Mmp13* RNA expression at E15.5. In the chromosome-12 network, upregulation of the *Dlk1* gene in the ng$^{\Delta Double}$/fg fetuses clearly affected the expression of other genes—*Apoa1*, *Daam1*, *Elovl1*, *Entpd5*, *Hoxa9*, *Trf*, *Hrasls3*, *Krt14*, *Mpo*, *Rb4*, *Serpina10*, *Slc16a1*, *Tgm2* and *TTR*—at E12.5 (**Supplementary Fig. 4**). It is known that the *Dlk1*-null mutant exhibits prenatal growth retardation, postnatal obesity, blepharophimosis and skeletal malformation[19]. Furthermore, *Dlk1* also contributes to granulocyte, megakaryocyte and B-cell clonogenic growth[20]; however, the molecular mechanisms involving *Dlk1* contributions remain unknown, and other genes in that imprinted cluster may contribute to differential gene expression on the array. Of course, we cannot rule out that indirect effects associated with the behavior of more generalized modulators of expression might also contribute to the differences. These microarray analyses revealed that in the ng$^{\Delta Double}$/fg fetuses, many of the genes that are differentially affected in the two different single mutant ng/fg conceptuses, and that might contribute to fetal development, are normalized. We speculate that *Igf2* and *Dlk1* play complementary, pivotal roles in hematological, skeletal and muscular development.

Here we demonstrate that fecund, bi-maternal mice can be consistently produced at a high rate equivalent to that of bi-parental embryo generation. This 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

© 2007 Nature Publishing Group http://www.nature.com/naturebiotechnology

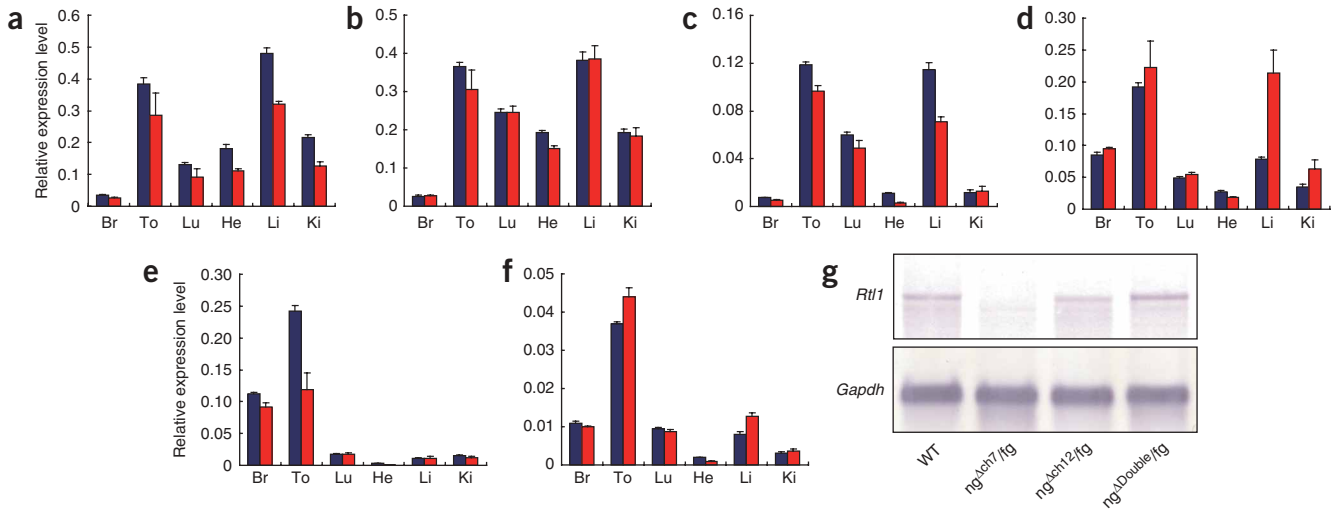

Figure 2 Graphical representations of the expression of the two sets of imprinted genes in the bi-maternal fetuses. (**a**–**f**) The expression of six imprinted genes, *Igf2* (**a**), *H19* (**b**), *Dlk1* (**c**), *Gtl2* (**d**), *Dio3* (**e**) and *Mirg* (**f**), in six different tissues of 15.5-d bi-maternal fetuses were analyzed by quantitative real-time PCR ($n = 3$). Br, brain; To, tongue; Lu, lung; He, heart; Li, liver and Ki, kidney. The values represent the levels of expression relative to that of the internal control gene (*Gapdh*). Wild-type ($n = 3$, dark blue); Bi-maternal mouse ($n = 3$, red). The values represent the means ± s.e.m. (**g**) Northern-blot analysis of *Rtl1* gene expression.

