Maternal Primary Imprinting Is Established at a Specific Time for Each Gene throughout Oocyte Growth*

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Received for publication, September 6, 2001, and in revised form, November 14, 2001
Published, JBC Papers in Press, November 16, 2001, DOI 10.1074/jbc.M108586200

Primary imprinting during gametogenesis governs the monoallelic expression/repression of imprinted genes in embryogenesis. Previously, we showed that maternal primary imprinting is disrupted in neonate-derived non-growing oocytes. Here, to investigate precisely when and in what order maternal primary imprinting progresses, we produced parthenogenetic embryos containing one genome from a non-growing or growth-stage oocyte from 1- to 20-day-old mice and one from a fully grown oocyte of adult mice. We used these embryos to analyze the expression of eight imprinted genes: Peg1/Mest, Peg3, Snrpn, Znf127, Ndn, Impact, Igf2r, and p57KIP2. The results showed that the imprinting signals for each gene were not all imposed together at a specific time during oocyte growth but rather occurred throughout the period from primary to antral follicle stage oocytes. The developmental ability of the constructed parthenogenetic embryos was gradually reduced as the nuclear donor oocytes grew. These studies provide the first insight into the process of primary imprinting during oocyte growth.

Genomic imprinting, a process that leads to the monoallelic expression of some specific genes in the diploid cells, is dependent on whether the allele is transmitted from the sperm or from the oocyte (1–3). This epigenetic mechanism renders the parental genomes functionally unequal. Consequently, uniparental embryos, containing only the paternal or maternal genome, die shortly after implantation (4–7). To date, about 40 imprinted genes have been identified, some of which have been shown to be involved in embryonic development and growth, placental differentiation, suppression of tumorigenesis, behavior, and genetic disorders, respectively (8).

Although little is known of the imprinting mechanisms, in the current model a multistep process is suggested (8). First, an imprinting signal for distinguishing the parental origin of the alleles in the embryo is constructed during gametogenesis of the parents. This process refers to a primary or gametic imprinting. Second, the imprinting signal on each gene is maintained after fertilization and leads to allele-specific gene expression until around the time of gastrulation. Third, this regulatory expression is sustained in the somatic cells throughout embryonic development, whereas the signals are erased in the germ cells. Finally, the imprinting signals of each gene are re-established in either the male or female germ line for the next generation.

Several studies have supported the idea that the imprinting signals are erased in the germ cells (9, 10). In those studies, allele-specific expression and DNA methylation of imprinted genes were shown to be disrupted in the early germ line. DNA methylation, a heritable epigenetic modification, acts in concert with other regulatory mechanisms to negatively control gene expression (11). In some of the imprinted genes, a gamete-derived methylation pattern from one parent precedes the allelic methylation differences observed in the somatic cells; for that reason, DNA methylation is proposed to be the imprinting signal (12–15).

On the other hand, we have also approached the erasure of the primary imprinting by analyzing mouse parthenogenetic embryos (PE)1 (non-growing/fully grown (ng/fg) PE) containing one haploid set of genomes from an ng oocyte from newborn mice and one from an fg oocyte from adult mice. The ng/fg PE developed to 13.5 days of gestation, 3 days longer than had been recorded previously for parthenogenetic development (16). Gene expression analysis in the ng/fg PE revealed that paternally expressed Peg1/Mest, Peg3, and Snrpn genes were expressed, whereas maternally expressed Igf2r and p57KIP2 genes were repressed in the alleles of the ng oocyte (17). These results indicated that maternal primary imprinting is disrupted in the ng oocyte genome and that acquisition of the primary imprinting during oocyte growth is required for the repression of Peg1/Mest, Peg3, and Snrpn genes and for the expression of Igf2r and p57KIP2 genes. These previous findings raised the interesting question of when precisely maternal primary imprinting is established during oocyte growth.

