Expression and methylation of imprinted genes during in vitro differentiation of mouse parthenogenetic and androgenetic embryonic stem cell lines

Piroska Szabó and Jeff R. Mann
Division of Biology, Beckman Research Institute of the City of Hope, 1450 East Duarte Road, Duarte, California 91010-0269, USA

SUMMARY

Messenger RNA and methylation levels of four imprinted genes, H19, Igf2r, Igf-2 and Snrpn were examined by northern and Southern blotting in mouse parthenogenetic, androgenetic and normal or wild-type embryonic stem cell lines during their differentiation in vitro as embryoid bodies. In most instances, mRNA levels in parthenogenetic and androgenetic embryoid bodies differed from wild type as expected from previously determined patterns of monoallelic expression in midgestation embryos and at later stages of development. These findings implicate aberrant mRNA levels of these genes in the abnormal development of parthenogenetic and androgenetic embryos and chimeras. Whereas complete silence of one of the parental alleles has previously been observed in vivo, we detected some mRNA in the corresponding embryonic stem cell line. This ‘leakage’ phenomenon could be explained by partial erasure, bypass or override of imprints, or could represent the actual activity status at very early stages of development. The mRNA levels of H19, Igf2r and Igf-2 and the degree of methylation at specific associated sequences were correlated according to previous studies in embryos, and thereby are consistent with suggestions that the methylation might play a role in controlling transcription of these genes. Paternal-specific methylation of the H19 promoter region is absent in sperm, yet we observed its presence in undifferentiated androgenetic embryonic stem cells, or before the potential expression phase of this gene in embryoid bodies. As such methylation is likely to invoke a repressive effect, this finding raises the possibility that it is part of the imprinting mechanism of H19, taking the form of a secondary imprint or postfertilization epigenetic modification necessary for repression of the paternal allele.

Key words: imprinting, methylation, androgenetic, parthenogenetic, embryonic stem cells, embryoid bodies, mouse genetics

INTRODUCTION

Endogenously imprinted genes are defined as those that exhibit inequality in activity of maternally and paternally derived alleles. The abnormal development of androgenetic, parthenogenetic and certain uniparental disomic embryos is thought to be due to aberrant total expression levels of these unusually expressed genes. For example, in normal embryos, if the paternal allele of an imprinted gene was active and the maternal silent, then in androgenetic embryos and embryos paternally disomic for the autosome region containing the gene, the total expression level would presumably be excessive, due to the presence of two active copies. Conversely, a lack of activity would be expected in their maternal counterparts; parthenogenetic and maternal disomic embryos (Cattanach and Kirk, 1985). To date, five imprinted genes have been reported: H19, (Bramman et al., 1990; Bartolomei et al., 1991); insulin-like growth factor 2 receptor, Igf2r, (Barlow et al., 1991); insulin-like growth factor-2, Igf-2, (DeChiara et al., 1991); small nuclear ribonucleoprotein N, Snrpn (Cattanach et al., 1992; Leff et al., 1992), and U2 small nuclear ribonucleoprotein auxiliary factor-related sequence, U2afbp-rs or sp2 (Hatada et al., 1993; Hayashizaki et al., 1994). For all of these genes, measurable RNA is derived entirely from either the maternal or paternal allele when examined in midgestation embryos and at later stages of development.

Here we describe the mRNA levels of H19, Igf2r, Igf-2 and Snrpn during in vitro differentiation of parthenogenetic and androgenetic embryonic stem (ES) cell lines. These studies have been carried out with a number of considerations in mind. Firstly, as an initial requirement in determining whether aberrant expression of any of these genes plays a role in the abnormal development observed in parthenogenetic and androgenetic embryos, and in chimeras containing parthenogenetic and androgenetic cell types (Barton et al., 1984; McGrath and Solter, 1984; Mann and Lovell-Badge, 1984; Surani et al., 1984; Kaufman et al., 1989; Mann et al., 1990; Barton et al., 1991; Mann and Stewart, 1991), their level of expression in parthenogenetic and androgenetic embryos and cell types must be characterized. Measurements in embryos are complicated by the fact that parthenogenones and androgenones are usually lethal at the peri-implantation stage, and consequently no quantitative information exists. However, this limitation could be circumvented to some extent by using ES cell lines derived from these embryos. ES cells are the in vitro counterpart of blastocyst inner cell mass (ICM) cells (Beddington and Robertson, 1989), and their differentiation in suspension as embryoid bodies (EBs) mimics, at a rudimentary level, events...
occurring at the late pre- and early postimplantation stages (Martin, 1975; Stevens, 1975; Doetschman et al., 1985). The developmental expression pattern of at least H19 is mimicked in EBs, in which RNA is first detected in the outer endoderm layer (Poirier et al., 1991). Also, parthenogenetic and androgenetic ES cells retain at least some of the genomic imprinting present in their ICM cell ancestors, in spite of many divisions without differentiation in vitro. This is most strikingly evident in androgenic ES cells, which induce the same abnormalities in chimeras as are observed when androgenetic cleavage stage eggs or ICM cells are used to form chimeras. By similar criteria, there is evidence for both imprinting loss and retention in parthenogenetic ES cells (Mann, 1992; J. Mann and C. Stewart, unpublished data).

Secondly, mRNA levels of imprinted genes during in vitro differentiation of parthenogenetic and androgenetic ES cells could provide clues as to the parental-specific expression level of these genes at very early stages of development, for which, to date, there is no information, and thereby provide insights into imprinting mechanisms. Whilst such an analysis would best be conducted on normal or wild-type embryos before and during gastrulation, quantitative allele-specific RNA analysis of small numbers of cells is technically difficult. This question of parental-specific expression patterns of imprinted genes during early development is of interest in that imprinting could be a multistep process that, although at least initiated in the germ line, might involve additional postfertilization epigenetic events necessary for the establishment of monoallelic expression (Allen and Mooslehner, 1992; Chaillet, 1992). There is a precedent for this in that the paternally derived X chromosome is active before it undergoes preferential inactivation in the trophectoderm and primitive endoderm (Takagi and Sasaki, 1975; Epstein et al., 1978; Kratzel and Gartler, 1978).

Thirdly, parthenogenetic and androgenetic ES cells and EBs are potentially useful for analyzing at least some aspects of the molecular mechanism of genomic imprinting, and therefore it is important to determine their status, with respect to what is known, from studies in embryos, of parental-specific epigenetic modifications of imprinted genes. DNA methylation is a strong candidate for the imprinting mechanism as it can be clonally inherited and can influence gene expression (Riggs, 1989; Efstratiadis, 1994). Also, methylation patterns of transgenes (Allen and Mooslehner, 1992; Chaillet, 1992) and imprinted genes (Sasaki et al., 1992; Stöger et al., 1993; Bartolomei et al., 1993; Brandeis et al., 1993; Ferguson-Smith et al., 1993) can be correlated with their level of expression. In the case of Ig2r, methylation of an intron II region has been suggested as the sole imprinting mechanism (Stöger et al., 1993). Here, we have examined whether similar correlations of methylation and mRNA levels also exist in parthenogenetic and androgenetic ES cells and EBs.

### MATERIALS AND METHODS

Two sets of cell lines were used. Each was composed of one parthenogenetic, one androgenetic and one wild-type cell line. Each of the two sets was analyzed for mRNA levels (in northern blots) and methylation patterns (in Southern blots) in differentiating EBs derived from ES cells at early passage numbers. This analysis was repeated in the same sets of cell lines at later passage numbers. In obtaining material for analysis, the three cell lines in each set were cultured at the same time and under the same conditions.

### Embryonic stem cell lines

The genotype of all cell lines was of the 129/Sv.C3+4/+ inbred strain (Stevens, 1970; Jackson Laboratory Stock No. JR0090 with the steel-J mutation bred out by intercrossing). Diploid 1-cell eggs for ES cell line derivation were obtained as follows: Parthenogenetic lines LG.1 (Pg-1) and LG.5 (Pg-2); ethanol induced activation of unfertilized eggs (Kaufman, 1982; Cuthbertson, 1983), followed by inhibition of second polar body extrusion by incubation for 4 hours in 5.0 µg/ml cytochalasin B (derived by J. Mann and C. Stewart). Wild-type lines W9.2 (Wt-1) and W9.5 (Wt-2); natural mating. Androgenetic lines LB.1 (Ag-1) and LB.4 (Ag-2); pronuclear transplantation (Mann et al., 1990). The sex chromosome constitutions of the cell lines are; Pg-1 and Pg-2, both XX; Wt-1, Wt-2, Ag-1 and Ag-2, all XY. Passage numbers (no.) at which analyses were conducted: early passage; Pg-1 no. 12, Pg-2 no. 2, Wt-1 no. 7, Wt-2 no. 8, Ag-1 no. 10 and Ag-2 no. 9. Later passage; Pg-1 no. 27, Pg-2 no. 32, Wt-1 no. 22, Wt-2 no. 23, Ag-1 no. 25 and Ag-2 no. 23. At early passage, all cell lines had a modal chromosome number of 40. At the later passages studied, all had retained this modal number except for Ag-1 (modal number of 41) and Pg-2 (predominantly polyploid). ES cells were cultured on mitomycin C inactivated STO fibroblast feeder cells transfected with the neomycin and leukemia inhibitory factor genes (kindly supplied by Frank Conlon and Elizabeth Robertson), under standard conditions (Mann et al., 1990).

### Production of embryoid bodies

Differentiation of ES cells was carried out in viscous methylcellulose medium. The following description is for one near confluent 6 cm dish of ES cells grown as described above. On day 0, fresh growth medium was added, then 3-5 hours later, the cells were trypsinized, dispersed into a single cell suspension, then transferred to a standard nongelatinized 10 cm tissue culture dish. This dish was incubated at 37°C for 1 hour to allow for removal of STO cells by differential adherence; during this time, a major proportion of the STO cells had firmly attached, whereas a minor proportion of ES cells had lightly attached, and were removed by moderate pipetting. The level of STO cell contamination in ES cells purified in this way is no more than 3%. ES cells were counted, pelleted, resuspended in 0.7% (w/v) methylcellulose medium at a concentration of 3.5x10^6 cells/ml, and 10 ml of this transferred to a 10 cm agarose-coated bacteriological dish. At day 4, the culture was split between two 10 cm dishes and grown for another 10 days, with medium replaced daily. Methylcellulose medium (500 ml) was made by mixing 150 g of 2.2% (w/v) methylcellulose (Sigma Chemical Co., viscosity of 2% aqueous solution equal to 400 centipoises), 268 ml of DMEM, a total volume of 20 ml of concentrated salt and glucose solutions to achieve standard DMEM concentrations, 50 ml fetal bovine serum, 5 ml each of nonessential amino acids, glutamine, penicillin and streptomycin at standard 100× concentrations, and 0.5 ml of 10^-1 M 2-mercaptoethanol.

### Northern blots

Total RNA was isolated with RNAzol™ B (Tel-Test Inc.). Then, poly(A)^+ RNA was purified using the PolyATract system (Promega Corp.), run on formaldehyde agarose gels, and transferred to GeneScreen™ nylon membrane (NEN Research Products). Hybridization of 32P-labelled probes was carried out at 63°C for 18 hours in 5x SSPE, 1% SDS, 8x Denhardt’s solution and 100 µg/ml denatured herring sperm DNA. Final wash was in 0.2x SSC, 1% SDS at 63°C. Conditions for Srpm were more stringent; hybridization in the above solution at 68°C, and a final wash in 0.1x SSC, 1% SDS at 68°C. Probe details are given in the following order: gene, size, method of production of gene fragment, primer sequences, 5′-3′, upper and lower, source of information for primer design, and method of probe
Imprinting in embryonic stem cells

RESULTS

mRNA levels of cell lineage indicator genes in embryoid bodies

A comparison of the mRNA levels of imprinted genes in parthenogenetic, androgenetic and wild-type EBs requires that all three follow a similar pathway of differentiation. No overt difference in morphology of the three types of EBs was observed during the culture period. However, to provide additional indications of differentiation, we also measured the mRNA levels of genes for which expression is limited to certain cell lineages during gastrulation. Results of hybridizations to poly(A)+ RNA of one set of cell lines at early passage are shown in Fig. 1. In wild-type EBs, mRNA levels of cell lineage indicator genes changed over the 14-day culture period as would be expected from previous observations of their tissue-specific expression patterns in embryos. