Mouse Parthenogenetic Embryos with Monoallelic 
H19 Expression Can Develop 
to Day 17.5 of Gestation

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In mammals, both maternal and paternal genomes are required for a fetus to develop normally to term. This requirement is due to the epigenetic modification of genomes during gametogenesis, which leads to an unequal expression of imprinted genes between parental alleles. Parthenogenetic mouse embryos that contain genomes from nongrowing (ng) and fully grown (fg) oocytes can develop into 13.5-day-old fetuses, in which paternally and maternally expressed imprinted genes are expressed and repressed, respectively, from the ng oocyte allele. The H19 gene, however, is biallelically expressed with the silent status Igf2 in such parthenotes. In this study, we examined whether the regulation of H19 monoallelic expression enhances the survival of parthenogenetic embryos. The results clearly show that the ngH19-KO/fgwt parthenogenetic embryos carrying the ng-oocyte genome that had been deleted by the H19 transcription unit successfully developed as live fetuses for 17.5 gestation days. Control experiments revealed that this unique phenomenon occurs irrespective of the genetic background effect. Quantitative gene expression analysis showed that day 12.5 ngH19-KO/fgwt parthenogenetic fetuses expressed Igf2 and H19 genes at <2 and 82% of the levels in the controls. Histological analysis demonstrated that the placenta of ngH19-KO/fgwt parthenotes was afflicted with atrophia with severe necrosis and other anomalies. The present results suggest that the cessation of H19 gene expression from the ng-allele causes extended development of the fetus and that functional defects in the placenta could be fatal for the ontogeny. © 2002 Elsevier Science (USA)

Key Words: parthenogenesis; genomic imprinting; epigenetic modification; oocytes; H19 gene; nuclear transfer; development; mouse.

INTRODUCTION

Mammalian parthenotes cannot be induced to develop normally to term. Attempts to induce such development have resulted in embryonic death before 10 days of gestation in mice (Surani and Barton, 1983; McGrath and Solter, 1984; Barton et al., 1984; Surani et al., 1986) and before 21 days of gestation in sheep (Hagemann et al., 1998) and pigs (Kure-bayashi et al., 2000). Although the precise mechanisms underlying the limited development of mammalian parthenotes are still unclear, one persuasive explanation is that the functions of the male and female genomes are definitively different as a result of epigenetic modifications that occur during gametogenesis (Monk, 1988; Surani et al., 1990; Sasaki et al., 1992; Brannan and Bartolomei, 1999). This hypothesis is supported by our recent results, which showed that the ability of maternal chromatin to support full-term development is attained during the last half of oocyte development (Bao et al., 2000). The epigenetic modifications during gametogenesis lead to parental allele-specific expression of the imprinted genes and result in the normal development of fertilized biparental embryos to term.

Previous studies (Kono et al., 1996; Obata et al., 1998) have provided direct evidence of the epigenetic modification that occurs in mammalian development as follows. The parthenogenetic mouse embryos that carry two sets of
haploid genomes from nongrowing and fully grown oocytes are able to develop to 13.5 days of gestation with a well-developed placenta. In these embryos, the paternally expressed genes, Peg1/Mest, Peg3, and Snrpn, are activated, while the maternally expressed genes, Igf2r and p57Kip2, are silent in the alleles derived from a nongrowing oocyte genome. However, of the genes analyzed, it was determined that the H19 gene was biallelically expressed while the Igf2 gene was repressed, as the enhancers that are shared by Igf2 and H19 act preferentially for the H19 gene (Bartolomei et al., 1993; Hark et al., 2000). So far, studies have shown that H19 is expressed in a broad range of tissues, although it is not expressed in neural tissues (Ohlisson et al., 1994; Svensson et al., 1995). This high expression of H19 is also detected in extraembryonic tissues. These findings imply that the biallelic expression of H19 is involved along with the silence of the Igf2 gene in the developmental arrest of the ngfg parthenogenotes.

To investigate this possibility, we constructed ngfg parthenogenetic oocytes using nongrowing oocytes derived from H19-null mutated mice (Ripoche et al., 1997) and assessed whether the parthenogenetic embryos can develop beyond 13.5 days of gestation.

