Regulated expression of two sets of paternally imprinted genes is necessary for mouse parthenogenetic development to term

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Abstract

Mouse parthenogenetic embryos (PEs) are developmentally arrested until embryo day (E) 9.5 because of genomic imprinting. However, we have shown that embryos containing genomes from non-growing (ng) and fully grown (fg) oocytes, i.e. ng^{wt}/fg^{wt} PE (wt, wild type), developed to E13.5. Moreover, parthenogenetic development could be extended to term by further regulation of *Igf2* and *H19* expression using mice with deletion of the *H19* transcription unit (*H19* Δ 13) together with its differentially unit (DMR). To gain an insight into the extended development of the parthenotes to term, we have here investigated the expression levels of paternally imprinted genes in ng^{H19 Δ 13}/fg^{wt} PE throughout their development. In ng^{H19 Δ 13}/fg^{wt} PEs that died soon after recovery, the expression of *Igf2* and *H19* was restored to the appropriate levels except for low *Igf2* expression in the liver after E15.5. Further, the paternally expressed *Dlk1* and *Dio3* were repressed, while the expression levels of the maternal *Gtl2* and *Mirg* were twice those of the controls. However, the above-mentioned four genes showed almost normal expression in the surviving ng^{H19 Δ 13}/fg^{wt} PEs. The methylation analysis revealed that the intragenic DMR of the *Dlk1-Gtl2* domain was hypermethylated in the ng^{H19 Δ 13}/fg^{wt} PEs that survived, but not in the PEs that died soon after recovery. The present study suggests that two sets of co-ordinately regulated but oppositely expressed genes, *Igf2-H19* and *Dlk1-Gtl2*, act as a critical barrier to parthenogenetic development in order to render a paternal contribution obligatory for descendants in mammals.

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Introduction

Parental genomes are required for both complete normal development and postnatal growth for genomic imprinting in mammals; this leads to the differential expression of genes between maternal and paternal alleles. Genomic imprinting acts as a strict barrier for parthenogenetic development; this is evident from results showing that parthenogenetic embryos could never develop beyond embryo day (E) 10 in mice (Surani & Barton 1983). To establish the parental-specific gene expression, certain loci of imprinted genes are differentially modified at some point either during spermatogenesis or oogenesis. In females, maternal-specific modifications are imposed during oocyte growth (Kono *et al.* 1996, Obata & Kono 2002); therefore, non-growing (ng) oocytes remain naïve at imprinting. A previous study by our group (Kono *et al.* 1996) demonstrated that an oocyte containing two haploid sets of genomes from ng and fully grown (fg) oocytes developed to E13.5 (ng^{wt}/fg^{wt} PE) (wt; wild type). Gene expression analysis showed that such types of parthenotes

were accompanied by the appropriate expression of maternally imprinted genes, which were epigenetically modified during oocyte growth.

To address the question of whether the collective expression of *Igf2* accompanied by *H19* would result in the extended development of parthenotes, we used mice with deletion of the *H19* transcription unit (*H19* Δ 13) together with its differentially methylated region (DMR) (Leighton *et al.* 1995) as an ng oocyte donor for the reconstruction of parthenogenetic embryos. In such types of parthenotes (ng^{H19 Δ 13}/fg^{wt} PE), the *Igf2* and *H19* genes were expressed and repressed respectively from the ng^{H19 Δ 13} alleles. These embryos successfully developed to term (Kono *et al.* 2004). With the exception of two pups, all parthenogenetic pups showed severe growth retardation and died before or shortly after their recovery from the uterus at 19.5 days of gestation. Two sets of co-ordinately regulated but oppositely expressed genes – *Igf2* and *H19* and *Dlk1* and *Gtl2* (Kobayashi *et al.* 2000, Schmidt *et al.* 2000) – were located on chromosome 7 and chromosome 12 respectively, and

both sets were paternally imprinted during spermatogenesis. The *Igf2* and *Dlk1* genes that were expressed from paternal alleles acted as a foetal growth factor (Feil *et al.* 1994, Leighton *et al.* 1995, Georgiades *et al.* 2000, Moon *et al.* 2002). The role of *H19* and *Gtl2* genes, which are maternally expressed non-coding RNAs, is not completely known. The reason for the survival of only two parthenotes, and not the others, remains to be clarified. To gain further insight into the extended development of the $\text{ng}^{\text{H19}\Delta 13}/\text{fg}^{\text{wt}}$ parthenogenetic embryo (PE), we carried out quantitative expression analysis of another set of paternally imprinted genes (*Dlk1*, *Gtl2*, *Mirg* and *Dio3*), which are members of the 1-Mb imprinted cluster on distal chromosome 12 (Lin *et al.* 2003).

