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

Yayoi Obata[1,6], Tomoko Kaneko-Ishino[2], Tsuyoshi Koide[3], Yasushi Takai[4,*], Takayuki Ueda[5], Ikuo Domeki[6], Toshihiko Shiroishi[3], Fumitoshi Ishino[4,7] and Tomohiro Kono[1,†]

[1]NODAI Research Institute, Tokyo University of Agriculture, Sakuragaoka, Setagaya-ku, Tokyo 156, Japan
[2]Tokai University, School of Health Science, Bohseidai, Isehara-shi, Kanagawa 259-11, Japan
[3]Mammalian genetics laboratory, National Institute of Genetics, Yata, Mishima-shi, Shizuoka 411, Japan
[4]Gene Research Center, Tokyo Institute of Technology, Nagatsuta-cho, Midori-ku, Yokohama, Kanagawa 226, Japan
[5]Research Laboratory for Molecular Genetics, Niigata University, Ichiban-cho, Asahimachidoori, Niigata 951, Japan
[6]Department of Zootechnical Science, Tokyo University of Agriculture, Sakuragaoka, Setagaya-ku, Tokyo 156, Japan
[7]PRESTO, Japan Science and Technology Corporation (JST), Hon-machi, Kawaguchi-shi, Saitama 332, Japan
*Present address: Department of obstetrics and Gynecology, the University of Tokyo, Hongo, Bunkyou-ku Tokyo 113, Japan
†Corresponding author (e-mail: tomohiro@nodai.ac.jp)

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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 p57^{KIP2}. 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 p57^{KIP2} 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*, p57^{KIP2}, *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., 1992), and the maternally expressed genes, *H19* (Bartolomei et al., 1991; Ferguson-Smith et al., 1991), *Igf2r* (Barlow et al., 1991) and p57^{KIP2} (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 p57^{KIP2} are repressed in the ng alleles of the parthenotes. The present study proposes that, during oocyte growth, imprints are established that lead to maternal-specific gene expression and repression.

MATERIALS AND METHODS

Production of reconstituted embryos

B6CBF1 (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 mM pyruvic acid, 26.7 mM $NaHCO_3$ and 5% fetal calf serum for 14 hours to progress into metaphase of the second meiosis (MII). The resultant MII chromosomes were transferred into ovulated fresh MII oocytes (Kwon and Kono, 1996). Cell fusion was induced with inactivated Senadai virus (HVJ, 2700 hemagglutinating activity U/ml). After artificial activation with 10 mM $SrCl_2$ in Ca^{2+}-free M16 medium for 1 hour (O'Neill et al., 1992), oocytes formed two second polar bodies and two female pronuclei were cultured for 3.5 days. Blastocysts derived from constituted oocytes were transferred into pseudopregnant females. The ng/fg PE at 9.5 and 12.5 dpc (day of plug is 0.5 dpc) were used for the molecular analysis. When analyses for allele-specific expression were required, JF1 (*Mus musculus molossinus*) mice and PWK (*Mus musculus musculus*) mice were used as non-growing oocyte donors.

Expression analysis by RT-PCR

Total RNA was isolated using the ISOGEN (Nippon Gene) from control and ng/fg PE at 9.5 and 12.5 dpc, which was based on the acid guanidine thiocyanate-phenol-chloroform extraction method. First-strand cDNA was synthesized from 1 μg of total RNA from each embryo by Superscript reverse transcriptase II (Gibco-BRL) according to manufacture's instructions. Genomic DNA and the cDNA were subjected to PCR, which was carried out using 1.25 U of Taq DNA polymerase (Takara), 1 pmol of each primer, 1.5 mM $MgCl_2$, and 250 μM dNTPs. For analysis of *H19* expression, cDNA was radiolabelled with [α-^{32}P]dCTP (0.3 μl/tube; 3000 Ci/mmol, NEN). The amplification consisted of a total of 30 cycles at 95°C for 30 seconds, 65°C for 30 seconds and 72°C for 60 seconds in a Perkin Elmer GeneAmp PCR system 2400. Primers used were:

Peg1/Mest, 5′-ATTCGCAACAATGACGGC-3′ and 5′-TGAGGTGGACTATTGTGTCACC-3′;

