Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells

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SUMMARY

Genomic imprinting is an epigenetic mechanism that causes functional differences between paternal and maternal genomes, and plays an essential role in mammalian development. Stage-specific changes in the DNA methylation patterns of imprinted genes suggest that their imprints are erased some time during the primordial germ cell (PGC) stage, before their gametic patterns are re-established during gametogenesis according to the sex of individuals. To define the exact timing and pattern of the erasure process, we have analyzed parental-origin-specific expression of imprinted genes and DNA methylation patterns of differentially methylated regions (DMRs) in embryos, each derived from a single day 11.5 to day 13.5 PGC by nuclear transfer. Cloned embryos produced from day 12.5 to day 13.5 PGCs showed growth retardation and early embryonic lethality around day 9.5. Imprinted genes lost their parental-origin-specific expression patterns completely and became biallelic or silenced. We confirmed that clones derived from both male and female PGCs gave the same result, demonstrating the existence of a common default state of genomic imprinting to male and female germlines. When we produced clone embryos from day 11.5 PGCs, their development was significantly improved, allowing them to survive until at least the day 11.5 embryonic stage. Interestingly, several intermediate states of genomic imprinting between somatic cell states and the default states were seen in these embryos. Loss of the monoallelic expression of imprinted genes proceeded in a step-wise manner coordinated specifically for each imprinted gene. DNA demethylation of the DMRs of the imprinted genes in exact accordance with the loss of their imprinted monoallelic expression was also observed. Analysis of DNA methylation in day 10.5 to day 12.5 PGCs demonstrated that PGC clones represented the DNA methylation status of donor PGCs well. These findings provide strong evidence that the erasure process of genomic imprinting memory proceeds in the day 10.5 to day 11.5 PGCs, with the timing precisely controlled for each imprinted gene. The nuclear transfer technique enabled us to analyze the imprinting status of each PGC and clearly demonstrated a close relationship between expression and DNA methylation patterns and the ability of imprinted genes to support development.

Key words: Genomic imprinting, Primordial germ cells, PGC clones, Imprinted genes, DNA methylation, Mouse

INTRODUCTION

The initialization and reprogramming processes of epigenetic information during germ cell development are not fully understood. In mammals, a parental-origin-specific gene regulation mechanism, known as genomic imprinting, plays an essential role in development, growth and behavior, by regulating the expression of two kinds of imprinted genes: paternally and maternally expressed genes (Pegs and Megs, respectively) (Surani et al., 1984; McGrath and Solter, 1984; Cattanach and Kirk, 1985; Barlow et al., 1991; Bartolomei et al., 1991; DeChiara et al., 1991; Kaneko-Ishino et al., 1995; Miyoshi et al., 1998). Parental imprinted memories persist in somatic cells after fertilization, while it is necessary for them to be erased and re-established during germ cell development to reflect the gender of the individual (Reik and Walter, 2001). The immigration of PGCs to the genital ridges starts at around day 10.5 of the embryonic stage and is completed by day 11.5 (Rugh, 1990; Yeom et al., 1996; Molyneaux et al., 2001), when differentiation of the testes and ovaries commences.

Previous studies have indicated that imprinted memories were erased from day 11.5 to day 15.5 PGCs, judging from changes in DNA methylation and the loss of the monoallelic expression of imprinted genes (Grant et al., 1992; Kafri et al., 1992; Brandeis et al., 1993; Szabo and Mann, 1995). Region 2 of the Igf2r gene, which shows the fully methylated pattern of maternal alleles and the unmethylated pattern of paternal alleles in somatic cells, becomes totally unmethylated in both
male and female germ cells by day 13.5 of gestation, indicating that DNA demethylation plays an important role in this process (Brandeis et al., 1993). Biallelic expression of Igf2r, Igf2, H19 and Snrpn was reported in day 11.5 PGCs and in day 12.5 to day 15.5 ovaries and testes, suggesting that erasure of genomic imprinting occurred before the PGCs reached the genital ridges (Szabo and Mann, 1995). Similar results were obtained in studies of EG cells (Labosky et al., 1994; Stewart et al., 1994; Tada et al., 1998) and of so-called ‘germ cell embryos’ (Kato et al., 1999). Region 2 of the Igf2r gene was completely unmethylated in EG cells derived from day 12.5 PGCs (Labosky et al., 1994). However, half of the EG cell lines derived from day 8.0 to day 8.5 PGCs showed the normal somatic cell pattern (paternally unmethylated and maternally methylated pattern) and the remaining half showed a completely unmethylated pattern, suggesting that the imprinted memories of some day 8.0 to day 8.5 PGCs had been erased (Labosky et al., 1994). Tada et al. (Tada et al., 1998) closely examined the DNA methylation status of several imprinted genes in EG cells from day 11.5 to day 12.5 PGCs of both males and females, and showed that these genes were totally unmethylated, except the H19 and Igf2 genes, suggesting the existence of similar epigenetic states between paternal alleles.

