Disruption of primary imprinting during oocyte growth leads to the modified expression of imprinted genes during embryogenesis

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SUMMARY

Parthenogenetic embryos, which contained one genome from a neonate-derived non-growing oocyte and the other from a fully grown oocyte, developed to day 13.5 of gestation in mice, 3 days longer than previously recorded for parthenogenetic development. To investigate the hypothesis that disruption of primary imprinting during oocyte growth leads to the modified expression of imprinted genes and this parthenogenetic phenotype, we have examined Peg1/Mest, Igf2, Peg3, Snrpn, H19, Igf2r and excess p57KIP2. We show that paternally expressed genes, Peg1/Mest, Peg3 and Snrpn, are expressed in the parthenotes, presumably due to a lack of maternal epigenetic modifications during oocyte growth. In contrast, the expression of Igf2, which is repressed in a competitive manner by transcription of the H19 gene, was very low. Furthermore, we show that the maternally expressed Igf2r and p57KIP2 genes were repressed in the alleles of the non-growing oocyte indicating maternal modifications during oocyte growth are necessary for its expression. Thus, our results show that primary imprinting during oocyte growth exhibits a crucial effect on both the expression and repression of maternal alleles during embryogenesis.

Key words: Parthenogenetic embryo, Imprinted gene, Primary imprinting, Oocyte growth, Igf2r, p57KIP2, H19, Igf2, Mouse

INTRODUCTION

The maternal and paternal genomes have complementary roles in mammalian development and both are required for development to term. In mice, parthenogenetic and androgenetic embryos die before day 10 of gestation and have distinctive phenotypes: 25-somite embryos with poor extraembryonic tissue and retarded embryos with proliferated trophoblast, respectively (Surani et al., 1984; Barton et al., 1984). This is due to genomic imprinting, gene expression being dependent on whether a parental allele is inherited from the spermatozoa or oocyte (DeChiara et al., 1991; Bartolomei et al., 1991; Ferguson-Smith et al., 1991). It is suggested that monoallelic expression is due to DNA methylation at the cytosine residue of the CpG dinucleotides in the regulatory domain of the imprinted genes (Zemel et al., 1992; Li et al., 1993; Ferguson-Smith et al., 1993; Bartolomei et al., 1993; Stöger et al., 1993; Sutcliffe et al., 1994). Less than 20 genes have been classified as imprinted genes (Nakao and Sasaki, 1996), but some of these genes have important roles in embryogenesis (Lau et al., 1994; Guillemot et al., 1995; Marahrens et al., 1997). That imprinted genes are responsible for the parthenogenetic and androgenetic development is supported by the observation that, in parthenogenetic embryos, the paternally expressed genes, Peg1/Mest (Kaneko-Ishino et al., 1995), Igf2 (Sasaki et al., 1992; Walsh et al., 1994), Peg3 (Kuroiwa et al., 1996) and Snrpn (Barr et al., 1995), are not expressed, whereas, in androgenetic embryos, the maternally expressed genes, H19 (Walsh et al., 1994) and Igf2r (Sasaki et al., 1995), are not expressed.

It has been suggested that the sex-specific epigenetic modifications that are imposed during gametogenesis act as primary markers to distinguish the maternal and paternal alleles. However, precisely when primary imprinting is established during gametogenesis is unknown. Recently, we have shown that a parthenogenetic embryo (ng/fg PE) containing one genome from a neonate-derived non-growing oocyte and the other from a fully grown oocyte developed to 13.5 days post coitum (dpc), 3 days longer than previously reported in mice (Kono et al., 1996). This suggests that
maternal primary imprinting occurs, at least in part, during oocyte growth, leading to the hypothesis that disruption of this process causes the modified expression of imprinted genes, which results in the parthenogenetic phenotype. To understand the molecular mechanisms underlying the extended development, we investigated the expression of the paternally expressed genes, Peg1/Mest (Sado et al., 1993; Kaneko-Ishino et al., 1995), Igf2 (DeChiara et al., 1991), Peg3 (Kuroiwa et al., 1996) and Snrpn (Cattanach et al., 1991), and the maternally expressed genes, H19 (Bartolomei et al., 1991; Ferguson-Smith et al., 1991), Igf2r (Hatada and Mukai, 1995) in 9.5 and 12.5 dpc ng/fg PE using RT-PCR and in situ hybridization procedures. The results clearly showed that Peg1/Mest, Peg3, Snrpn and H19 are expressed, while Igf2, Igf2r and Peg3 are repressed in the ng alleles of the parthenotes. The present study proposes that, during oocyte growth, imprints are repressed in the ng alleles of the parthenotes. The present study proposes that, during oocyte growth, imprints are repressed in the ng alleles of the parthenotes.

