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A functional analysis of imprinting in parthenogenetic embryonic stem cells

Nicholas D. Allen[1,*], Sheila C. Barton[2], Kathy Hilton[2], Mike L. Norris[1] and M. Azim Surani[2]

[1]AFRC Babraham Institute, Babraham, Cambridge, CB2 4AT, UK
[2]Wellcome/CRC Institute of Cancer and Developmental Biology, Tennis Court Road, Cambridge CB2 1QR and Physiological Laboratory, University of Cambridge, Cambridge, UK

*Author for correspondence

SUMMARY

A detailed analysis of the developmental potential of parthenogenetic embryonic stem cells (PGES) was made in vivo and in vitro, and a comparison was made with the development of cells from parthenogenetic embryos (PG). In vivo, in chimeras with normal host cells (N), PGES cells showed a restricted tissue distribution consistent with that of PG cells, suggesting faithful imprinting in PGES cells with respect to genes involved in lineage allocation and differentiation. Restricted developmental potential was also observed in teratomas formed by ectopic transfer under the kidney capsule. In contrast, the classic phenotype of growth retardation normally observed in PG↔N chimeras was not seen, suggesting aberrant regulation in PGES cells of genes involved in growth regulation.

We also analysed the expression of known imprinted genes after ES cell differentiation. *Igf2, H19* and *Igf2r* were all appropriately expressed in the PGES derived cells following induction of differentiation in vitro with all-*trans* retinoic acid or DMSO, when compared with control (D3) and androgenetic ES cells (AGES). Interestingly, *H19* was found to be expressed at high levels following differentiation of the AGES cells. Due to the unexpected normal growth regulation of PGES↔N chimeras we also examined *Igf2* expression in PGES derived cells differentiated in vivo and found that this gene was still repressed.

Our studies show that PGES cells provide a valuable in vitro model system to study the effects of imprinting on cell differentiation and they also provide invaluable material for extensive molecular studies on imprinted genes. In addition, the aberrant growth phenotype observed in chimeras has implications for mechanisms that regulate the somatic establishment and maintenance of some imprints. This is of particular interest as aberrant imprinting has recently been invoked in the etiology of some human diseases.

Key words: embryonic stem cells, parthenogenesis, imprinting

INTRODUCTION

During mouse development, the dramatic consequences of genomic imprinting have been clearly demonstrated by the failure of parthenogenetic/gynogenetic (PG/GG; two maternal chromosome sets) and androgenetic embryos (AG; two paternal chromosome sets) to develop to term and by the different capacities of cells with these uniparental genotypes to participate in development in chimeras with normal cells (Surani et al., 1990). This developmental failure is also seen for embryos carrying certain uniparental disomies (Cattanach and Beechey, 1990). Four of the imprinted genes that contribute to some of the observed phenotypes have recently been identified; the maternal alleles of *Igf2* (DeChiara et al., 1991; Ferguson-Smith et al., 1991) and *Snrpn* (Leff et al., 1992; Cattanach et al., 1993), and the paternal alleles of *Igf2r* (Barlow et al., 1991) and *H19* (Bartolomei et al., 1991) are inactive.

Imprinting has two major effects as observed in PG↔N chimeras. Firstly, PG cells affect growth in the fetus and postnatally (Fundele et al., 1990). Secondly, the cell lineages in which PG cells can participate are restricted; most notably they fail in trophectoderm and primitive endoderm, which results in failure of the extraembryonic tissues in whole PG/GG conceptuses (Surani and Barton, 1983). Whole PG/GG embryos can develop normally up to the 40-somite stage when provided with a normal trophectoderm and endoderm (Barton et al., 1985; Gardner et al., 1990). However, all PG/GG embryos die shortly after this stage. In chimeras, PG cells are allocated initially randomly in the embryo proper (Clarke et al., 1988; Thomson and Solter, 1989) but this is followed by a progressive elimination of PG cells, which is most pronounced between days 13 and 16 of gestation (Fundele et al., 1990). The loss of PG cells is not random and is most pronounced in tissues of the mesoderm and endoderm lineages. The loss of PG cells in skeletal muscle development has been of particular interest.

The mechanism of genomic imprinting is at present poorly understood. Presumably, it involves a germline-specific epigenetic marking process since the expression of imprinted genes is determined by their parental origin. Heritable epigenetic modifications that could be employed in imprinting mechanisms include allele-specific DNA methylation and chromatin structural modifications such as those detected by DNase1

hypersensitivity assays. Indeed, allele-specific methylation of the *Igf2, Igf2r* and *H19* genes and DNase1 hypersensitivity in the *H19* promoter have recently been reported (Sasaki et al., 1992; Stöger et al., 1993; Ferguson-Smith et al., 1993). However, for each gene, some of the allelic differences detected were actually acquired after fertilisation and therefore do not constitute germline-specific imprints. These observations demonstrate the progressive nature of the imprinting process, and support a model of imprinting that involves at least three phases; germline-specific marking, somatic establishment, and maintenance (Allen and Mooslehner, 1992). Thus, imprinting of genes is likely to be influenced by the major epigenetic programming that occurs genome wide, early in development. For example, parental gametes show varying degrees of DNA methylation. However, soon after fertilisation, there is a striking decline in the levels of genomic DNA methylation that reaches a minimum by the blastocyst stage (Howlett and Reik, 1989; Sanford et al., 1987; Kafri et al., 1992). A major de novo methylation event then occurs after implantation that affects numerous methylatable sites at the onset of gastrulation (Kafri et al., 1992). The importance of somatic control of imprinting is also highlighted by the fact that imprinting can exhibit tissue specificity. For example, *Igf2* is not imprinted in the leptomeninges and choroid plexus (DeChiara et al., 1991), *H19* is expressed in androgenetic trophoblast and embryonic cells (R. Ohlsson, unpublished data; N. D. A., A. S., unpublished observation) and *Snrpn* seems to be imprinted principally in the brain (Leff et al., 1993; Cattanach et al., 1993).

