INTRODUCTION

Development of the mammalian germ line is accompanied by a series of characteristic genomic modifications. These modifications include extensive demethylation of the genome, erasure of allele-specific methylation associated with imprinted genes, and the re-activation of the X chromosome. The allele-specific differential methylation is involved in regulating the monoallelic expression, and thus the gene dosage, of imprinted genes, which underlies functional differences between parental genomes. However, when the imprints are erased in the germ line, the parental genomes acquire an equivalent epigenetic and functional state. Therefore, one of the reasons why primordial germ cells are unique is because this is the only time in mammals when the distinction between parental genomes ceases to exist.

To test how the potentially imprint-free primordial germ cell nuclei affect embryonic development, we transplanted them into enucleated oocytes. Here we show that the reconstituted oocyte developed to day 9.5 of gestation, consistently as a small embryo and a characteristic abnormal placenta. The embryo proper also did not progress much further even when the inner cell mass was ‘rescued’ from the abnormal placenta by transfer into a tetraploid host blastocyst. We found that development of the experimental conceptus was affected, at least in part, by a lack of gametic imprints, as judged by DNA methylation and expression analysis of several imprinted genes. The evidence suggests that gametic imprints are essential for normal development, and that they cannot be initiated nor erased in mature oocytes; these properties are unique to the developing germ line.

SUMMARY

There are distinctive and characteristic genomic modifications in primordial germ cells that distinguish the germ cell lineage from somatic cells. These modifications include, genome-wide demethylation, erasure of allele-specific methylation associated with imprinted genes, and the re-activation of the X chromosome. The allele-specific differential methylation is involved in regulating the monoallelic expression, and thus the gene dosage, of imprinted genes, which underlies functional differences between parental genomes. However, when the imprints are erased in the germ line, the parental genomes acquire an equivalent epigenetic and functional state. Therefore, one of the reasons why primordial germ cells are unique is because this is the only time in mammals when the distinction between parental genomes ceases to exist.

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Key words: Primordial germ cells, Totipotency, Imprinting, Mouse

INTRODUCTION

Development of the mammalian germ line is accompanied by a series of characteristic genomic modifications. These modifications include extensive demethylation of the genome, erasure of allele-specific methylation of imprinted loci, and re-activation of the silent X chromosome (Monk and McLaren, 1981; Monk et al., 1987; Kafri et al., 1992; Brandeis et al., 1993). The erasure of allele-specific methylation results in the imprinted genes having the potential for biallelic expression or repression (Szabo and Mann, 1995; Shemer, 1997; Tada et al., 1998). Later during gametogenesis, germ line-specific epigenetic modifications are re-introduced, which are inherited after fertilisation by developing embryos and they survive into adulthood (Barlow, 1997; Mertineit, 1998; Reik and Walter, 1998). The differential DNA methylation of parental alleles is responsible for the monoallelic expression of imprinted genes (Li et al., 1993), although other forms of epigenetic modifications are not excluded from playing a significant role. Functional differences between parental genomes during mouse development occur because of the differential expression of imprinted genes (Solter, 1988; Surani, 1986, 1998). However, with the erasure of gametic imprints in primordial germ cells by about day 12.5-13.5 of development, the homologous chromosomes attain an equivalent epigenetic status for probably the only time in mammalian development.

The reasons for the extensive genomic modifications in the germ line may be to prevent inheritance of aberrant epigenetic modifications by subsequent generations, as well as to restore totipotency. Previous studies have shown that embryonic germ (EG) cells have a similar epigenotype to that of PGCs from which they are derived (Matsui et al., 1992, Resnick et al., 1992; Stewart et al., 1994; Tada et al., 1998). EG cells can contribute to most if not all the somatic tissues, as well as the germ line, and to this extent they are totipotent (Labosky et al., 1994; Stewart et al., 1994; Tada et al., 1998). Both male and female day 11.5-12.5 EG cells undergo comparable epigenetic changes (Tada et al., 1998). What is
also striking is that EG cells have a dominant trans-modification activity which is capable of reprogramming a somatic nucleus. This was deduced from experiments using EG-somatic cell hybrids which resulted in an extensive epigenetic modification of the somatic nucleus so that it resembled the germ cell nucleus (Tada et al., 1997). Consequently, the EG-somatic hybrid cells were also able to differentiate into a variety of cell types.

Recently, transplantation of somatic nuclei into enucleated oocytes has resulted in development of viable adults (Wilmut et al., 1997; Wakayama et al., 1998). This has provided unequivocal evidence that the determined and totipotent states are indeed reversible. It is most likely that erasure of epigenetic modifications is a primary prerequisite in this reprogramming event. Indeed, genome-wide demethylation similar to that observed in PGCs does occur during preimplantation development, except for the imprints which are not erased (Kafri et al., 1992; 1993; Brandeis et al., 1993; Tremblay et al., 1995; Olek and Walter, 1997). Apart from gametic imprints, it is perhaps necessary that most of the epigenetic modifications acquired during gametogenesis by sperm and oocytes are erased before development of pluripotent epiblast cells can occur. The same mechanism may also be involved in reprogramming of a somatic nucleus when transplanted to a mature oocyte, although the fate of gametic imprints in these experiments is unknown.