Materials and Methods

Production of PEs

Serial nuclear transfer was conducted to reconstruct oocytes of the two classes of PE (Kono *et al.* 1996). The genome types of these two classes are shown in Fig. 1. The fusion of the diplotene oocytes with enucleated germinal vesicle (GV) oocytes was induced by an inactivated Sendai virus. The reconstituted oocytes were then cultured for 14 h in α -minimum essential medium (α -MEM) (GIBCOBRL, Grand Island, NY, USA). Throughout the experiment, the GV oocytes were manipulated in a medium containing 200 mM dbcAMP and 5% calf serum and released from the medium 1 h after fusion with an ng oocyte. A set of MII (metaphase of the second meiotic division) chromosomes from the reconstituted oocytes was transferred into a freshly ovulated MII oocyte. These reconstructed oocytes were then artificially activated with 10 mM SrCl₂ in Ca²⁺-free M16 medium (Whittingham 1971) for 2 h. These embryos were cultured in M16 medium for 3.5 days in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 37 °C. Blastocysts that were obtained from the constructed oocytes were transferred into the uterine horns of CD-1 female mice at day 2.5 of pseudopregnancy.

Quantitative gene expression analysis

Total RNAs were extracted from the major organs of the $\text{ng}^{\text{H19}\Delta 13}/\text{fg}^{\text{wt}}$ PEs, the $\text{ng}^{\text{H19}\Delta 13}/\text{fg}^{\text{wt}}$ PE survivors and

controls at E12.5, 15.5, 18.5 and 19.5 by using an RNeasy mini kit (Qiagen, Tokyo, Japan). The cDNAs were synthesized using a SuperScript RNaseH⁺ reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) in a reaction solution (20 μ l) containing total RNA (1 μ g) prepared from the tissues. Next, this synthesized cDNA was used for the quantitative analysis of the expression of imprinted genes by means of real-time quantitative RT-PCR (LightCycler System; Roche Molecular Biochemicals, Mannheim, Germany) using a ready-to-use reaction mixture kit (LightCycler FirstStart DNA Master SYBR Green I; Roche Molecular Biochemicals). The primer sequences used for the PCR reaction, the PCR conditions and the product sizes are listed in Table 1. The *Gapdh* gene was used as a loading control.

DNA preparation and bisulphite modification

DNA was isolated from the tongue ($n = 3$) and intestine ($n = 1$) of the $\text{ng}^{\text{H19}\Delta 13}/\text{fg}^{\text{wt}}$ PE with growth retardation at E18.5, and the intestine ($n = 1$) and tail ($n = 1$) of the $\text{ng}^{\text{H19}\Delta 13}/\text{fg}^{\text{wt}}$ PE that survived (survivors) at 19.5 and adult Kaguya respectively (Kono *et al.* 2004). As a control, tongue and tail at E18.5 ($n = 3$) and adult tail ($n = 1$) were collected from wt and used for DNA preparation. Methylation analysis was carried out by subjecting the samples to bisulphite modification, PCR amplification, subcloning and sequencing for the intergenic germline-derived (IG)-DMR of *Dlk1-Gtl2* (Takada *et al.* 2002). The isolated DNA was treated with sodium bisulphite using a CpGenome modification kit (CHEMICON, CA, USA).

Staining (cartilage and bone)

Embryos at E18.5 were eviscerated and fixed in 90% ethanol for 2 weeks. Cartilage samples were stained with 0.01% Alcian blue (Sigma) in 80% ethanol and 20% glacial acetic acid. They were then rehydrated with a series of 70%, 40% and 15% ethanol incubations for 2 h with each concentration. For bone staining, samples were incubated with 0.001% Alizarin red (Sigma) in 1% KOH for 2–5 days. The samples were rinsed several times with 1% KOH and stored in glycerol.