MATERIALS AND METHODS

Oocyte Collection

MF1 mice, whose H19 transcription unit was targeted with a Py-ty-neo cassette, and B6CBF1 (C57BL/6J × CBA) were used as oocyte donors. Fully grown germinal vesicle (GV) oocytes were collected from the ovarian follicles of adult females 44-48 h after an eCG injection, and nongrowing stage oocytes, which are at the diploene stage of the first meiosis, were collected from the ovaries of 1-day-old pups.

Embryo Production

Nuclear transfer was carried out as outlined previously (Kono et al., 1996; Obata et al., 1998). Oocytes (ng19KO/fg19 wt) containing a haploid set of genomes from H19-null mutant females and another genome from ovulated MII oocytes of wild-type B6CBF1 mice were constructed by nuclear transfer. Fusion of the diploene oocytes with enucleated GV oocytes was induced with an inactivated Sendai virus (HVJ, 2700 hemagglutinating activity unit/ml). After the fusion, the reconstituted oocytes were cultured for 14 h in Waymouth medium (MB752/1; GIBCO). The GV oocytes were manipulated in a medium containing 200 μM db-cAMP and 5% calf serum throughout the experiment and were released from the medium 1 h after fusion with a nongrowing stage oocyte. A set of MII chromosomes from reconstituted oocytes was transferred into an enucleated MII oocyte that had been collected from the oviducts of superovulated B6CBF1 mice 15-16 h after hCG injection. ng19/ fg19 parthenogenotes were also produced from wild-type MF1 outbreed and B6CBF1 mice as described above. The reconstructed oocytes were artificially activated with 10 mM SrCl2 in Ca2+-free M16 medium for 2 h (Bos-Mikich et al., 1995).

MF1 and B6CBF1 embryos produced by in vitro fertilization were used as controls.

In Vitro Culture and Embryo Transfer

These embryos were manipulated in M2 medium and cultured in M16 medium (Whittingham, 1971) for 3 days in an atmosphere of 5% CO2, 5% O2, and 90% N2 at 37°C. Blastocysts obtained from the constructed oocytes were transferred into the uterine horns of CD-1 female mice at 2.5 days of pseudopregnancy. The postimplantation development was assessed by an autopsy performed between 15.5 and 18.5 days of gestation.

Gene Expression Analysis

Total RNA was isolated from the control and parthenogenetic embryos at 12.5 and 17.5 days of gestation by using the SV Total RNA Isolation Kit (Promega). First-strand cDNA was synthesized from 1 μg of total RNA from each embryo by Superscript reverse transcriptase II (Gibco-BRL) according to the manufacturer’s instructions. The cDNA was subjected to PCR, which was carried out in a 50-μl reaction buffer containing 1.25 U of Taq DNA polymerase (Takara), 1 pmol of each primer, 1.5 mM MgCl2, and 250 μM dNTPs. Quantitative analysis of gene expression was performed by means of Real-Time quantitative PCR (LightCycler System; Roche Molecular Biochemicals, Germany) using a ready-to-use reaction mixture kit (LightCycler FirstStart DNA Master SYBR Green I; Roche Molecular Biochemicals). The primers used for Igf2 were 5'-CTACTTACAGGCTTCAAG-3' and 5'-AGTGGTTTGCTTGACATCTCC-3'; and those used for H19 were 5'-CATGCTGGCCCTTGAAG-3' and 5'-TTGGTCCAGGATGATG-3'. The quantity of PCR products was detected by monitoring the luminous intensity. To provide a standard, day 12.5 fetuses from normal fertilized embryos were analyzed by the same procedure.

Histological Analysis

The live fetuses and placentas at 17.5 days of gestation derived from ng19KO/fg19 wt parthenogenetic embryos were fixed with 4% paraformaldehyde and processed for wax embedding. Serial sections prepared in the cross planes were mounted on slides and stained with hematoxylin and eosin.