Kato et al. (Kato et al., 1999) produced germ cell embryos by transplantaing the nuclei of day 14.5 to day 16.5 male PGCs into enucleated oocytes, and showed the expression pattern of imprinted genes from an imprint-free genome. Some imprinted genes, such as p57Kip2, Igf2, Igf2r, Snrpn, Nnat, Peg3, Peg10, Dlk1, (proximal 6), (sub-proximal 6), (sub-distal 11), Mest, H19, Igf2, H19 and Snrpn, were silenced in these embryos, while the DNA methylation status of the DMRs of several imprinted genes correlated very well to the loss of parental-origin-specific expression among the day 11.5 PGC clones, which demonstrated the existence of a default state of genomic imprinting common to female and male germ cell lines. Interestingly, the process of genomic imprinting memory erasure is seen in the day 11.5 PGC clones, which show several intermediate patterns of genomic imprinting. This conclusion was supported by the fact that the loss of DNA methylation in the DMRs of several imprinted genes correlated very well to the loss of parental-origin-specific expression among the day 11.5 PGC clones and that DNA methylation status in PGC clones represented well that of donor PGCs themselves.

**MATERIALS AND METHODS**

**Preparation of recipient oocytes**

Recipient oocytes for nuclear transfer were collected from mature B6D2F1 females that were superovulated by consecutive injections of 7.5 IU eCG and 7.5 IU hCG at 46- to 52-hour intervals. Cumulus-enclosed oocytes retrieved 14-17 hours after hCG injection were treated with 0.1% bovine testicular hyaluronidase in CZB medium until the cumulus cells were completely dispersed. After washing, oocytes were placed in a drop containing 6 μg/ml cytochalasin D for 5 minutes and enucleated with a glass pipette, by aspirating the metaphase II plate with a small volume of the surrounding cytoplasm.

**PGC preparation and nuclear transfer**

PGCs for nuclear donors were collected from the gonads of day 11.5 to day 13.5 fetuses of C57BL/6 (Mus musculus musculus) × JF1 (Mus musculus molossinus) F1 shortly before nuclear transfer. Two or three fetal gonads were placed in a 3 μl drop of HEPES-CZB containing 10% polyvinylpyrrolidone in a micromanipulation chamber, and punctured using a fine disposable needle to allow the PGCs to spread into the medium. The nucleus was removed from the donor cells by gently aspirating it in and out of the injection pipette (4-5 μm inner diameter). The donor nuclei were injected deep inside the ooplasm using a Piezo-driven micropipette. Oocytes injected with donor nuclei were incubated in CZB medium for 1-2 hours under 5% CO2 in air at 37°C, and then activated by treatment with 10 mM SrCl2 for 6-7 hours. The activation medium also contained 6 μg/ml cytochalasin D to prevent polar body extrusion. After washing, the oocytes were cultured in CZB medium for 48 or 72 hours under 5% CO2 in air at 37°C.

**Embryo transfer**

After 72 hours in culture, embryos that had developed to the morula or blastocyst stages were transferred into the uteri of day 2.5 pseudopregnant ICR females (Clea Japan, Tokyo, Japan). Some embryos were cultured for 48 hours and those that reached the four-cell stage were transferred into the oviducts of day 0.5 pseudopregnant females. Recipient embryos were killed on days 9.5-11.5 and their uteri were examined for live or dead fetuses.

**Quantitative RT-PCR**

Genomic DNA and total RNA were prepared from E9.5 embryos and placentas in both PGC clones and Dmnn1 mutant mice, using ISOGEN (Nippon Gene), as described previously (Kaneko-Ishino et al., 1995). cDNA was synthesized from 1 μg of total RNA using Superscript II reverse transcriptase (Life Technologies) with oligo(dT) as a primer. Gene expression levels were measured with an ABI PRISM 7700 using SYBR Green PCR Core Reagents (Applied Biosystems), designed to detect cDNAs. Target cDNA fragments were cloned into plasmids to be used as standards in the quantitative analysis of gene expression. Twelve imprinted genes (six Pegs and six Megs) were selected from eight different chromosomal imprinted regions: Mest (sub-proximal 6), Igf2 (distal 7), Peg3 (proximal 7), Nnat (sub-distal 2), Dlk1 (distal 12), Peg10 (proximal 6), Grb10 (proximal 11), H19.
Allelic analysis of gene expression

