Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells

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Genomic reprogramming of primordial germ cells (PGCs), which includes genome-wide demethylation, prevents aberrant epigenetic modifications from being transmitted to subsequent generations. This process also ensures that homologous chromosomes first acquire an identical epigenetic status before an appropriate switch in the imprintable loci in the female and male germ lines. Embryonic germ (EG) cells have a similar epigenotype to PGCs from which they are derived. We used EG cells to investigate the mechanism of epigenetic modifications in the germ line by analysing the effects on a somatic nucleus in the EG-thymic lymphocyte hybrid cells. There were striking changes in methylation of the somatic nucleus, resulting in demethylation of several imprinted and non-imprinted genes. These epigenetic modifications were heritable and affected gene expression as judged by re-activation of the silent maternal allele of *Peg1/Mest* imprinted gene in the somatic nucleus. This remarkable change in the epigenotype of the somatic nucleus is consistent with the observed pluripotency of the EG-somatic hybrid cells as they differentiated into a variety of tissues in chimeric embryos. The epigenetic modifications observed in EG-somatic cell hybrids *in vitro* are comparable to the reprogramming events that occur during germ cell development.

Keywords: cell fusion/demethylation/embryonic germ cell/epigenetic reprogramming/imprinting

Introduction

Development of primordial germ cells (PGCs) is accompanied by major epigenetic modifications of the genome, including genome-wide demethylation in mice (Monk *et al.*, 1987). This is partly to ensure that no aberrant epigenetic modifications are transmitted to subsequent generations and to obtain an equivalent epigenetic state in the germ line of male and female embryos. Indeed, this is probably the only stage in the life of a mammal when the homologous chromosomes are indistinguishable as far as their epigenetic status is concerned. The subsequent

germ line-specific epigenetic modifications that result in the differences between parental alleles are responsible for the preferential expression of one parental allele of imprinted genes throughout development and in adulthood (Efstratiadis, 1994).

DNA methylation that occurs primarily but not exclusively from gastrulation onwards, is an important component of the allele-specific parental imprints (Li *et al.*, 1993) and of regulation of cell type-specific gene expression (Yeivin and Razin, 1993). Genome-wide demethylation, on the other hand, is evident in pre-implantation embryos and in PGCs (Monk *et al.*, 1987; Kafri *et al.*, 1992; Ueda *et al.*, 1992). During pre-implantation development, genome-wide demethylation may be essential for the restoration of pluripotency in the developing epiblast cells. However, while genome-wide demethylation and bi-allelic expression of some imprinted genes are features of pre-implantation development (Monk *et al.*, 1987; Kafri *et al.*, 1992; Latham *et al.*, 1994), this is crucially without incurring loss of underlying parental imprints (Brandeis *et al.*, 1993; Kafri *et al.*, 1993; Tremblay *et al.*, 1995). The loss of allele-specific imprints does occur in cells or embryos with a null mutation of the DNA methyltransferase gene (Li *et al.*, 1993). However, loss of allele-specific parental imprints also occurs naturally during demethylation in PGCs. Consistent with the loss of allele-specific methylation imprints (Brandeis *et al.*, 1993), at least some imprinted genes have the potential for bi-allelic expression in PGCs (Szabo and Mann, 1995).

Recently, embryonic germ (EG) cells have been derived directly from PGCs at different stages of development (Matsui *et al.*, 1992; Stewart *et al.*, 1994). There are notable similarities in the epigenotype of EG cells and PGCs (Labosky *et al.*, 1994; T.Tada, M.Tada, K.Hilton, S.C.Barton, T.Sado, N.Takagi and M.A.Surani, in preparation). It seemed therefore that EG cells could be used to investigate the mechanism of epigenetic modifications that occur in PGCs. Furthermore, there are advantages in their use as EG cells can be manipulated easily and cultured *in vitro*, and used to generate chimeric embryos. In this context, our recent study showed that erasure of parental imprints occurs at several imprinted loci in EG cells from 11.5–12.5 days post-coitum (dpc) male and female embryos, with the process being almost if not entirely complete in the latter (T.Tada, M.Tada, K.Hilton, S.C.Barton, T.Sado, N.Takagi and M.A.Surani, in preparation).

To examine the nature of epigenetic changes in germ cells, we investigated the fate of the somatic nucleus in a model system consisting of hybrid cells between EG cells from female embryos and thymic lymphocytes. We demonstrate that the EG cells induce reprogramming of the somatic nucleus in hybrid cells resulting in an extensive demethylation of a number of loci. Furthermore, demethylation of a silent maternal allele of an imprinted gene

Fig. 1. Experimental strategy for generating EGF×T hybrid cells. **(A)** *In vitro* system to study: (I) the demethylation/reprogramming of lymphocyte genome from transgenic mice carrying the ROSA26 transgene; (II) the erasure of parental imprints from the lymphocyte genome using the maternally inherited mutant allele of *Peg1/Mest*. **(B)** G-banded metaphase chromosome spread and karyotype of the EGF×T2 hybrid clone. The translocated chromosome between chromosome 2 and X chromosome is indicated (X in A, arrow in B).

resulted in its re-activation. Consequently, the EG–somatic hybrid cells are pluripotent as they differentiate into many cell types in developing chimeric embryos. The experimental system we describe here should provide a valuable adjunct to the understanding of the mechanism of epigenetic reprogramming in germ cells.