RESULTS

Development of Parthenogenetic Embryos

For developmental assessment, we constructed 789 oocytes (ng19KO/fg19 wt) using nongrowing stage oocytes from H19-null mutant newborn mice. After maturation in vitro and artificial activation, 87% of the oocytes resulted in diploid one-cell parthenogenetic embryos that formed two second-polar bodies and pronuclei, and 88% of them developed to the blastocyst stage in vitro (Table 1). In all, 505 blastocysts derived from ng19KO/fg19 wt oocytes were transferred to 43 recipients (Table 2). Since ng19/fg19 parthenogenotes can develop to 13.5 days of gestation (Kono et al., 1996), the autopsy to assess postimplantation development was carried out between 15.5 and 18.5 days of gestation. A total of 42 fetuses were estimated to have developed up to day 14.5 of gestation. At 17.5 days of gestation, 4 live fetuses were recovered from 166 embryos transferred (Figs. 1a-1d). In
these fetuses, the skin was wrinkled and thickened, and the subcutaneous veins were no longer distinctly visible. The eyelids were fused and thickened, and the fingers and toes were parallel. The umbilical hernia had already repositioned. However, the weight of the fetuses (503 mg, (n/H11005 4)) was significantly (P/H11021 < 0.01) less than that of the controls, which was 708 mg (n/H11005 12) at stage 25–26. When the remaining fetuses were autopsied at 18.5 days of gestation, only two dead fetuses, which were estimated as day 17.5 fetuses (Figs. 1e and 1f), were recovered, suggesting that term development was difficult for them.

To demonstrate that the present results were due to the monoallelic expression of the H19 gene irrespective of the genetic background effect and of the particular case, we constructed an adequate number of ng wt/fg wt parthenotes using both MF1 outbreed and B6CBF1 mice (Tables 1 and 2). The 185 and 239 blastocysts that were obtained from the MF1 outbreed and B6CBF1 mice, respectively, were transferred to the recipient females as before. The results from the autopsy at 14.5 days of gestation confirmed that ng wt/fg wt parthenotes cannot develop beyond 13.5 days of gestation. Further, we have confirmed that fg H19KO/fg wt parthenogenetic embryos die before day 10 of gestation (Tables 1 and 2). These results suggest that the extended development of the parthenogenetic embryos to 17.5 days of gestation was caused by the monoallelic expression of the H19 gene and by a unique phenomenon seen in the ng H19-KO/fg wt parthenogenetic mouse embryos.

Expression of Igf2 and H19

To obtain further insight into the mechanism underlying the extended development of the parthenogenetic embryos, quantitative expression analysis was performed in individual ng H19-KO/fg wt parthenotes (n = 10) by using quantitative Real-Time PCR (Fig. 2). The level of Igf2 in these parthenotes at day 12.5 of gestation was found to be <2% that of the level in the control fetuses at day 12.5 of gestation. The expression level is not significantly different from that of the level in day 12.5 of ng wt/fg wt parthenotes (n = 7), suggesting that this leaking expression did not affect the extended development in ng H19-KO/fg wt parthenotes. The low expression of Igf2 may correlate with the significantly lighter weight of the parthenotes. The day 17.5 parthenotes (n = 3) expressed Igf2 at a level equal to that of the day 17.5 controls, which was about 20% in the level in the control fetuses at day 12.5 of gestation.

In contrast, H19 gene expression in ng H19-KO/fg wt parthenotes, which have a single expression allele, was only

### TABLE 1
Development of Constructed Parthenogenetic Mouse Embryos in Vitro

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>No. of oocytes constructed</th>
<th>No. of oocytes activated (%)</th>
<th>No. of embryos developed to blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng H19-KO/fg wt</td>
<td>789</td>
<td>698 (88)</td>
<td>613 (88)</td>
</tr>
<tr>
<td>ng wt/fg wt (MF1)</td>
<td>282</td>
<td>254 (90)</td>
<td>201 (79)</td>
</tr>
<tr>
<td>ng wt/fg wt (B6CBF1)</td>
<td>458</td>
<td>424 (93)</td>
<td>326 (77)</td>
</tr>
<tr>
<td>fg H19KO/fg wt</td>
<td>133</td>
<td>123 (92)</td>
<td>113 (92)</td>
</tr>
</tbody>
</table>