To address this question, we utilized the parthenogenetic embryos containing one haploid set of genomes from the growth-stage oocyte from juvenile mice and one from an fg oocyte from adult mice. The oocytes were classified into non-growing (1d(day)-ng and 5d-ng), early growing (eg), middle growing (mg), and late growing (lg) stages. They were obtained from 1-, 5-, 10-, 15-, and 20-day-old mice, respectively. Using these oocytes, six types of parthenogenetic embryos, 1d-ng/fg PE, 5d-ng/fg PE, eg/fg PE, mg/fg PE, lg/fg PE, and fg/fg PE, were produced and subjected to gene expression and developmental analyses. The expression analysis of eight imprinted genes included paternally expressed Peg1/Mest (18), Peg3 (19),

* This work was supported by grants from the Ministry of Education, Science, Culture and Sports of Japan, the Ministry of Agriculture of Japan, and the Japanese Society for the Promotion of Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PE, parthenogenetic embryos; ng, non-growing; fg, fully grown; eg, early growing; mg, middle growing; lg, late growing; GV, germinal vesicle; dpc, days post-coitum; M II, metaphase in the second meiotic division; Dnm, DNA methyltransferase.
mice 14 h after injection of human chorionic gonadotropin, which was
second meiotic division (M II), were collected from the oviducts of adult
ovulated fg oocytes, which had progressed to the metaphase in the
5-, 10-, 15-, and 20-day-old mice, respectively, as reported previously.

1d-ng, 5d-ng, eg, mg, and lg oocytes were isolated from ovaries from 1-
and a freshly ovulated fg oocyte of JF1 mice, were transferred into an
recipient either an ng oocyte or a nucleus of a growth-stage oocyte derived
transfer (Fig. 1). Enucleated fully grown GV oocytes, which had re-
produced by serial nuclear
containing one haploid set of genomes from a various growth-stage

grown oocytes of JF1 mice. For the production of control parthenoge-
netic embryos (fg/fg PE) and control biparental embryos, the M II
chromosomes of the constructed oocytes were transferred into M II

donated from B6CBF1 mice. The constructed embryos were transferred
into pseudopregnant females and analyzed at 9.5 and 12.5 days post-
coeitum (dpc; the day of the plug was 0.5 dpc).

Expression Analysis by Reverse Transcriptase-PCR—Total RNA was
isolated using ISOGEN (Nippon Gene) from constructed embryos at 9.5
dpc. First strand cDNA was synthesized from 1 μg of total RNA from
each embryo by Superscript Reverse Transcriptase II (Invitrogen). One-
hundredth of the cDNA was subjected to PCR. Polymorphisms of Igf2r
and p57Kip2 genes in the JF1, PWK, and B6CBF1 mice were detected by
length polymorphisms using PCR products (17). The following primers
were used: β-actin, 5'-gtggtctagttgctagttg-3' and 5'-ctcacaagct-
ccctagag-3'; Peg1/Mest, 5'-tttgttttcctttcattc-3' and 5'-tcggctcctgag-
gac-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atactggcattgctcgtgtg-3' and 5'-gctgtgctatgttgctctagacttc-
3'; Zmf127, 5'-ttcctctgtgccagttgaagt-3' and 5'-cttcctgtgccagttgaagt-
3'; Igf2r, 5'-tggtgcagacattgaa-3' and 5'-tggtgcagacattgaa-3'; Snrpn, 5'-atac
tested paternally expressed Peg1/Mest, Peg3, Snrpn, Znf127, Ndn, and Impact genes were expressed in the 1d-ng/fg PE (Fig. 2c). These expression patterns in the 1d-ng/fg PE are responsible for the lack of primary imprinting on the 1d-ng oocyte genome according to our previous report (17). Although recent studies of mammalian cloning have suggested that epigenetic marks are sensitive to nuclear manipulation and in vitro culture (27), no unexpected epimutation was seen in control biparental embryos or in control fg/fg PE. This gave us confidence in our data.