Results

Cloning of female EG-somatic hybrid cells

The TMA-58G EG cells were derived from PGCs of female 12.5 dpc embryos heterozygous for the Rb(X.2)2Ad translocation and characterized extensively (Tada *et al.*, in preparation). Here we studied the consequences of cell fusion between the EG cells and somatic cells. The somatic cells were thymic lymphocytes from (129/Sv×129/Sv-ROSA26)F1 female mice carrying a *neo/lacZ* transgene that is expressed ubiquitously (Friedrich and Soriano, 1991). Therefore it was possible to isolate hybrid clones expressing *neo* by their resistance to G418 selection

following electrofusion (see Figure 1A and Materials and methods). As a control, when thymic lymphocytes alone were subjected to a similar procedure, no colonies were detected. The morphology of EG–lymphocyte hybrid cells was similar to that of the EG cells and remained so without any detectable morphological changes.

Ten metaphases in each hybrid clone were subjected to cytogenetic analysis by the G-banding method. This clearly demonstrated that there was a full tetraploid complement of chromosomes in these hybrid cells at least at early passage (Figure 1B). Furthermore, synchronous replication of four X chromosomes was revealed by the R-banding method, indicating that all four X chromosomes were active in hybrid cells (data not shown).

Demethylation of maternally expressed imprinted genes

First, we examined the *H19* gene (Bartolomei *et al.*, 1991; Ferguson-Smith *et al.*, 1993), and in particular, the *HhaI* sites clustered in a 3.8 kb *SacI* fragment upstream to the

Fig. 2. Demethylation of maternally expressed imprinted genes in EGF×T hybrid cells. DNA was extracted from thymus (T), female EG cells (EGF) and their 5-independent fusion clones (EGF×T-1, 2, 4, 5 and 7). Methylated DNA fragments are indicated with arrows and digested fragments with methylation-sensitive restriction enzymes are indicated with circles. (A) *H19*; DNA was digested with *Bam*HI and the methylation-sensitive restriction enzyme *Hha*I (H), and hybridized with 3.8 kb *Sac*I fragment. (B) *p57^{Kip2}*; DNA was digested with *Bam*HI and the methylation-sensitive restriction enzyme *Eag*I, and hybridized with the 0.5 kb *Xho*I–*Eag*I fragment in the 5' region of mouse cDNA. The fully methylated fragment is detected at ~3 kb (arrow). (C) *Igf2r* region 2; DNA was digested with *Pvu*II and the methylation-sensitive restriction enzyme *Mlu*I, and hybridized with a 330 bp fragment (Stöger *et al.*, 1993). (D) *Igf2r* region 1; DNA was digested with *Stu*I and the methylation-sensitive restriction enzyme *Not*I, and hybridized with a 190 bp fragment (Stöger *et al.*, 1993). Uncut fragment with *Not*I is detected at 1.2 kb (arrow) and cut fragment is at 0.5 kb (circle).

transcription start site that are the key paternal-allele-specific methylation imprints (Tremblay *et al.*, 1995). A 10 kb and a 2.7 kb *Bam*HI fragment represent methylated *Hha*I sites, whereas ~8 kb, 0.7 kb and 0.4 kb bands appeared when these sites were unmethylated (Figure 2A). As expected, a methylated paternal and a demethylated maternal allele was obvious in the DNA samples from thymic lymphocytes. However, in both the EG cells and hybrid cell clones, only the unmethylated DNA bands were prominent. Note that for comparison with samples from hybrid cell clones, we have always used a 1:1 mixture of DNA from the EG cells and lymphocytes (1:1 DNA mixture). These results clearly

indicate that in the DNA from hybrid cells, the paternally inherited *H19* allele in the somatic nucleus was demethylated at these critical sites.

Similar results were obtained with the *p57^{Kip2}* gene, that is maternally expressed and paternally methylated (Hatada and Mukai, 1995). Using a 0.53 kb *Xho*I–*Eag*I fragment of the 5' region of mouse *p57^{Kip2}* cDNA as a probe, the *Eag*I sites were undermethylated in the EG cells and in hybrid cell clones. The degree of undermethylation was similar to that observed in the MatDpDist7 embryo, which has two maternal copies and no paternal copy of the gene (Figure 2B).

Finally, we examined the *Igf2r* gene, particularly the intronic CpG island 27 kb downstream of the transcription start site that is methylated only on the expressed maternal allele (Brandeis *et al.*, 1993; Stoger *et al.*, 1993). In this region, methylated (2.9 kb) and unmethylated (2.0 kb) DNA fragments were detected following restriction enzyme digestion with *PvuII* and the methylation-sensitive enzyme *MluI*. Both bands representing the maternal and paternal alleles respectively, were detected in samples from thymic lymphocytes. However, the 2.0 kb band was predominant in EG and hybrid cell clones, indicating dominant demethylation of the *MluI* site (Figure 2C). Quantitative densitometric analysis of the 2.0 kb and 2.9 kb bands indicated >90% demethylation at this site in the EG and hybrid cell clones instead of the 50% found in thymic lymphocytes. Analysis of the *NotI* site in the promoter region again showed that it is unmethylated in the EG cells and hybrid cell clones (Figure 2D).