MATERIALS AND METHODS

Production of reconstituted embryos

B6CFlf1 (C57BL/6J × CBA) mice were used as oocyte donors. Fully grown germinal vesicle (GV) stage oocytes were collected from ovarian follicles 44-48 hours after injection of PMSG. Non-growing primary oocytes were obtained from ovaries of 1-day-old mice. Parthenogenetic embryos (PE) containing genomes from non-growing (ng) and fully grown oocytes (fg) were produced by serial nuclear transfer as described previously (Kono et al., 1996). Enucleated fully grown GV oocytes that received non-growing oocytes were cultured in Waymouth 752/1 medium (Gibco-BRL) supplemented with 0.23 ng/ml E2 and 250 μl CaCl2, and 250 μM dNTPs. For analysis of placental polymorphism) and LP (length polymorphism) analyses using PCR products. After RT-PCR, the Peg3 products were digested with TaqI at 65°C for 4 hours and separated on a 3% agarose gel in 0.5 TBE, which was run at 3500 V for 4 hours at 10°C. Length polymorphisms in each products of Igf2 (unpublished data) and Peg3 (Hatada and Mukai, 1995) genes were detected by a 3% agarose gel electrophoresis in 0.5x TBE.

Expression analysis by in situ hybridization

Embryos were dissected from the uterus of recipient mice at 9.5 and 12.5 dpc. Then, embryos were fixed with 4% paraformaldehyde overnight at 4°C and processed for wax embedding. Each riboprobe was prepared from each cDNA cloned into pBluescript SK or pDIRECT, T7 and T3 RNA polymerase (Promega). For analysis of placental growth, spgnotiophoblast-specific RNA probe, 4311 (Lescisn et al., 1988), was synthesized using SP6 RNA polymerase from EcoRI linearized plasmid. In situ hybridization was carried out as described (Walsh et al., 1994).

Analysis of embryonic and placental weight

ng/fg PE and control biparental embryos were dissected at 12.5 dpc in PBS and examined for a heartbeat and yolk sac circulation. After overnight fixation with 4% paraformaldehyde, embryonic and placental weights were measured as previously described (Baker et al., 1993). Statistical comparisons between the weights of ng/fg PE and control biparental embryos were analyzed by Student’s t-test.

RESULTS

Peg1/Mest, Peg3 and Snrpn are expressed by the non-growing oocyte alleles in ng/fg PE

The ng/fg PE were produced by standard micromanipulation (Kono et al., 1996: Fig. 1A). To investigate the expression of the paternally expressed genes, Peg1/Mest, Peg3 and Snrpn, mRNA transcripts from the ng/fg PE were amplified using RT-PCR. The results showed that these three imprinted genes were expressed in ng/fg PE both at 9.5 (n=5) and 12.5 dpc (n=3),

Igf2, 5'-CTACTTCAGCAGCCCTCAG-3' and 5'-GATGGTG-CTGTA CATCCTCC-3';

Peg3, 5'-TGGTGTCACAGATGACC-3' and 5'-TTGCTCTT-CCTCCCTCAGG-3';

Snrpn, 5'-ATACTCGCATGGCTCTGAGT-3' and 5'-TGGAGGAGGCATTGCTCT-3';

H19, 5'-TGTAAACCTTTTGGGCAATGCTGC-3' and 5'-TATGATGACCAGACCTGTTG-3';

Igf2r, 5'-TTGGACTCAATAAGAGCTCTT-3' and 5'-GGTACTCTTGTTTGGGTA-3';

Peg3, 5'-GCCGGTGTGACCGTGGGAA-3' and 5'-AGAGGAGGCTGTCTCTCAGG-3';