In this study, we examined the developmental potential of male PGC nuclei from day 14.5-16.5, after transplantation into enucleated oocytes. As discussed above, a number of genomic modifications in the germ cell lineage are consistent with the restoration of totipotency. However, germ cells are essentially devoid of gametic imprints and this may allow examination of how the imprint-free PGC nucleus affects development. Our findings demonstrate that the reconstituted zygotes developed to form a conceptus, referred to here as germ cell (GC) conceptus, with a distinct phenotype, consistent with the absence of imprints in the donor nuclei.

MATERIALS AND METHODS

Animals

(C57Bl/6 × CBA/Ca) F1 mice (GPI-1BB) (B6/CBA F1) were generated from parental stocks (Bantin and Kingman Ltd) and the MF1 outbred albino mice (GPI-1BB) were from Olac Ltd. The ROSA26 transgenic mice were a kind gift from Dr Philip Soriano (Friedrich and Soriano, 1991), which were bred into the 129/Sv background (GPI-1AA). The inbred 129/Sv × 129/Sv (GPI-1AA) mice were also bred from stocks.

Isolation of PGCs and nuclear transplantation into oocytes

Isolation of PGCs and transplantation of PGC nuclei into oocytes was carried out essentially as described previously (Tsunoda et al., 1989; Kato and Tsunoda, 1995). Briefly, PGCs were isolated from the genital ridges of fetal male mice at day 14.5-16.5. The embryos were derived following mating of the ROSA26 or the 129/Sv mice. PGCs were stored in PBl+10% fetal calf serum at room temperature. Recipient oocytes were recovered from the superovulated B6/CBA F1 females. 14-19 hours after injection of human gonadotropin. The oocytes were denuded of cumulus cells by treatment with hyaluronidase. The recipient oocyte nuclei at the MII chromosome stage were first removed as described. A single PGC was then fused with the recipient oocyte using Sendai virus and cultured for 20 minutes followed by an electrical stimulus (DC pulse of 150V for 50 milliseconds) to activate the oocyte. This was repeated twice at 20 minute intervals using a 50 V DC pulse for 50 milliseconds. The oocytes were then examined after 5-6 hours culture for the presence of the donor nucleus in the activated oocyte. The reconstituted oocytes were cultured in vitro for up to 5 days until the blastocyst stage. Some of the developing preimplantation GC embryos (either day 0.5 or day 2.5), were transferred to pseudopregnant F1 recipients obtained by mating with vasectomised males.

Chimeras and rescue of GC ICM in tetraploid (4n) blastocysts

The inner cell mass was isolated by immunosurgery from GC blastocysts obtained after 5-6 days of culture in vitro (Solter and Knowles, 1975). Tetraploid blastocysts (from CB6/CBA F1 mating) were prepared by fusing 2-cell embryos (Nagy et al., 1990) which were then allowed to develop to the blastocyst stage in vitro. The ICM from GC blastocysts was then injected into the tetraploid host blastocysts. Alternatively, the GC ICMs were transferred into normal diploid CB6/CBA F1 blastocysts. All the reconstituted blastocysts were transferred to day 2.5 pseudopregnant recipients for development in vivo for varying durations.

Histology, in situ hybridisation, X-gal staining and Gpi analysis

Normal, GC and androgenetic day 9.5 conceptuses were fixed, embedded and sectioned (10 mm sections) as described by Wilkinson and Nieto (1993). The sections were stained with the standard histological stains, haemotoxylin and eosin.

The appropriate clones for detecting expression of imprinted genes were cloned into pBluescript (SK–) (Strategene) and were used to prepare sense and antisense RNA probes by in vitro transcription using the DIG RNA labelling kit (Boehringer Mannheim). In situ hybridisation was carried out essentially as described previously (Wilkinson and Nieto, 1993). The sections from control and GC conceptuses were hybridised with the probes and adsorbed with alkaline phosphatase-conjugated anti-DIG antibody. Alkaline phosphatase activity was detected by using BM purple AP substrate (Boehringer Mannheim) followed by counter stain with 0.5% eosin.

For X-gal staining, embryos were rinsed in PBS and fixed for 3-4 hours in a fixative containing 1% formaldehyde, 0.2% glutaraldehyde, 0.02% NP40 and 1 mM MgCl2 in PBS. The specimens were rinsed in PBS and then stained in a reaction mixture containing 1 mg/ml 5bromo-4-chloro-3-indolyl-β-D-galactopyranoside in dimethyl formamide, 5 mM potassium ferrocyanide, 5 mM potassium ferrocyanide and 2 mM MgCl2 in PBS for 24-48 hours at room temperature.