normally regulated by elements that are paternally methylated/maternally unmethylated at the regions on chromosomes 7 and 12. Restoration of regulation of imprinted gene expression at these two regions is essential and sufficient for normal term development. These results provide a framework to further elucidate the role of epigenetic modifications in maternal and paternal genomes during mammalian development.

It has been argued that lack of male-derived factors, like sperm RNAs, may contribute to the low success rate of somatic cell nuclear transfer or of previous attempts to generate bi-maternal mice from ng$^{\Delta ch7}$/fg bi-maternal embryos (which resulted in the isolation of one adult mouse[21]). However, our data clearly demonstrate that transcripts specific to male germ cells are unnecessary for mammalian development after gamete fusion. This 'sperm-free' mouse production system may enable further characterization of differential imprinting programs.

METHODS

Nuclear transfer. Nuclear transfer was performed using a previously described method[3–6,13,22]. In brief, fully grown germinal vesicle (GV) oocytes were

© 2007 Nature Publishing Group http://www.nature.com/naturebiotechnology

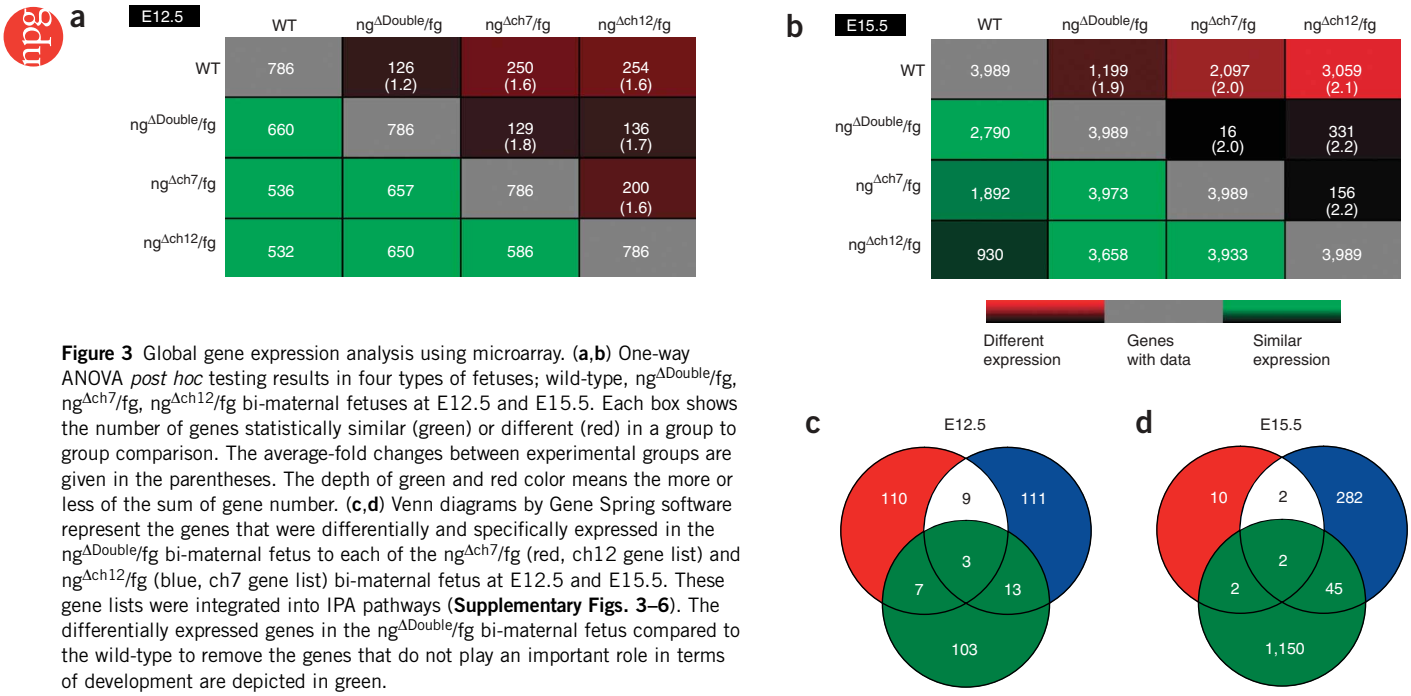

Figure 3 Global gene expression analysis using microarray. (**a**,**b**) One-way ANOVA *post hoc* testing results in four types of fetuses; wild-type, ng$^{\Delta Double}$/fg, ng$^{\Delta ch7}$/fg, ng$^{\Delta ch12}$/fg bi-maternal fetuses at E12.5 and E15.5. Each box shows the number of genes statistically similar (green) or different (red) in a group to group comparison. The average-fold changes between experimental groups are given in the parentheses. The depth of green and red color means the more or less of the sum of gene number. (**c**,**d**) Venn diagrams by Gene Spring software represent the genes that were differentially and specifically expressed in the ng$^{\Delta Double}$/fg bi-maternal fetus to each of the ng$^{\Delta ch7}$/fg (red, ch12 gene list) and ng$^{\Delta ch12}$/fg (blue, ch7 gene list) bi-maternal fetus at E12.5 and E15.5. These gene lists were integrated into IPA pathways (**Supplementary Figs. 3–6**). The differentially expressed genes in the ng$^{\Delta Double}$/fg bi-maternal fetus compared to the wild-type to remove the genes that do not play an important role in terms of development are depicted in green.