Figure 1 *H19* genotypes of $\text{ng}^{\text{wt}}/\text{fg}^{\text{wt}}$ and $\text{ng}^{\text{H19}\Delta 13}/\text{fg}^{\text{wt}}$ PE. Only ng alleles are shown. Reproduced with permission from Leighton *et al.* (1995).

Table 1 Real-time RT-PCR conditions for *Gapdh* and imprinted genes.

Genes	Primer	MgCl ₂	Annealing temp.	Product size
<i>H19</i>	5'-CATGTCTGGGCCTTTGAA-3' 5'-TTGGCTCCAGGATGATGT-3'	4 nM	55 °C	245 bp
<i>Igf2</i>	5'-AGGGGAGCTTGTGACACG-3' 5'-GGGTATCTGGGGAAGTCGTC-3'	2 mM	59 °C	225 bp
<i>Gtl2</i>	5'-AAGCACCATGAGCCACTAGG-3' 5'-TTGCACATTCCTGTGGGAC-3'	3 mM	62 °C	288 bp
<i>Dlk1</i>	5'-ACTTGCGTGGACCTGGAGAA-3' 5'-CTGTTGGTTGCGGCTACGAT-3'	2 mM	62 °C	221 bp
<i>Mirg</i>	5'-CCTTCCTGGATCTCTCGCTT-3' 5'-GTGGGAGTTGAAACATGGGT-3'	2 mM	62 °C	162 bp
<i>Dio3</i>	5'-TGTACCTGACCACCGTTCA-3' 5'-GGTGACCTTGTGTAGTACTC-3'	4 nM	60 °C	209 bp
<i>Rasgrf1</i>	5'-ACCCAGGACTTTGATACCGA-3' 5'-GTGTGAAGACTGAGCCCTTC-3'	2 mM	57 °C	207 bp
<i>Gapdh</i>	5'-GTCGTGGAGTCTACTGGTGTC-3' 5'-GAGCCCTTCCACAATGCCAAA-3'	2 mM	60 °C	240 bp

Statistical analysis

The data regarding the rate of development of the embryos were analyzed by Chi-square analysis (a *P* value of less than 0.01 was considered to be statistically significant).

Results

In vivo growth of the PEs

In order to obtain the foetal growth curve of the $ng^{H19\Delta13}/fg^{wt}$ PEs, we recovered the PEs at E12.5, E15.5 and E18.5 (Fig. 2A). At 12.5 days of gestation, the mean body weight of the $ng^{H19\Delta13}/fg^{wt}$ PEs (98 ± 1.8 mg, $n = 5$) was 15% less than that of the controls derived from fertilized embryos (98 ± 1.8 mg, $n = 5$ vs 115 ± 1.2 mg, $n = 5$), but the difference was not significant. However, the mean body weight gain of the $ng^{H19\Delta13}/fg^{wt}$ PEs was significantly greater than that of the ng^{wt}/fg^{wt} PEs (77 ± 3.0 mg, $n = 5$); this was 67% of the mean weight of the controls. It should be noted that thereafter the mean body weight of the $ng^{H19\Delta13}/fg^{wt}$ PEs was definitely reduced from 74% (311 ± 11.1 mg, $n = 8$), which was the mean weight of the controls at E15.5, to 55% (726 ± 15.5 mg, $n = 7$), which was the mean weight of the controls at E18.5 (Fig. 2B). These results showed that severe foetal growth retardation occurs mainly in the latter half of gestation. The cartilage and bone staining experiment revealed that the $ng^{H19\Delta13}/fg^{wt}$ PE at E18.5 formed a delayed ossification pattern and had an abnormally shaped thoracic cage (Fig. 2C).

