INTRODUCTION

Cells that give rise to the primordial germ cells (PGCs) of the mouse are first identified by lineage tracing as a population of cells within the proximal margin of the 6.5 days post coitum (p.c.) epiblast, close to the junction with the extraembryonic ectoderm (Lawson and Hage, 1994). By 7.5 days p.c. PGCs have migrated to the extraembryonic mesoderm at the posterior of the primitive streak near the base of the allantois where they can be identified by staining for endogenous alkaline phosphatase (AP) (Ginsburg et al., 1990). The PGCs then become associated with the hindgut endoderm and migrate through the gut mesentery and begin to arrive at the genital ridges by 10.5 days p.c. During the process of moving from the posterior primitive streak to the genital ridges PGCs increase in number from about 150 cells at 8.5 days p.c. to approximately 25,000 at 13.5 days p.c. (Tam and Snow, 1981). By 13.5 days p.c., PGCs within the genital ridge cease dividing; those in the female enter meiosis and those in the male undergo mitotic arrest (Ginsburg et al., 1990).

Given the importance of PGCs to the continuation of the species, it is of interest to define the in vivo factors that control their proliferation, survival and migration. Progress has come from the study of mouse mutants, such as Dominant White-spotting (W) and Steel (Sl), in which PGCs do not follow their normal tightly regulated pattern of proliferation and differentiation, resulting in severe defects in fertility in homozygotes (Mintz and Russell, 1957; McCoshen and McCallion, 1975). The finding that W and Sl encode, respectively, the c-kit tyrosine kinase transmembrane receptor (Chabot et al., 1988; Geissler et al., 1988) and its ligand, Steel factor (SF, also known as stem cell factor, mast cell growth factor and c-kit-ligand), (Williams et al., 1990; Flanagan and Leder, 1990; Zsebo et al., 1990; Huang et al., 1990,) suggests that polypeptide growth factors, their receptors and downstream intracellular signalling pathways play key roles in regulating the proliferation pattern and germ cell competence. A significant difference was observed between embryonic stem cell and embryonic germ cell lines in their ability to maintain the methylation imprint of the Igf2r gene in culture. This may illustrate a fundamental difference between these two cell types.

SUMMARY

Primordial germ cells of the mouse cultured on feeder layers with leukemia inhibitory factor, Steel factor and basic fibroblast growth factor give rise to cells that resemble undifferentiated blastocyst-derived embryonic stem cells. These primordial germ cell-derived embryonic germ cells can be induced to differentiate extensively in culture, form teratocarcinomas when injected into nude mice and contribute to chimeras when injected into host blastocysts. Here, we report the derivation of multiple embryonic germ cell lines from 8.5 days post coitum embryos of C57BL/6 inbred mice. Four independent embryonic germ cell lines with normal male karyotypes have formed chimeras when injected into BALB/c host blastocysts and two of these lines have transmitted coat color markers through the germline. We also show that pluripotent cell lines capable of forming teratocarcinomas and coat color chimeras can be established from primordial germ cells of 8.0 days p.c. embryos and 12.5 days p.c. genital ridges. We have examined the methylation status of the putative imprinting box of the insulin-like growth factor type 2 receptor gene (Igf2r) in these embryonic germ cell lines. No correlation was found between methylation pattern and germ cell competence. A significant difference was observed between embryonic stem cell and embryonic germ cell lines in their ability to maintain the methylation imprint of the Igf2r gene in culture. This may illustrate a fundamental difference between these two cell types.

Key words: primordial germ cells, embryonic germ cells, embryonic stem cells, imprinting, growth factors, insulin-like growth factor type 2 receptor gene (Igf2r), methylation

INTRODUCTION

Cells that give rise to the primordial germ cells (PGCs) of the mouse are first identified by lineage tracing as a population of cells within the proximal margin of the 6.5 days post coitum (p.c.) epiblast, close to the junction with the extraembryonic ectoderm (Lawson and Hage, 1994). By 7.5 days p.c. PGCs have migrated to the extraembryonic mesoderm at the posterior of the primitive streak near the base of the allantois where they can be identified by staining for endogenous alkaline phosphatase (AP) (Ginsburg et al., 1990). The PGCs then become associated with the hindgut endoderm and migrate through the gut mesentery and begin to arrive at the genital ridges by 10.5 days p.c. During the process of moving from the posterior primitive streak to the genital ridges PGCs increase in number from about 150 cells at 8.5 days p.c. to approximately 25,000 at 13.5 days p.c. (Tam and Snow, 1981). By 13.5 days p.c., PGCs within the genital ridge cease dividing; those in the female enter meiosis and those in the male undergo mitotic arrest (Ginsburg et al., 1990).