Gpi patterns were analysed according to the procedures of Eicher and Washburn (1978). One volume of red blood cells was mixed with four volumes of distilled water and in the case of homogenised tissue samples, 20 volumes of distilled water. Erythrocytes was conducted on Titan III iso-vis cellulose acetate plates (Helena Laboratories, USA) with tris-glycine buffer (pH 8.5) at 180 V for 45 minutes. The plates were impregnated with the staining mixture in 1% agarose at 37°C for several minutes and fixed. The intensity of the Gpi activity was visually assessed.

DNA probes and methylation analysis

Genomic DNA was prepared from GC and control embryos, digested with the appropriate restriction endonucleases, separated on agarose gels and transferred to Hybond-N+ by alkali blotting. Radioactive probes were made using MegaPrimer DNA labelling system (Amersham) and blots were hybridised with 32P-labelled DNA. The DNA probes used for the analysis of methylation of
imprinted loci were exactly as described previously (Tada et al., 1997, 1998).

RESULTS

Preimplantation development of oocytes with the PGC donor nucleus

In this study, we transplanted the mitotically arrested male PGCs from day 14.5-16.5 fetal gonads into enucleated oocytes, to see the influence of the epigenotype of the PGC nucleus on embryonic development. Male primordial germ cells begin to enter into G0 mitotic arrest after day 12.5 of gestation, an optimum cell cycle stage for nuclear transplantation into oocytes (Campbell et al., 1996). The female PGCs enter into meiotic arrest after day 13.5 and therefore they are unsuitable for this experiment. In a similar experiment previously, no postimplantation embryos were recovered (Tsunoda et al., 1989). Here, we used PGCs from the 129/Sv strain of mice with appropriate markers to verify the genotype of the resulting embryos (see Methods). The PGCs were isolated and fused with enucleated recipient oocytes as described previously (Fig. 1).

Fig. 1. (A) Reconstitution of a zygote following transplantation of day 14.5-16.5 donor PGC nuclei into enucleated oocytes. The recipient oocyte was first enucleated. A PGC was then fused with the enucleated oocyte. After the entry of the donor nucleus into the recipient cell, the oocyte was activated. (B) Preimplantation development of GC embryos. A pronucleus-like structure (Nu) is observed in some of the activated oocytes following transformation of the diploid PGC donor nucleus in the oocyte cytoplasm. Most of these embryos reach the morula stage. About 40% of them go on to develop as blastocysts; one is seen hatching from the zona pellucida.

Fig. 2. Postimplantation development of a GC embryo at day 9.5 of gestation. (A) The GC embryo is apparently normal but it is growth retarded. (B) Histology of day 9.5 conceptuses. Note the abnormal placental development the GC compared with the control. The GC embryos were confirmed to be of the donor PGC nucleus genotype. The placenta of the GC conceptus resembles that observed in androgenones. Scale bars, 1.0 mm.
1A). Nearly 70% of them developed a single pronucleus-like structure after activation of the oocyte and a large proportion of them developed to the blastocyst stage (Fig. 1B; Table 1). The age of donor PGCs did not have a significant effect on the rate of development. An additional 165 reconstituted oocytes of mixed genetic background (129/Sv × M. spretus F1 and 129/Sv × MF1) also showed similar rates of development to the blastocyst stage (55-68%). However, of the 122 reconstituted oocytes with PGCs derived from (129 × M. spretus)F1 embryos, 89% reached the morula stage but only 21 (17%) reached the blastocyst stage.

Postimplantation development of GC conceptus

Next, the GC morulae or blastocysts were transferred to pseudopregnant recipients. Overall, 64% of them implanted and 43% of them had fetal tissues (Table 2). Development was unaffected by the age of the donor nucleus obtained between day 14.5-day 16.5. On day 9.5 of gestation, the majority of embryos were at the 20-25 somite stage (Fig. 2A). Although apparently normal, they were considerably growth retarded. On day 10.5 and day 11.5, slightly larger embryos were seen, but it seemed unlikely that the GC embryos would develop much further.

To determine if there were any recognisable defects in GC conceptuses a number of them were examined histologically at day 9.5 of gestation. There was a striking and consistent defect in placental development (Fig. 2B). In particular, the chorion seemed to be developmentally arrested at day 8.5. There was also a thick layer of trophoblast giant cells. Most significantly, there was usually only a small and discrete layer of diploid trophoblast with an evident lack of the spongiotrophoblast. The allantois was well developed and seemed to be fusing with the chorion but without interacting with it to become labyrinthine trophoblast. The placental phenotype was reminiscent of placental development in the Mash2-/- conceptus; Mash2 is an imprinted gene with maternal allele-specific expression, encoding a helix-loop-helix transcription factor with a crucial role in development of the trophoblast lineage (Guillemot et al., 1994, 1995). A similar placental phenotype is also observed in the AG conceptus which probably has two repressed paternal alleles of Mash2 (Fig. 2B; S. C. B., unpublished). In contrast to the placenta, the GC embryo was usually well formed although small and a little developmentally retarded but more advanced than the chorion stage would suggest.