Expression of the imprinted genes in $ng^{H19\Delta13}/fg^{wt}$ PE

To gain a better understanding of the organ-specific gene expression, we carried out a quantitative expression analysis by real-time RT-PCR using major organs, i.e. the brain, tongue, heart, liver and leg (muscle and bone) at E12.5, E15.5 and E18.5 in controls and $ng^{H19\Delta13}/fg^{wt}$ PEs. During the process of development, the expression levels of *H19*

and *Igf2* were slightly reduced in the $ng^{H19\Delta13}/fg^{wt}$ PEs in most tissues as compared with the expression levels observed in the controls (Fig. 3A and B). *H19* and *Igf2* gene expressions were measured; the expression patterns obtained were similar to those of the controls (Fig. 3A and B) except for the low expression of the *Igf2* in the liver. In all organs of the $ng^{H19\Delta13}/fg^{wt}$ PEs, *Gtl2* was overexpressed to approximately twice the control level, showing a peak at E15.5 (Fig. 3C). However, the *Dlk1* expression was repressed in the above-mentioned five organs, with expression at levels below 20% of the controls (Fig. 3D). With regard to the tested organs, the expression levels of the paternally expressed *Dio3* in the $ng^{H19\Delta13}/fg^{wt}$ PEs were approximately 70% of the controls, with the exception of the tongue (<50%; Fig. 3E), while the maternally expressed *Mirg* was overexpressed in most tissues of the $ng^{H19\Delta13}/fg^{wt}$ PEs (Fig. 3F). The other paternally imprinted gene, namely, *Rasgrf1* (de la Puente *et al.* 2002, Yoon *et al.* 2002), which is expressed from the paternal allele in the brain and stimulates growth hormone expression from the pituitary, was examined. The data showed that *Rasgrf1* was completely repressed in the $ng^{H19\Delta13}/fg^{wt}$ PEs (Fig. 3G).

We also carried out gene expression analysis in the survivor PEs in order to understand whether the expressions of the paternally imprinted genes were involved in the successful survival of the $ng^{H19\Delta13}/fg^{wt}$ PE. The data clearly indicated that the expression of both paternally expressed *Dlk1* and *Dio3* and maternally expressed *Gtl2* and *Mirg* were corrected in the survivor PEs, suggesting that both sets of paternally imprinted genes are certainly involved in the extended development of the $ng^{H19\Delta13}/fg^{wt}$ PE (Fig. 4).

Methylation analysis of the IG-DMR of the *Dlk1-Gtl2* domain

The expressions of *Dlk1*, *Gtl2*, *Dio3* and *Mirg* that are regulated by the methylation status of IG-DMR, located

Figure 2 The prenatal growth of parthenogenetic $ng^{H19\Delta13}/fg^{wt}$ embryos. (A) Live embryos of two genotypic parthenotes – ng^{wt}/fg^{wt} and $ng^{H19\Delta13}/fg^{wt}$ – were recovered at 12.5, 15.5 and 18.5 days of gestation. (B) The mean body weights of these parthenogenetic embryos were measured at E12.5, E15.5 and E18.5. The average body weight for each parthenogenetic embryo is shown, and data are expressed as means $\pm$ S.E.M. E12.5 (controls: $n = 5$; ng^{wt}/fg^{wt} : $n = 5$; $ng^{H19\Delta13}/fg^{wt}$: $n = 5$); E15.5 (controls: $n = 10$; $ng^{H19\Delta13}/fg^{wt}$: $n = 8$); E18.5 (controls: $n = 5$; $ng^{H19\Delta13}/fg^{wt}$: $n = 7$; $**P < 0.01$). (C) The cartilage and bone staining of the controls (wt) and $ng^{H19\Delta13}/fg^{wt}$ PEs at E18.5.

upstream of *Gtl2*, were normally expressed in the survivor $ng^{H19\Delta13}/fg^{wt}$ PEs; this perhaps resulted in normal birth. To gain further insight into the correction of the gene expressions, we analysed the methylation status of the IG-DMR of the *Dlk1-Gtl2* domain. The analysis of the tail of Kaguya showed that the IG-DMR of the *ng* allele was completely methylated and that of the *fg* allele was also partially methylated (Fig. 5). Further, the intestine of another survivor $ng^{H19\Delta13}/fg^{wt}$ PE at E19.5 PE also showed complete methylation of the IG-DMR in the *ng* allele. However, the IG-DMR was completely unmethylated in the tongue and the intestine in the growth-retarded $ng^{H19\Delta13}/fg^{wt}$ PE at E18.5 (Fig. 5). In the controls, the analysis of all tissues exhibited parental allele-specific methylation status with hypermethylated paternal allele and hypomethylated maternal allele. The results thus obtained gave an answer as to why *Dlk1* was expressed only in Kaguya, revealing the importance of normal expression patterns of imprinted genes located in the *Dlk1-Gtl2* domain in the normal development and survival of Kaguya (Kono *et al.* 2004). However, the reason for IG-DMR methylation remained unclear in only a few cases.