Given the importance of PGCs to the continuation of the species, it is of interest to define the in vivo factors that control their proliferation, survival and migration. Progress has come from the study of mouse mutants, such as Dominant White-spotting (W) and Steel (Sl), in which PGCs do not follow their normal tightly regulated pattern of proliferation and differentiation, resulting in severe defects in fertility in homozygotes (Mintz and Russell, 1957; McCoshen and McCallion, 1975). The finding that W and Sl encode, respectively, the c-kit tyrosine kinase transmembrane receptor (Chabot et al., 1988; Geissler et al., 1988) and its ligand, Steel factor (SF, also known as stem cell factor, mast cell growth factor and c-kit-ligand), (Williams et al., 1990; Flanagan and Leder, 1990; Zsebo et al., 1990; Huang et al., 1990,) suggests that polypeptide growth factors, their receptors and downstream intracellular signalling pathways play key roles in regulating the proliferation, migration and differentiation of PGCs.

Previous work from our laboratory (Matsui et al., 1991,1992) and others (Dolci et al., 1991; Godin et al., 1991; Resnick et al., 1992) has shown that specific soluble and membrane-associated polypeptide growth factors are necessary for PGCs to survive and proliferate in culture. When PGCs from 8.5 days p.c. embryos are cultured with a mixture of membrane associated and soluble SF, leukemia inhibitory
factor (LIF) and basic fibroblast growth factor (bFGF). PGCs continue to divide in culture and give rise to lines of undifferentiated cells. These cells have been termed embryonic germ cells or EG cells to distinguish them from blastocyst-derived embryonic stem (ES) cells (Resnick et al., 1992). Once established, these EG cells can proliferate without added GFG and SF, but they still require feeder cells and serum factors. EG cells can differentiate into embryoid bodies in vitro and form teratocarcinomas in vivo. Recent studies have shown that EG cell lines derived from 129/Sv 8.5 days p.c. PGCs can contribute to the germline of chimeric mice (Stewart et al., 1994) and we show here that the same is true for EG cell lines derived from C57BL/6 PGCs.

One of the events associated with germ cell differentiation is a change in the methylation status of paternally imprinted genes (Barlow, 1993). Because it is not feasible to isolate large numbers of primordial germ cells from very early embryos, we have studied EG cell lines established from PGCs at various times during development in the hope of gaining some information about the imprinting process in vivo. We have analyzed the methylation state of the putative imprinting box of the maternally expressed insulin-like growth factor 2 receptor (Igf2r) gene (Stöger et al., 1993) in different EG cell lines and show that the methylation of region 2 of the Igf2r gene in most EG cell lines differs from that characteristic of normal somatic cells and ES cells. Moreover, in one cell line this methylation is lost in culture. However, there appears to be no correlation between the methylation pattern and the ability of the cells to contribute to the germline of chimeric mice. This illustrates a difference between ES cells and EG cells, a distinction that may reflect the difference in the developmental program of an inner cell mass cell versus a PGC.

MATERIALS AND METHODS

Mouse embryos and cell lines

C57BL/6, BALB/c, Swiss Webster, ICR, (C57BL/6 × DBA)F1 and nude mice were obtained from Jackson Labs, Harlan Labs or Taconic Farms. Noon on the day of plug is 0.5 days post coitum (p.c.). The ES cell lines tested were all derived from 129/Sv mice. Cell lines D3, J1 and R1 were kindly provided by Drs Thomas Farms. Noon on the day of plug is 0.5 days post coitum (p.c.). The ES cell lines tested were all derived from 129/Sv mice. Cell lines D3, J1 and R1 were kindly provided by Drs Thomas Farms. Noon on the day of plug is 0.5 days post coitum (p.c.). The ES cell lines tested were all derived from 129/Sv mice. Cell lines D3, J1 and R1 were kindly provided by Drs Thomas Farms. Noon on the day of plug is 0.5 days post coitum (p.c.). The ES cell lines tested were all derived from 129/Sv mice. Cell lines D3, J1 and R1 were kindly provided by Drs Thomas Farms. Noon on the day of plug is 0.5 days post coitum (p.c.).