To test if developmental retardation and lethality in the GC embryo were primarily due to the placental abnormality, we introduced the inner cell masses (ICMs) isolated from GC blastocysts, into tetraploid host blastocysts (Nagy et al., 1990; Guillemot et al., 1994). The donor ICMs were of the 129/Sv × ROSA26 genotype with GPI-1A and the host tetraploid blastocysts were either of the MF1 or B6/CBAF1 genotype with GPI-1B. Hence we could type our embryos by GPI analysis and by X-gal staining. In such reconstituted blastocysts, normal placentae develop from tetraploid cells but the fetuses which go on to term, develop from the diploid ICMs. Using this approach, embryos lacking MASH2 could be rescued as they developed to term (Guillemot et al., 1994). We examined the fate of our 4n GC ICM reconstituted blastocysts between day 11.5-13.5 of gestation. We recovered 13 fetuses from 55 reconstituted blastocysts on d11.5 and six of these were apparently healthy (Fig. 3B). However, no fetuses were obtained from 15 reconstituted blastocysts when examined for development between day 12.5 and 13.5. The fetuses were GPI-1A and they stained uniformly for X-gal, which confirmed that they were indeed derived from the GC ICM and of the PGC donor nucleus genotype. While the overall growth of the GC fetuses was improved in 4n GC

### Table 1. Preimplantation development of GC embryos

<table>
<thead>
<tr>
<th>PGC strain</th>
<th>Age (days)</th>
<th>1-cell</th>
<th>2-cell</th>
<th>4-cell</th>
<th>8-cell</th>
<th>Morula</th>
<th>Blastocyst (%)</th>
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<tbody>
<tr>
<td>129 × 129</td>
<td>14.5</td>
<td>87/173</td>
<td>79</td>
<td>59</td>
<td>55</td>
<td>49</td>
<td>15 (17%)</td>
</tr>
<tr>
<td></td>
<td>15.5</td>
<td>247/379</td>
<td>246</td>
<td>229</td>
<td>226</td>
<td>220</td>
<td>117 (47%)</td>
</tr>
<tr>
<td></td>
<td>16.5</td>
<td>73/85</td>
<td>73</td>
<td>69</td>
<td>69</td>
<td>69</td>
<td>29 (40%)</td>
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<tr>
<td>Total (%)</td>
<td></td>
<td>407/637</td>
<td>398</td>
<td>357</td>
<td>350</td>
<td>338</td>
<td>161</td>
</tr>
<tr>
<td>129 × Rosa 26</td>
<td>14.5</td>
<td>30/51</td>
<td>28</td>
<td>23</td>
<td>22</td>
<td>22</td>
<td>16 (53%)</td>
</tr>
<tr>
<td></td>
<td>15.5</td>
<td>305/420</td>
<td>296</td>
<td>278</td>
<td>264</td>
<td>263</td>
<td>202 (66%)</td>
</tr>
<tr>
<td></td>
<td>16.5</td>
<td>107/134</td>
<td>104</td>
<td>95</td>
<td>93</td>
<td>93</td>
<td>60 (56%)</td>
</tr>
<tr>
<td>Total (%)</td>
<td></td>
<td>422/605</td>
<td>428</td>
<td>396</td>
<td>379</td>
<td>378</td>
<td>278</td>
</tr>
<tr>
<td>Rosa 26 × 129</td>
<td>15.5</td>
<td>47/72</td>
<td>45</td>
<td>44</td>
<td>40</td>
<td>40</td>
<td>29 (62%)</td>
</tr>
<tr>
<td></td>
<td>16.5</td>
<td>18/37</td>
<td>17</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>9 (50%)</td>
</tr>
<tr>
<td>Total (%)</td>
<td></td>
<td>65/109</td>
<td>62</td>
<td>60</td>
<td>56</td>
<td>56</td>
<td>38</td>
</tr>
</tbody>
</table>

### Table 2. Postimplantation development of GC embryos

<table>
<thead>
<tr>
<th>Age of PGC (days)</th>
<th>Number transferred</th>
<th>Day of gestation (No. of conceptuses)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>14.5</td>
<td>10</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>11.5</td>
</tr>
<tr>
<td>15.5</td>
<td>116</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>10.5</td>
</tr>
<tr>
<td>16.5</td>
<td>13</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>10.5</td>
</tr>
<tr>
<td>Total</td>
<td>204</td>
<td>–</td>
</tr>
</tbody>
</table>
ICM conceptuses compared to the complete GC conceptuses described above, the experimental embryos were still substantially smaller than the controls (Fig. 3C,D). This suggested that we had probably reached the limit of GC embryonic development. In a further experiment, the contribution of GC cells to chimeric embryos with wild-type cells was not excluded from any somatic cell lineage during development or in the adults (Fig. 3E-H). In this respect at least, GC embryonic cells are similar to the Dnmt1-/- ES cells that have lost their inherited allele-specific imprints and regained their ability to differentiate by reintroduction of the wild-type Dnmt1 cDNA (Li et al., 1993; Tucker et al., 1996). Interestingly, they did not show the restricted allocation patterns observed for androgenetic (AG) and gynogenetic (GG) ES cells in chimeras with normal blastocysts (see Discussion).

