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Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (*Igf2r*) gene compared with embryonic stem (ES) cell lines

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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 germline 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 FGF 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 chimeras (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 parentally 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)F_1 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 Doetschman, Rudolph Jaenisch and Janet Rossant, respectively. SC6 was derived from AB-1 cells obtained from Dr Allan Bradley. TL1 was derived in this laboratory.

Tumors were initiated in nude mice by the subcutaneous injection of 1×10^6 cells at each of four locations on the back. Tumors were dissected and processed for histological analysis as described (Matsui et al., 1992).

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 Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS) and dissociated with 0.25% trypsin, 1 mM EDTA (GIBCO) and gentle pipetting. This single cell suspension is then plated in 0.1% gelatin-coated 24-well dishes (Corning) with irradiated *Sl/Sl*^{4} 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 embryo 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)F_1 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 Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS) and lysed in TENS (100 mM EDTA, 50 mM Tris-Hcl pH8.0, 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) and then with chlorform:isoamyl alcohol (24:1). Genomic DNA was ethanol precipitated and spooled onto a capillary tube. Southern blots were performed as described (Stöger et al., 1993). Briefly, DNA was dissolved in 10 mM Tris-HCl pH 7.6, 1 mM EDTA (TE), digested with the restriction enzymes *Hpa*II and *Pvu*II, 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* receptor (pPP4, see Stöger et al., 1993). A probe from region 1 of the *Igf2r* gene was used to check for complete digestion of genomic DNA (pX*Igf2r*, see Stöger et al., 1993). All Southern blots were repeated with at least two independent restriction digests of the genomic DNA.

PCR for Zfy

DNA from EG cell lines was analyzed by PCR for the presence of a Y chromosome. Primers used were 5′-AAGATAAGCTTA-CATAATCACATGGA and 3′-CCTATGAAATCCTTTGCTGCA-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, scalded with hot water for thirty seconds, skinned and eviscerated. Cartilage was stained with Alcian Blue and bone counterstained with Alizerin 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)F_1 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 differentiate into functional germ cells. The results of these matings are shown in Table 2. Male chimeras from two independent cell lines ($TGC^{8.5}10$ and $TGC^{8.5}19$) were able to transmit the C57BL/6 genome through the germline (Table 2; Fig. 1). No female chimeras gave birth to pigmented pups.

Table 2. Germline transmission of the EG cell lines $TGC^{8.5}10$ and $TGC^{8.5}19$

	Number of chimeras	Number of pups
$TGC^{8.5}10$ male chimeras transmitting only BALB/c genome	5 individuals	123 pups
$TGC^{8.5}10$ male chimeras transmitting both C57BL/6 and BALB/c genomes	Male #3 Male #12 Male #13 Male #14	1 of 71 pups pigmented 5 of 21 pups pigmented 1 of 32 pups pigmented 1 of 18 pups pigmented
$TGC^{8.5}19$ male chimeras transmitting only BALB/c genome	5 individuals	68 pups
$TGC^{8.5}19$ male chimeras transmitting both C57BL/6 and BALB/c genomes	Male #1	2 of 62 pups pigmented

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 newborn mice did not give rise to EG cell lines under the conditions used previously. However, we were able to derive EG cell lines from the genital ridges of 12.5 days p.c embryos, at a time when most of the PGCs have migrated into them. At 12.5 days p.c. it is possible to identify the sex of the genital ridge by its morphology, so male and female genital ridges were pooled separately. The frequency of EG cell line formation is much lower with genital ridges from 12.5 days p.c. embryos compared to posterior tissue of 8.5 days p.c. embryos. 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. Two karyotypically normal cell lines were tested and shown to form differentiated tumors in nude mice and contribute to coat color chimeras. Table 1 shows the frequency of chimerism for these two EG cell lines ($TGC^{12.5}1$ and $TGC^{12.5}2$). The chimeras did not show a bias toward maleness; of the 9 surviving adult chimeras from both cell lines, there are 6 females and 3 males. These were bred to test for germline transmission but no pigmented pups resulted from these matings. Two of eleven identified chimeras from these two cell lines exhibited skeletal abnormalities and died soon after birth (within one to two days). The skeleton of one of these two newborns exhibited malformations of the rib cage and sternum (data not shown).

Imprinting of the Igf2 receptor gene

In the somatic tissues of the adult mouse, the *Igf2r* gene is parentally imprinted with expression only from the maternal 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 maternally 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

Table 1. Chimeras from blastocyst injection of EG cell lines

	Cell line	Passage number	Blastocysts injected	Pups born	Total chimeras	Chimeras not surviving
EG cell lines derived from 8.5 days p.c. embryos	$TGC^{8.5}10$	P6-12	181	63	21	5
	$TGC^{8.5}11$	P6-9	41	18	3	1
	$TGC^{8.5}12$	P8-10	62	36	3	3
	$TGC^{8.5}19$	P7-10	129	39	7	0
EG cell lines derived from 12.5 days p.c. genital ridges	$TGC^{12.5}1$	P6-13	193	94	8	2
	$TGC^{12.5}2$	P8-12	156	64	3	0

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

Fig. 1. Germline transmission of TGC$^{8.5}$10. Male chimera from EG cell line TGC$^{8.5}$10, ICR random bred female, and pups from two separate litters. Pigmented pups (agouti and non-agouti) illustrate germline transmission of the C57BL/6 derived EG cell line.