Changes in the expression patterns were first seen in three paternally expressed genes: the Snrpn, Znf127, and Ndn genes in the 5d-ng/fg PE, which were constructed with an ng oocyte from the primordial follicles of 5-day-old mice and an adult-derived fg oocyte (Fig. 2d). These genes were repressed in two of the four 5d-ng/fg PE, indicating that the Snrpn, Znf127, and Ndn genes began to be imprinted in the earliest stage of follicular genesis. The proportion of fetuses in which Snrpn, Znf127, and Ndn genes were repressed increased to four of the five eg/fg PE, which were constructed with an eg oocyte from the secondary follicles of 10-day-old mice and an fg oocyte from adults (Fig. 2e). This indicates that the primary imprinting of Snrpn, Znf127, and Ndn genes is completed in the eg oocyte genome. The shift from expression to repression of Snrpn was always accompanied by the repression of Znf127 and Ndn in individuals (Fig. 2, c–g). This is presumably due to the fact that the expression of these genes is under the coordinate control of a common regulatory element in the upstream region of the Snrpn gene (28). In some of the eg/fg PE, the Peg3 gene was repressed, and the Igf2r and p57kip2 genes, which were repressed in the 5d-ng alleles, were expressed from both eg and fg alleles (Fig. 2e). Such Peg3 repression and Igf2r and p57kip2 expression by the eg alleles are the results of acquisition of the imprints. The repression of the Peg1/Mest gene was first detected in the mg/fg PE, which were constructed with an mg oocyte from the tertiary follicles of 15-day-old mice and an fg oocyte from adults (Fig. 2f). In the lg/fg PE, which were constructed with an lg oocyte from the antral follicles of 20-day-old mice and an fg oocyte from adults, the Peg1/Mest and Peg3 genes were repressed and the Igf2r and p57kip2 genes were expressed biallelically, except for only one case in the Peg3 gene (Fig. 2g). These expression patterns of the seven imprinted genes tested (the Impact gene being the exception) coincided exactly with the control fg/fg PE (Fig. 2b). The Impact gene, however, was still expressed in all of the lg/fg PE (Fig. 2g), and it was repressed in the fg/fg PE (Fig. 2b), indicating that the Impact gene is imprinted after the oocyte has grown to the final stage of the antral follicle. Thereafter, we confirmed that the Impact gene is imprinted in the lg oocytes derived from 23-day-old mice (data not shown). With these findings taken together, it was found that the epigenetic modifications for each im.

Fig. 2. Reverse transcriptase-PCR analysis of eight imprinted genes in (a) three control biparental embryos, (b) three control fg/fg PE, (c) five 1d-ng/fg PE, (d) four 5d-ng/fg PE, (e) five eg/fg PE, (f) six mg/fg PE, and (g) seven lg/fg PE. The establishment of maternal primary imprinting was shown by the repression of paternally expressed genes and biallelic expression of maternally expressed genes in the constructed parthenogenetic embryos. All of the paternally expressed genes, Peg1/Mest, Peg3, Snrpn, Znf127, Ndn, and Impact, were expressed in the control biparental embryos (panel a, lanes 1–3) but not in the fg/fg PE (panel b, lanes 1–3). The allele-specific expression analysis by length polymorphisms between B6CBF1 (B) and JF1 (J) or PWK (P) mice showed that maternally expressed Igf2r and p57kip2 were expressed biallelically in all fg/fg PE (panel b, lanes 1–3). The Igf2r expression was shown for a B6CBF1-derived 240-bp band and a JF1-derived 220-bp band. The p57kip2 expression was shown for a B6CBF1-derived 222-bp band and a PWK-derived 198-bp band. In control biparental embryos (panel a, lanes 1–3), Igf2r and p57kip2 genes expressed exclusively from maternal alleles (JF1- and PWK-derived bands, respectively). sp, sperm.

Fig. 3. Progress in primary imprinting during oocyte growth in eight imprinted genes. The number of oocytes observed to establish primary imprinting is indicated in each frame. The percentage is calculated by the imprinted alleles of the analyzed alleles. P, paternally expressed gene; M, maternally expressed gene; Chr, chromosome; Prox, proximal region; Dist, distal region.
printed gene progress independently in a specific sequence during oocyte growth.

To further understand maternal primary imprinting, we investigated the changing aspect of the developmental competence of the oocyte genome. When the developmental ability was assessed at 9.5 and 12.5 dpc, 59 and 29% of implanted 1d-ng/fg PE, respectively, developed into living fetuses with well developed placenta (Fig. 4, a and b). Developmental ability was not reduced in 5d-ng/fg PE. However, all of the eg/fg PE but one (1/32) died before 12.5 dpc, although up to 9.5 dpc, they developed as well as 1d-ng/fg PE did (Fig. 4b). The percentage of implanted embryos developing into living fetuses and into a stage of 20 or more somites (scored stage 15) was significantly reduced in mg/fg PE and lg/fg PE (Fig. 4b). Notably, the phenotype of lg/fg PE was close to that of fg/fg PE, showing typical abnormalities such as small embryo proper and retarded extraembryonic tissue. Thus, the parthenogenetic development was gradually reduced as the nuclear donor oocytes grew, corresponding to the progress of maternal primary imprinting.