β-actin, 5'-GCTGTGCTATGTGCTTAGAATCTC-3' and 5'-CTCATGATTACAGTCCCGCAG-3'.
Disruption of primary imprinting

but not in the control parthenogenetic embryos (fg/fg PE) at 9.5 dpc (n=3; Fig. 1B). The level of expression of these genes was estimated to be similar to the control biparental embryos at the corresponding stages (Fig. 1B). The expression of Peg1/Mest (n=9) and Peg3 (n=9), which are mainly expressed in the mesodermal tissue (Sado et al., 1993; Kaneko-Ishino et al., 1995; Kuroiwa et al., 1996), was observed by in situ hybridization analysis (Fig. 2I,III). Strong signals were detected in the mesodermal tissue of the ng/fg PE and control embryos (Fig. 2I,III), but not in the parthenogenetic embryos (fg/fg PE) in which the genomes were derived solely from fully grown oocytes (data not shown). The transcripts of the Peg3 gene were shown to be derived from the ng allele in the ng/fg PE by the use of the DNA polymorphisms present in the alleles (Fig. 1C; n=3). These results indicate that primary imprinting during oocyte growth acts normally to repress the expression of Peg1/Mest, Peg3 and Snrpn from the maternal alleles after implantation (Table 1).

Expression of the Igf2 and H19 genes are reciprocal in the ng/fg PE

All of the paternally expressed genes are not activated in the ng allele since Igf2 (DeChiara et al., 1991) was either not detected or only detected at a low level in the ng/fg PE (Fig. 1B; n=12). In situ hybridization experiments failed to detect Igf2 transcripts in the ng/fg PE (Fig. 2II; n=15), except in the choroid plexus and leptomeninges of the brain (data not shown), where Igf2 is biallelically expressed (DeChiara et al., 1991). Although maternal repression during oocyte growth is

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<tr>
<td>Peg1/Mest</td>
<td>Chr. 6 Prox</td>
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<td>maternal repression</td>
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<tr>
<td>Peg3</td>
<td>Chr. 7 Prox</td>
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<td>Snrpn</td>
<td>Chr. 7 Prox</td>
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<td>Igf2</td>
<td>Chr. 7 Dist</td>
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<td>maternal activation*</td>
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<td>H19</td>
<td>Chr. 7 Dist</td>
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<td>p57KIP2</td>
<td>Chr. 7 Dist</td>
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<td>Ig2r</td>
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<td>maternal activation</td>
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* through H19 repression by epigenetic modification during spermatogenesis.
one mechanism of ensuring monoallelic expression patterns, \textit{Igf2} appears to be regulated differently. \textit{Igf2} and \textit{H19} genes are located in tandem on the distal region of chromosome 7 and the enhancer sequence, which is present downstream of the \textit{H19} gene, is functional for both genes, though preferentially for \textit{H19} (Leighton et al., 1995). The repression of \textit{Igf2} seen in the ng/fg PE (Figs 1B, 2II) may be explained in terms of the locus of the enhancer that is predominantly used for the expression of \textit{H19} gene on the same chromosome. To reveal this, we examined the allele-specific expression of the \textit{H19} gene using SSCP analysis with the DNA polymorphism present in the allele. The results showed that the \textit{H19} gene is expressed equivalently by both the ng and fg alleles in the ng/fg PE (Fig. 3A; \(n=4\)), suggesting that the \textit{H19} gene expression results in the transcriptional silencing of the \textit{Igf2} in the ng allele (Table 1). Alternatively, the \textit{Igf2} gene may be maternally repressed during an earlier stage of oogenesis or after the ng alleles were transferred, either during oocyte maturation or the subsequent embryonic development, although this was unlikely.

\textbf{Igf2r and p57\textsuperscript{KIP2} are repressed in the ng allele of ng/fg PE}

To understand further the regulatory expression by maternal primary imprinting, we analyzed two maternally expressed genes, \textit{Igf2r} and p57\textsuperscript{KIP2}, in addition to \textit{H19}. The allele-specific analysis of \textit{Igf2r} and p57\textsuperscript{KIP2} expression was examined by length polymorphism to show whether these genes are expressed from the ng alleles in the ng/fg PE. Maternal \textit{Igf2r} expression is regulated by a gene silencer that is proposed to be inactivated by maternal imprinting (Stöger et al., 1993). The \textit{Igf2r} gene was expressed from the fg allele (240 bp fragment) but not the ng allele in the ng/fg PE (Fig. 3B; \(n=4\)), which suggests that the \textit{Igf2r} expression is caused by primary imprinting during oocyte growth. Furthermore, p57\textsuperscript{KIP2} was also expressed solely from fg allele in the ng/fg PE (Fig. 3C; \(n=4\)). This indicates that, like \textit{Igf2r}, expression of the p57\textsuperscript{KIP2} gene from the fg allele is a result of maternal epigenetic modifications during oocyte growth (Table 1).