Igf2, 5′-CTACTTCAGCAGGCCTTCAAG-3′ and 5′-GATGGTTGCTGTACATCTCC-3′;

Peg3, 5′-TGGTGCAGACATTGAAGACC-3′ and 5′-TTGCTCTCTTCCTCCTCAGG-3′;

Snrpn, 5′-ATACTGGCATTGCTCGTGTG-3′ and 5′-TGGAGGAGGCATGCCTATAG-3′;

H19, 5′-TGTAAACCTCTTTGGCAATGCTGCC-3′ and 5′-TATTGATGGACCCAGGACCTCTGGT-3′;

Igf2r, 5′-TTCGACCTATAAGAAGCCTT-3′ and 5′-GGGTACTTTGCTTTTGGGTA-3′;

p57^{KIP2}, 5′-GCCGGGTGATGAGCTGGGAA-3′ and 5′-AGAGAGGCTGGTCCTTCAGC-3′;

β-actin, 5′-GCTGTGCTATGTTGCTCTAGACTTC-3′ and 5′-CTCAGTAACAGTCCGCCTAGAAGC-3′.

Polymorphic analysis

Polymorphisms of *Peg3*, *H19*, *Igf2r* and p57^{KIP2} genes between JF1, PWK and B6CBF1 were detected by RFLP (restriction enzyme fragment polymorphism), SSCP (single-strand conformation polymorphism) and LP (length polymorphism) analyses using PCR products. After RT-PCR, the *Peg3* products were digested with *Taq* I at 65°C for 4 hours and separated on a 3% agarose gel in 0.5× TBE. Prior to SSCP analysis of *H19* gene, the sample was heated at 80°C for 5 minutes, transferred immediately to 4°C for denaturing and loaded onto the 10% polyacrylamide gel with 10% glycerol in 0.5× TBE, which was run at 3500 V for 4 hours at 10°C. Length polymorphisms in each products of *Igf2r* (unpublished data) and p57^{KIP2} (Hatada and Mukai, 1995) genes were detected by a 3% agarose gel electrophoresis in 0.5× 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 labeled with ^{35}S-UTP (1000-1500 Ci/mmol, NEN). *Igf2* antisense and sense probes were synthesized from a human cDNA *IGF2* cloned into pGem-3 (Ohlsson et al., 1989) using SP6 (Takara) and T7 RNA polymerase (Promega), respectively. The *Peg1* and *Peg3* probes were prepared from each cDNA cloned into pBluescript SK or pDIRECT, following linearization with *Bam*HI (antisense) or *Xho*I (sense), using T7 and T3 RNA polymerase (Promega). For analysis of placental growth, spongiotrophoblast-specific RNA probe, 4311 (Lescisin et al., 1988), was synthesized using SP6 RNA polymerase from *Eco*RI 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 was 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),