PGC culture

Cultures were initiated as described (Matsui et al., 1992) by dissecting C57BL/6 8.5 days p.c. embryos free of extraembryonic tissues. Fragments comprising the posterior third of the embryo (from the base of the allantois to the first somite) were then pooled, rinsed with Dulbecco’s Ca2+-, Mg2+-free phosphate-buffered saline (PBS) and dissociated with 0.25% trypsin, 1 mM EDTA (GIBCO) and sterile pipetting. This single cell suspension is then plated in 0.1% gelatin-coated 24-well dishes (Corning) with irradiated SI/SII m220 cells as feeder layers at a concentration of approximately 0.5 embryo equivalents per well. The cultures were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Specialty Media, Lavallette, NJ) supplemented with 4.5 g/l glucose, 0.01 mM non-essential amino acids (GIBCO), 2 mM glutamine (GIBCO), 50 µg/ml gentamycin (Sigma), 15% fetal bovine serum (selected batches, Hyclone) and 0.1 mM 2-mercaptoethanol (Sigma). For these primary cultures, the medium was additionally supplemented with soluble recombinant rat SF at 60 ng/ml, bFGF at 20 ng/ml (GIBCO) and LIF at 20 ng/ml. After 6 days some of the cultures are stained for alkaline phosphatase (AP) as described (Matsui et al., 1992) in order to assess the survival and proliferation of PGCs. After 10 days, parallel cultures are dissociated into single cells and plated onto mouse embryonic fibroblast (mef) feeder layers with LIF (ESGRO, GIBCO 1000 U/ml). These cultures were monitored for the appearance of colonies of EG cells. Individual EG colonies were isolated with a micropipette and lines established. EG cultures were then maintained in the same manner as ES cell lines with irradiated mefs as feeder cells and LIF (Smith et al., 1988; Williams et al., 1988). Cultures from 12.5 days p.c. genital ridges or 8.0 days p.c. embryos were initiated and maintained in a similar manner.

Blastocyst injection

All EG cell lines were karyotyped (Robertson, 1987) and only lines with 80% or more of the cells containing 40 chromosomes were used for blastocyst injection. Ten to twenty EG cells at passage numbers 6 to 10 were injected into 3.5 days p.c. blastocysts from BALB/c mice. Foster mothers were (C57BL/6 × DBA)F1 females mated to vasectomized Swiss Webster males. Injected blastocysts were transferred to the uterus of 2.5 days p.c. foster mothers (Hogan et al., 1986) and chimeric pups were identified by their coat color. Chimeras were bred to either BALB/c or ICR mice and germline transmission was judged on the day of birth by the presence of eye pigment.

Southern analysis

EG cell lines from passage number 6 to 10 were cultured without feeder layers on gelatin-coated 6 cm tissue culture dishes (Corning) for at least two passages in order to remove contaminating feeder cells while differentiation was inhibited by the continued presence of LIF in the medium. Cultures were dissociated with 0.25% trypsin, 1 mM EDTA, washed 3× with Dulbecco’s Ca2+-, Mg2+-free phosphate-buffered saline (PBS) and lysed in TENS (100 mM EDTA, 50 mM Tris-HCl pH 8.0, 100 mM NaCl) with 1% SDS. The lysate was incubated overnight at 55°C with 0.5 mg/ml Proteinase K. The lysates were extracted first with phenol, then with phenol:chloroform:isoamyl alcohol (25:24:1), digested with either HpaII or PvuII, and separated on a 0.8% agarose gel. Southern blots were generated by standard methods (Sambrook et al., 1989) and filters were hybridized with an intron-specific probe covering region 2 of the Igf2r gene (see Stöger et al., 1993). A probe from region 1 of the Igf2r gene was used to check for complete digestion of genomic DNA (pXLig2r, see Stöger et al., 1993). All Southern blots were repeated with at least two independent restriction digests of the genomic DNA.

PCR for Zfhy

DNA from EG cell lines was analyzed by PCR for the presence of a Y chromosome. Primers used were 5′-AGATAAGCTTTA-CATAACACATGGA and 3′-CCTAGGAATCTTTTGCTGCA-CATGT (Nagamine et al., 1989). PCR reactions were cycled at 94°C for 45 seconds, 62°C for 25 seconds and 72°C for 1 minute for 30 cycles. The presence of a Y chromosome was indicated by a 600 base pair reaction product.