collected into M2 medium from the ovarian follicles of B6D2F1 (C57BL/6N × DBA) female mice, 44–48 h after they were injected with equine chorionic gonadotropin. Ovulated metaphase II oocytes were also collected from superovulated B6D2F1 mice, 16 h after they were injected with human chronic gonadotropin. We collected ng oocytes that were in the diplotene stage of the first meiosis from the ovaries of 1-d-old newborn mice. Tested paternal methylation imprints in the ng^{WT} genome had already been erased at this stage (data not shown), although we cannot formally rule out that as yet unidentified paternal methylation imprints might persist. The GV oocytes were manipulated in M2 medium containing 200 μM dbcAMP and 5% FCS throughout the experiment. ng oocytes were fused with enucleated GV oocytes by using inactivated Sendai virus (HVJ, 2,700 hemagglutinating activity unit/ml). The reconstructed oocytes were cultured for 14 h in α-MEM medium (GIBCO) containing 5% FCS. A spindle from the reconstructed oocytes was again transferred into ovulated MII oocytes, followed by treatment with 10 mM $SrCl_2$ in Ca^{2+}-free M16 medium for 2 h. These embryos were cultured in M16 medium for 3.5 d in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 37 °C. The embryos that developed to the blastocyst stage were transferred into the uterine horns of recipient female mice at 2.5 d of pseudopregnancy. The ng/fg bi-maternal embryos therefore consist of two sets of haploid maternal genome; maternal methylation imprints have been acquired in the fg oocyte genome, whereas imprints have been erased but maternal methylation not yet acquired in the ng oocytes. Therefore unlike parthenogenetic/gynogenetic embryos, only bi-maternal embryos are able to express the normally paternally expressed imprinted genes such as *Peg1/Mest*, *Peg3*, *Impact*, *Peg10* and others that are usually repressed by maternal methylation. To distinguish the genotype of each bi-maternal mouse, we detected the neo cassette sequence for the *H19* locus and the deleted sequence of IG-DMR by PCR using specific primers.