Polymorphisms in 12 imprinted genes between JF1 and C57BL/6 were detected by RFLP (restriction fragment length polymorphism), LP (length polymorphism) and SSP (single site polymorphism) analyses. For RT-PCR, 1 ng of cDNA in a 100 μl reaction mixture containing 1× ExTaq buffer (TaKaRa), 2.5 mM dNTP mixture, primers and 2.5 U of ExTaq (TaKaRa) was subjected to 30 PCR cycles. PCR was carried out on a Perkin Elmer GeneAmp PCR system 9600 under the following conditions: 96°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The following primers were used for DNA amplification:

**Most**, 5′-GATCTGCCAAGTACGCCG-3′ and 5′-ATCCAGATCGACACTTGTTG-3′; 5′-TTGAGATATAGGGACCCAGCCT-3′ and 5′-GGACACAGGCTTCGAGG-3′; 5′-CAAGCTCCTGAC-3′ and 5′-AGCGTACCC-3′; and 5′-TTGACAAGCTCCCTTGAC-3′

For RFLP analysis of **Peg5**, 5′-TTGCACTAAGTTGCACT-3′ and 5′-GAATACGGTCTTGATGGAG-3′; and 5′-TTGCACTAAGTTGCACT-3′ and 5′-GGTTGATTTGAGGGTT-3′.

**Allelic analysis of gene expression**

Polymorphisms in 12 imprinted genes between JF1 and C57BL/6 were detected by RFLP (restriction fragment length polymorphism), LP (length polymorphism) and SSP (single site polymorphism) analyses. For RT-PCR, 1 ng of cDNA in a 100 μl reaction mixture containing 1× ExTaq buffer (TaKaRa), 2.5 mM dNTP mixture, primers and 2.5 U of ExTaq (TaKaRa) was subjected to 30 PCR cycles. PCR was carried out on a Perkin Elmer GeneAmp PCR system 9600 under the following conditions: 96°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1 minute. For Peg5, two rounds of PCR were performed consisting of up to 35 and 12 cycles for the first (using F1 and R1) and second (using F1 and R2) rounds, respectively, under the same conditions used for **H19** and **Peg10**. Amplified fragments were cloned into plasmids and sequenced.

**RESULTS**

**Developmental potential of PGC clones**

When day 12.5 to day 13.5 PGCs were used as donor cells, 50-60% of oocytes developed to the morula/blastocyst stage and were transferred to pseudopregnant recipients. Overall 60% implanted and 10% gave rise to conceptus (Fig. 1A). The day 12.5 to day 13.5 PGC clones (Fig. 1B, upper column) were growth-retarded compared with control embryos produced by in vitro fertilization (Fig. 1B, lower column) on both day 10.5 and 11.5 of pregnancy. Apparently, they showed early embryonic lethality around embryonic day 9.5 and no further development was observed in any of these embryos. This situation was very different from that for somatic clones from cumulus, tail tip and Sertoli cells, in which using the same technique, the majority (>70%) of day 9.5 embryos survive to term, although the overall birth rate is low (2-3% per transfer) (Wakayama et al., 1998; Wakayama and Yanagimachi, 1999; Ogura et al., 2000). The development of day-14.5 to day 15.5 PGC clones was almost the same (data not shown), although the recovery rate of the morula/blastocyst stage and the implantation rates were higher than those of the day 12.5 to day 13.5 PGCs (Fig. 1A).

Regarding retarded growth and early embryonic lethality, these PGC clone embryos resembled germ cell embryos produced by the simple nuclear transplantation technique using day 14.5 to day 16.5 male PGCs, as reported previously by Kato et al. (Kato et al., 1999). They reported that the majority of clones never develop to term, at least partly because of loss of genomic imprinting memory (see next paragraph). Our results indicated that even the somatic cloning technique incorporating

**Methylation analysis of PGC clone embryos and PGCs**

Genomic DNA and RNA were isolated from day 11.5 to day 13.5 PGC clone embryos using ISOGEN as described in the RT-PCR section. To isolate genomic DNA of day 10.5 to day 12.5 PGCs, 300-500 cells were collected from the gonads of day 10.5 to day 12.5 fetuses of C57BL/6 (Mus musculus musculus) x JF1 (Mus musculus molossinus) F1. The sex of each fetus was determined by genomic PCR of Hprt and Sry. Purified genomic DNA (1 μg) was treated with a sodium bisulfite solution as described previously (Raizis et al., 1995). The H19 promoter region, Peg10 intron2 and 5′ upstream region of Peg5 were amplified by PCR with specific primers (5′-GGAATATTTTTGTGGTGGAG-3′ and 5′-AATTTGGGTTGAGATGAATAATTTAGG-3′ for H19; 5′-CAAGTGAACGTGCCTCTGACTTTCTAATGG-3′ and 5′-AATTTGGGTTGAGATGAATAATTTAGG-3′ for Peg10, and 5′-GAGTTATAAGTTTATTTGGAATAAG-3′ (F1), 5′-TACCTTTAAATACCTCTCTCACCAGAC-3′ (R1), 5′-CAACACCAACTAAAATCTCTC-3′ (R2) for Peg5), during which cytosine was converted to uracil.