Skeletal preparations

Skeletal preparations were performed essentially as described (McLeod, 1980). Briefly, newborn mice were first killed using Metofane, then incubated in distilled water at room temperature
overnight, scalped with hot water for thirty seconds, skinned and eviscerated. Cartilage was stained with Alcian Blue and bone counterstained with Alizarin Red. Skeletons were cleared with KOH and glycerin then stored in glycerin.

RESULTS

EG cell lines derived from C57BL/6 8.5 days p.c.
PGCs can contribute to the germline of chimeric mice

Initial experiments from our laboratory to derive EG cells in culture used embryos from matings of ICR random bred females with (C57BL/6 × DBA)F1 males (Matsui et al., 1991, 1992). Here, we have used the inbred mouse strain C57BL/6 to control for any genetic differences that may affect the derivation of EG cell lines. C57BL/6 blastocysts have been used previously to generate totipotent ES lines (Ledermann and Burki, 1991).

EG cell lines were first obtained from 8.5 days p.c. C57BL/6 embryos and early passage EG cells (passage number 6-10) were used for both blastocyst injection and genomic DNA isolation. Cell lines were also tested for the presence of a Y chromosome by performing PCR with primers specific for Zfy (see Methods). All cell lines were karyotyped and male cell lines with a normal karyotype were injected into BALB/c blastocysts. Table 1 shows the number of chimeras obtained from four of these EG lines. Male and female chimeras were bred to either BALB/c or ICR mice to test for the ability of the cells to transmit the C57BL/6 genome through the germline (Table 2; Fig. 1). No female chimeras gave birth to pigmented pups.

Generation of EG cell lines from gonadal PGCs

To ask whether PGCs become restricted in their ability to form EG cell lines after entering the gonad, we repeated these experiments with germ cells from later stage C57BL/6 embryos. Germ cells from the gonads of 15.5 days p.c. embryos and early passage EG cells (passage number 6-10) were used for both blastocyst injection and genomic DNA isolation. The results of these experiments are shown in Table 2. Male chimeras from two independent cell lines (TGC8.510 and TGC8.519) were able to transmit the C57BL/6 genome through the germline (Table 2; Fig. 1). The contribution of these EG cells to the viable chimeras ranges from <5% to approximately 40% as judged by coat color.

Imprinting of the Igf2r receptor gene

In the somatic tissues of the adult mouse, the Igf2r gene is parentally imprinted with expression only from the paternal allele (Barlow et al., 1991). Recent work has identified a specific region in the Igf2r gene, region 2, within the second intron 27 kilobases downstream of the transcriptional start site of the gene, that is hypermethylated only on the paternally inherited allele (Stöger et al., 1993). This methylation pattern may be one factor regulating the transcription of Igf2r, as mice that possess two mutated copies of the methyltransferase gene embryo. From several separate experiments, ten cell lines were obtained from the equivalent of 6/100 of a genital ridge (approximately 432 PGCs, as calculated from Tam and Snow, 1981) as compared to more than 20 from the equivalent of the posterior portion of 1/6 of an 8.5 days p.c. embryo (approximately 24 PGCs, as calculated from Tam and Snow, 1981). All ten cell lines were derived from male gonads; no cell lines were obtained from female gonads.

Table 1. Chimeras from blastocyst injection of EG cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Passage number</th>
<th>Blastocysts injected</th>
<th>Pups born</th>
<th>Total chimeras</th>
<th>Chimeras not surviving</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG cell lines derived from 8.5 days p.c. embryos</td>
<td>TGC8.510</td>
<td>P6-12</td>
<td>181</td>
<td>63</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>TGC8.511</td>
<td>P6-9</td>
<td>41</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>TGC8.512</td>
<td>P8-10</td>
<td>62</td>
<td>36</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>TGC8.519</td>
<td>P7-10</td>
<td>129</td>
<td>39</td>
<td>7</td>
</tr>
<tr>
<td>EG cell lines derived from 12.5 days p.c. genital ridges</td>
<td>TGC12.51</td>
<td>P6-13</td>
<td>193</td>
<td>94</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>TGC12.52</td>
<td>P8-12</td>
<td>156</td>
<td>64</td>
<td>3</td>
</tr>
</tbody>
</table>

The contribution of these EG cells to the viable chimeras ranges from <5% to approximately 40% as judged by coat color.