Considering the dynamic pattern of epigenetic changes during early development, it is of particular interest to examine the stability of imprinting in embryonic stem cells (ES cells). ES cells are pluripotent and possibly even totipotent (Nagy et al., 1993). However, during the establishment and maintenance of ES cell lines it is possible that the normal epigenetic programming events of the embryo are disrupted. Defective epigenetic programming may account for the loss of embryos and neonatal mice that are derived exclusively from ES cells, even though the same cells can give germline transmission from a chimera (Nagy et al., 1990). ES cells are derived from epiblast cells of blastocysts at a time when genome-wide DNA methylation levels are minimal (Monk et al., 1987; Howlett and Reik, 1989; Kafri et al., 1992). Whether the pluripotent state of these cells is linked somehow to the exceedingly low levels of DNA methylation is unknown. The properties of parental imprints could be examined by comparing ES cells derived from PG, AG and normal blastocysts. Studies on AG derived ES cells (AGES) have previously been reported (Mann et al., 1990). In these studies, the AGES cells showed properties that were remarkably similar to those observed with AG inner cell mass cells of the same genotype (Mann and Stewart, 1991; Barton et al., 1991), even after extensive culture in vitro. Therefore, paternal imprints are apparently stable in AGES cells. PG derived ES cells (PGES) have also been described previously (Robertson et al., 1983). Although their phenotypic properties have not been reported in detail, some contribution to skeletal muscle in adult chimeras with normal embryos was observed (Evans et al., 1985), a property rarely exhibited by PG ICM cells in similar studies. This observation may represent an epigenetic instability in these cells affecting imprinting. Interestingly, genetic instability was also observed in PGES cells with variable deletions on one of the X chromosomes (Robertson et al., 1983). We report on a detailed investigation on the phenotypic properties of a number of different PGES cell lines, and also on an analysis of the expression of *Igf2, H19* and *Igf2r* in PGES cell derivatives after differentiation.

MATERIALS AND METHODS

Animals

The non-albino (C57Bl/6J × CBA/Ca)F_1 mice (GPI-1BB) were bred from parent stocks from Bantin and Kingman (UK). The outbred albino CFLP mice (Bantin and Kingman, UK) were selected and bred to be GPI-1AA. The transgenic mouse line ROSA26 was a kind gift from Dr P. Soriano; it was made in a 129/sv embryonic stem cell line (ES) and was since maintained on a mixed C57Bl/6J and CBA/Ca background by crossing with (C57Bl/6J × CBA/Ca)F_1 mice.

Derivation of parthenogenetic embryonic stem cell lines (PGES)

Diploid parthenogenetic embryos were produced as described previously, by activation of (C57Bl/6J × CBA/Ca)F_1 superovulated unfertilised oocytes by a brief exposure to 7% ethanol in T6 embryo culture medium + 4% BSA and subsequent culture in medium containing 5 μg/ml cytochalasin B for 4 hours (Barton et al., 1987). Diploid parthenogenetic embryos were cultured in vitro until expanded blastocysts started to hatch (day 5). Hatching blastocysts were transferred on to mitomycin C (0.2 mg/ml) -treated sln feeder cells in gelatinised tissue culture wells (50 blastocysts per 1.5 cm well) and cultured for 3 days in ES medium (1:1 DMEM : Hams F12, 10% fetal calf serum with supplements of sodium pyruvate, 20 mM L-glutamine, nucleosides, non-essential amino acids, sodium bicarbonate, β-2 mercaptoethanol) following standard procedures (Robertson, 1987). After 3 or 4 days, ICM outgrowths were harvested in 0.1% trypsin/EDTA, disaggregated by mouth pipetting and plated onto feeder cells in ES medium. Clones resembling ES cells in morphology were then picked and disaggregated a second time. Clones obtained following the second dissaggregation were designated PGES passage 1 (e.g. PK1-1°); they were then expanded and passaged prior to freezing or use.

For comparison, androgenetic ES cells (AGES) were also derived. Androgenetic embryos were made by nuclear transplantation as described previously (Barton et al., 1991) and cultured to the blastocyst stage. AGES cells were derived in the same way as the PGES cells. Two control cell lines were used, D3 (gift from A. Gossler) and CCE (gift from M. Evans; both are 129 genotype).

Generation and analysis of chimeras

Chimeras were made by injecting 10-15 PGES cells into the blastocoel cavity of recently cavitated (day 4) CFLP blastocysts (PGES→CFLP; Bradley, 1987). For chimeras with tetraploid (4^n) host embryos, day-2 fertilised embryos were made tetraploid by electrofusion of 2-cell blastomeres in 0.3 M D-glucose using a BTX200 electro-cell manipulator (Skatron, UK). PGES↔4^nCFLP chimeras were made by aggregating a clump (5-15 cells) of PGES cells between a sandwich of two 4^nCFLP compacting embryos after removing zona pelucidae with acid tyrode's solution (Nagy et al., 1990), or by simple coculture of PGES cells with 4^nCFLP embryos (Wood et al., 1993). For parthenogenetic embryo (PG)↔4^nCFLP chimeras, synchronous PG (8 cells) and 4^nCFLP (4 cells) embryos were aggregated. Composite embryos were transferred to the uterine horns of recipient females (F_1 females mated to vasectomised males) on the third day of pseudopregnancy, counting the day of finding the vaginal plug as day 1. Injected blastocysts were transferred immediately after operation. Aggregates were cultured to the blastocyst stage before transfer.

The percentage contribution of PGES cells in chimeras was studied

by GPI analysis as described previously (Fundele et al., 1990). The control HM1 chimera was a kind gift from I. Rosewell. The percentage chimerism in the R26PG-1 was assessed by the proportion of cells expressing the *lacZ* reporter gene in tissue sections. Tissues were dissected after fixation by perfusion with 4% paraformaldehyde, tissue slices were stained as described previously (Wood et al., 1993).

Teratomas were produced by transferring ES cell aggregates under the kidney capsule of isogenic adult mice. Mice were killed after 4 weeks and teratomas were recovered and fixed in 4% paraformaldehyde. Tissue was wax imbedded, sectioned and counterstained with eosin and haemotoxylin.

ES cell culture and differentiation

ES cells were maintained in ES medium on feeder cells, as described for the derivation of cell lines. For all molecular analysis, ES cells were cultured in ES medium containing 10^3 units/ml of recombinant LIF (ESGRO, BRL) in the absence of feeder cells (Smith, 1991). Feeder cells were diluted from these cultures by passaging at least 3 times prior to use. To differentiate ES cells, cells were trypsinised and then allowed to form aggregates on bacteriological dishes in ES cell medium (without LIF) or in the presence of 0.3 μM all-*trans* retinoic acid (RA) or 1% dimethyl sulphoxide (DMSO). After 4 days in culture, aggregates were transferred to gelatinised tissue culture dishes and cultured for a further 4 days in ES medium.