### Expression of imprinted genes in the GC conceptus

Next, we asked if the GC phenotype is affected, at least in part, by the lack of gametic imprints in the donor PGC nucleus. Expression of some well characterised imprinted genes was examined in the GC conceptuses by in situ hybridisation.

The upstream region of H19 gene in the GC conceptus, which was consistent with the inheritance of the potentially imprint-free PGC epigenotype (Li et al., 1993; Tucker et al., 1996; Ainscough et al., 1997) (Fig. 4). Igf2 and H19 are linked mechanistically so that their expression is mutually exclusive in cis (Ainscough et al., 1997). These results were therefore consistent with the probable biallelic expression of H19 and no Igf2 expression in the GC conceptus, a conclusion which was also supported by the methylation status of the H19 gene in the GC conceptus (see below). Similarly, the imprinted gene, Igf2r, was also repressed in the GC conceptus (Fig. 4). This result could also be explained by the erasure of methylation in the donor PGC nucleus, since the expression of the maternal allele is dependent on the methylation of an intronic CpG island in the developing oocyte (Stöger et al., 1993). Our findings on these three imprinted genes were identical to the observations in Dnmt1-/- embryos where the imprints were erased (Li et al., 1993; Tucker et al., 1996).

The p57kip2 and Mash2 genes, located 300-500 kb upstream of Igf2, show maternal allele-specific expression (Guillemot et al., 1995; Hatada and Mukai, 1995; Paulsen et al., 1998). The p57kip2 gene was also clearly repressed in the GC conceptus (Fig. 4). Furthermore, we detected Mash2 in the control conceptus in the extraembryonic tissues (Guillemot et al., 1995; Tanaka, 1997), but it was undetectable in the GC conceptus (data not shown). The placental phenotype of a GC conceptus in which the diploid trophoblast cells failed to proliferate, was consistent with the lack of Mash2 in the knockout mice (Guillemot et al., 1994). However, the absence of Mash2 in the GC conceptus could simply be because the trophoblast cells where the gene is expressed failed to proliferate in the GC conceptus (Guillemot et al., 1994,1995).

Four further genes, Peg3 (Kuroiwa et al., 1996), Peg1/Mest (Kaneko-Ishino et al., 1995), Nnat (Kikyo et al., 1997) and Srrpn (Ozçelik et al., 1992) showed expression in the GC conceptus (data not shown). Furthermore, the pattern of expression of imprinted genes in the GC conceptus was similar to that in the control conceptus. None of the imprinted genes examined exhibited any ectopic expression. To further elucidate the imprinted status of the loci and to see if the expression analyses were consistent with the epigenetic status, we performed DNA methylation analyses.

### Methylation analysis of imprinted loci

The parental alleles of imprinted genes show differential methylation, a heritable epigenetic modification that normally reflects their mono allelic expression (Barlow et al., 1997; Reik and Walter, 1998, 1999). We therefore carried out methylation analysis of some of the imprintable loci in GC embryos. The expression and methylation analyses were in agreement and indicated erasure of parental imprints in GC embryos.

DNA from day 9.5 GC and 129/Sv normal embryos was compared for imprint associated allele-specific methylation. The upstream region of H19 was analysed using DNA digested with HindIII or HindIII + CfoI (a methylation-sensitive restriction endonuclease and isoschizomer of HhaI). The HindIII digestion of DNA from control embryos gave a single band of 4.3 kb which was partially digested by CfoI in the double digest, and gave bands of 4.3, 2.6, and 2.4 kb (Fig. 5), a pattern expected for the differential methylation of H19 parental alleles (Ferguson-Smith et al., 1993; Ainscough et al., 1997). By contrast, the HindIII/CfoI double digest of GC embryonic DNA showed full digestion of the 4.3 kb band. This complete digestion by CfoI meant that the site closest to the probe was unmethylated and lacking the paternal-specific methylation at this CfoI site. The lack of methylation observed at this site probably indicated biallelic expression of H19 in these embryos and correlated with the lack of Igf2 expression in GC embryos since these genes show mutually exclusive expression in cis (Ainscough et al., 1997).