**Discussion**

It has been believed that during ontogeny, the parental imprint is erased in the early gametogenesis, and that the new imprint is established in the subsequent stage according to the embryonal sex. Evidence showing the erasure of the parental imprint comes from the expression and/or methylation analysis of imprinted genes in primordial germ cells (9, 10), primordial germ cell-derived cloned embryos (29), and ng/fg PE (17). Here we showed, for the first time, the process of primary imprinting in mouse oogenesis. Based on follicular development, the eight imprinted genes were classified into four groups: 1) the *Snrpn*, *Znf127*, and *Ndn* genes, which are imprinted in the primordial to primary follicle stages; 2) the *Peg3*, *Igf2r*, and *p57Kip2* genes, which are imprinted in the secondary follicle stage; 3) the *Peg1/Mest* gene, which is imprinted in the tertiary to early antral follicle stage; and 4) the *Impact* gene, which is imprinted in the antral follicle stage (Fig. 3). Our data demonstrated that the establishment of maternal primary imprinting extends beyond 9.5 dpc and that the percentage of those developing to the 9.5 dpc embryonal stage was reduced from 60 to 15% as the oocytes grew (Fig. 4). These observations correspond well with our recent report (24) demonstrating that fertilized embryos containing the maternal genome from a growth-stage oocyte extended their developmental competence gradually. In that previous investigation, the first embryos capable of full term development contained maternal genomes from fg oocytes from juvenile mice. On the other hand, there was no significant difference in the developmental ability between 1d-ng/fg PE and 5d-ng/fg PE. This suggests that the primary imprinting of some genes, which are necessary for the extended parthenogenetic development beyond 9.5 dpc, had not been established in the 5d-ng oocyte genome. Our findings that only the *Snrpn*, *Znf127*, and *Ndn* genes are imprinted in the 5d-ng oocyte genome do not contradict the developmental competence of 5d-ng/fg PE because a deficiency in these genes caused lethality in the postnatal stage (28). However, eg oocytes isolated from 10-day-old or older mice lost the ability to support parthenogenetic development beyond 9.5 dpc (Fig. 4), suggesting that some genes causing this retardation are imprinted during the early phase of oocyte growth.

Here, we showed that among the four groups (1) *Snrpn*, *Znf127*, and *Ndn*; 2) *Peg3*, *Igf2r*, and *p57Kip2*; 3) *Peg1/Mest*; and 4) *Impact*, the genes are imprinted in the aforementioned order during oocyte growth. It has long been proposed that molecules of imprinting signals are included in DNA methylation because a gamete-derived methylation pattern from one parent precedes the allelic methylation differences observed in imprinted genes (12–15). If DNA methylation functions as the imprinting signals, the timing of DNA methylation may determine the sequence of the primary imprinting. By now, three DNA methyltransferases (Dnmts) have been identified in mammals (31). Dnmt1, which is the predominant maintenance and de novo DNA methyltransferase (32), was reported to be localized in the nucleus and cytoplasm of eg oocyte from 7-day-old mice (33). However, the deletion of oocyte-specific Dnmt1 promoter did not affect the oocyte-specific methylation patterns...
of the \textit{Snrpn} gene (34). Whether or not Dnmt3a and Dnmt3b, which are de novo DNA methyltransferases (35), are responsible for gamete-specific methylation has not yet been understood. On the other hand, a methylation-independent molecular system for genomic imprinting has also been suggested. Allele-specific methylation of the \textit{H19} gene is completely erased in primordial germ cells prior to 13.5 dpc. However, when paternal imprinting is established during spermogenesis, the paternally derived \textit{H19} allele is methylated before the maternally derived \textit{H19} allele (36). Moreover, Dnmt1-dependent DNA methylation in the postimplantation stage is not required for the imprinted expression of the \textit{Mash2} gene (37) or for preferential inactivation of the paternal X chromosome in the extraembryonic tissues of an XX embryo (38, 39). Also, Dnmt3-dependent DNA methylation in the postimplantation stage is not required for the imprinted methylation of the \textit{Igf2r} or \textit{H19} gene (35). Thus, the molecular mechanisms of genomic imprinting have not yet been understood clearly. In any case, there must be some mechanisms explaining sex-specific and imprinted gene-specific epigenetic modification. Further studies on DNA methylation might provide insight into the molecular mechanisms for the primary imprinting.

Acknowledgment—We thank Dr. Hatada for helpful discussion.

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