DNA fragments were amplified using ExTaq (TaKaRa, Kyoto, Japan) for 31-35 cycles under the following conditions: 96°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1 minute. For Peg5, two rounds of PCR were performed consisting of up to 35 and 12 cycles for the first (using F1 and R1) and second (using F1 and R2) rounds, respectively, under the same conditions used for **H19** and **Peg10**. Amplified fragments were cloned into plasmids and sequenced.
an ‘initiation’ or ‘reprogramming’ step in unfertilized eggs did not improve the development of embryos produced from PGCs, and support the idea of Kato et al.

Interestingly, the development of day 11.5 PGC clones was significantly improved compared with that of clones from day 12.5 to day 13.5 PGCs. They appeared normal, even at the day 11.5 embryonic stage (Fig. 1B, middle column), although they were still a little smaller than IVF-derived controls. Regardless of extended growth, the implantation rate of the day 11.5 PGCs seemed lower than those of later stage PGCs. It should be noted that the remnants of imprinted memories in some embryos could explain the expanded growth of the day 11.5 PGC clones. PGC clones from day 10.5 PGCs could not be obtained because only 30% of them reached the two-cell stage and few embryos developed to the morula/blastocyst stage (data not shown). This is probably due to technical limitations of somatic cloning using rapidly proliferating cell populations (Campbell et al., 1996; Ogura et al., 2001). Only a part of the day 10.5 PGCs may be at G0/G1 stage, but other factors might also have affected their embryonic development.

**Default states of gene expression when genomic imprinting is lost in both male and female day 12.5 PGC clone embryos**

The expression levels and imprint status of imprinted genes were analyzed in day 12.5 and day 13.5 PGC clones (Fig. 2A-L, lanes 10-18). Twelve imprinted genes (six Pegs and six Megas) were selected from eight different chromosomal imprinted regions (see Materials and Methods), in order to represent the properties of several imprinted regions in the genome. The parental-origin-specific expression was determined by analyzing DNA polymorphism between B6 (Mus musculus musculus) and JF1 (Mus musculus molossinus). Conversion from monoallelic to biallelic expression was observed for Mest, Peg3, Nnat, H19, and Meg3 (Fig. 2, white bars), while Igf2, Dlk1, Igf2r, p57Kip2, Grb10 and Mash2 showed non-expression patterns (Fig. 2, black bars). These results are consistent with a previous study (Kato et al., 1999).

In this study, we have conclusively demonstrated the biallelic expression of about half of the imprinted genes examined, as well as the silenced state of the remaining half, confirming that genomic imprinting memories were completely erased in these embryos. We have previously reported that the maternally imprinted expression pattern was not established in non-growing oocytes (Obata et al., 1998). It should be noted that the expression patterns of non-growing alleles are almost the same as those in the day 12.5 PGC clones in this study and in germ cell embryos (Kato et al., 1999). Importantly, the day 12.5 female PGC clones (five shown in red, lanes 12-16) showed patterns identical to those in the day 12.5 male PGC clones (two shown in blue, lanes 10-11). This evidence demonstrates that there is a default state of genomic imprinting common to both male and female germlines at day 12.5 at least. It should be indicated that biallelic gene expression does not necessarily result in a twofold increase in the expression level.

**Process of genomic imprinting erasure represented in day 11.5 PGC clones**

Surprisingly, imprinted patterns were dramatically altered in the day 11.5 PGC clone embryos (Fig. 2A-L, lanes 1-9). A total of nine embryos produced from day 11.5 male (three shown in blue) and female (six shown in red) PGCs were examined, and each had a totally different imprinted status. The results are aligned in descending order of the number of genes that maintained a monoallelic expression pattern (Fig. 2M). We only counted a gene as biallelic when over 25% expression from the originally repressed alleles was detected compared with normally expressed alleles. In the day 11.5 PGC clones E1 and E2, 11 out of 12 imprinted genes were preserved and 10 were preserved in E3, indicating that the normal imprinted gene expression profiles observed in somatic cells (with the exceptions of the Peg3 and Nnat genes) were conserved (Fig. 2B,C). The imprinting of three to nine genes was maintained in E4-E7, and no imprinting except the Peg10 gene was detected in E8 or E9 (Fig. 2D). These patterns corresponded to intermediate states between that in normal somatic cells and that in the clones from day 12.5 to day 13.5 PGCs. Apparently, loss of imprinted expression proceeds in a step-wise manner coordinated specifically for each imprinted gene (see also Fig. 5). These results indicate that what we observed in the day 11.5
PGC clones was the process of erasing genomic imprinting, and each clone represented an intermediate state in this process.