For the Igf2r gene, digestion of normal and GC embryo DNA with PvuII or PvuII + MluI (a methylation sensitive restriction endonuclease) was carried out (Stöger et al., 1993) (Fig. 5). The double digestion of DNA from control embryos cleaved 50% of the 3.4 kb PvuII band as has been described for the maternal-specific methylation seen in region 2 (Stöger et al., 1993). However, the DNA from GC embryos was again fully digested by MluI indicating biallelic demethylation of Igf2r region 2 and an absence of any imprint. Methylation of region 2 is directly correlated with expression of Igf2r; therefore, the lack of methylation in both alleles in the GC conceptus was consistent with its lack of expression described above (Stöger et al., 1993).
For *Nnat*, the 5’ region is known to be methylated on the silent maternal allele (Kikyo et al., 1997). In the DNA from control embryo, the 7 kb *BamHI* fragment was partially digested with the methylation sensitive enzyme *EagI* while in DNA from GC embryos, the *BamHI* fragment was almost completely digested giving a 2.8 kb band (Fig. 5). Thus, *Nnat*...
was also devoid of a maternal allele-specific methylation imprint in GC embryos. This was consistent with the observed expression of Nnat in a GC conceptus with a strong probability for biallelic expression.

We have some evidence for the methylation of p57kip2 (which is less well characterised), using the same BamHI/EagI blot described above, probed with the 5' end of the p57Kip2 cDNA. The 3.0 kb BamHI fragment was partially digested into 1.8 and 0.95 kb bands in the control 129 DNA, while DNA from GC embryos showed higher methylation of EagI sites, since no band was visible at 0.95 kb and most of the 3.0 kb band remained undigested (Fig. 5). We cannot precisely map the location of this increased methylation at the p57kip2 locus in GC embryos because the genomic structure and sequence of mouse p57kip2 are not yet available. However, there is one EagI site within the cDNA sequence that lies just upstream of the repeat region characterised as showing paternal-specific methylation on the inactive allele in normal mouse embryos (Hatada and Mukai, 1995). Thus, the observed increase in methylation of p57kip2 correlated with the absence of expression in the GC embryos described above.

We have also obtained preliminary evidence for the methylation status of Peg1/Mest (Kuriwa et al., 1996) and Peg3 (Kaneko-Ishino, 1995), which indicated that both alleles of these genes were demethylated as opposed to the differential methylation in control embryos (data not shown). Therefore it was likely that both these genes were also biallelically expressed in the GC embryo.

The combined expression and methylation analysis suggested that instead of the usual monoallelic expression, there was probably biallelic expression of H19 and Nnat, as well as biallelic repression of Igf2, Mash2, p57kip2 and Igf2r. It was also likely that Peg1, Peg3 and Snrpn were expressed from both alleles. To obtain additional information on the expression of both alleles in the GC conceptus, requires polymorphic markers to distinguish between expression of the two alleles. For this reason, we attempted to generate GC embryos with donor nuclei from the germ cells of day 15.5 129/Sv x M. spretus hybrid embryos as described above. However, only 15% of these reconstituted zygotes (21/137) developed to the blastocyst stage. A further 59 preimplantation embryos were transferred to pseudopregnant recipients but none of them developed to day 9.5. Development of the reconstituted eggs was possibly affected by the genetic background of the donor nucleus.

**DISCUSSION**

This study demonstrates that transplantation of a PGC nucleus into an enucleated oocyte resulted in the development of a GC conceptus with a distinct phenotype. This at least in part could be attributed to the absence of appropriate allele-specific methylation and expression of imprinted genes, suggesting that the appropriate gene dosage for imprinted genes is critical for normal development. The lack of imprints is known to result in either biallelic expression or repression of specific imprinted genes (Li et al., 1993). Our analysis of expression and DNA methylation of several such genes in the GC conceptus is consistent with the inheritance of an imprint-free epigenotype from the PGC cells. Thus, some imprinted genes in the GC conceptus were repressed and others were probably expressed biallelically (Table 4). The epigenetic modifications that constitute imprints occur later, primarily during gametogenesis, and this is necessary for the appropriate monoallelic expression of imprinted genes in normal embryos (Table 4). The donor PGC nuclei we used here had skipped this latter stage. What is also important to note is that the PGC nuclei evidently did not acquire the allelic imprints necessary for monoallelic expression when transplanted into enucleated oocyte. This explains why there was either repression or biallelic expression of imprinted genes in the GC conceptus.

The placental abnormality observed in the GC conceptus is reminiscent of the loss of Mash2 function, a maternally expressed imprinted gene which is necessary for normal development of the trophoblast lineage (Guillemot et al., 1994, 1995). Although we did not detect Mash2 in the GC conceptus, this is probably because the trophoblast cells where the gene
is expressed, failed to proliferate in the GC conceptus (Guillemot et al., 1994, 1995; Tanaka et al., 1997). However, additional factors must also be implicated for the lack of normal development. Clearly, as the p57kip2 gene in addition to Igf2r and Igf2 were also repressed, they will also contribute to the GC embryonic lethality. Aberrant expression of other imprinted genes resulting in both the gain (such as Nnat) or loss of function (such as Igf2r) must affect the GC conceptus. Genetic studies have also shown that paternal or maternal duplications of specific chromosomal regions with imprinted genes is incompatible with normal development (Cattanach and Jones, 1995). This is evidently due to biallelic expression or repression of imprinted genes present within these regions.