Demethylation of paternally expressed imprinted genes

The preferential expression of the paternal *Igf2* allele is accompanied by methylation of several *HpaII* sites ~3 kb upstream of the first exon (Sasaki *et al.*, 1992). The thymic lymphocyte DNA showed the fully methylated paternal 2.2 kb DNA fragment, and partially methylated maternal DNA fragments between 2.0–0.5 kb, with *EcoRI* and *HpaII* digestion. The fully methylated 2.2 kb band was diminished in the EG cells and hybrid cell clones (Figure 3A).

Peg3, with preferential expression of the paternal allele (Kuroiwa *et al.*, 1996) has a methylated maternal allele (L.-L.Li, E.B.Keverne, S.Aparicio, S.Viville, S.C.Barton, F.Ishino and M.A.Surani, in preparation). A *SacII* site of *Peg3* is methylated on the maternal allele (represented by the 20 kb band) and unmethylated on the paternal allele (represented by the 9.0 kb band) in thymic lymphocytes. Methylation status at the *SacII* site was similar in both the EG cells and hybrid cell clones. Again, demethylation of the maternal allele in lymphocyte DNA was evident as shown by a single 9.0 kb band in the EG cells and hybrid cell clones (Figure 3B).

Finally, *Peg1/Mest*, which shows expression of the paternal allele (Ishino-Kaneko *et al.*, 1995), has a *SmaI* site in the promoter region that is only methylated on the maternal allele (L.Lefebvre, S.Viville, S.C.Barton and M.A.Surani, in preparation). Methylated and unmethylated bands were detected at 3.0 and 2.1 kb respectively, in thymic lymphocytes. As indicated by the dominant 2.1 kb band in hybrid cell clones and the EG cells, the maternal allele in the lymphocyte nucleus became unmethylated (Figure 3C). Although a faint 3.0 kb band was detected in hybrid cells, densitometric analysis revealed that ~80% of DNA was demethylated in the hybrid cell clones again indicating that demethylation was dominant at this site after fusion of lymphocytes with EG cells.

Demethylation of non-imprinted genes

In addition to the analysis of genes that are subject to parental imprinting, we examined four well-characterized non-imprinted genes that were previously investigated during gametogenesis and early embryogenesis (Singer-Sam *et al.*, 1990; Kafri *et al.*, 1992). The CpG sites of

Aprt, *Pgk-2* and β *globin* are fully methylated in post-implantation embryos but unmethylated in PGCs of 12.5 dpc embryos (Kafri *et al.*, 1992). *Pgk-1* is an X-linked gene that is methylated on the inactive X chromosome (Singer-Sam *et al.*, 1990).

The *HhaI* site in the 3' region of the *Pgk-2* gene was hypermethylated in thymic lymphocytes. This was indicated by a 10 kb band, but unmethylated in the EG cells and hybrid cell clones (note the ~5.0 kb bands in Figure 4A). The *HhaI* site in an intron between exon 2 and 3 of β *globin* was methylated in thymic lymphocytes (8.0 kb band) and undermethylated in the EG cells and in hybrid cell clones (2.0 kb band in Figure 4B). In the analysis of the X-linked *Pgk-1* gene, a *HpaII* site in the 5' region of exon 1 showed that in female thymic lymphocytes, random X-inactivation was accompanied by methylated (at ~2.0 kb) and unmethylated (at ~0.8 kb) DNA fragments. The unmethylated DNA band was predominant not only in the EG cells but also in hybrid cell clones (Figure 4C). This demethylation is consistent with the cytogenetic data showing the re-activation of the inactivated X chromosome derived from the lymphocyte in hybrid cells. Finally, a *HpaII* site in exon 4 of the *Aprt* gene was substantially methylated in the DNA from lymphocytes, whereas DNA from both the EG and hybrid cell clones was unmethylated as shown by a band of <0.5 kb (Figure 4D).

DNA demethylation of minor satellite DNA

The bulk DNA methylation status in hybrid cells was analysed using the minor satellite DNA probe, MR150, which is scattered around the chromosome arms and centromeres (Pietras *et al.*, 1983). After digestion with the methylation-sensitive restriction enzyme, *HpaII*, the lymphocyte DNA remained uncut and was detected as a higher molecular band at >20 kb. However, the DNA from hybrid cell clones showed <2.5 kb ladder bands that were similar to DNA from EG cells (Figure 5). These ladder bands were more obvious in DNA from hybrid cells than in 1:1 DNA mixture. Nevertheless, there was residual DNA methylation in hybrid cell clones as judged by comparison with the methylation-insensitive isoschizomer *MspI* digestion.

Reprogramming of a silent imprinted allele and its expression

To investigate if the observed demethylation can cause re-activation of a silent allele, we used thymic lymphocytes from mice in which the *Peg1/Mest* gene was mutated so that exons were deleted and replaced with a *IREs*/ β -*geo* cassette (henceforth called *Peg1* ^{β geo}). The mutant *Peg1* ^{β geo} paternal allele is active but the maternal allele is methylated and repressed (Lefebvre *et al.*, 1997). As before, hybrid cells were made by fusion between the EG cells and thymic lymphocytes (Figure 1A).