**Progression of DNA demethylation in day 11.5 PGC clones**

To confirm this idea, we analyzed the methylation pattern of the DMRs of several imprinted genes that have been implicated in parental memory in somatic cells. Paternal alleles of *H19* are fully methylated and maternal alleles are non-methylated in normal somatic cells that show imprinted maternal expression (Bartolomei et al., 1993; Ferguson-Smith et al., 1993). We carried out allele-specific methylation analysis by the bisulfite method combined with DNA polymorphism analysis between C57BL6 (*Mus musculus musculus*) and JF1 (*Mus musculus molossinus*). Blue and red bars show paternal and maternal allelic expression profiles, respectively, that are similar to normal imprinted states. In the 11.5 PGC clone embryos, allele-specific expression of Pegs (A-D,J,L) and Megs (E-I,K) starts to convert to two of the so-called default states: biallelic expression (white bars) or non-expression (black bars). The timing of this erasing process differs with the individual imprinting genes, but the lack of distinction between male (sample number written in blue) and female (red) germ cells indicates that the erasure process is simultaneous in both germ lines. The ratios of imprinted gene expression in erased PGC clones and *Dnmt1 c/c* embryos (light-gray bars) were essentially the same. Placental expression was examined in the case of *Mash2*. (M) The numbers of genomic imprinted genes showing an imprinted monoallelic expression pattern.

**Fig. 2.** PGC clone embryo expression ratios and imprinting status. Relative expression levels in PGC clone embryos at day 9.5 were estimated by quantitative RT-PCR. The expression levels of IVF control embryos are shown as 1. Allelic expression was determined by DNA polymorphism analysis between C57BL6 (*Mus musculus musculus*) and JF1 (*Mus musculus molossinus*). Blue and red bars show paternal and maternal allelic expression profiles, respectively, that are similar to normal imprinted states. In the 11.5 PGC clone embryos, allele-specific expression of Pegs (A-D,J,L) and Megs (E-I,K) starts to convert to two of the so-called default states: biallelic expression (white bars) or non-expression (black bars). The timing of this erasing process differs with the individual imprinting genes, but the lack of distinction between male (sample number written in blue) and female (red) germ cells indicates that the erasure process is simultaneous in both germ lines. The ratios of imprinted gene expression in erased PGC clones and *Dnmt1 c/c* embryos (light-gray bars) were essentially the same. Placental expression was examined in the case of *Mash2*. (M) The numbers of genomic imprinted genes showing an imprinted monoallelic expression pattern.
Biallelic expression was observed in E4 and E5 embryos, but the levels of paternal expression were 27% and 59% that of maternal expression, respectively (Fig. 3C). Correspondingly, paternal-specific methylation was absent in 20% and 27% of these embryos. The degree of DNA methylation of paternal alleles was dramatically reduced in E7 and E8, which showed almost equal biallelic expression patterns. There were no apparent changes in non-methylated maternal alleles in any of these embryos. The loss of DNA methylation correlated well with changes in the pattern of H19 expression, supporting the postulate that the order of the embryos in Fig. 2 represents the progression of the erasure of parental imprinted memories.

Similar results were also obtained from analyses of DMRs in Igf2r (Stoger et al., 1993) and Nnat. As shown in Fig. 2, Nnat lost monoallelic expression more rapidly than other imprinted genes; 60-100% of expression from maternal alleles was detected in E2-E8. Actually, demethylation of maternal alleles was observed in E2, and was almost complete in E4-E8 (data not shown). DNA methylation of region 2 of Igf2r was maintained in E1-E4, and was completely lost in E7-E8, in which Igf2r expression was silenced completely (data not shown).

In the case of Peg10, which was the most resistant to the erasure process (Fig. 2), the correspondence between monoallelic expression and DNA methylation was rather low (Fig. 3B,C). Although expression of maternal alleles was only observed in E5 and E8, at levels of 18% and 22%, respectively (Fig. 3C), DNA demethylation of repressed maternal allele was detected in E4, E5 and E8 at the 20%, 50% and 60% levels, respectively. Therefore, reactivation from maternal alleles seemed more tolerant to DNA demethylation levels of the DMR in Peg10.