Table 2. Germline transmission of the EG cell lines TGC8.510 and TGC8.519

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Number of chimeras</th>
<th>Number of pups</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGC8.510 male chimeras transmitting only BALB/c genome</td>
<td>5 individuals</td>
<td>123 pups</td>
</tr>
<tr>
<td>TGC8.510 male chimeras transmitting both C57BL/6 and BALB/c genomes</td>
<td>Male #3</td>
<td>1 of 71 pups pigmented</td>
</tr>
<tr>
<td></td>
<td>Male #12</td>
<td>5 of 21 pups pigmented</td>
</tr>
<tr>
<td></td>
<td>Male #13</td>
<td>1 of 32 pups pigmented</td>
</tr>
<tr>
<td></td>
<td>Male #14</td>
<td>1 of 18 pups pigmented</td>
</tr>
<tr>
<td>TGC8.519 male chimeras transmitting only BALB/c genome</td>
<td>5 individuals</td>
<td>68 pups</td>
</tr>
<tr>
<td>TGC8.519 male chimeras transmitting both C57BL/6 and BALB/c genomes</td>
<td>Male #1</td>
<td>2 of 62 pups pigmented</td>
</tr>
</tbody>
</table>
display an altered methylation pattern in this region as well as
downregulated expression of *Igf2r* (Li et al., 1993). Fig. 2
shows the methylation pattern of *Igf2r* region 2 for some of the
EG cell lines tested. Half of the 8.5 days p.c. derived EG cell
lines (9 out of 18), including TGC8.5 10, one of the two cell
lines to transmit through the germline, show both a 3 kilobase
and a 500 base pair hybridizing fragment implying that they
carry one methylated and one non-methylated allele (Fig.
2C,D). This pattern of methylation is characteristic of somatic
cells (Fig. 2A, mefs) and 5 different pluripotent ES cell lines
(Fig. 2A for D3 and R1, and data not shown for J1, SC6 and
TL1. The remaining cell lines, including TGC8.519, the other
totipotent cell line, have a different pattern of methylation in
which both alleles are unmethylated (Fig. 2C, D; Table 3) and
show only a 500 base pair hybridizing fragment.

In order to evaluate the methylation pattern of *Igf2r* region
2 during PGC maturation, EG cell lines were established from
PGCs at different times. EG cell lines from earlier PGCs (8.0
days p.c.) were generated and these cell lines were assayed for
their methylation pattern. The results were similar to those with
the EG cell lines derived from 8.5 days p.c. PGCs. Some cell
lines (10 out of 18) showed both alleles to be unmethylated
(for example TGC8.59, TGC8.010, TGC8.014, TGC8.015 and
TGC8.016 in Fig. 2B; Table 3) whereas others (8 out of 18)
showed a methylated and an unmethylated allele (for example
TGC8.512 in Fig. 2B; Table 3). All ten EG cell lines derived
from 12.5 days p.c. genital ridges showed an unmethylated
pattern for region 2 (Fig. 2E).

Occasionally, when longer exposures of Southern blots were
examined, some cell lines showed a faint 3 kb hybridizing
fragment that was much less intense than the 3 kb fragment of
either ES cells or fibroblasts. We interpret this as partial methylation of region 2, due to heterogeneity in the cultures. A summary of the methylation pattern of region 2 and the sex of the cell lines is presented in Table 3. There appears to be no correlation between the methylation pattern and the sex of the cells.

In order to test the possibility that the methylation pattern of region 2 of the Igf2r could change as ES and EG cell lines are grown in culture, several lines were cultured to late passage numbers (between 17 and 34). We have cultured ES cells to later passages (p = 17) and have never seen alteration of the normal somatic pattern of methylation. Two of the three EG cell lines tested (TGC8.519 and TGC12.58) had both alleles of Igf2r unmethylated at early passage. As shown in Fig. 3, both of these cell lines maintained this methylation pattern in culture, even as late as passage 34. In contrast, TGC8.35, which had partial methylation at early passages (p = 7, 11, 15), lost this methylation and the 3 kb hybridizing fragment by late passage numbers (p = 20, 26, 29).