First, we examined the reproducibility of the *Peg1* ^{β geo} re-activation in hybrid cells. For this purpose, we compared lymphocytes carrying the *Peg1* ^{β geo} allele inherited from either the father (active) or the mother (inactive) after fusion with the EG cells and cultured under identical conditions for 7 days without selection. These cells were then used to produce embryoid bodies (EBs) which should be chimeric as they consist of normal EG cells and hybrid

Fig. 3. Demethylation of paternally expressed imprinted genes in EGF×T hybrid cells. (A) *Igf2*; DNA was digested with *EcoRI* and the methylation-sensitive restriction enzyme *HpaII* (M), and hybridized with the 3.1 kb *EcoRI* fragment. Hypermethylated DNA fragment at 2.2 kb (arrow) was obvious only in the DNA from thymus and the 1:1 DNA mixture. (B) *Peg3*; DNA was digested with *KpnI* and the methylation-sensitive restriction enzyme *SacII*, and hybridized with a 1.3 kb *NheI* fragment. (C) *Peg1/Mest*; DNA was digested with *XbaI* and the methylation-sensitive restriction enzyme *SmaI*, and hybridized with the 3.0 kb *XbaI* fragment.

cells. No differences in the number of EBs or their phenotype were detected, and both sets of EBs were positive to an equal extent for X-gal staining (data not shown). This experiment suggests that not only the active paternal *Peg1*^{*βgeo*} allele showed expression as expected but the silent maternal *Peg1*^{*βgeo*} allele was also evidently re-activated to an equal extent. This latter expression could most readily be accounted for by postulating re-activation of the silent maternal allele in hybrid cells following fusion of lymphocyte with EG cell.

The fate of the silent maternal *Peg1*^{*βgeo*} allele derived from lymphocytes in hybrid cells was further characterized.

Following electrofusion between the EG cells and lymphocytes carrying the maternal *Peg1*^{*βgeo*} allele, the cultures were subjected to selection with G418 for 7–8 days, starting 2 days after cell fusion. This was done to allow for re-activation of the mutant allele and therefore of the *neo* gene. Individual colonies were examined cytogenetically to confirm 1:1 fusion product of the EG cell and thymic lymphocyte. The cells were also examined for the presence of four intact chromosomes 6 (on which *Peg1/Mest* is located) which was observed in a hybrid clone. The maternally inherited silent *Peg1*^{*βgeo*} allele was methylated in lymphocytes (Figure 7C). However, in the hybrid

Fig. 4. Demethylation of non-imprinted genes in EGFxT hybrid cells. (A) *Pdk-2*; DNA was digested with *Bam*HI and the methylation-sensitive restriction enzyme *Hha*I, and hybridized with a 430 bp fragment containing only one *Hha*I site (Kafri *et al.*, 1992). (B) *βglobin*; DNA was digested with *Bam*HI and the methylation-sensitive restriction enzyme *Hha*I, and hybridized with a 420 bp fragment (Kafri *et al.*, 1992). (C) *Pdk-1*; DNA was digested with *Eco*RI and the methylation-sensitive restriction enzyme *Hpa*II, and hybridized with a 170 bp fragment (Singer-Sam *et al.*, 1990). (D) *Aprt*; DNA was digested with *Sma*I and the methylation-sensitive restriction enzyme *Hpa*II, and hybridized with a 300 bp fragment (Kafri *et al.*, 1992). Fully and partially methylated fragments were detected only in thymus DNA, but the *Hpa*II site located within the probe fragment was always methylated. When this stage-specific methylation site is demethylated, the small fragment marked with a circle is detected.

clone, EGFxTP1-/+ at passage 4, Southern hybridization analysis showed that the two *Sma*I sites of *Peg1*^{βgeo} allele were demethylated, consistent with our findings described above (Figure 3C). Moreover, this allele was expressed appropriately following differentiation of the hybrid cells in chimeric embryos *in vivo* (see below).

Developmental potential of hybrid cells

The EG-somatic hybrid cells were phenotypically similar to the EG cells. To examine if the hybrid cells were capable of contributing to normal embryonic development as seen with the EG cells alone (M.Tada, T.Tada and N.Takagi, in preparation), we introduced the hybrid cells (EGFxT2) carrying the ROSA26 transgene into host diploid blastocysts and examined the embryos at 9.5 and 10.5 dpc as shown in Figure 1A(I). Since the lymphocytes contained the ubiquitously expressed ROSA26 transgene, we could assess the contribution of the hybrid cells following X-gal staining. Three out of the 52 (6%) embryos analysed were positive for X-gal staining (Table I). The degree of contribution of the tetraploid hybrid cells was

Fig. 5. Demethylation of mouse repetitive DNA in EGFxT hybrid cells. DNA (2 μg) was diluted and divided equally. One half was digested with the methylation-sensitive restriction enzyme *Hpa*II and the other half was digested with the methylation insensitive isoschizomer *Msp*I. The minor satellite sequence MR150 was oligo-synthesized and end-labelled as a probe.

modest, as previously observed when tetraploid and diploid cells were present in chimeras (James *et al.*, 1995). Nevertheless, EGFxT2 hybrid cells did colonize a variety of tissues (Figure 6A). To increase the contribution of the hybrid cells, they were also injected into tetraploid host blastocysts. Fourteen out of the 44 embryos (32%) were positive for X-gal staining at 7.5–9.5 dpc (Table I), and the contribution of hybrid cells was less severely affected in this case. In the 8.5 dpc chimeric egg-cylinder, hybrid cells contributed significantly to the embryonic ectoderm and visceral endoderm (Figure 6B). In more advanced 9.5 dpc chimeras, X-gal positive cells were found in the yolk-sac mesoderm and embryonic ectoderm and mesoderm (Figure 6C).