Comparison of imprinted gene expression of PGC clones with that in Dnmt1 KO embryos

Conversion from normal monoallelic expression to biallelic or non-expression has been reported for several imprinted genes in Dnmt1 mutant embryos, which lack activity of the major
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DNA methyltransferase (Li et al., 1992; Li et al., 1993). Therefore, we compared the results for the PGC clones with those of Dnmt1 c/c embryos (Fig. 2, lane 20). It has been reported that only 2% of genomic DNA methylation remained in Dnmt1 c/c embryos (Lei et al., 1996). This activity is lower than in Dnmt1 n/n and s/s embryos, which had 30% and 5-10% DNA methylation levels compared with wild-type embryos, respectively (Li et al., 1992; Li et al., 1993). The imprinted gene expression profiles were very similar. This again indicates the importance of DNA demethylation in the erasure step of genomic imprinting, and that of DNA methylation in the maintenance of memory in somatic cells. However, different levels of expression of the Mash2, Meg3, Peg3 and Peg10 genes were observed in PGC clones and Dnmt1 c/c mutants. Mash2 expression in placentas totally disappeared in the PGC clone embryos, suggesting that the parental imprint on the Mash2 gene was completely erased. However, no such dramatic decrement was found in Dnmt1 c/c embryos. This result is consistent with the previous reports by Caspary et al. (Caspary et al., 1998) and Tanaka et al. (Tanaka et al., 1999) using Dnmt1 s/s embryos. The residual DNA methylation level (2%) might be sufficient for Mash2 imprinting or perhaps mechanism(s) other than DNA methylation play an important role in this. The expression of the latter three genes decreased to about 50%, although they changed from monoallelic to biallelic patterns. On the contrary, approx. twofold increments were detected in Dnmt1 c/c embryos. These results also suggested that other mechanism(s) regulate the final gene expression levels during this process in PGCs together with DNA demethylation.

DNA methylation in day 10.5 to day 12.5 PGCs

Do PGC clones really represent the imprinting status of donor PGCs, and exactly when does the erasing process start during PGC development? To address these questions, we further examined the DNA methylation of three imprinted genes that had fast, intermediate and slow erasing of imprinted expression, were analyzed using bisulfite-treated genomic DNA from PGCs isolated from the genital ridges of day 10.5 to day 12.5 embryos. The results were consistent with the DNA methylation status of day 11.5 and day 12.5 PGC clones shown in Fig. 3.

DISCUSSION

Erasure of genomic imprinting in PGCs, EG cells, germ cell embryos and PGC clones

It is widely accepted that genomic imprinting memories are erased in PGCs and that DNA demethylation is an important factor in this process (Grant et al., 1992; Kafri et al., 1992; Brandeis et al., 1993; Szabo and Mann, 1995; Kato et al., 1999). However, it is not known when this erasing process starts or how it progresses in the PGCs. In this study, we approached this problem by analyzing clone embryos produced from day 11.5 to day 13.5 PGCs. Our results demonstrated that the day 12.5 to day 13.5 PGC clones showed the default states of gene expression when genomic imprinting was lost, and confirmed that imprint-free embryos never develop to term, as indicated previously by Kato et al. (Kato et al., 1999). We also demonstrated several intermediate states of the erasure process.
in the day 11.5 PGC clones. Combined with DNA methylation analyses of day 10.5 to day 12.5 PGCs themselves, it is highly possible that the erasing process has already started in gonadal PGCs at 10.5 dpc.

In a previous report, PGCs isolated from day 11.5 embryos showed bi-allelic expression of Igf2r, Igf2, H19 and Snrpn, and it was concluded that erasure occurred before the PGCs reached the genital ridges (Szabo and Mann, 1995). These authors used a mixture of one hundred PGCs, and their data showed biased expression between paternal and maternal alleles of Igf2 and H19. From our data, the day 10.5 to day 11.5 PGCs are heterogeneous, judging from DNA methylation status; they seem to be at different stages in the erasure process, and to possess the ability to show imprinted expression patterns that differ from each other. Therefore, in retrospect, these authors’ results might be interpreted as demonstrating the process of erasing imprinting in these genes.

Somatic cloning techniques and nuclear transplantation experiments enable us to examine the developmental potential of nuclei from single donor cells and the gene expression profiles of these embryos. However, it is important to note that the PGC clones represent the property of PGC nuclei in embryonic development, and not that of PGCs themselves. Therefore, we should be careful in interpreting the information from PGC clones by comparing the results with those from PGCs themselves. There is apparent discord between our results and those for the expression of Igf2 and Igf2r in the default state. In the PGC clones, these genes showed no expression, while it was reported that they showed bi-allelic expression in PGCs. The reason for this discrepancy remains unknown. However, it is accepted that the loss of DNA methylation silenced the expression of both Igf2 and Igf2r. Other reports demonstrated the loss of expression of these genes in Dmnt1 knockout mice (Li et al., 1992; Li et al., 1993). We have previously analyzed the gene expression profiles in non-growing oocytes combined with fully grown oocytes, and demonstrated that the maternal-specific imprints were established during oocyte maturation (Obata et al., 1998). In the non-growing/fully grown reconstituted conceptus produced in a nuclear transfer experiment, Peg genes from non-growing alleles except Igf2 were expressed and Meg genes (including Igf2r) except H19 were silenced. There was also no expression of Igf2 and Igf2r in germ cell embryos produced from male day 14.5 to day 16.5 PGCs (Kato et al., 1999). These results also indicate that these genes are silenced in imprint-free genomes and are consistent with our results. Thus, it is possible that the basal expression of these genes in the day 11.5 PGCs was very low; therefore, they showed no differences between parental alleles. Alternatively, DNA demethylation might reactivate silenced alleles in PGCs, while these non-methylated states induce silenced states in embryonic development.