The methylation of p57kip2 in the GC conceptus is consistent with previous evidence from day 11.5-12.5 male and female EG cells, which showed that while the p57kip2 was unmethylated in undifferentiated and imprint-free EG cells, both alleles became methylated after differentiation of EG cells (Tada et al., 1998). Furthermore, in embryos derived from the non growing (ng) and fully grown (fg) oocytes (Kono et al., 1998), the p57kip2 allele inherited from the imprint-free ng nucleus was repressed while that from fg nuclei was expressed. All the evidence suggests that activation of the p57kip2 gene requires passage through the developing oocyte. This is particularly interesting because both p57kip2 and the H19 genes are present within the same imprinted chromosomal region, and they both show expression of the maternal allele (Paulsen et al., 1998). Therefore it is striking that repression of H19 apparently requires passage through oogenesis to be active during development (Obata et al., 1998). Imprinting in some instances is clearly needed to ensure activation rather than repression of the appropriate parental allele, as is the case for Igf2r (Wutz et al., 1997). The effect could be indirect through regulation of closely linked imprinted genes (Barlow et al., 1997). Whether this is the case also for p57Kip2 (and perhaps Mash2) which is repressed in the GC conceptus is yet unknown.

Additional investigation will also be needed to address the relationship, if any, between imprinting of Mash2 and the closely upstream p57kip2 as both genes show maternal allele-specific expression. These genes reside within a 300-500 kb region upstream of Igf2 in which a large number of other imprinted genes with maternal allele-specific expression have been detected (Paulsen et al., 1998). In particular it will be important to know if they are also mechanistically linked as observed for a cluster of paternal allele-specific gene expression (which includes SNRPN) on the human chromosome 15q, which appears to be regulated by an imprinting centre (Dittrich et al., 1996).

Analysis of GC conceptuses and of the developmental potential of GC cells as whole embryos and chimeras suggest that they also differ from androgenones (AG: duplicated paternal genome) and gynogenes (GG: duplicated maternal genome) (Solter, 1988; Surani et al., 1986). For example, GC cells apparently show uniform distribution in chimeras. However, this uniform distribution of GC cells is unlike that of the androgenetic (AG) and gynogenetic (GG) cells which show non-uniform and reciprocal distribution of AG and GG cells.

### Table 4. Expression from imprint-free genome and the predicted influence of gametic imprint

<table>
<thead>
<tr>
<th>Gene</th>
<th>Imprint-free genome in PGC*</th>
<th>Expression in GC embryo</th>
<th>Origin of gametic imprint and its influence in normal embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>H19</td>
<td>On (demethylated)</td>
<td>On (biallelic)</td>
<td>Paternal imprint Off (methylated)</td>
</tr>
<tr>
<td>Igf2</td>
<td>Off</td>
<td>Off</td>
<td>Paternal allele On (reciprocal to H19)</td>
</tr>
<tr>
<td><strong>P57Kip2</strong></td>
<td>Off (methylated)</td>
<td>Off</td>
<td>Maternal imprint On (methylated)</td>
</tr>
<tr>
<td>Mash2</td>
<td>Off</td>
<td>Off</td>
<td>Maternal imprint On</td>
</tr>
<tr>
<td>Igf2r</td>
<td>Off (DMR2 demethylated)</td>
<td>Off</td>
<td>Maternal imprint On (methylated DMR2)</td>
</tr>
<tr>
<td>Nnat</td>
<td>On (demethylated)</td>
<td>On (biallelic)</td>
<td>Paternal imprint Off (methylated)</td>
</tr>
<tr>
<td>{Peg1/Mest}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>{Snrpn}</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*There are equivalent changes in male and female primordial germ cells to generate imprint-free genomes.
†Igf2 expression is the opposite of H19 in cis because of a mechanistic link regulating their expression.
§Prediction from current study.
¶Nnat is a representative of genes that normally show expression of paternal allele, which include Peg1/Mest, Peg 3 and Snrpn.
**p57kip2 is demethylated after erasure of imprints but it becomes methylated if the imprint-free cells undergo differentiation (Tada et al., 1998).
Equivalent epigenetic modifications occur in male and female primordial germ cells (PGCs) during the erasure of gametic imprints. Furthermore, erasure of gametic imprints and DNA methylation can in some instances result in repression of imprinted genes. Thus, after erasure of imprints, repression is the default state for Igf2r, Igf2, p57kip2 and possibly Mash2. These genes were indeed repressed in the germ cell (GC) conceptus. They (except for Igf2) acquire the potential for expression of their maternal alleles in normal embryos only after their passage through oogenesis. By contrast, the default state after the erasure of imprints for the H19 gene is biallelic expression (and repression for the closely linked Igf2 gene). The H19 gene requires passage through spermatogenesis for the repression of the paternal allele, and expression of the closely linked Igf2 gene. For other genes (which show paternal allele-specific expression in normal embryos) represented by Nnat, and including Peg1/Mest, Peg3 and Snrpn, erasure of imprints also establishes biallelic expression as the default state. These genes need to pass through oogenesis for the maternal allele to be repressed. All the findings are consistent with the observation that the GC conceptus phenotype is the result of inheritance of imprint-free epigenotype of the PGC nucleus. Therefore genes including Igf2r, Igf2r, p57kip2 and Mash2 were repressed in the GC conceptus. By contrast, other genes such as H19 and Nnat, as well as Peg1/Mest Peg3 and Snrpn were demethylated. They probably show biallelic expression in the GC conceptus instead of the usual monoallelic expression in normal embryos. This contrasts with the situation in androgenones (two paternal genomes) and gynogenomes (two maternal genomes) (see text for details).
Androgenetic cells contribute substantially to mesodermal tissue such as muscle while GG cells contribute relatively more to the neural tissues. By contrast, GC cells can differentiate into many diverse cell types in chimeras. In this respect at least, GC embryonic cells are similar to the Dmnt−/− ES cells that have lost their inherited allele-specific imprints and regained their ability to differentiate by reintroduction of the Dmnt cDNA (Tucker et al., 1996).