Northern analysis

RNA was prepared from cell cultures by the method of Chomczynski and Sacchi (1987). RNA was electrophoresed on 1% formaldehyde gels and subsequently transferred to HybondN+ filters. Northern blots were probed with radiolabeled *Igf2*, *H19*, *Igf2r* and *GAPDH* cDNA probes. The *Igf2* probe was first competed with 400 μg/ml sonicated genomic mouse DNA prior to hybridisation, on account of repetitive elements present within the *Igf2* 6th exon.

RESULTS

Derivation of parthenogenetic ES cells

Diploid parthenogenetic embryos were cultured in vitro for 5 days to the expanded blastocyst stage. Approximately 100 blastocysts were plated on to mitomycin C treated Sln feeder cells in ES cell culture medium. The majority of blastocysts hatched and formed healthy outgrowths under these conditions. Compared with the outgrowth of normal F_1 embryos, the development of the parthenogenetic embryoid body was more extensive and the outgrowth of giant cells from the trophectoderm was restricted. Embryoid bodies were picked individually after 3 days culture, then disaggregated and plated on to feeder cells following standard procedures (Robertson, 1987). Twenty four PGES clones were obtained following the second disaggregation.

Analysis of PGES cells in chimeric mice

The behaviour of five independent PGES cell clones was analysed in chimeric mice. The PGES cells, which carry the GPI-1B allozyme and non-albino coat colour markers, were injected into the blastocoel cavity of CFLP blastocysts, which carry the GPI-1A allozyme marker and are albino. The number of chimeras obtained and the passage number of the ES cells used is given in Table 1. Eighteen female and 26 male chimeras survived to weaning age. The participation of PGES cells in the chimeras varied from as little as 5% to more than 70% as judged by coat colour and GPI analysis. All chimeras developed and grew well postnatally.

Table 1. PGES→CFLP chimeras produced by blastocyst injection

PGES line	Passage no.	No. transferred	No. born	Chimeras born
PK1	4	23	21	14 (67%)
PK 2	6	25 (+6 control)	18	5* (42%)
PK 8	3	19(+10 control)	24 (2 dead)	12*(85%)
PK19	2	31	24 (1 dead)	14 (61%)
PK22	2	35(+6 control)	21	6* (40%)

*% chimeric of number born, percentages adjusted to take into account the number of unoperated control blastocysts transferred.

No significant growth retardation was apparent in PGES→N chimeras, irrespective of their degree of chimerism or the PGES cell line used. To confirm this, whole litters were weighed between days 10 and 12 post-partum and weights of chimeric and non-chimeric mice were compared using an unpaired, 2-tail students *t*-test. This compares with a significant growth retardation that was observed in control PG↔N chimeras (Fig. 1).

The participation of PGES cells in different tissues of the mice was determined by GPI allozyme analysis. Six male chimeras were killed at 18-20 days of age, three from line PK1 and three from line PK8; the mice were chosen as representatives of relatively high, medium and low PGES contribution to coat colour in these two lines. A seventh male (chimera 8-M4) was killed at three months; this was the most heavily pigmented (80%) of all chimeras born. Participation of PGES cells was found in all tissues tested, though not in every animal (Table 2). The contribution to skeletal muscle and testis was considerably lower than in the other tissues tested. For skeletal muscle, GPI-AB heterodimers as well as GPI-BB were detected in samples from five of the animals (GPI-AB heterodimers result from fusion between host and PGES derived myoblasts). In a control male chimera made by blastocyst injection of HM1 cells (GPI-1A) into F_1 blastocysts (GPI-1B) a very good contribution of ES cells was seen in skeletal muscle samples as well as in all other tissues tested.

A more detailed analysis was made of the female chimeras, which were also tested for germline transmission of the PGES cells. Germline transmission is the most important functional

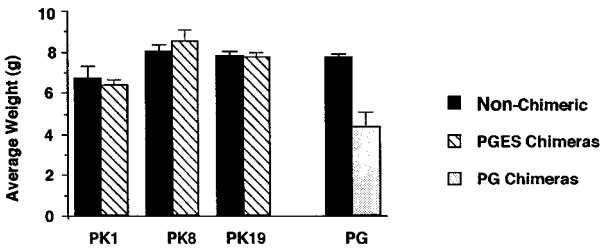

Fig. 1. PGES→N chimeras show no growth retardation compared with PG↔N chimeras. When the weights of non-chimeric and chimeric PGES→N littermates were compared, no significant differences were detected using an unpaired 2-tail students *t*-test (for three PGES↔N litters, PK1-4, PK-8-3, PK19-2, *n* (chimeric/non-chimeric) = 10/8, 8/10, 14/9, and *P*=0.9, 0.45, 0.54 respectively). A significant difference in body weight was detected between non-chimeric (*n*=8) and chimeric (*n*=10) PG↔N littermates, *P* = 0.001.

Table 2. Percentage contribution of parthenogenetic embryonic stem cells (GPI-1B) in male injection chimeras with CFLP blastocysts (GPI-1A)

Chimera	Cell line	% Non-albino	Age	Muscle 1	Muscle 2	Muscle 3	Heart	Liver	Brain	Spleen	Blood	Lung	Gonad
1-M1*	PK1-4°	50	18 day	10	ND	15	50	35	30	50	40	50	<10
1-M2	PK1-4°	30	18 day	0	ND	10	45	25	20	50	50	10	<10
1-M3	PK1-4°	10	18 day	0	ND	0	50	65	25	50	50	15	20
8-M1	PK8-3°	30	18 day	10	<10	<10	25	50	10	25	20	40	20
8-M2*	PK8-3°	50	18 day	20	0	0	35	30	25	60	ND	50	<10
8-M3	PK8-3°	15	18 day	<10	0	0	20	0	0	15	10	0	<10
8-M4*	PK8-3°	80	3 month	25	20	35	50	70	50	70	70	50	<10
HM1-F1*	HM1	35	6 month	50	50	60	50	10	40	20	15	15	<10

*GPI-1AB heterodimers were observed in muscle samples.
Muscle samples 1, 2 and 3 are forelimb, hindlimb and tongue, respectively.

criterion for genetic normality of the PGES cell lines and it is known from previous studies that parthenogenetic cells can give rise to a functional germline. Female chimeras were crossed with CFLP males and non-albino offspring were found in the litters of five chimeras, from three of the PGES cell lines used (Table 3). Three of the germline chimeras were females with relatively low overall levels of PGES contribution (1-F2, 1-F3 and 19-F4). PK8, the PGES line that gave the highest overall levels and rate of chimerism in injected blastocysts, yielded no germline chimeras. For line PK2, only one female chimera reached adulthood and she was not germline chimeric. Lines PK8 and PK2, which did not transmit through the germline, nevertheless had the normal complement of 40 chromosomes.