### TABLE 2
Postimplantation Development of Constructed Parthenogenetic Mouse Embryos

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Day of biopsy (dpc)</th>
<th>No. of embryos transferred</th>
<th>No. of pregnant/recipient(s)</th>
<th>No. of implantation (%)</th>
<th>No. of fetuses developed to 12.5 dpc &lt; 14.5 dpc &lt; 16.5 dpc &lt; 17.5 dpc</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ng H19-KO/fg wt</td>
<td>15.5</td>
<td>104</td>
<td>7/8</td>
<td>60 (58)</td>
<td>D6, D7, L2, D9, D2, L1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.5</td>
<td>79</td>
<td>6/7</td>
<td>35 (44)</td>
<td>D14, D12, D2, L4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17.5</td>
<td>166</td>
<td>13/14</td>
<td>117 (70)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18.5</td>
<td>156</td>
<td>12/14</td>
<td>96 (62)</td>
<td>D18, D8, D2, D2</td>
<td></td>
</tr>
<tr>
<td>ng wt/fg wt (MF1)</td>
<td>14.5</td>
<td>185</td>
<td>12/15</td>
<td>127 (69)</td>
<td>D59, 0</td>
<td></td>
</tr>
<tr>
<td>ng wt/fg wt (B6CBF1)</td>
<td>14.5</td>
<td>239</td>
<td>16/18</td>
<td>155 (65)</td>
<td>D70, 0</td>
<td></td>
</tr>
<tr>
<td>fg H19KO/fg wt</td>
<td>12.5</td>
<td>113</td>
<td>8/10</td>
<td>74 (65)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Note. dpc, day post coitum; D, dead fetuses; L, live fetuses.
slightly lower (82%) than that of day 12.5 controls (Fig. 2). This level of H19 gene expression was almost half the level of ng\textsuperscript{wt}/fg\textsuperscript{wt} parthenotes, both of whose maternal alleles were active. The expression of H19 in day 17.5 ng\textsuperscript{H19-KO}/fg\textsuperscript{wt} parthenotes was 88, 153, and 258% of the level in the day 17.5 and 12.5 controls and day 12.5 ng\textsuperscript{wt}/fg\textsuperscript{wt} parthenotes, respectively.

**Histological Analysis**

The placenta of ng\textsuperscript{H19-KO}/fg\textsuperscript{wt} parthenotes was afflicted with atrophia with severe anomalies (Fig. 3). Histological analysis showed that severe necrosis had occurred throughout the tissue, especially in the labyrinthine layer. Thrombus and agglutination of red blood cells were also seen in some blood vessels. A number of endometrial glycogen cells, which were vacuolated, were also distributed in the labyrinthine layer. The border between the spongiotrophoblast and labyrinth layers was disrupted. The necrosis and vacuolation led to severe defects in the distribution of the maternal and fetal blood vessels in the spongiotrophoblast and labyrinthine layers, respectively. In contrast, no cardiac defect, which are the most likely reason for fetal death at 17.5 days of gestation, was detected, and neither were any histological anomalies (Fig. 4). This result suggests that defects in the placenta, rather than in the fetus itself, caused the ng\textsuperscript{H19-KO}/fg\textsuperscript{wt} parthenotes to fail to develop to term.