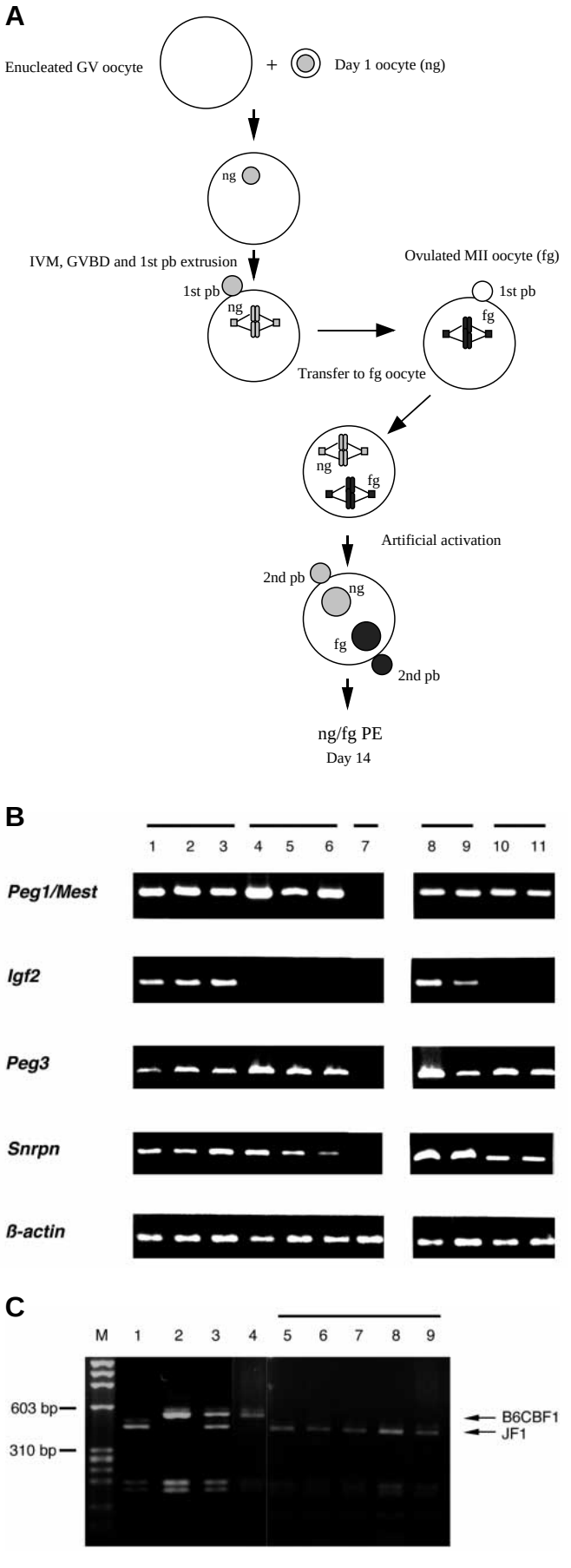

Fig. 1. Analysis of paternally expressed genes in ng/fg PE. (A) Schematic diagram showing the production of oocytes containing genomes from non-growing and fully grown oocytes (see Materials and methods). (B) RT-PCR analysis of *Peg1/Mest*, *Igf2*, *Peg3* and *Snrpn* expression. Expression was examined in control biparental embryos (lane 1-3, 8, 9), ng/fg PE (lane 4-6, 10, 11) and control parthenogenetic embryos (fg/fg PE; lane 7) at 9.5 (lane 1-7) and 12.5 (lane 8-11) dpc. The expression of genes in ng/fg PE was similar those in controls without *Igf2* gene, approximately one-tenth to one-hundredth versus control (data not shown). (C) Allele-specific analysis of *Peg3* expression. Genomic PCR products digested with *Taq* I were shown for JF1 (lane 1; 410 bp and 83 bp fragments), B6CBF1 (lane 2; 493 bp fragment) and the interspecific hybrid (JF1 × B6CBF1; lane 3). *Taq* I polymorphism of RT-PCR products in control biparental embryo (JF1 female × B6CBF1 male) showed that Peg3 was expressed only from paternal allele (lane 4). In ng/fg PE (ng, JF1; fg, B6CBF1) at 9.5 dpc *Peg3* was expressed only from ng allele (lane 5-9). ϕ×174/*Hae*III digests were used as molecular mass markers.

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 at 9.5 dpc (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

Table 1. Regulatory expression in the imprinted genes by primary imprinting

Imprinted genes	Mapping	Expressed allele	Mode of regulation
Peg1/Mest	Chr. 6 Prox	paternal	maternal repression
Peg3	Chr. 7 Prox	paternal	maternal repression
Snrpn	Chr. 7 Prox	paternal	maternal repression
Igf2	Chr. 7 Dist	paternal	paternal activation*
H19	Chr. 7 Dist	maternal	paternal repression
$p57^{KIP2}$	Chr. 7 Dist	maternal	maternal activation
Igf2r	Chr. 17 Prox	maternal	maternal activation

* through *H19* repression by epigenetic modification during spermatogenesis.

one mechanism of ensuring monoallelic expression patterns, *Igf2* appears to be regulated differently. *Igf2* and *H19* genes are located in tandem on the distal region of chromosome 7 and the enhancer sequence, which is present downstream of the *H19* gene, is functional for both genes, though preferentially for *H19* (Leighton et al., 1995). The repression of *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 *H19* gene on the same chromosome. To reveal this, we examined the allele-specific expression of the *H19* gene using SSCP analysis with the DNA polymorphism present in the allele. The results showed that the *H19* gene is expressed equivalently by both the ng and fg alleles in the ng/fg PE (Fig. 3A; *n*=4), suggesting that the *H19* gene expression results in the transcriptional silencing of the *Igf2* in the ng allele (Table 1). Alternatively, the *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.