The female chimeras were killed between 6-7 months of age for GPI analysis (Table 4). Again, most notable was the poor participation of PGES cells in skeletal muscle, for which four different muscle samples were analysed (fore-limb, hind-limb, back and tongue); detectable levels of GPI-1B and GPI-1AB heterodimers were only found in muscles of two of the females analysed. In contrast, good PGES participation was seen in the other tissues tested. When chimeras derived from the five different PGES cell lines are compared, no significant difference in the pattern of PGES cell participation is apparent. However, a greater overall contribution of PGES cells to the chimeras, both male and female, was observed for the PK8 ES line.

Development of PGES↔tetraploid CFLP chimeras

To test further the developmental potential of the PGES cells we attempted to make embryos derived predominately or exclusively from PGES or PG cells by aggregation with tetraploid host embryos. In such aggregations, the tetraploid host cells contribute fully to the development of the extraembryonic membranes and cooperate with experimental cells during early postimplantation development before being gradually selected against in the embryo proper. In this way, mice derived entirely from ES cells have been generated previously, and this has also been repeated in our laboratory (Nagy et al., 1990, 1993). If the PGES cells were substantially altered due to changes in the imprinted status of some important genes, then the phenotype of embryos derived from PGES cells could be strikingly different from pure PG embryos, as well as from PG embryos derived from reconstituted blastocysts (Barton et al., 1985; Gardner et al., 1990).

For this experiment we initially used the PK8 ES cell line since these cells participated better overall in chimeras with normal CFLP embryos. However, we subsequently used the PK1 ES cells because of their germline transmission, thus proving their totipotency. Tetraploid aggregations were also made with control PG embryos, but no PG midgestation embryos could be recovered. For the PK1 ES cell line we did not succeed in significantly rescuing parthenogenetic development beyond that previously observed for PG embryos (Barton et al., 1985; Gardner et al., 1990). Out of 217 PK1↔4n CFLP embryos transferred to pseudopregnant recipient foster mothers, only five embryos containing PK1 cells were recovered between days 13 and 17 of gestation (Table 5). Embryo A was dying and morphologically similar to a day-13 embryo although it was recovered on day 17 of gestation (Table 6). The remaining embryos recovered were chimeric (embryos C,D,E and F). Three additional embryos recovered were predominately host cell derived (embryos B,G and H).

Table 3. Germline transmission of PGES cells

Chimera	PGES cells and passage no.	% Non-albino	GPI-1a% blood	Germline transmission
1-1	PK1-4	20	50	0/7
1-2	PK1-4	15	65	3/24
1-3	PK1-4	<10	35	1/9
2-1	PK2-6	40	<10	0/11
8-1	PK8-3	15	15	0/27
8-3	PK8-3	65	50	0/32
8-4	PK8-3	40	50	0/13
8-5	PK8-3	50	40	0/26
19-1	PK19-2	60	40	0/10
19-2	PK19-2	70	50	0/13
19-3	PK19-2	<10	0	0/7
19-4	PK19-2	<10	0	3/14
19-5	PK19-2	15	25	3/40
22-1	PK22-2	20	65	0/8
22-3	PK22-2	35	20	0/12
22-4	PK22-2	15	25	8/28
R26PK-1	R26PK-4	35	ND	1/10

Table 4. Percentage contribution of parthenogenetic embryonic stem cells (GPI-1B) in adult (6 month) female injection chimeras with CFLP blastocysts (GPI-1A)

Chimera	Cell line	% Non-albino	Muscle 1	Muscle 2	Muscle 3	Muscle 4	Heart	Liver	Brain	Spleen	Blood	Kidney	Ovaries
1-F1	PK1-4°	10	<10	0	0	0	50	40	35	50	50	20	15
1-F2	PK1-4°	15	ND	ND	0	ND	15	15	15	50	60	10	10†
1-F3	PK1-4°	<10	0	0	0	0	0	0	0	50	35	15	10†
1-F4	PK1-4°	<10	0	0	0	0	0	0	0	0	0	0	0
2-F1	PK2-6°	30	0	0	0	<10	40	0	25	30	<10	0	20
8-F5	PK8-3°	35	0	0	0	0	50	60	35	50	40	15	35
19-F1*	PK19-2°	30	<10	0	<10	0	50	0	50	50	40	40	45
19-F2*	PK19-2°	50	20	<10	<10	<10	50	10	30	50	50	40	15
19-F3	PK19-2°	<10	0	0	0	0	<10	0	0	0	0	0	0
19-F5	PK19-2°	10	ND	ND	0	ND	50	45	40	50	25	30	35†
22-F1	PK22-2°	10	0	0	0	0	15	0	0	50	50	25	<10
22-F3	PK22-2°	30	0	0	0	0	35	0	15	50	50	20	10
22-F4	PK22-2°	10	ND	ND	0	ND	30	35	<10	35	40	0	10†
R26PK-1	R26PK-4°	35	0	0	0	0	25	20	15	20	ND	15	ND†

Parthenogenetic contribution was assessed by GPI-allozyme analysis except for R26PK-1 which was assessed by the proportion of cells expressing the *lacZ* reporter gene in tissue sections.

Muscle samples 1, 2, 3 and 4 are forelimb, hindlimb, back and tongue, respectively. For R26PK-1, four additional muscle samples were assayed and all were negative for *lacZ* staining. Good staining in muscle was observed in control ROSA26 muscle.

*GPI-1AB heterodimers in muscle samples; †Germline chimeras, see table 3.

For the PK8 ES cells, slightly better development was observed; out of 147 PK8↔4n CFLP embryos transferred, four predominately ES cell derived embryos were recovered together with two chimeras, as judged by GPI-isozyme analysis (Tables 5 and 6). The PK8 derived day-13 embryos had very good overall morphology compared with controls, however, one consistent phenotype was a slight extension of the hindbrain region with an enlarged fourth ventricle, appearing translucent in the whole-mount embryos (Fig. 2).