Discussion

In the present study, quantitative gene expression analysis of the two sets of co-ordinately regulated but oppositely expressed genes provided further insight into the characteristics of the $ng^{H19\Delta13}/fg^{wt}$ PEs, which were able to develop to term. Using mutant mice we successfully regulated the imprinted expressions of both *Igf2* and *H19* in the $ng^{H19\Delta13}/fg^{wt}$ PE, since the *Igf2* and *H19* genes are regulated by DMR located upstream of *H19* sharing the same endoderm-derived tissue-specific enhancers located downstream of *H19* (Feil *et al.* 1994, Sasaki *et al.* 1995, Thorvaldsen *et al.* 1998, Hark *et al.* 2000). In addition to being a major foetal growth regulator, *Igf2* is a common

regulator of haematopoietic stem cell differentiation (Zhang & Lodish 2004). We also confirmed that the *Igf2* expression was repressed in the ng^{wt}/fg^{wt} PE (data not shown) and, as expected, it recovered in the $ng^{H19\Delta13}/fg^{wt}$ PE. However, similar to the ng^{wt}/fg^{wt} PE, the $ng^{H19\Delta13}/fg^{wt}$ PE continued to exhibit growth retardation (Fig. 2) (Kono *et al.* 2004). The *Igf1* and *Igf2* genes are known to play important regulatory roles in foetal growth and development (Allan *et al.* 2001). The expression levels of *Igf1*, *Igf1r* and *Igf2r* in the two types of parthenotes at E12.5 did not differ from those of the controls (data not shown). These findings suggest that additional factors may be involved in the growth of the two types of parthenotes. Although *H19* encodes an untranslated maternal RNA, the correct imprinted expression of the gene in various tissues may be involved in foetal development (Kono *et al.* 2002) and tumorigenesis (Juan *et al.* 2000, Lottin *et al.* 2002). In the present study, *H19* expression increased with gestation in most tissues, except the brain and heart, and the highest expression of *Igf2* was observed at E15.5, after which the levels decreased, although a developmental increase was observed in certain important tissues (Fig. 3).

Paternally imprinted *Dlk1* and *Gtl2* are also important with regard to foetal lethality and growth retardation in maternal disomic mice (Georgiades *et al.* 2000, Schmidt *et al.* 2000, Lin *et al.* 2003). They are another set of co-ordinately regulated but oppositely expressed genes, located on the distal region of chromosome 12. In the present study, we observed the increased expression of *Dlk1* in the controls at E15.5, while the gene expression was repressed in all parthenotes, which showed retardation throughout development and were estimated to be unable to survive beyond E19.5 (Figs 3 and 4). The repression of *Dlk1* is thought to be a major reason for developmental failure in the $ng^{H19\Delta13}/fg^{wt}$ PE, since the paternally expressed *Dlk1* encodes a cell-surface transmembrane protein and is essential for normal development (Moon *et al.* 2002), haematopoiesis (Doggett *et al.* 2002, La

Figure 3 Quantitative analysis of the expression of imprinted genes in five types of tissues at 12.5, 15.5 and 18.5 days of gestation of $ng^{H19\Delta13}/fg^{wt}$ PEs. Total RNA was extracted from each tissue sample at 12.5, 15.5 and 18.5 days of gestation of the $ng^{H19\Delta13}/fg^{wt}$ PE and from the controls (wt). The cDNAs were synthesized using a SuperScript II RnaseH reverse transcriptase kit in a reaction solution (20 μ l) containing the total RNA (1 μ g) prepared from each tissue. The expression levels of the seven imprinted genes – (A) *H19*, (B) *Igf2*, (C) *Gtl2*, (D) *Dlk1*, (E) *Dio3*, (F) *Mirg* and (G) *Rasgrf1* – were analyzed quantitatively using real-time RT-PCR. The expression of each gene is shown as the average of the expression levels relative to that of *Gapdh*, which was used as an internal control. Data are expressed as means $\pm$ s.e.m. ($n = 5$).