Similar experiments were carried out with the hybrid cells (EGFxTP1-/+) carrying the maternally inherited *Peg1*^{βgeo} allele as shown in Figure 1A(II). When these hybrid cells were injected into tetraploid host blastocysts, 3 out of 35 embryos transferred to foster mothers gave some embryonic development on 9.5 dpc, and a further 16 yolk-sac membranes were obtained. The contribution of EGFxT2 hybrid cells carrying ROSA26 transgene to the three primary germ layers of developing embryos was evident when injected into tetraploid blastocysts (Figure 6B and C). By contrast, *Peg1*^{βgeo} was re-activated and expressed appropriately in the differentiated derivatives of hybrid cells in the mesoderm, the yolk-sac mesoderm and allantois in chimeric embryos (Figure 7A and B). This expression pattern is comparable to that of *Peg1*/*Mest* in early post-implantation embryos (Sado *et al.*, 1993; Ishino-Kaneko *et al.*, 1995).

Discussion

This study demonstrates that the EG-lymphocyte hybrid cells display phenotypic properties that are similar to those

Table I. Early post-implantation development of diploid/tetraploid and tetraploid/tetraploid chimeras with female EG cell $\times$ thymic lymphocyte hybrid cells

Hybrid cell clone-passage (Blastocysts)	Recipients	Age of embryos (days)	No. of embryos transfected		No. of chimeric (non-chimeric) embryos			
			Transferred	Implanted	Normal	Retarded	Abnormal	Resorbed ^a
EGF $\times$ T2-5 (Diploid blastocysts)	1	9.5	9	8	0 (6)	0	0	0 (2)
	2		9	9	1 (8)	0	0	0
	3	10.5	9	8	0 (6)	0	0	0 (2)
	4		8	7	0 (7)	0	0	0
	5		9	9	1 (5)	1	0	1 (1)
	6		8	7	0 (6)	0	0	0 (1)
EGF $\times$ T2-4 (Tetraploid blastocysts)	1-1	7.5	8	6	0	0	1 (3)	0 (2)
	1-2	8.5	8	7	0	0	3 (3)	0 (1)
	1-3		8	7	0	0	2 (3)	0 (2)
	1-4	9.5	8	6	0	1	0 (2)	0 (3)
EGF $\times$ T2-6 (Tetraploid blastocysts)	2-1	8.5	6	6	0	1	3 (1)	0 (1)
	2-2		6	5	0	0	3 (1)	0 (1)
EGF $\times$ TP1-/+2 (Tetraploid blastocysts)	1	9.5	8	3	0	0	0	3
	2		9	8	0	0	2	6
	3		9	2	0	0	1	1
	4		9	3	0	0	0	3

^aChimerism in resorbed embryos was assessed by the contribution of EG hybrid cells in the yolk sac.

of EG cells, including pluripotency, as they contribute to many tissues in chimeric embryos. Perhaps their full developmental potential is only restricted because they are tetraploid. This phenotype contrasts markedly with that of non-dividing, non-adhesive thymic lymphocytes. There are at least two, although not mutually exclusive, possibilities to explain the observed phenotypic properties of our hybrid cells. First, there could be repression of lymphocyte-specific gene activity by *trans*-acting factors originating from EG cells, implying that the EG cell nucleus must be present continuously to maintain the phenotype of hybrid cells. Second, lymphocyte-specific properties may be lost after fusion with EG cells because of the extensive epigenetic changes of the lymphocyte genome. If the latter occurs, it is possible that a new heritable epigenetic modification of the somatic nucleus was induced by the EG cell in hybrid cells. We propose that both of these events probably occur sequentially following EG–lymphocyte cell fusion.

The primary focus of our studies was to examine methylation changes in the somatic nucleus. Previous studies have already demonstrated the repression of lymphocyte-specific gene expression following fusion with embryonal carcinoma (EC) cells (Miller and Ruddle, 1977; Martin *et al.*, 1984). This phenomenon, sometimes referred to as extinction, is well recognized even in hybrid cells between two somatic cells (reviewed by Boshart *et al.*, 1993). In an EC–lymphocyte hybrid clone, it appeared that there was virtual suppression of lymphocyte-specific gene expression as judged by the analysis of proteins using two-dimensional gels (Forejt *et al.*, 1984). A similar phenomenon probably occurs in the EG–lymphocyte hybrid cells because expression of the thymocyte-specific antigen, Thy-1.2 (Ingraham *et al.*, 1986), was reduced in the EG as well as hybrid cells when compared with the expression in lymphocytes (data not shown). These and other such studies argue for repression of the somatic cell gene expression in hybrid cells containing a pluripotential

Fig. 6. The contribution of EGF $\times$ T hybrid cells carrying the ROSA26 transgene in chimeric embryos. (A) A 10.5 dpc chimeric embryo formed with diploid host embryo and tetraploid EGF $\times$ T2 hybrid cells. (B) A 8.5 dpc egg-cylinder embryo formed with tetraploid host embryo and tetraploid EGF $\times$ T2 hybrid cells. (C) A 9.5 dpc chimeric embryo and the yolk sac formed with tetraploid host embryo and tetraploid EGF $\times$ T2 hybrid cells.

nucleus. It is possible that repression of the somatic nucleus is essential before major genome-wide epigenetic modifications can occur to prevent inappropriate gene expression while these modifications proceed.