Differentiation of PGES cells in teratomas

The differentiation of ES cells was also analysed in teratomas, produced by transfer of ES cell aggregates under the kidney capsule of isogenic adult host mice. Six ES cell lines were used for comparison, two parthenogenetic (PK8 and PK19), two androgenetic (AK12 and AK27) and two normal diploid ES cell lines (D3 and CCE). All ES cell lines gave mixed teratomas; however, each varied in its composition depending on its genotype. Fig. 2 shows three representative tissue sections from PK19 (A), AK12 (B), and CCE (C) derived teratomas. Most strikingly, the PGES (Fig. 3A) teratomas contained very little skeletal muscle; up to 5% skeletal muscle could be found in some sections but in the majority, no muscle was seen. This contrasts with AGES teratomas (Fig. 3B), which were composed predominately of skeletal muscle with some cartilage, and control teratomas (Fig. 3C), which were up to 25% muscle.

Analysis of *Igf2, H19* and *Igf2r* expression in differentiated PGES cells

The expression of the imprinted genes *Igf2, H19* and *Igf2r* was studied following differentiation of ES cells in vitro. *Igf2* and *Igf2r* expression was of particular interest since no growth retardation of the PGES↔normal chimeric mice was observed. For these studies, three ES cell lines were compared, PK1, D3 and AK1.

ES cells were induced to undergo differentiation in vitro under three conditions. Cells were allowed to form aggregates on bacteriological dishes in ES cell medium (without LIF), and in the presence of 0.3 μM all-*trans* retinoic acid (RA) or 1% dimethyl sulphoxide (DMSO) (Rudniki and McBurney, 1987). After 4 days in culture, aggregates were transferred to gelatinised tissue culture dishes and cultured for a further 4 days in ES medium before RNA was prepared. Northern analysis, controlled by hybridisation with *GAPDH*, showed that *Igf2* expression was repressed in PK1 cells compared with D3 or AK1 cells (Fig. 4). Very little expression was detected in the differentiated PK1 cultures with DMSO or RA, although some expression could be detected after a long exposure of the films.

Table 5. Production of PGES↔tetraploid CFLP chimeras

PGES cells and passage no.	No. chimeras transferred	Day analysed*	Embryos/ implantations	Embryo designation
PK1-7	31	d17	1/15	A
PK1-7	20	d16	3/17	B, C, D
PK1-8	26	d15	0/14	
PK1-9	20	d14	0/13	
PK1-8	120	d13	4/72	E, F, G, H
PK8-5	53	d15	1/19	I
†PK8-5	29	d14	1/25	J
PK8-4	13	d13	2/7	K, L
†PK8-5	32	d11	6/28	M, N, O, P, Q, R
PG 8-cell	38	d13	0/30	

*Period of gestation with day 1=day of vaginal plug of recipient foster mother

†Chimeras made by blastocyst injection of PK8 cells.

Table 6. Appearance of PGES↔tetraploid CFLP chimeras

Embryo	Age	PGES	Description (% GPI-1B)
A	d17	PK1	Dying, appearance of d13 (>90% PGES)
B	d16	PK1	All GPI-1A, probable diploid host
C	d16	PK1	Chimeric, 70% PGES derived
D	d16	PK1	Chimeric, 50% PGES derived
E	d13	PK1	Retarded, chimeric limbs and tail, 60% PGES*
F	d13	PK1	Dead d12, 50% PGES
G,H	d13	PK1	Dying tetraploid
I	d15	PK8	Limbs >90% PGES*
J	d14	PK8	Normal d14, >90% PGES
K,L	d13	PK8	Normal d13, >90% PGES
M,N	d11	PK8	Dying chimera, 50% PGES
O,P,Q,R	d11	PK8	Dying tetraploid

*Embryos E and H were prepared for in situ hybridisation analysis after sampling limbs, tail and branchial arches for GPI analysis. Cells in sections appeared highly picnotic and good in situ data was not obtained.

In contrast, *Igf2* was induced at high levels in differentiated AK1 cells and D3 cells. In all expressing cultures, multiple transcripts were observed, reflecting splice variants and usage of the different *Igf2* promoters (Rotwein and Hall, 1990). For each cell line, the level of *Igf2* induction was dependent on the treatment given. Interestingly, the AK1 cells responded more to RA than to DMSO, whereas the D3 cells responded more to DMSO than to RA. In contrast, the reciprocally imprinted genes *Igf2r* and *H19* were expressed in the PK1 cultures as well as in the D3 cultures, and no *Igf2r* expression was seen in the AK1 cultures. However, *H19* expression was seen in the differentiated AK1 cells; for RA-treated cells this was in fact higher than the expression seen in D3 cells.

It is likely that some of the expression differences observed reflect some differences in the cell types that result from each treatment. At the time that RNA was prepared, no major differences in the cell types present in each culture were observed; however after prolonged culture, significant differences in the differentiated cell types were seen (N. D. A., unpublished observation). (D3 cultures were very heterogeneous, with a high proportion of parietal endoderm-like cells; the AK1 cultures contained a large proportion of fused myoblasts, together with some parietal endoderm. In contrast, PK1 cells developed poorly in long term cultures; some fibroblast and epithelium-like cells were seen together with some neuronal and glial-like cells.)

To extend these studies further, we looked at the expression of *Igf2* and *H19* in ES cells differentiated in vivo and then selected in vitro. For this purpose, additional PGES cell lines were made (R26PGES), derived from the transgenic mouse line ROSA26, which expresses *lacZ* and neomycin ubiquitously (Friedrich and Soriano, 1991). One R26PGES cell line was tested in adult chimeras and was found to behave similarly to the other PGES cell lines described in detail above. Firstly, there was no growth retardation, and secondly, no R26PGES cells were detected in skeletal muscle (eight samples tested) but R26PGES cells were found in heart, brain, kidney, spleen, and liver and the cells were transmitted through the germline (Tables 3 and 4). To analyse gene expression in R26PGES cells differentiated in vivo, R26PGES↔CFLP chimeras were made and allowed to develop to day 13 of gestation. R26PGES derived cells were then selected from the control CFLP cells in fetal chimeras by virtue of their G418 resistance in cell culture. Disaggregated embryo chimeras were placed under selection for 10 days in 300 μg/ml G418. After this time a proportion of the selected cells were stained for β-galactosidase activity to confirm that the cells were R26PG derived (data not shown). RNA was then prepared from cultures and was studied by northern analysis. As controls, cells were selected from normal ROSA26↔CFLP and ROSA26PG↔CFLP chimeras. Fig. 5 shows that *Igf2* expression was repressed in cells selected from the R26PGES↔CFLP chimeras (lanes 1-4) and from the R26PG↔CFLP chimeras (lanes 5 and 6), whereas *Igf2* expression was seen in cultures from the normal R26 selected cells (lanes 7-12). In contrast to the in vitro differentiation study (Fig. 4), fewer different *Igf2* transcripts were observed in the control R26 cultures, the majority were expressed from promoter 3. *H19* expression was seen in all the selected cultures. In all cultures, a wide selection of cell types was observed, typical of midgestation embryo primary cell cultures. In these experiments, it is unlikley that the 10-day in vitro culture period has significantly affected the expression of