The work by Kono and colleagues on the female germ line using nuclear transplantation studies is informative in the context of this work (Kono et al., 1996; Obata et al., 1998). They combined genomes from non growing (ng) and fully grown (fg) oocytes, from which they obtained more advanced embryonic development to day 13.5 of gestation. This is the result of a combination of an imprint-free ng genome with the fg genome carrying the maternal allele-specific imprints. However, it is intriguing to note that embryos with the imprint-free ng/fg genomes did not progress beyond the 8-cell stage (Kono et al., 1996). This could be because the ng oocytes are in meiotic arrest and the ng/fg embryos are XX, while the male PGCs are XY and in mitotic arrest. The placental abnormality observed in the GC conceptus could potentially be affected if there was X chromosome inactivation in the extraembryonic lineage. However, evidence suggests that the Xist gene that is required for X inactivation, is silenced in the germ line during the erasure process when the inactive X chromosome is reactivated (Tada et al., 1997 and unpublished observations). The erasure process in the male PGCs is therefore compatible with sustaining the X chromosome in an active state in the GC conceptus.

The wider significance of imprinting for mammalian development remains unresolved. One widely accepted view is that imprinting is a consequence of the parent offspring conflict through which parental genomes may counteract each other especially to regulate embryonic growth (Moore and Haig, 1991). These ‘push-pull’ effects may explain some of the growth phenotypes associated with imprinted genes, but perhaps not others, such as behavioural phenotypes seen with the Peg1/Mest gene (Lefebvre et al., 1998). Considering these ideas, Jaenisch (1997) proposed that a mammalian genome free through which parental genomes may counteract each other especially to regulate embryonic growth (Moore and Haig, 1991). These ‘push-pull’ effects may explain some of the growth phenotypes associated with imprinted genes, but perhaps not others, such as behavioural phenotypes seen with the Peg1/Mest gene (Lefebvre et al., 1998). Considering these ideas, Jaenisch (1997) proposed that a mammalian genome free

In conclusion, evidence suggests that the germ cell and oocyte are apparently unique in that they are able to erase epigenetic modifications, such as DNA methylation, from differentiated cell nuclei. However, one significant difference between germ cells and oocytes is that the imprints are not erased in the oocyte, but they are in primordial germ cells (Kafri et al., 1993; Szabo and Mann 1995; Shemer et al., 1997; Olek and Walter 1997; Tada et al., 1997, 1998). Mechanisms evidently exist in the oocyte and early embryo which are capable of erasing genome-wide DNA methylation patterns such as those acquired during spermatogenesis and oogenesis. These demodifications occur in the preimplantation embryo without affecting the allele-specific methylation associated with imprinted genes, which is critical for normal development. The same mechanism may also restore totipotency to the somatic nucleus after transplantation into the oocyte without affecting the gametic imprints. The imprints are likely to be present in most donor somatic nuclei, as they are known to persist into adulthood in the form of stable and heritable DNA methylation modifications. By contrast, the PGC nucleus lacked these imprints (Szabo and Mann 1995; Shemer et al., 1997; Tada et al., 1997, 1998), a notion which is consistent with our observations on the phenotype and the epigenotype of the GC conceptus. Thus, the imprint-free PGC genome could not acquire the appropriate allele-specific methylation for imprinted genes when transplanted into oocytes. The erasure and re-initiation of parental imprints are events that are unique to the developing germ line.

We thank Anne McLaren, Justin Ainscough, Li Lan Li, Masako and Takashi Tada as well as other members of the group for valuable comments throughout this study. Y. K. was supported by the Human Science Foundation and Narashige Zoological Science Award and W. M. R. was supported by a Hitchings-Ellion Fellowship from the Burroughs-Wellcome Fund. This work was supported by a grant from the Wellcome Trust to M. A. S.

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