Igf2r and p57^{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, *Igf2r* and p57^{KIP2}, in addition to *H19*. The allele-specific analysis of *Igf2r* and p57^{KIP2} expression was examined by length polymorphism to show whether these genes are expressed from the ng alleles in the ng/fg PE. Maternal *Igf2r* expression is regulated by a gene silencer that is proposed to be inactivated by maternal imprinting (Stöger et al., 1993). The *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 *Igf2r* expression is caused by primary imprinting during oocyte growth. Furthermore, p57^{KIP2} was also expressed solely from fg allele in the ng/fg PE (Fig. 3C; *n*=4). This indicates that, like *Igf2r*, expression of the p57^{KIP2} gene from the fg allele is a result of maternal epigenetic modifications during oocyte growth (Table 1).

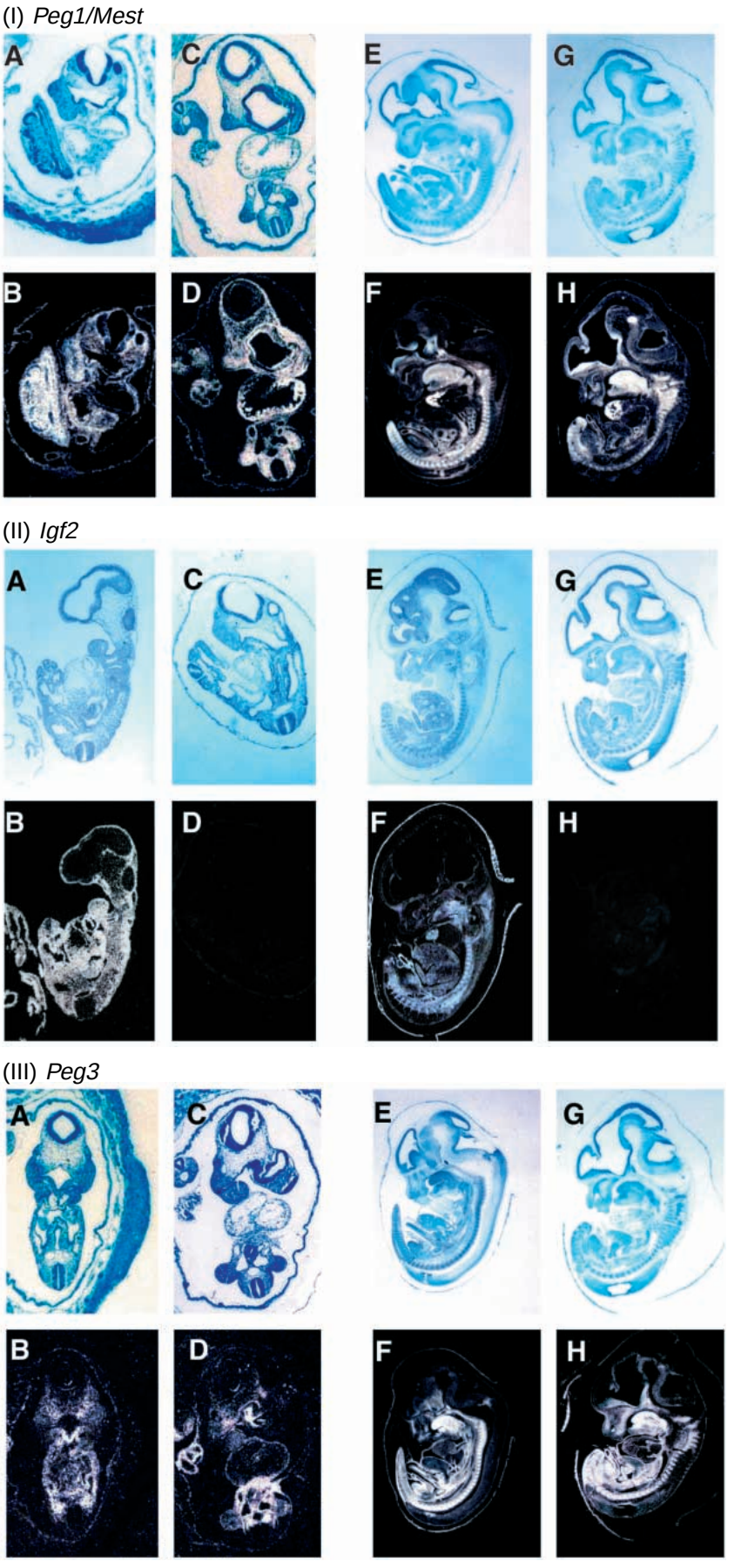

Fig. 2. In situ hybridization analysis of the *Peg1/Mest* (I), *Igf2* (II) and *Peg3* (III) expression. Control biparental embryos (A,B,E,F) and ng/fg PE (C,D,G,H) were sectioned at 9.5 (A-D) and 12.5 (E-H) dpc. Strong signals were seen in tongue, heart and hypothalamic region for *Peg1/Mest*, and in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