\textbf{Fig. 2.} In situ hybridization analysis of the \textit{Peg1/Mest} (I), \textit{Igf2} (II) and \textit{Peg3} (III) expression. Control biparental embryos (A,B,E,F) and ng/fg PE (C,D,G,H) were sectioned at 9.5 (A-D) and 12.5 (E-H) dpc. Strong signals were seen in tongue, heart and hypothalamic region for \textit{Peg1/Mest}, and in hypothalamus, pituitary gland, tongue and gut for \textit{Peg 3}. 

\(\text{ng/fg PE}\)
ng/fg PE are smaller than biparental control embryos in weights

The weight of ng/fg PE at 12.5 dpc was significantly reduced to about 70% of that reached by biparental controls (P<0.001). The developmental stage of the ng/fg PE were estimated to be at stage 20-21 in 12.5 dpc controls (Theiler, 1989), which are characterized by the digits of the hand plate, pigmented eyes and sinus sigmoideus. The placental weight was also reduced by 78% (P<0.01; Fig. 4); however, development of the spongiotrophoblast tissue, which is essential for functional placenta, was similar to control (Fig. 5).

**DISCUSSION**

Parthenogenetic and gynogenetic diploid mouse embryos die at or before 10 dpc (Surani et al., 1984; Barton et al., 1984). However, parthenogenetic mouse embryos (ng/fg PE) which contain one genome from a non-growing oocyte (ng) and the other from a fully grown oocyte (fg) develop up to 13.5 dpc (Kono et al., 1996). We have proposed that this extended parthenogenetic development may be induced by modified expression of imprinted genes, due to a lack of primary imprinting during oocyte growth. To understand the role of maternal imprinting in the regulation of gene expression, we investigated the expression of known imprinted genes in the ng/fg PE. Table 1 summarizes the consequences of the modified gene expression in the imprinted alleles as revealed by the gene expression patterns in the ng/fg PE. Gene...
expression of Peg1/Mest, Peg3, Snrpn, Igf2r and p57Kip2 from maternal alleles was shown to be altered as a result of the disruption of the primary imprinting during oocyte growth, but not the Igf2 and H19 genes, which are regulated by paternal epigenetic modifications during spermatogenesis.

We have shown that the paternally expressed genes, Peg1/Mest (Sado et al., 1993; Kaneko-Ishino et al., 1995), Peg3 (Kuriwa et al., 1996) and Snrpn (Cattanach et al., 1992) are expressed in the ng/fg PE both at 9.5 and 12.5 dpc. The level of expression of these genes was similar to the control biparental embryos at the corresponding stages. These genes are thought to be expressed from ng alleles since an analysis of the allele-specific expression showed that the Peg3 gene was expressed by the ng allele. This is the first case that shows that paternally expressed genes can be expressed from the maternal alleles and suggests that the expression of Peg1/Mest, Peg3 and Snrpn is normally regulated by a mechanism of maternal repression that is established during the period of oocyte growth.

However, this is not the case for all of the paternally expressed genes; the Igf2 gene (DeChiara et al., 1991) was not expressed in the ng/fg PE at either 9.5 and 12.5 dpc. The reason for silence of Igf2 in the ng allele may be explained by the enhancer competition model (Bartolomei et al., 1993). Igf2 and H19 genes, which lie 90 kb apart on the distal end of chromosome 7, share enhancers, which are at +9 and +11 kb relative to the start of transcription of the H19 gene but are preferential for H19 (Yoo-Warren et al., 1988; Leighton et al., 1995). Our finding that H19 was expressed from both alleles supports the view that paternal repression with methylation of the upstream region of the promoter is the mechanism governing H19 imprinting (Elson and Bartolomei, 1997). This hypermethylation of the H19 promoter is thought to prevent it binding to the enhancers situated in the 3′ region of the gene. The enhancers are thus able to engage the expression of Igf2 from the paternal allele. Therefore, according to the enhancer competition model, biallelic expression H19 should lead to a lack of Igf2 expression. Thus, our results lend support to the enhancer competition model and further experiments using H19 mutants should reveal more about the relationship between the expression of H19 and Igf2.