The actual timing of DNA demethylation in the PGCs is also important. Labosky et al. (Labosky et al., 1994) examined EG cells (Matsui et al., 1992; Resnick et al., 1992) derived from PGCs at developmental stages from day 8.0 to day 12.5. They showed that region 2 of the Igf2r gene was completely demethylated in EG cells from day 12.5 PGCs. However, half of the EG cell lines from the day 8.0 to day 8.5 PGCs had normal methylation patterns, as observed in somatic cells, and the remaining half was totally unmethylated, suggesting that the imprinted memories of some day 8.0 to day 8.5 PGCs have been erased. However, it is still possible that the DNA methylation status of imprinted genes was altered during the establishment and subsequent cell culture of EG cells.

Recently, Sato et al. (Y. Matsui, personal communication) analyzed the DNA methylation status of region 2 of the Igf2r gene in PGCs isolated from Oct4/GFP transgenic mice by the HpaII-PCR method. They showed that DNA demethylation of HpaII site 3 did not occur in migrating PGCs at days 8.5 or 9.5, and was first detected in day 11.5 PGCs in the genital ridges. Their findings are consistent with our data. Combining this information, it is likely that the process of imprinting memory erasure, including Igf2r, starts around day 10.5 of gestation (Fig. 5). To determine the precise timing of the initiation of imprinting memory erasure, methylation analysis of migrating PGCs or non-gonadal PGCs at day 10.5 is necessary, using PGCs isolated from Oct4/GFP transgenic mice that also have DNA polymorphism, allowing discrimination between the alleles derived from each parent.

**Fig. 5.** Possible scheme for genomic imprinting memory erasure in PGCs. The erasure process is divided into two patterns, which proceed to bi-allelic expression as one default state (four Pegs, and H19 and Meg3) or non-expression as the other default state (four Megs, and Igf2 and Dlk1). In the former pattern, status conversion timing depends on individual genes; in the latter pattern, however, conversion occurred almost simultaneously in all genes examined.
Process of genomic imprinting erasure

Day 11.5 PGCs showed a variety of states, ranging from almost normal imprinted gene expression patterns (E1-E3) to nearly complete loss of monoallelic expression (E8-E9). These changes in the expression pattern are associated with a decrement in DNA methylation in DMRs. As DNA demethylation in the same regions was also observed in day 10.5 and day 11.5 PGCs themselves, we concluded that the changes in the gene expression profile seen in the day 11.5 PGC clone embryos arose from erasure of genomic imprinting memories. Assuming that the process starts when the PGCs enter the genital ridges, the variable imprinting observed in these embryos could represent temporal stages in donor PGC immigration. It is known that PGCs arrive at, and begin to enter, the genital ridges around day 10.5, and immigration is completed by day 11.5 (Rugh, 1990; Yeom et al., 1996; Molyneaux et al., 2001). This means that individual PGCs in the genital ridges must differ by at most 24 hours in development time. Therefore, the sequence in Fig. 2 presumably represents the time course of the erasure process (see also Fig. 5).

All imprinted genes (including Mash2) in the PGC clones were observed in one of two default states: biallelic expression or non-expression (Figs 2, 5). It should be noted that the genes in these two categories showed completely different features. Biallelic conversion occurred at different times, whereas the conversion to non-expression in six genes seemed to occur synchronously (Fig. 5). Biallelic expression may be explained by the differing degree in the progression of DNA demethylation among the imprinted genes, such as Nnat, H19 and Peg10 (Fig. 4). In the case of Peg10, however, gene sensitivity to the DNA demethylation level may also be an important factor for the erasure of genomic imprinting (Fig. 3B). On the contrary, conversion to the non-expressed state also appears in genes with different sensitivities to DNA methylation: Igf2 and Igf2r (sensitive) (Li et al., 1993), p57Kip2 (resistant) (Caspary et al., 1998) and Mash2 (highly resistant) (Caspary et al., 1998; Tanaka et al., 1999). Therefore, it seems likely that other factors co-operate with DNA methylation to produce changes in imprinting.