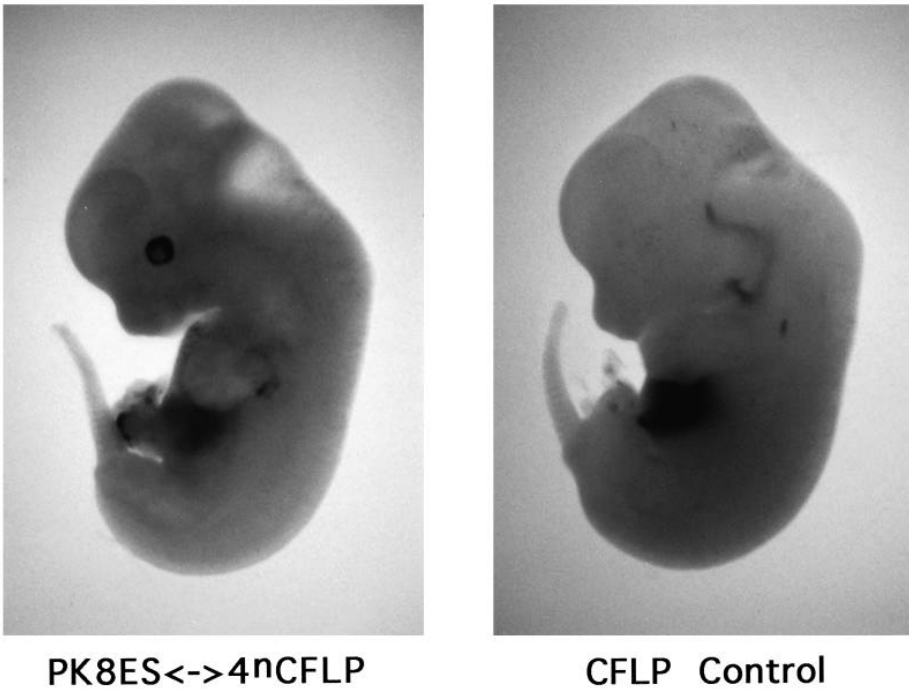

Fig. 2. Left, a PK8ES derived embryo obtained by aggregation with tetraploid CFLP host embryos (albino) and recovered on day 13 of gestation. Right, a CFLP control embryo from the same litter. Note the uniformly pigmented eyes in the PK8ES embryo, no participation of CFLP cells could subsequently be detected by *GPI* isozyme analysis.

Igf2 or *H19*, since *Igf2* repression has been shown to be stable in cell lines derived from embryos maternally disomic for distal chromosome 7, which includes these two genes (Eversole-Cire et al., 1993).

DISCUSSION

Comparisons between PGES↔CFLP and PG embryo↔CFLP chimeras

The participation of parthenogenetic cells (PG) from activated embryos in chimeras with normal host embryos has been analysed extensively in the past (Fundele et al., 1989, 1990; Thomson and Solter 1988; Nagy et al., 1987, 1989; Clarke et al., 1988; Paldi et al., 1989; Thomson and Solter 1989; Jägerbauer et al., 1992). The two most apparent phenotypes of these chimeras are, firstly, a selection against the participation of PG cells in certain tissues, most notably in skeletal muscle, and secondly, growth retardation that is proportional to the overall contribution of PG cells in chimeras; higher contribution of PG cells results in greater growth retardation and a high rate of infant mortality.

In this study we have characterised the behaviour of parthenogenetic ES cells in chimeric mice. Since the pluripotency of normal ES cell lines is known to vary and to gradually decrease with passage number, the PGES→CFLP chimeras were made with very early passage PGES cells. In chimeras, the PGES cells resembled PG cells in their tissue distribution, most notably with a poor contribution to skeletal muscle. However, higher overall levels of PGES cells were detected in this study than in our previous studies of PG↔N chimeras (Fundele et al., 1989). Thus, for skeletal muscle, whereas eight out of 21 PGES chimeras tested showed some contribution to skeletal muscle, in the previous study only one of 13 PG chimeras tested showed a contribution to skeletal muscle. In both studies, GPI-1AB heterodimers derived from fused myoblasts were detected.

We also assessed the developmental potential of two PGES cell lines in tetraploid chimeras, which allows the development of totally ES cell derived mice (Nagy et al., 1990). The best previously recorded parthenogenetic development is a day-12, 44-somite embryo, derived from a triple tissue blastocyst reconstitution experiment in which parthenogenetic primitive ectoderm was provided with fertilised derived primitive endoderm and trophectoderm (Gardner et al., 1990). This represented a slight improvement on earlier blastocyst reconstitution experiments in which several 35- to 40-somite parthenogenetic embryos were obtained (Barton et al., 1985). The four day-13, predominately PK8 ES cell derived embryos, obtained with the tetraploid chimeras might represent an improvement in parthenogenetic embryo development. However, PK8 was also the cell line that deviated most from the expected parthenogenetic phenotypes in the diploid chimeras. Overall,