Fig. 7. Demethylation and gene re-activation of a silent maternal allele of *Peg1/Mest* (*Peg1*^{βgeo}) in EGF×TP1-/+ hybrid cells. **(A)** Abnormal 9.5 dpc chimeric embryo and the yolk sac from the tetraploid host embryo and tetraploid EGF×TP1-/+ hybrid cells. **(B)** The inside view of exocoelom (Exc) of a 9.5 dpc abnormal chimeric embryo. Strong expression of the *Peg1*^{βgeo} mutant allele was detected with X-gal within the first 3 h of staining time. Staining was seen in mesodermal tissues, inside layer of yolk sac (YsMe) and allantois (Al), but not in embryonic ectoderm (EmE) and endoderm layer of yolk sac (End). **(C)** Southern blot analysis of the methylation pattern of the *Peg1/Mest* and *Peg1*^{βgeo} alleles in EGF×TP1-/+ cells. DNA was extracted from thymus of m-/p+ heterozygote for *Peg1/Mest* (T-/+), EG cells (EGF) and their fusion clone (EGF×TP1-/+). DNA was digested with *Hind*III and the methylation-sensitive restriction enzyme *Sma*I and hybridized with the 3.0 kb *Xba*I fragment of the upstream region including exon 1. One of the two *Sma*I sites in the *Hind*III fragment is identical to that analysed in Figure 4C. The 13.2 kb and 10.6 kb *Hind*III fragments represent the wild-type *Peg1/Mest* (wt) and the mutant *Peg1*^{βgeo} (KO) alleles respectively. The methylated fragment is detected only at 10.6 kb in thymus DNA, and *Sma*I digested fragments are marked with circles.

DNA methylation, a heritable epigenetic modification, regulates expression of imprinted and other genes in mammals (Li *et al.*, 1993). Imprinting, which is a multi-step process, first requires erasure of allele-specific epigenetic modification in both male and female PGCs (Brandeis *et al.*, 1993; T.Tada, M.Tada, K.Hilton, S.C. Barton, T.Sado, N.Takagi and M.A.Surani, in preparation). This is followed by appropriate methylation of genes that constitutes a sex-specific switch of the epigenotype in gametes, a process that is crucial for subsequent embryonic development (Brandeis *et al.*, 1993; Stoger *et al.*, 1993; Kono *et al.*, 1996). We detected extensive demethylation of the thymic lymphocyte genome in hybrid cells, a reprogramming event that renders the lymphocyte genome similar to the EG cell nucleus, at least at the imprinted and other loci we examined. Clearly, the demethylation activity from EG cells is dominant in these hybrid cells.

The reprogramming activity of EG cells is comparable to that in PGCs. For example, in EG-somatic hybrid cells, we observed re-activation of the *Peg1*^{βgeo} allele, extensive demethylation of many loci and re-activation of the inactive X chromosome derived from the somatic nucleus. This is similar to the loss of allele-specific methylation imprints (Brandeis *et al.*, 1993), re-activation of the inactive X chromosome (Monk and McLaren, 1981) and the potential for bi-allelic expression of four imprinted genes (Szabo and Mann, 1995) *in vivo* in PGCs. It is therefore likely that what we are witnessing *in vitro* is probably what occurs in PGCs. Against the background of marked demethylation activity, selective methylation of specific sites cannot be excluded. When examining the genome-wide methylation status in hybrid cells using a minor satellite sequence (MR150), we detected obvious general demethylation in the DNA from the EG hybrid

cells, but there was not a total lack of methylation in the DNA from both the EG and hybrid cells. Low degree of methylation of *Igf2r* region 2, *Peg1/Mest*, β globin and *Pgk-1* was detected in the hybrid cells and EG cells, suggesting that both the demethylation and methylation activities are present. In this context, we did also detect DNA methyltransferase RNA by Northern hybridization in EG cells (data not shown), although it is not known if the enzyme itself is present and if so whether it is biologically active. Other pluripotential cells such as embryonic stem (ES) cells (Lei *et al.*, 1996) and EC cells (Stewart *et al.*, 1982; Frank *et al.*, 1991) clearly have the potential for DNA methylation. Based on the methylation analysis of hybrid cells, we suggest that while EG cells have a dominant demethylation activity, they also have methylation activity, albeit at a low level. Methylation profiles in the hybrid cells may be controlled by the dominant demethylation and minor methylation activity. Recent advances on the mechanism of demethylation suggest that in a cell-free system, an active process of demethylation is mediated by an RNA molecule (Weiss *et al.*, 1996), a system that could be functioning in the EG cells. Weiss *et al.* (1996) also demonstrated the existence of putative protein inhibitors (demethylation protection factors) that protect genes from indiscriminate demethylation activity. During pre-implantation development, allele-specific imprints are resistant to genome-wide demethylation (Brandeis *et al.*, 1993; Kafri *et al.*, 1993; Tremblay *et al.*, 1995). This situation is different in germ cells where there is erasure of all parental imprints (Brandeis *et al.*, 1993; T.Tada, M.Tada, K.Hilton, S.C. Barton, T.Sado, N.Takagi and M.A.Surani, in preparation). In EG-somatic cell hybrids, the allele-specific methylation patterns of imprinted genes in the somatic genome were also erased. This suggests that PGCs and EG cells may lack functional factors needed to protect against the demethylation activity that are perhaps present in pre-implantation embryos and in somatic cells. The existence of such demethylation protection factors is also evident from studies on demethylation of the *in vitro* methylated *Aprt* gene. Demethylation occurred when the methylated gene was transfected into the F9 embryonic teratocarcinoma (EC) cells, but not when transfected into L-cell fibroblasts or L8 myoblasts (Frank *et al.*, 1991). However, the methylated *Aprt* gene was demethylated by extracts from L8 myoblast cells but only if pre-treated with proteinase K (Weiss *et al.*, 1996). This indicated the presence of a protein factor that protects against the demethylation activity. Taking all these findings into consideration, the loss of allele-specific methylation from lymphocytes may be a consequence of fusion with EG cells that lack functional demethylation protection factors.