**DISCUSSION**

The present experiment provided the parthenotes an opportunity to develop to 17.5 days of gestation. The phenotypes were quite similar to those of the control...
fetuses at the corresponding stage and were estimated to be at stage 25 (Theiler, 1989). This extended development of mouse parthenotes is 4 days longer than that observed previously with nongrowing and fully grown oocyte genomes. The deletion of the transcription unit in the H19 gene and the corresponding monoallelic expression of H19 gene may be responsible for this extended development of the parthenotes. Assuming that silencing the H19 gene in the ng allele results in the extended development of ng H19-KO/fg wt parthenotes, the question remained as to what the mechanism of this extension of development might be. It is known that the H19 gene is expressed abundantly in a broad range of tissues of both endodermal and mesodermal origin in developing mouse embryos (Ohlsson et al., 1994; Svensson et al., 1995), but the 2.5-kb H19 spliced and polyadenylated RNA does not encode a protein. Earlier studies have been unable to identify any function for the H19 transcript, except one report that found that growth retardation was induced when an H19 expression construct was transfected into human embryo tumor cells (Hao et al., 1993). It has recently been shown that the H19 transcript is associated with polysomes in a variety of cells and that there is a reciprocal correlation in trans between cytoplasmic H19 and Igf2 mRNA levels, suggesting that the H19 transcript represses Igf2 expression in trans (Li et al., 1998). In contrast, the replacement of the H19 gene with a protein-coding gene does not affect the expression of Igf2 (Jones et al., 1998). The deletion of a silencer element located between 11.7 and −2.9 kb from the transcription start site of H19 has been found to cause a loss of imprinting after paternal inheritance, but the expression of the neighboring imprinted Igf2 gene is not affected (Drewell et al., 2000). Thus, the function of H19 is still not clear and appears to be quite complicated. For the H19-deletion mice used here as ng oocyte donors, it has been reported that homozygous mutants are viable and exhibited an 8% overgrowth (Ripoche et al., 1997). To
correlate the extended development of parthenotes with the expressions of Igf2 and H19 genes, we detected the levels of the imprinted genes in ng\textsuperscript{19K0}/fg\textsuperscript{wt} and ng\textsuperscript{wt}/fg\textsuperscript{wt} parthenotes and in the control fetuses. The expression of Igf2 gene in both ng\textsuperscript{19K0}/fg\textsuperscript{wt} and ng\textsuperscript{wt}/fg\textsuperscript{wt} parthenotes was a very small percentage (<2%) of that of the controls. The normal level of Igf2 expression detected in day 17.5 ng\textsuperscript{19K0}/fg\textsuperscript{wt} parthenotes is not likely to be the primary reason for the extended development, as mice carrying Igf2 deletions are viable and fertile (Baker et al., 1993), albeit with a reduction in body weight. Similar expression is seen in H19 deletion mice. The maternal Igf2 allele, which is normally silent, is altered to have an active status in cis (Ripoche et al., 1997). Thus, the Igf2 expression, although at a low level, may provide feasible circumstances under which the embryo can develop to day 17.5 of gestation.

It is expected that maternally expressed imprinted genes could be expressed in parthenogenetic embryos at twice the level of the controls. As expected, ng\textsuperscript{wt}/fg\textsuperscript{wt} parthenotes expressed H19 gene at 240% of the levels found in the controls, while ng\textsuperscript{19K0}/fg\textsuperscript{wt}, which has a single H19 gene allele, expressed it at a level almost equal to the level found in the controls, suggesting that the expression of H19 gene at an appropriate dose from a single allele is involved in the extended development beyond day 13.5 of gestation.

So far, studies have shown that overexpression of the H19 gene itself does not have deleterious effects. No obvious phenotype has been detected with an excess gene dosage of H19 in transgenic experiments using a 130-kb YAC clone (Ainscough et al., 1997). An earlier report based on results in transgenic mice suggested that ectopic expression of the H19 gene can cause prenatal lethality (Brunkow and Tilghman, 1991), but this suggestion was later retracted by the same group (Pfeifer et al., 1996). However, we cannot remove the possibility that it is H19 itself that appears to be responsible for the extended development of ng\textsuperscript{19K0}/fg\textsuperscript{wt} parthenotes. It is possible that the biallelic expression of the gene itself is responsible for the developmental arrest at day 13.5 of gestation in ng\textsuperscript{wt}/fg\textsuperscript{wt} parthenotes. Although the mechanism is not clear, it is supposed that the transcripts facilitate or block the binding of transcription factors and thus alter the DNA conformation. Therefore, the biallelic expression may have deleterious effects at the cis and/or trans positions on a certain gene that regulates embryo development (Forne et al., 1997; Duvillie et al., 1998).

On the other hand, although the reason for death at 17.5 days of gestation is not clear, histological analysis suggests that a defect of the placenta led to death. Severe ischemic necrosis was observed throughout the placental tissues, suggesting that this placental defect causes hypoxiaosis and metabolic defects in the parthenotes. In contrast, no anomaly was detected in the cardiac tissue. These observations suggest that defects in the placenta, rather than in the fetus itself, are the main reason that the ng\textsuperscript{19K0}/fg\textsuperscript{wt} parthenotes failed to develop to term.

Thus, together with our previous work, the present study clearly demonstrates that epigenetic modifications that occur during oocyte growth, namely maternal primary imprinting, have a critical effect on the development of parthenogenetic mouse embryos, and that an alteration of imprinting status leads to extended parthenogenetic development. However, it remains unclear whether further alterations of the imprint status of the ng allele can bring about term development of parthenogenetic mouse embryos.

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