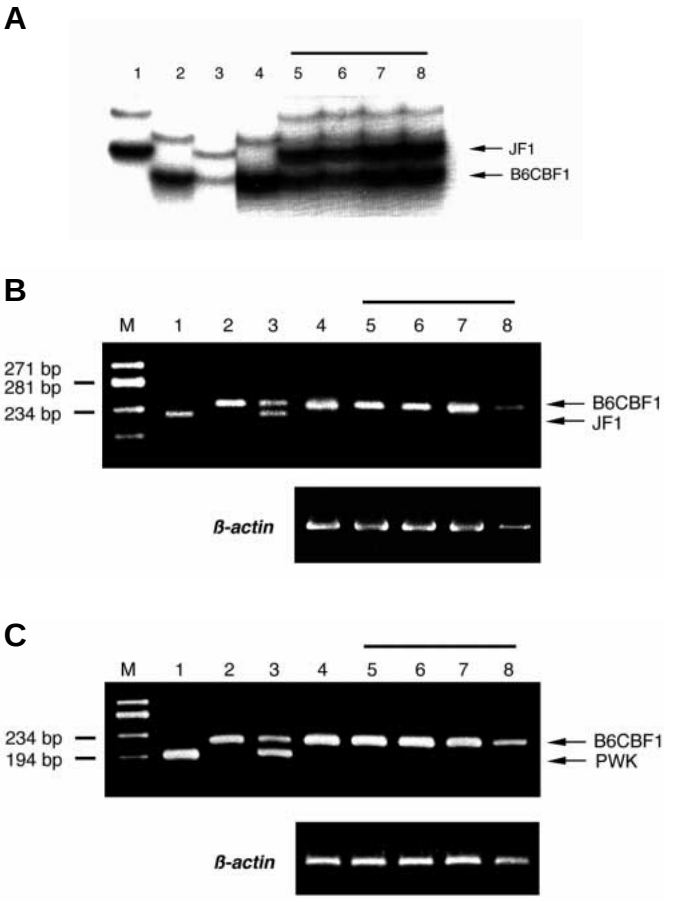

Fig. 3. Analysis of maternally expressed genes in ng/fg PE. (A) SSCP analysis of *H19* expression. Genomic PCR products were shown for JF1 (lane 1), B6CBF1 (lane 2) and the interspecific hybrid (JF1×B6CBF1; lane 3). RT-PCR products of control biparental embryos (B6CBF1 female × JF1 male; lane 4) and ng/fg PE (lane 5-8). Biallelic expression was seen in ng/fg PE and, in control embryos, the expression was exclusively from maternal allele. (B) LP analysis of *Igf2r* expression. Genomic PCR products were shown for JF1 (lane 1), B6CBF1 (lane 2; amplified a 240 bp fragment) and the interspecific hybrid (JF1 × B6CBF1; lane 3). RT-PCR products of control biparental embryos (B6CBF1 female × JF1 male) and ng/fg PE were shown in lane 4 and lane 5-8, respectively. ϕ×174/*Hae* III digests were used as molecular mass markers. In both of control and ng/fg PE, the *Igf2r* expression was observed only from B6CBF1 (fg) derived allele. (C) LP analysis of $p57^{KIP2}$ expression. Genomic PCR products were shown for PWK (lane 1; 198 bp fragment), B6CBF1 (lane 2; 222 bp fragment) and the interspecific hybrid (PWK × B6CBF1; lane 3). RT-PCR products of biparental control embryos (B6CBF1 female × PWK male; lane 4), and ng/fg PE (lane 5-8).