**DISCUSSION**

**Derivation of EG cell lines by treating PGCs with growth factors**

Previous work from our laboratory (Matsui et al., 1991, 1992; Labosky et al., 1994) and others (Dolci et al., 1991; Godin et al., 1991; Resnick et al., 1992) and the data reported here show that the combination of SF, LIF and bFGF allows the establishment of EG cell lines. The mechanism by which this is achieved is not clear. One possibility is that this combination of growth factors somehow alters the developmental program of the PGCs so that they now behave like the epiblast or inner mass cells from which they were derived. Alternatively, the combination of growth factors may block the terminal differentiation of the PGCs, arresting them as an immature or precursor PGCs. At present the only difference that we have observed between EG cells and blastocyst-derived ES cells is the pattern of DNA methylation within region 2 of the Igf2r gene. Both cell types differentiate in vitro and in vivo, and both EG cells and ES cells can differentiate into functional sperm and thereby transmit their genome through the germline (Table 2; Matsui et al., 1992; Labosky et al., 1994; Stewart et al., 1994). Our results regarding the methylation pattern of region 2 of the Igf2r gene support the second alternative, that the EG cells resemble an immature germ cell rather than an inner cell mass cell, since most of the EG cell lines have a pattern of methylation more similar to that of late germ cells (Brandeis et al., 1993) than somatic cells and ES cells. Additionally, we have used our culture conditions with epiblasts from earlier embryos (6.5 days p.c.) and so far have been unable to establish stem cell lines, further supporting the theory that the EG cell lines derived from 8.0 and 8.5 days p.c. PGCs do not resemble epiblast cells.

Our data here suggest that PGCs from 8.5 days p.c. embryos and 12.5 days p.c. genital ridges are not equivalent in their response to the combination of SF, bFGF and LIF. Although pluripotent EG cell lines can be derived from both 8.5 and 12.5 days p.c. germ cells, the process is much more efficient with younger PGCs. This is illustrated by the observation that only ten cell lines were obtained from approximately four hundred 12.5 days p.c. PGCs while over twenty cell lines were obtained from an estimated twenty four 8.5 days p.c. PGCs. It appears that germ cells in 15.5 days p.c., newborn and 8 days postnatal

![Fig. 2. Methylation status of region 2 of the Igf2r gene. Southern blots of genomic DNA from different cell lines digested with restriction enzymes HpaII and PvuII and hybridized with a probe for region 2 of the Igf2r gene. (A) D3 and R1, two different ES cell lines, and mouse embryo fibroblasts (mef). (B) EG cell lines derived from 8.0 days p.c. primordial germ cells. (C) EG cell lines derived from 8.5 days p.c., primordial germ cells. (D) The two EG cell lines derived from 8.5 days p.c. primordial germ cells which transmitted through the germline. (E) EG cell lines derived from 12.5 days p.c. genital ridges.](image-url)
gonads do not respond to this combination of growth factors since we have been unable to establish EG cell lines from these stages with C57BL/6 inbred mice. Germ cells become postmitotic after they reach the genital ridge and it is possible that as they become quiescent they no longer respond to SF, LIF and bFGF perhaps because they downregulate one or more receptors for these factors. In support of this hypothesis, it has been shown (Manova and Bachvarova, 1991) that c-kit, the receptor for SF, is down regulated in PGCs shortly after they reach the genital ridge.

Our data raise the possibility that the EG cell lines derived from 12.5 days p.c. PGCs may be qualitatively different from the EG cell lines derived from 8.5 days p.c. PGCs. The EG cell lines derived from 12.5 days p.c. PGCs, while pluripotent, do not appear to be totipotent. A sex bias is not seen in chimeras after blastocyst injection and these resulting chimeras are less extensive than chimeras from EG cell lines derived from 8.5 days p.c. PGCs (as judged by coat color contribution) even when as many as 20 EG cells are used for each blastocyst injection (Table 2). Possible reasons for a qualitative difference are discussed below.

**Imprinting of the Igf2r gene in EG cells**

We have shown here that in approximately half of the EG cell lines derived from 8.0-8.5 days p.c. PGCs and all of the EG cell lines derived from 12.5 days p.c. genital ridges the methylation of region 2 of the Igf2r gene differs from normal somatic cells. Instead of the normal region 2 methylation pattern, with the maternal allele methylated and the paternal allele unmethylated, these EG cell lines have both alleles unmethylated in the majority of the cells.