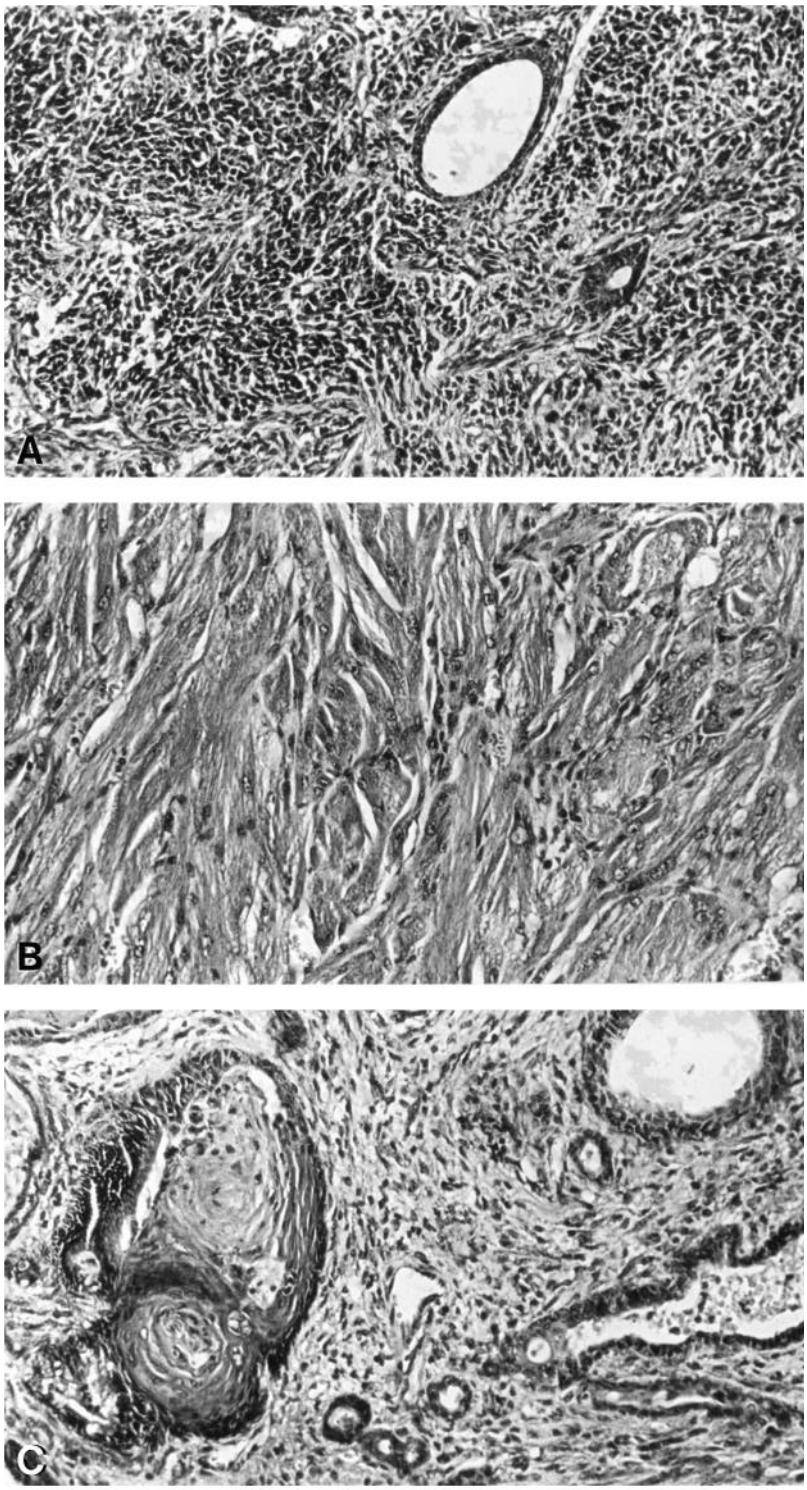

Fig. 3. Representative tissue sections of PGES (A), AGES (B) and D3 (C) derived teratomas produced by ectopic transfer of ES cell aggregates under the kidney capsule of adult host mice. PGES (PK19) derived teratomas were heterogenous in composition, but largely devoid of striated muscle (A), as compared with AGES (AK12) derived teratomas in which striated muscle predominated (B). Control ES (CCE) derived teratomas were highly heterogenous (C), including up to 25% striated muscle.

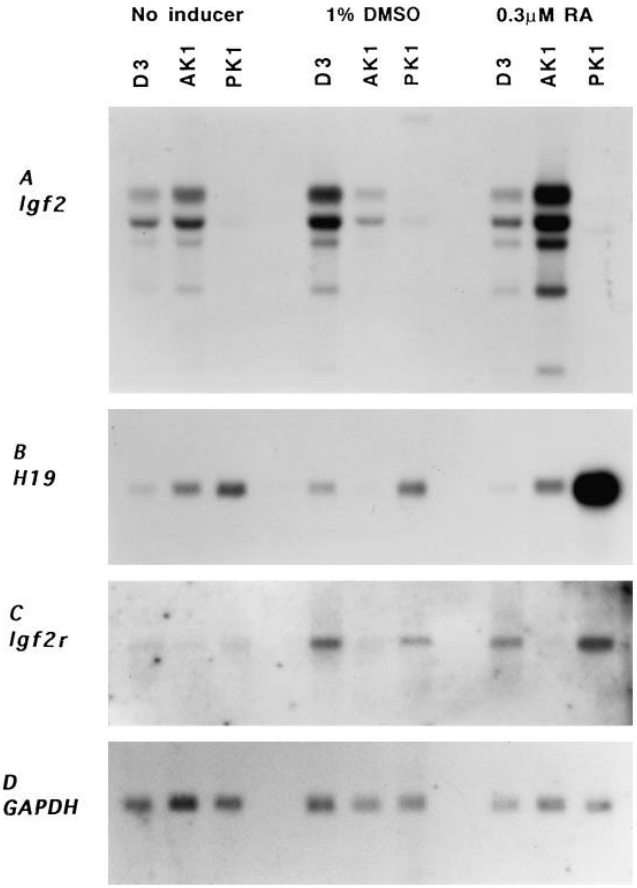

Fig. 4. Northern analysis of *Igf2*, *H19* and *lgf2r* expression in differentiated ES cell cultures. Control D3, AK1 and PK1 ES cells were allowed to form simple embryoid bodies (EB) for four days in bacteriological culture wells in the absence of LIF, and in the presence of either 1% DMSO, or 0.3 μM all-*trans* retinoic acid. EBs were then plated for a further 4 days on tissue culture plates before RNA was prepared for northern analysis. The probes used were *Igf2*, *H19* and *Igf2r* cDNAs; and hybridisation to GAPDH cDNA is shown as a loading control.