Although the DNA methylation patterns of PGC clones and PGCs themselves showed good agreement, it is apparent that the DNA methylation patterns observed in these PGC embryos did not precisely mirror those of single donor PGCs. If the initial DNA methylation patterns of donor nuclei were completely preserved in the PGC clone embryos, only the DNA methylation pattern from each parental allele should appear. However, we detected several intermediate demethylation patterns from the imprinted parental alleles in the cases of H19, Igf2r, Nnat and Peg10. These results indicate that either demethylation continues during PGC clone development for at least three to four cell divisions, or that the methylated patterns might not be stably maintained only in the day 11.5 PGC clones, although the latter is unlikely. Therefore, we could not ascertain in these experiments whether active or passive demethylation accounts for the patterns observed. However, this does not affect the conclusion that the initial change in the erasure process proceeds in at least day 10.5 and day 11.5 PGCs.

In the default state, most Megs become silenced while most Pegs show biallelic expression, with the exceptions of H19, Igf2 (Leighton et al., 1995), Meg3 (Miyoshi et al., 2000) and Dlk1 (Kobayashi et al., 2000; Schmidt et al., 2000; Takada et al., 2000), suggesting that there are different control mechanisms for the latter genes. In these cases, apparent DMRs are found only in the upstream regions of Megs, and Pegs seem to be reciprocally regulated under the control of Meg regions. The timings of the conversion to the biallelic form in the two Megs (H19 and Meg3) and to non-expression in the two Pegs (Igf2 and Dlk1) appear to be coordinated, suggesting additional common regulatory networks in both sets of genes. Recently, an insulator model (improved enhancer competition model) suggesting that binding of CTCF protein to specific sites in the DMR of H19 regulated both H19 (paternal repression and maternal expression) and Igf2 (paternal expression and maternal repression) simultaneously was put forward (Bell and Felsenfeld, 2000; Hark et al., 2000).

In our experiment, loss of imprinting of H19 did not seem to be completely coordinated with that of Igf2 (Fig. 2I,J, Fig. 5). In three embryos (E4–E6), the former gene converted to biallelic expression, but the latter gene maintained paternal expression. The same situation was observed in Meg3 and Dlk1 (Fig. 2K,L and Fig. 5). This discrepancy in timing between Megs and Pegs is probably explained by incomplete biallelic expression in E4–6. In these embryos, only 30-60% of repressed paternal alleles were expressed; therefore, it is possible that half of the cells became biallelic, while the remaining half kept the monoallelic expression pattern.

Somatic cloning method to elucidate the mechanism of genomic imprinting

We previously reported that imprinting status was almost normal in Sertoli clones (Ogura et al., 2000). Mouse somatic clones almost always have large placentas. Although lots of gene expression is abnormal in the term placentas of these clones, expression levels and monoallelic expression patterns of imprinted genes, such as Igf2, Igf2r and H19, were normal (Inoue et al., 2002). Moreover, most imprinted genes examined in this study showed normal imprinting expression in the Sertoli clone embryos on day 9.5 (Inoue et al., 2002). These data indicate that the genomic imprinted memories cannot be perturbed during nuclear transfer, even by the reprogramming process, and are normally maintained in the somatic clones.

This idea is supported by an X-inactivation study of cloned mice (Eggan et al., 2000). In the extra-embryonic tissues of the female, inactivation always occurs in the paternally derived X chromosome, while random inactivation occurs in embryonic cells after implantation. When clone mice are produced from female somatic cells, donor cells have either the paternal or maternal active X chromosome. In the placentas of these somatic clones, the memories of X-inactivation of somatic cells were maintained and nonrandom expression of either the paternal or maternal X chromosome was observed according to donor cell type. Recently, clone mice produced from cultured ES cells have shown abnormal expression of some imprinted genes, such as H19 and Igf2 (Humphreys et al., 2001). However, it is highly possible that the abnormal expression in ES clones simply reflects the properties of ES cells used as donors. We did not detect such abnormalities in the somatic clones from cumulus, tail tip or Sertoli cells, as described above (Inoue et al., 2002). This kind of abnormality was also observed in ES cells after successive passages (Dean et al., 2002).
et al., 1998). In summary, the application of cloning techniques gives us important knowledge of basic biology (Eggen et al., 2000). This study also demonstrates the potential of PGC cloning for elucidating the reprogramming process of genomic imprinting. The findings also give rise to a novel paradox: cloned animals can be born from somatic cells, but not from germ cells. The crucial roles of genomic imprinting as an epigenetic mechanism in mammalian development should be revisited.

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