Genotyping. Genotyping of blastocysts or animals was conducted by PCR amplification of DNA using four primer sets: IG-DMR KO (5′-GAC ATG GGA CTT GAA AGC AAC T-3′;5′-GCA CAA GTC ACC ATA GTT ACA GAC-3′), IG-DMR (5′-CAA TTC ACG GTA TAT GAG TCC TAT C-3′;5′-TGC AAG TAC CAG ATT CCA TCA-3′), *neo* (5′-GAT CGG CCA TTG AAC AAG AT-3′; 5′-CTC GTC CTG CAG TTC ATT CA-3′) and *H19* (5′-TAT CGG GGA AAC TGG GGA AGA-3′; 5′-CTC TAA ACC TTC GTT TAG AAG -3′). Furthermore, the genotypes of the representative bi-maternal offspring were also verified by Southern blot hybridization of genomic DNA using H19 5′-probe (419 bp) and IG-DMR 5′-probe (431 bp). The probe primers used for PCR were as follows: H19, 5′-CCT ACA GAG GCA TCA GGT GAT-3′ and 5′-TAG GAT CAG GCT TGG GAG AGT-3′; IG-DMR, 5′- AGA GGA GCA AGG ACC TTC CTT -3′ and 5′-TAG GTT CAA GAT GGC CAC GAG-3′. In $\Delta ch7^{-/-}$ mice harboring a 13-kb deletion including the *H19* transcription unit and its DMR on chromosome 7, the *Pst*I fragment is 3.3 kb (mutant allele) due to the homozygotic deletion event. In $\Delta ch12^{+/-}$ mice heterozygously carrying a 4.15-kb deletion of the IG-DMR on chromosome 12, the *Nde*I fragments are 4.1 (mutant allele) and 8.2 (wild-type allele) kb due to the heterozygotic deletion event. In wild-type mice, the fragments digested with *Pst*I and *Nde*I are 5.8 (wild-type allele) and 8.2 (wild-type allele) kb, respectively. These differences were used to distinguish between the mutant and the wild-type allele in bi-maternal offspring.

Gene expression analyses. Total RNA was extracted using an RNeasy Mini Kit (Qiagen) from each organ, brain, tongue, lung, heart, liver and kidney of wild-type (wt, B6D2F1 × C57BL/6N) and bi-maternal $ng^{\Delta Double}$/fg fetuses. The cDNAs were then synthesized using the SuperScript III RNaseH reverse transcriptase kit (Invitrogen) in a reaction solution (20 μl) containing the total RNA (1 μg) prepared from each fetus. 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 our previous reports[13,23].

To conduct the northern-blot hybridization analysis for *Rtl1*, we isolated total and poly $(A)^+$ RNA from wild-type and bi-maternal $ng^{\Delta ch7}$/fg, $ng^{\Delta ch12}$/fg, $ng^{\Delta Double}$/fg fetuses at E15.5 as described previously ($n = 3$). We used probes as described previously for *Rtl1* (ref. 24).

Histology and physiological analysis. We performed histological analysis of many organs of wild-type and bi-maternal mice at 8 weeks after birth ($n = 3$). Brain, tongue, pituitary, thyroid gland, lung, trachea, thymus, heart, pancreas, liver, spleen, kidney, adrenal, stomach, duodenum, intestinum jejunum, ileum, cecum, colon, intestinum rectum and bladder were fixed with 10% formalin and observed after hematoxylin/eosin (HE) staining.

Moreover, we performed some physiological analyses—blood property tests, serum biochemical tests, pulse rate and blood pressure measurements. Content of leukocytes, erythrocytes, hemoglobin, MCV, MCH, MCHC and platelets in blood of wild-type and bi-maternal mice at 8 weeks after birth were examined by using a hematology inspection apparatus ADVIA 120 (Bayer Medical) ($n = 3$). Pulse rate and blood pressure measurements were examined by using MK-2000 blood pressure monitor (Muromachikikai) ($n = 3$).

Behavioral examination. Animals were examined for ability to exercise in a rotarod test at 5 and 8 weeks after birth ($n = 6$). Mice were placed on the rotating rotarod, and how long they remained on the rotarod was recorded. The rotation speed of the rotarod was 10 r.p.m.

In addition, animals were tested for learning and memory (reference memory) in a Multiple T-Maze at 6 weeks after birth ($n = 6$). Mice were trained three times per day for four consecutive days, and memory was tested consisting of three runs on that day. Time to reach the goal box and correct decision were monitored.