In the context of this study, it is important to consider the properties of pluripotential cells such as ES and EC cells. ES cells apparently retain at least some of the appropriate parental imprints (Mann and Stewart, 1991; Allen *et al.*, 1994), suggesting that the germline-specific modifications are protected. Although the fate of parental imprints in ES-somatic cell hybrids has not been examined, we have analysed several EC cell lines of different origins for their effects on EC-lymphocyte hybrid cells. What is striking is that methylation of some imprinted genes in lymphocytes was preserved, but only in cases where the

EC cells were derived from more advanced embryos, and not if the EC cells were derived from ovarian teratocarcinoma (M.Tada, M.Tada, K.Hilton, S.C.Barton, T.Sado, N.Takagi and M.A.Surani, unpublished data). It appears that the activity of demethylation protection factors may be developmentally regulated so that EG cells may differ from ES cells, just as we detect differences between EC cells of diverse origin.

While focusing on DNA methylation, it is important to note that other epigenetic modifications may also occur in the somatic nucleus in hybrid cells. This could include chromatin structural and replication timing changes in the somatic nucleus to resemble the status in the EG cell nuclei. Several rounds of cell cycle are perhaps required to complete the reprogramming or remodelling of the somatic cell nucleus into a pluripotential nucleus. This idea is supported by the finding that several cell divisions are necessary to change the replication timing of a lymphocyte X chromosome from late S-phase (inactive state) to early S-phase (active state) in EC-lymphocyte hybrid cells (Takagi *et al.*, 1983).

Finally, consistent with the epigenetic modifications in the somatic nucleus, we demonstrated that EG-lymphocyte hybrid cells participate in the development of chimeric embryos. Furthermore, we demonstrated re-activation of the silent maternal *Peg1*^{*βgeo*} allele; this allele was subsequently expressed appropriately in differentiated mesoderm derivatives of hybrid cells present in chimeric embryos. The epigenetic changes induced in the somatic genomes in hybrid cells are therefore likely to be heritable. However, the unequivocal verification of heritability of epigenetic reprogramming of the somatic nucleus will require development of techniques for subsequent elimination of the EG nucleus from hybrid cells. While EC-somatic hybrid cells can also differentiate into a variety of cell types in ectopic sites by tumour formation (Miller and Ruddle, 1976), they failed to contribute to a chimeric embryo (Martin *et al.*, 1984). It is not possible at present to consider whether there are fundamental differences between our hybrid cells using EG cells and those with EC cells. This could in any case depend on the origin of EC cells, because the pluripotency of hybrid cells may depend on the precise epigenetic changes induced in the somatic genomes. We propose that the *in vitro* system described here may provide further insights into the concept that defines the state of pluripotency, as well as on the mechanism of epigenetic modifications in the germ line. These changes are critical for the sex-specific epigenotype switching in germ cells and for the subsequent development in mammals.

Materials and methods

EG cell line

Female mice homozygous for the Rb(X.2)2Ad translocation were mated with 129/Sv male mice. The EG cell line TMA-58G was established from the 12.5 dpc female embryos. The EG and hybrid cells were maintained in ES medium containing Dulbecco's modified Eagle's medium (MEM) supplemented with 20% fetal bovine serum (FBS), 10^{-4} M 2-mercaptoethanol, non-essential amino acid, 1000 units/ml recombinant leukaemia inhibitory factor (LIF; AMRAD), on inactivated PEF feeder cells. EG cells <10 passages were used for all the experiments here.

Mouse

Two types of female mice were used to obtain thymuses for this study. One was (129/Sv×129/Sv-TgR(ROSA26)26Sor)F1 mice (Friedrich and Soriano, 1991) and another was female heterozygous mice inheriting a *Peg1/Mest* mutant allele from their mothers or fathers (Lefebvre *et al.* in preparation). In this mutant allele, the exons 3–8 of *Peg1/Mest* were replaced with IRES/ β -geo (*neo/lacZ*) by homologous recombination. Expression of *lacZ* was subject to appropriate imprinting and tissue-specific expression as judged by *lacZ* expression (Lefebvre *et al.*, 1997) based on the previously reported analysis for *Peg1/Mest* (Sado *et al.*, 1993; Ishino-Kaneko *et al.*, 1995).