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 TGC$^{8.5}$10, one of the two cell lines to transmit through the germline, show both a 3 kilobase and a 500 base pair hybridizing fragment implying that they carry one methylated and one non-methylated allele (Fig. 2C,D). This pattern of methylation is characteristic of somatic cells (Fig. 2A, mefs) and 5 different pluripotent ES cell lines (Fig. 2A for D3 and R1, and data not shown for J1, SC6 and TL1. The remaining cell lines, including TGC$^{8.5}$19, 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 TGC$^{8.0}$9, TGC$^{8.0}$10, TGC$^{8.0}$14, TGC$^{8.0}$15 and TGC$^{8.0}$16 in Fig. 2B; Table 3) whereas others (8 out of 18) showed a methylated and an unmethylated allele (for example TGC$^{8.0}$12 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

Table 3. The methylation status and sex of all EG cell lines

Cell line	Y chromosome	Methylation of region 2
EG cell lines derived from 8.0 days p.c. PGCs		
TGC$^{8.0}$1	+	met/unmet (partial)
TGC$^{8.0}$2	+	met/unmet (partial)
TGC$^{8.0}$3	+	met/unmet (partial)
TGC$^{8.0}$4	+	unmet
TGC$^{8.0}$5	+	met/unmet (partial)
TGC$^{8.0}$7	–	unmet
TGC$^{8.0}$8	+	unmet
TGC$^{8.0}$9	+	unmet
TGC$^{8.0}$10	+	unmet
TGC$^{8.0}$11	+	unmet
TGC$^{8.0}$12	+	met/unmet (somatic)
TGC$^{8.0}$13	+	met/unmet (somatic)
TGC$^{8.0}$14	+	met/unmet (partial)
TGC$^{8.0}$15	+	unmet
TGC$^{8.0}$16	+	unmet
TGC$^{8.0}$17	+	met/unmet (partial)
TGC$^{8.0}$18	+	unmet
TGC$^{8.0}$19	+	unmet
EG cell lines derived from 8.5 days p.c. PGCs		
TGC$^{8.5}$1	+	met/unmet (partial)
TGC$^{8.5}$2	+	met/unmet (partial)
TGC$^{8.5}$3	+	met/unmet (somatic)
TGC$^{8.5}$4	–	unmet
TGC$^{8.5}$5	+	met/unmet (partial)
TGC$^{8.5}$6	+	unmet
TGC$^{8.5}$8	–	unmet
TGC$^{8.5}$9	–	met/unmet (somatic)
TGC$^{8.5}$10	+	met/unmet (somatic)
TGC$^{8.5}$11	+	met/unmet (partial)
TGC$^{8.5}$14	–	met/unmet (partial)
TGC$^{8.5}$15	–	unmet
TGC$^{8.5}$16	–	unmet
TGC$^{8.5}$17	–	unmet
TGC$^{8.5}$18	–	unmet
TGC$^{8.5}$19	+	unmet
TGC$^{8.5}$21	+	met/unmet (somatic)
TGC$^{8.5}$22	–	unmet
EG cell lines derived from 12.5 days p.c. germ cells		
TGC$^{12.5}$1	+	unmet
TGC$^{12.5}$2	+	unmet
TGC$^{12.5}$3	+	unmet
TGC$^{12.5}$4	+	unmet
TGC$^{12.5}$5	+	unmet
TGC$^{12.5}$6	+	unmet
TGC$^{12.5}$7	+	unmet
TGC$^{12.5}$8	+	unmet
TGC$^{12.5}$9	+	unmet
TGC$^{12.5}$10	+	unmet
Other cells		
mouse embryo fibroblasts		met/unmet (somatic)
J1		met/unmet (somatic)
R1		met/unmet (somatic)
D3		met/unmet (somatic)
SC6		met/unmet (somatic)
TL1		met/unmet (somatic)

Footnote: Examples of the different methylation patterns can be seen in the following figures:
somatic pattern in Fig. 2A for ES cells and mefs
partial methylation pattern in Fig. 3A for TGC$^{8.5}$5
unmethylated pattern in Fig. 2D for TGC$^{8.5}$19.