Microarray analysis. Total RNA was extracted from four types of whole fetus, $ng^{\Delta ch7}$/fg, $ng^{\Delta ch12}$/fg, $ng^{\Delta Double}$/fg bi-maternal, and wild type, at E12.5 and E15.5 using a commercially available acid-phenol reagent (TRIzol; Invitrogen). Total RNA from three replicates of each embryo was used for the One-Cycle Eukaryotic Target Labeling Assay (Affymetrix). Fifteen μg per replicate of fragmented cRNA samples were serially hybridized to GeneChip Mouse Genome 430 2.0Array (Affymetrix) that contain 45,101 probe sets and then processed according to the manufacturer's instructions.

Briefly, GeneChip Operating Software 1.3 (GCOS; Affymetrix) was first used to quantify microarray signals with default analysis parameters. GCOS output files were then loaded into GeneSpring v7.3 (Agilent Technologies) with per chip normalization to the 50th percentile and per-gene normalization to average expression level of wild type. The first simply identified those transcripts having a raw signal of at least 50 in expression level of wild-type embryo. A filtered list was created of all genes detected (GCOS "P" and "M" call) in all replicates. The GCOS output files of all genes after GeneSpring normalization were also used for a one-way ANOVA analysis with *post hoc* testing using Tukey's true test for significance ($P = 0.01$ at E12.5 and $P = 0.001$ at E15.5, respectively).

After obtaining the Venn diagram using Gene Spring software, we constructed gene lists that represent differentially and specifically expressed genes in the $ng^{\Delta Double}$/fg bi-maternal fetus to each of the $ng^{\Delta ch7}$/fg and $ng^{\Delta ch12}$/fg bi-maternal fetuses at E12.5 and E15.5. The list of the differentially expressed genes in the $ng^{\Delta Double}$/fg bi-maternal fetus was compared to the wild type to remove the genes that do not play an important role in term development. After removal, we called these lists the ch12 gene list and ch7 gene list. All genes of the chr12 and chr7 gene lists were integrated into IPA pathways based on biological relationships from previous references.

IPA. IPA version 3.0 was used to search for possible biological pathways and the interrelationships between network genes in the subsets of candidate genes with particularly interesting patterns. A detailed description of IPA can be found at http://www.Ingenuity.com/. 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.

To build networks, the program queries the Ingenuity Pathways Knowledge Base for interactions between Focus Genes and all other gene objects stored in the knowledge base and generates a set of networks. IPA then computes a score for each network according to the fit of the network to the set of focus genes. Biological functions are calculated and assigned to each network.

Statistical analysis. Statistical analyses of all data for comparison were carried out using analysis of one-way analysis of variance (ANOVA) and Fisher PLSD

© 2007 Nature Publishing Group http://www.nature.com/naturebiotechnology

test by using the statistical analysis software Statview (Abacus Concepts). $P < 0.05$ was considered significant.

Note: Supplementary information is available on the Nature Biotechnology website.

ACKNOWLEDGMENTS
We thank Shirley Tilghman, Princeton University, for the gift of mutant mice and Hidehiko Ogawa, Yayoi Obata and Yusuke Sotomaru for discussions. This work was supported by grants from the Bio-oriented Technology Research Advancement Institution of Japan, the Ministry of Education, Science, Culture and Sports of Japan, and the UK Biotechnology and Biological Sciences Research Council.

AUTHOR CONTRIBUTIONS
M.K. and T.K. designed the experiments, analyzed the data and wrote the manuscript. M.K., Q.W., N.T., S.M., K.Y. and M.I. performed experiments. A.C.F.-S. provided mutant mice and wrote the manuscript.

COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

Published online at http://www.nature.com/naturebiotechnology
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. Surani, M.A., Barton, S.C. & Norris, M.L. Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature* **308**, 548–550 (1984).
2. McGrath, J. & Solter, D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* **37**, 179–183 (1984).
3. Kono, T., Obata, Y., Yoshimzu, T., Nakahara, T. & Carroll, J. Epigenetic modifications during oocyte growth correlates with extended parthenogenetic development in the mouse. *Nat. Genet.* **13**, 91–94 (1996).
4. Obata, Y. *et al.* Disruption of primary imprinting during oocyte growth leads to the modified expression of imprinted genes during embryogenesis. *Development* **125**, 1553–1560 (1998).
5. Obata, Y. & Kono, T. Maternal primary imprinting is established at a specific time for each gene throughout oocyte growth. *J. Biol. Chem.* **277**, 5285–5289 (2002).
6. Kono, T. *et al.* Birth of parthenogenetic mice that can develop to adulthood. *Nature* **428**, 860–864 (2004).
7. Tremblay, K.D., Saam, J.R., Ingram, R.S., Tilghman, S.M. & Bartolomei, M.S. A paternal-specific methylation imprint marks the alleles of the mouse H19 gene. *Nat. Genet.* **9**, 407–413 (1995).
8. Pearsall, R.S. *et al.* A direct repeat sequence at the Rasgrf1 locus and imprinted expression. *Genomics* **55**, 194–201 (1999).
9. Lin, S.P. *et al.* Asymmetric regulation of imprinting on the maternal and paternal chromosomes at the Dlk1-Gtl2 imprinted cluster on mouse chromosome 12. *Nat. Genet.* **35**, 97–102 (2003).
10. Leighton, P.A., Ingram, R.S., Eggenschwiler, J., Efstratiadis, A. & Tilghman, S.M. Disruption of imprinting caused by deletion of the H19 gene region in mice. *Nature* **375**, 34–39 (1995).
11. Clapcott, S.J., Peters, J., Orban, P.C., Brambilla, R. & Graham, C.F. Two ENU-induced mutations in Rasgrf1 and early mouse growth retardation. *Mamm. Genome* **14**, 495–505 (2003).
12. Brambilla, R. *et al.* A role for the Ras signalling pathway in synaptic transmission and long-term memory. *Nature* **390**, 281–286 (1997).
13. Kawahara, M., Wu, Q., Yaguchi, Y., Ferguson-Smith, A.C. & Kono, T. Complementary roles of genes regulated by two paternally methylated imprinted regions on chromosomes 7 and 12 in mouse placentation. *Hum. Mol. Genet.* **15**, 2869–2879 (2006).
14. Zeng, F. & Schultz, R.M. RNA transcript profiling during zygotic gene activation in the preimplantation mouse embryo. *Dev. Biol.* **283**, 40–57 (2005).
15. Su, Y.Q. *et al.* Selective degradation of transcripts during meiotic maturation of mouse oocytes. *Dev. Biol.* **302**, 104–117 (2007).
16. Fielder, P.J., Thordarson, G., English, A., Rosenfeld, R.G. & Talamantes, F. Expression of a lactogen-dependent insulin-like growth factor-binding protein in cultured mouse mammary epithelial cells. *Endocrinology* **131**, 261–267 (1992).
17. Delany, A.M., Rydziel, S. & Canalis, E. Autocrine down-regulation of collagenase-3 in rat bone cell cultures by insulin-like growth factors. *Endocrinology* **137**, 4665–4670 (1996).
18. Stickens, D. *et al.* Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. *Development* **131**, 5883–5895 (2004).
19. Moon, Y.S. *et al.* Mice lacking paternally expressed Pref-1/Dlk1 display growth retardation and accelerated adiposity. *Mol. Cell. Biol.* **22**, 5585–5592 (2002).
20. Sakajiri, S. *et al.* Dlk1 in normal and abnormal hematopoiesis. *Leukemia* **19**, 1404–1410 (2005).
21. Ostermeier, G.C., Miller, D., Huntriss, J.D., Diamond, M.P. & Krawetz, S.A. Reproductive biology: delivering spermatozoan RNA to the oocyte. *Nature* **429**, 154 (2004).
22. Kono, T., Sotomaru, Y., Katsuzawa, Y. & Dandolo, L. Mouse parthenogenetic embryos with monoallelic H19 expression can develop to day 17.5 of gestation. *Dev. Biol.* **243**, 294–300 (2002).
23. Wu, Q. *et al.* Regulated expression of two sets of paternally imprinted genes is necessary for mouse parthenogenetic development to term. *Reproduction* **131**, 481–488 (2006).
24. Seitz, H. *et al.* Imprinted microRNA genes transcribed antisense to a reciprocally imprinted retrotransposon-like gene. *Nat. Genet.* **34**, 261–262 (2003).

© 2007 Nature Publishing Group http://www.nature.com/naturebiotechnology

Copyright of Nature Biotechnology is the property of Nature Publishing Group and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.