Thymic lymphocyte

Thymic lymphocytes were obtained from 6- to 7-week-old female mice carrying the *neo/lacZ* marker. After dissection, the thymuses were squirted in and out of a 2.5 ml syringe through a 18-gauge needle several times to obtain a single cell suspension.

Cell fusion and selection

The EG cells and thymic lymphocytes were mixed in the ratio of 1:5, and washed three times in PBS. These cells were suspended in 0.3 M mannitol buffer at a concentration of 1×10^6 cells/ml. Hybrid cells were produced by electrofusion ($E = 2.5$ – 3.0 kV/cm) using Electro Cell Manipulator 200/Microslide with a 1 mm electrode gap (BTX). After 1 day of culture in the ES medium, cells were selected on G418-resistant PEFs with culture medium containing 250 μ g/ml G418 for 7–10 days. The rate of derivation of hybrid clones was comparable to the rate obtained between EC cells and thymic lymphocytes (Takagi *et al.*, 1983). Hybrid cell clones were picked and expanded. Any contaminating EG cells were eliminated following disaggregation of cells and further selection for 3–5 days in the G418 medium. Isolated clones were positive for X-gal staining, indicating that they were hybrid cells. Furthermore, with the aid of chromosome polymorphism, 1:1 fusion of the EG cell and lymphocyte was identified cytogenetically.

Chromosome analysis

Chromosomes of the EG and hybrid cells were examined by the replication (R) banding. The duration of BrdU (150 μ g/ml) incorporation was 7–8 h and Colcemid (0.3 μ g/ml) was present in the medium for the final 1 h of incubation. Cells were treated with 0.075 M KCl for 8 min at room temperature and then fixed in 3:1 methanol:acetic acid. Slides of chromosomes prepared by an air-drying method were stained with acridine orange solution and examined under the fluorescence microscope to determine X-chromosome activity. These specimens were also stained with Hoechst 33258 (10 ng/ml) in McIlvaine buffer (pH 4.4) for G-banding to examine the chromosome constitution of hybrid cells.

Chimera production

Both normal diploid (2n) and tetraploid (4n) blastocysts were used to make chimeric embryos with hybrid cells. On day 3.5 of pregnancy, diploid blastocysts were flushed from the uteri of (C57BL/6×CBA)F1 females naturally mated with (C57BL/6×CBA)F1 males. Hybrid cells were injected into the blastocoel cavity of well-expanded blastocysts. Tetraploid embryos were produced by electrofusion between blastomeres of 2-cell stage embryos (James *et al.*, 1995). After 3 days in culture, hybrid cells were injected into tetraploid blastocysts. Two hybrid clones, EGF×T2 and EGF×TP1–/+, were used as donor cells.

X-gal staining

Cultured cells were rinsed with PBS and then fixed for 5 min at 4°C in 4% paraformaldehyde or in a fixative containing 1% formaldehyde, 0.2% glutaraldehyde, 0.02% NP40 and 1 mM MgCl₂ in PBS. Embryos were fixed with this fixative for 3–4 h at 4°C. The specimens were washed with PBS and then stained in a reaction mixture containing 1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside in dimethyl formamide, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM MgCl₂ in PBS for 24–48 h at room temperature.

Southern blot hybridization analysis

Genomic DNA was prepared from thymic lymphocytes, EG cells at passage 9 and hybrid clones at passage 4 (Sambrook *et al.*, 1989). DNA was digested and separated on 1% or 1.2% agarose gels and then transferred to Hybond-N+ by alkali blotting. Radioactive probes were made using Megaprime DNA labelling systems (Amersham) and blots were hybridized with ³²P-labelled DNA overnight at 42°C following prehybridization treatment. The membranes were washed twice in 2×

SSC/0.1% SDS at 65°C for 30 min and twice in 0.1× SSC/0.1% SDS at 65°C for 30 min.

Probes

Six DNA probes for the methylation analyses were generated by PCR amplification and agarose gel purification. In the *Igf2r* region 2, a *MluI* site was analysed using a 330 bp fragment (5' primer, AATCGCATTA-AACCTCCGAACCT; 3' primer, TAGCACAATCCAATTGTGCT-GCG) and in the *Igf2r* region 1, a *NotI* site was analysed using a 190 bp fragment (5' primer, CGGGCCGACTCAGGTCACGTGACGC; 3' primer, TGTGGCGTGGGGCTCAGGCGGCTGG) (Stoger *et al.*, 1993). For the non-imprinted genes, *Pgk-2*, β major globin gene, *Pgk-1* and *Aprt*, one methylation site in the probe fragment was analysed using primer sets reported by Kafri *et al.* (1992) and Singer-Sam *et al.* (1990). Other probes used for methylation analysis are described in the figures and their legends. The minor satellite sequence MR150 was oligo-synthesized and gel-purified; 66 mer, 5'-GACTGAAAAACACATT-CGTTGGAAACGGGATTTGTAGAACAGTGTATATCAATGAGTTA-CAATGAG-3' (Pietras *et al.*, 1983), and end-labelled as a probe.

Embryoid body formation

Mixtures of 2×10^5 hybrid cells were cultured in a 500 μ l drop of DMEM medium containing 5% FBS and 5% of FCS under suspension conditions in a bacterial dish for 7 days.

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