Figure 4 Quantitative analysis of the expression of paternally imprinted genes at 19.5 days of gestation of $ng^{H19\Delta13}/fg^{wt}$ PEs and $ng^{H19\Delta13}/fg^{wt}$ survivors. The expression of four imprinted genes – (A) *Dlk1*, (B) *Gtl2*, (C) *Dio3* and (D) *Mirg* – at E19.5 of the $ng^{H19\Delta13}/fg^{wt}$ PE ($n = 3$), $ng^{H19\Delta13}/fg^{wt}$ survivor ($n = 1$) and controls ($n = 3$) were analysed by quantitative real-time PCR. The expression of each gene is shown as the average of the expression levels relative to *Gapdh*. Data are expressed as means $\pm$ S.E.M.

Motte-Mohs *et al.* 2005, Li *et al.* 2005) and ossification (Moon *et al.* 2002). The maternally expressed *Gtl2* encodes an untranslated RNA and is enhanced in the $ng^{H19\Delta13}/fg^{wt}$ PE (Figs 3 and 4); however, normal expression of *Gtl2* was observed in some important tissues of other survivor with an apparently normal phenotype at birth (Fig. 4) (Kono *et al.* 2004). These results suggest that the correct expressions of *Dlk1* and *Gtl2* also play important roles in the development and survival of the ng/fg PE. Moreover, the increased expression of the maternally expressed *Mirg* and the decreased expression of the paternally expressed *Dio3* were also invested in the $ng^{H19\Delta13}/fg^{wt}$ PE (Figs 3 and 4). *Dlk1-Gtl2* also belongs to members of the 1-Mb imprinted cluster, which is located on mouse distal chromosome 12 (Lin *et al.* 2003). The phenotype of the $ng^{H19\Delta13}/fg^{wt}$ PE resembled that of the mice with *mUPD* for chromosome 12 (Georgiades *et al.* 2000) rather than that of the *Dlk1* null mice (Moon *et al.* 2002). However, there is no evidence for the involvement of *Dio3*, *Mirg*, etc. in foetal development.

The present results, together with the results of our previous study (Kono *et al.* 2004), demonstrated that the normal level of paternally expressed *Dlk1* was invested in Kaguya, which survived and grew up to adulthood with normal reproductive ability. We carried out further methylation analysis of the IG-DMR of *Dlk1-Gtl2*. Interestingly, we observed a higher frequency of hypermethylated patterns in the $ng^{H19\Delta13}$ allele and partial methylation in the *fg* allele (Fig. 5). In contrast, the DMR of the $ng^{H19\Delta13}/fg^{wt}$ PE with growth retardation was completely hypomethylated. The reason for the different IG-DMR methylation

patterns of *Dlk1* in survivor $ng^{H19\Delta13}/fg^{wt}$ PEs is unknown. However, the data on both methylated *ng* and *fg* alleles suggests that the unexpected methylation of DMR could occur after oocyte reconstruction, but not during oogenesis. These results indicate that the normal expression of *Dlk1* together with the neighbouring imprinted genes is an important factor in the normal development and survival of $ng^{H19\Delta13}/fg^{wt}$ PEs.

The present study demonstrated that the expression of the paternally imprinted genes regulates foetal growth after mid gestation; its inappropriate expression leads to foetal growth retardation and lethality in the $ng^{H19\Delta13}/fg^{wt}$ PE. Therefore, both paternally and maternally expressed genes act as a barrier to parthenogenesis in mammals. Together with the results of our previous studies, the present findings support the idea that adequate correction of the expression dosage of the imprinted genes (*Igf2-H19* and *Dlk1-Gtl2*) by gene manipulation may promote long-term normal development in the $ng^{H19\Delta13}/fg^{wt}$ PE in mice.

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