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 ($TGC^{8.5}19$ and $TGC^{12.5}8$) 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, $TGC^{8.5}5$, 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

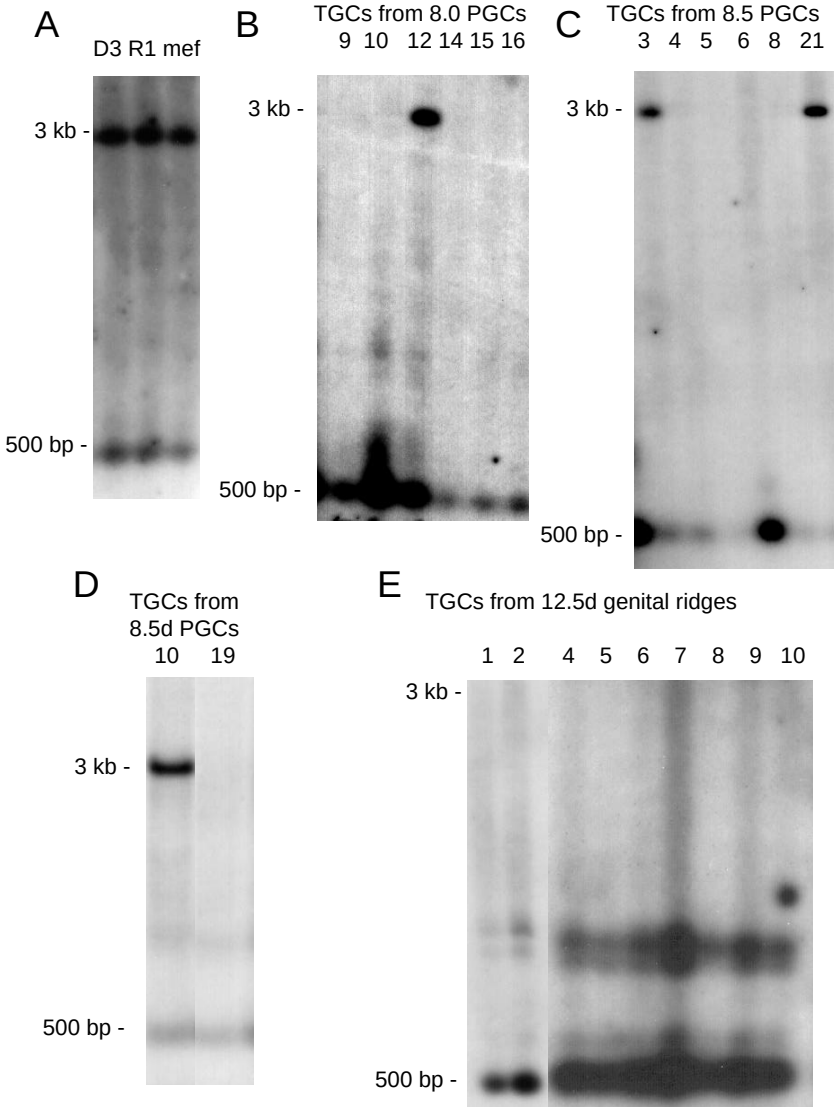

Fig. 2. Methylation status of region 2 of the *Igf2r* gene. Southern blots of genomic DNA from different cell lines digested with restriction enzymes *Hpa*II and *Pvu*II and hybridized with a probe for region 2 of the *Igf2r* gene. (A) D3 and R1, two different ES cell lines, and mouse embryo fibroblasts (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.

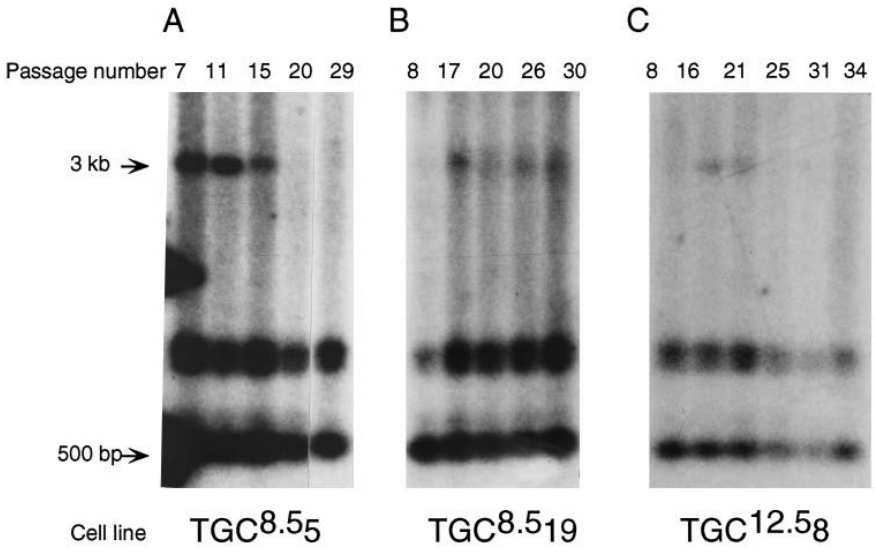

Fig. 3. Temporal changes in methylation status of region 2 of the *Igf2r* gene in EG cell lines. Southern blots of genomic DNA from different cell lines digested with restriction enzymes *Hpa*II and *Pvu*II and hybridized with a probe for region 2 of the *Igf2r* gene. (A) $TGC^{8.5}5$ at passage numbers 7, 11, 15, 20, 29; (B) $TGC^{8.5}19$ at passage numbers 8, 17, 20, 26, 30; (C) $TGC^{12.5}8$ at passage numbers 8, 16, 21, 25, 31, 34.

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 allelle 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 ($TGC^{8.5}5$) 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 $TGC^{8.5}10$ and $TGC^{8.5}19$ (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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