Stöger et al. (1993) have suggested that the maternal expression of Igf2r is dependent on the maternal allele-specific methylation of an intronic site of region 2 that may be active as a gene silencer when it is not methylated. In blastocysts derived from ng/fg oocytes, the intronic site 3 of region 2 in the ng allele remained unmethylated (Kono et al., 1996). Igf2r gene was not expressed by the ng allele in the ng/fg PE, suggesting that the regulatory region of Igf2r is unmethylated in the ng allele. This was apparently due to the bypassing of the period during oogenesis when the imprint is established. Unexpectedly, p57Kip2 gene is also repressed in the ng allele in ng/fg PE, suggesting that repression of the p57Kip2 gene is due to lack of maternal imprinting signal during oocyte growth. The similar mechanisms that regulate expression of the Igf2r gene from the maternal allele may govern the p57Kip2 expression (Wutz et al., 1997). Here, we showed that maternal epigenetic modifications during oocyte growth regulate Igf2r and p57Kip2 expression in a positive fashion, but the regulatory elements of the p57Kip2 gene are unclear.

Studies of gene expression in the ng/fg PE show that epigenetic changes during oocyte growth have dramatic effects on the expression of maternal and paternal genes. The ng/fg PE provides a closer balance to the normal pattern of expression of imprinted genes in the biparental embryo. Can this altered pattern of gene expression explain the extended development of ng/fg PE? This possibility is supported by the observation that mouse embryos with maternal duplication of the region containing Peg1/Mest, Peg3 or Snrpn genes die in mid-gestation (Cattanach and Beechey, 1990; McLaughlin et al., 1996). It is possible to consider that the other paternally expressed genes including unidentified one are also expressed by the ng alleles in the ng/fg PE. It is likely that lack of maternal repression of genes that would normally be paternally expressed enabled the embryos to develop beyond that seen in control parthenotes (fg/fg PE). Another question is whether the extended development of ng/fg PE is achieved by the successful placentation with functional spongiotrophoblast. In chimeras that were constituted with primitive endoderm and trophectoderm derived from fertilized blastocysts and primitive ectoderm derived from parthenogenetic blastocysts, the development was slightly extended but arrested at 11.5 dpc (Gardner et al., 1990). This shows that extraembryonic tissues that derived from biparental embryos are unable to rescue parthenogenetic development beyond 11.5 dpc. Therefore, the extended development of ng/fg PE up to 13.5 dpc could be accomplished not only by placentation but also by enhanced viability of foetus itself that was induced by the default maternal imprinting during oocyte growth.

The development of ng/fg PE was vastly improved compared to standard parthenotes but the embryonic and placental weight remained about 70% less than controls. There are a number of possible explanations for this reduction. First, we show that ng/fg PE do not express Igf2, which is known to be an important regulator of fetal growth. Disruption of the paternal Igf2 gene is not lethal to the heterozygous mouse but affected the fetal body mass, which is about 75% of wild type at 11.0 dpc (DeChiara et al., 1990; Baker et al., 1993). Second, the reduced placental development may not support normal rate of fetal growth. It is known that hypotrophy of the placenta frequently leads to growth impairment (Zechner et al., 1996). Third, growth retardation may result from the requirement of other unknown genes or abnormal levels of expression of maternal genes.

The ng/fg PE die at a specific time during development and it is not clear why death occurs at 13.5 dpc. As discussed above, the placenta of the ng/fg PE were small in size but they have the proliferated spongiotrophoblastic tissue. Although we cannot be certain, this would suggest that the placenta is functioning and supports the possibility that the limiting factor is embryonic survival. Further work on genes important for placentation, Mash2 (Guillemot et al., 1995) and Xist (Marahrens et al., 1997) etc., may help to clarify any role for the placenta in embryonic death. The demise of ng/fg PE around 13.5 dpc may be attributed to disrupted expression of some other imprinted genes. For example, genes such as H19 have been shown to double their transcripts due to expression by both alleles, which may have a detrimental effect on development (Brunkow and Tilghman, 1991). The Igf2r and p57Kip2 transcripts, which are essential genes in embryogenesis negatively affecting the cell cycle and cell proliferation (Yan et al., 1997; Zhang et al., 1996).
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