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, pigmentated eyes and sinus sigmoideus. The placental weight was also reduced by 78% (P<0.01; Fig. 4); however, development of the

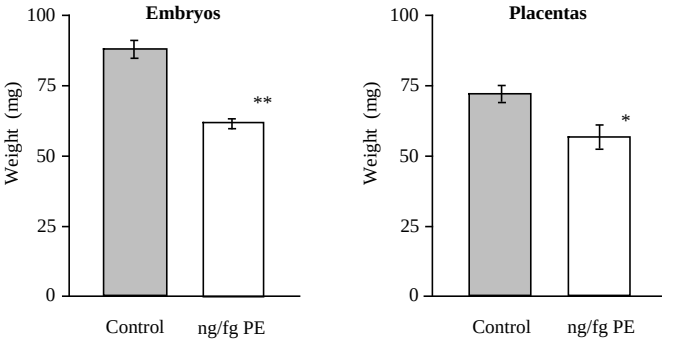

Fig. 4. Embryonic and placental weight at 12.5 dpc. Experimental data are expressed as the mean ± s.e. All embryos were living at recovery. Embryonic weights: biparental control embryo, 88.2±2.54 mg (n=11); ng/fg PE, 61.7±1.36 mg (n=7; **P<0.001). Placental weights: biparental control embryo, 72.2±2.93 mg (n=11); ng/fg PE, 56.5±4.06 mg (n=7; *P<0.01).

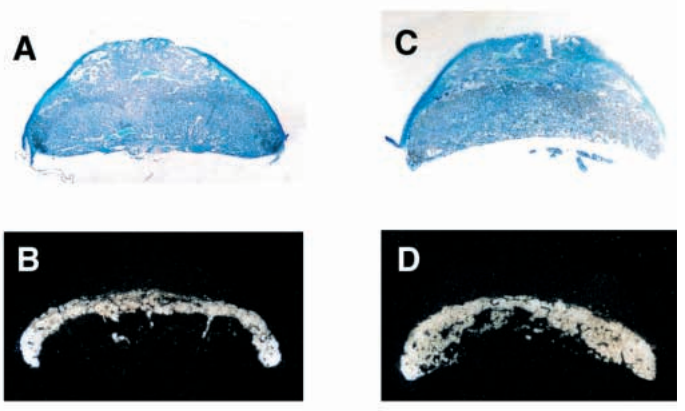

Fig. 5. Differentiation of the spongiotrophoblast in biparental control embryo (A,B) and ng/fg PE (C,D) and at 12.5 dpc. Only the spongiotrophoblastic tissue was labeled. (A,C) Bright-field image of the section; (B,D) dark-field image of the section.

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 p57^{KIP2} 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* (Kuroiwa 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, p57^{KIP2} gene is also repressed in the ng allele in ng/fg PE, suggesting that repression of the p57^{KIP2} 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 p57^{KIP2} expression (Wutz et al., 1997). Here, we showed that maternal epigenetic modifications during oocyte growth regulate *Igf2r* and p57^{KIP2} expression in a positive fashion, but the regulatory elements of the p57^{KIP2} 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 73% 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 p57^{KIP2} transcripts, which are essential genes in embryogenesis negatively affecting the cell cycle and cell proliferation (Lau et al., 1994; Yan et al., 1997; Zhang et al.,

1997), are predicted to be present at the same level as in the control biparental embryos. These results indicate that not only disruption of maternal imprinting but also some other regulatory mechanism for gene expression, which is induced by epigenetic modification during spermatogenesis, is required for the development of the ng/fg PE beyond 13.5 dpc.

Our findings show that maternal imprinting occurs during oocyte growth and that disrupting this process leads to altered expression patterns of imprinted genes during embryogenesis. Furthermore, primary imprinting during oocyte growth occurs by mechanisms that can either activate or repress subsequent embryonic gene expression. Insight into the molecular mechanisms responsible for the extended development of ng/fg PE would provide an approach to understand the mechanisms of primary imprinting during gametogenesis and its role on embryonic development.

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