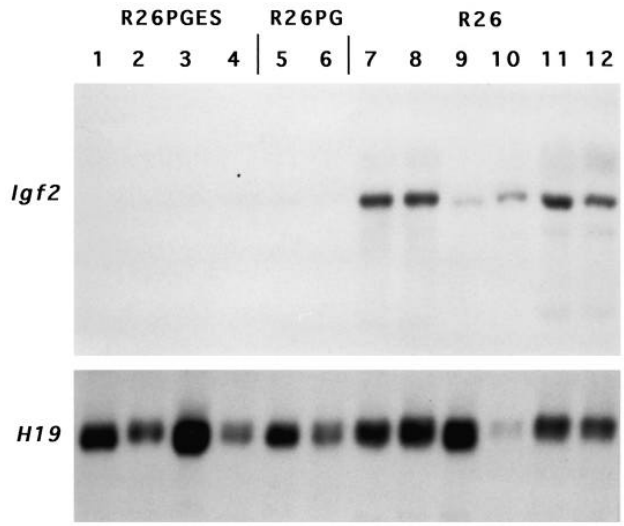

Fig. 5. Northern analysis of *Igf2 and H19* expression in R26PGES (lanes 1-4), R26PG (lanes 5 and 6) and R26 derived cells (lanes 7-12), differentiated in chimeras in vivo and then isolated from host cells under G418 selection in vitro for 10 days. The probes used were *Igf2* and *H19* cDNAs.

PK8 cells contributed better in chimeras, with more GPI-AB heterodimers being observed in muscle samples. PK8 cells also failed to be transmitted through the germline, indicating a general loss of totipotency. Such a loss of totipotency may involve altered epigenetic programming, which also affects imprinting, thereby allowing better rescue in fetal development in the tetraploid assay. In contrast, the PK1 ES cells were not significantly rescued in the tetraploid chimeras and gave good germline transmission from diploid chimeras.

In direct contrast to studies of PG↔N chimeras, we saw no evidence of pre- or postnatal growth retardation with any of the PGES cell lines tested. Prenatal growth retardation in chimeras with PG cells is observed as early as day 13 of gestation and continues throughout fetal and neonatal life (Fundele et al., 1990). While there may be some general epigenetic changes in ES cells as a consequence of their adaptation to in vitro tissue culture conditions (without necessarily affecting imprinted genes), the absence of growth retardation in the PGES→CFLP chimeras could suggest a disruption of normal imprinting in one or more genes involved in growth regulation.

One candidate gene for aberrant imprinting affecting fetal growth could be *Igf2*, which is repressed in parthenogenetic embryos and in embryos maternally disomic for distal chromosome 7 (Ferguson-Smith et al., 1991; R. Ohlsson, unpublished data). However, *Igf2* expression may not be involved in the discrepancy between PG and PGES phenotypes, since *Igf2* repression was observed using various differentiation strategies both in vitro and in vivo. Importantly, the deficiency of *Igf2* expression was inversely correlated with high levels of *H19* expression. Since *Igf2* and *H19* are apparently co-expressed in embryonic tissues during development (Poirier et al., 1991; Lee et al., 1990), the reciprocal expression of *Igf2* and *H19* favour the notion that PGES retain their parental imprints, at least for these two genes, after differentiation of PGES cells. The differences in *Igf2* and *H19* expression cannot therefore be merely a reflection of different cell types obtained after PGES cell differentiation when compared with the AK1 and D3 ES cells.

In addition to *Igf2*, we studied *Igf2r* and found it to be appropriately expressed following in vitro differentiation of PK1, AK1 and D3 ES cell cultures. However, we did detect quantitative differences in the levels of *Igf2r* expression in PK1 cultures compared with D3 cultures, which depended on the differentiation treatment given. Thus PK1 cells expressed more *Igf2r* than D3 cells when cultured in retinoic acid but expressed less *Igf2r* than D3 cells when cultured with DMSO. *Igf2r* has a role in regulating levels of IGF II by removing any excess ligand and thereby regulating growth. Since *Igf2r* is normally only active when maternally inherited, the levels of this receptor in PG cells should be in excess compared to normal cells. Such an excess of IGF II receptor may cause a functional deficiency of IGF II ligand resulting in growth retardation of PG↔N chimeras. Although expression per se of *Igf2* and *Igf2r* was appropriate in the PGES cells when compared with control

and androgenetic cells, it is still possible that small quantitative changes in the expression of these genes may affect the phenotype observed in chimeras.

It is also noteworthy that no growth retardation was previously observed in chimeras made with embryos maternally disomic for distal chromosome 7 (Ferguson-Smith et al., 1991). Since mice carrying an *Igf2* mutation are growth retarded, the *Igf2* deficit in chimeras is presumably compensated for by host cells (DeChiara et al., 1990). In addition to chromosome 7, it is important to note that imprinted genes on chromosome 11 also apparently affect size (Cattanach and Beechey, 1990). It is therefore possible that the imprinting of genes on this chromosome could be affected in PGES cells.

In our differentiation studies, we also showed that *H19* is expressed in androgenetic cells despite the fact that this gene is normally repressed when inherited paternally. However, this is consistent with our previous observations that *H19* is not imprinted in the trophoblast of a wholly androgenetic conceptus and that in chimeras, *H19* expression appears in AG cells in the embryo proper as they start to undergo differentiation (R. Ohlsson, unpublished data; A. S., N. D. A., unpublished observations).

In conclusion, the PGES cells carefully analysed here provide a valuable new in vitro model system for studies on imprinting in the mouse. In particular, since the PGES cells closely resemble PG cells in their developmental potential in vitro as well as in vivo, they can be used to study aspects of cell differentiation and committment related to imprinting. These cells are also valuable in the molecular analysis of imprinting since epigenetic changes in imprinted genes associated with differentiation and the loss of totipotency can be analysed extensively under defined differentiation conditions (R. Feil and N. D. A., unpublished data). The anomalous growth regulation observed in our chimeras is also of great interest. While this may reflect possible changes in imprinting, it is likely that there are also more general epigenetic modifications that affect non-imprinted genes in ES cells. During the derivation of all types of cell lines, there may be a certain degree of adaptation to culture conditions involving subtle changes in epigenetic modifications; some such effects may influence phenotype. Nevertheless, the identification of aberrant imprinting in the PGES and AGES cells will be of great interest, especially since loss of imprinting of *Igf2* and *H19* has recently been observed in some cancers (Rainier et al., 1993; Ogawa et al., 1993; Feinberg, 1993).

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