There are several possible explanations for these methylation differences. First, it may reflect the in vivo methylation status of the Igf2r gene in the founding PGCs. The mouse embryo undergoes massive general demethylation of genomic DNA at the 8- to 16-cell stage and a subsequent remethylation, which is completed by implantation (Monk et al., 1987). In germ cells, the general methylation changes are not identical to those seen in the rest of the embryo (Monk et al., 1987; Kafri et al., 1992). While the DNA in the somatic cells of the embryo has begun to be remethylated by 4.5 days p.c., germ cells remain relatively unmethylated at 12.5-13.5 days p.c.. By 15.5 days p.c. they are partially remethylated, with the process completed by 18.5 days p.c.. These observations on general demethylation of genomic DNA do not apply to region 2 of the Igf2r gene since the maternal-specific methylation of this region, directly inherited from the oocyte, is preserved through preimplantation (Stöger et al., 1993; Brandeis et al., 1993). However, region 2 does become unmethylated in both male and female germ cells by 13.5 days p.c. (Brandeis et al., 1993), implying that the genomic imprint for Igf2r is erased sometime between the blastocyst and 13.5 days p.c.. If the methylation pattern for Igf2r region 2 of our EG cell lines represents the methylation pattern of founding PGCs, the absence of methylation reported here in most EG cell lines reflects erasure of the Igf2r genomic imprint. Our results suggest that some PGCs have erased their imprint by 8.0-8.5 days p.c., with all having done so by 12.5 days p.c.

At present we cannot distinguish between an erased or an androgenetic imprint of the Igf2r gene (both alleles unmethylated), but our observations for the Igf2r gene may reflect a general absence or erasure of imprinting signals in the EG cells and not simply an androgenetic pattern. In support of this speculation, our results showing that EG cells from 8.5 days p.c. PGCs can form healthy chimeras and in some cases contribute to the germline illustrates that these EG cells differ from androgenetic ES cells (Mann et al., 1990; Mann and Stewart, 1991). However, we cannot eliminate the possibility that some of the EG cells from gonadal 12.5 days p.c. PGCs have acquired an androgenetic imprint since some of the skeletal defects of the chimeras obtained with these EG cells resemble the defects of androgenetic ES cell chimeras, although they are less severe and not as prevalent.

An alternative explanation for the unmethylated pattern in the EG cells is that methylation of Igf2r region 2 is not stable during in vitro culture. We have shown here that the somatic pattern of methylation seen in blastocyst-derived ES cells remains stable for up to 17 passages in culture. In addition, the unmethylated pattern in EG cells remains stable in several different lines for up to 34 passages. However, one EG cell line (TGC9.5) that displayed methylation at early passage (p=7, 11, 15) lost this methylation by later passages (p=20, 26, 29).
This observation implies that the methylation differences between different EG cell lines may not pre-exist before the lines were established but instead are the result of a difference in their response to in vitro culture. It is also possible that the methylation is unstable in EG cells because of their origin from PGCs. This instability may reflect an in vivo difference between PGC-derived EG cell lines and inner cell mass-derived ES cell lines, reflecting the difference in the developmental program of the founding cells. It is also noteworthy that, once unmethylated, the methylation of region 2 is not regained, implying that this erased pattern may be the normal end result for a PGC as it reaches the gonad.

The difference in the methylation pattern of region 2 in TGC8.510 and TGC8.519 (Fig. 2D) implies that the imprinted status of the Igf2r gene in an EG cell line does not affect its ability to pass through the germline. This result is perhaps not surprising in view of the fact that, although parthenogenetic embryos cannot form normal mice (Stevens, 1978; Clarke et al., 1988; and Fundele et al., 1990), it has been observed that parthenogenetic ES cells can contribute to chimeras and form functional germ cells giving rise to normal offspring (J. Mann and C. Stewart, unpublished data).

In conclusion, the two hypotheses that we have proposed for the differences in methylation of region 2 of the Igf2r gene between ES cells and EG cells may not be mutually exclusive. It is possible that the methylation differences between the various early passage EG cell lines reflect the changing methylation status of the migrating PGCs. It is also possible that the heterogeneity and plasticity of this methylation pattern in vitro is another property of PGCs. PGCs are programmed to erase their imprint in vivo and may be carrying out that program in vitro. In the future, the identification of specific regions such as region 2 in the Igf2r gene that regulate other imprinted genes will allow the generalities of these ideas to be explored.

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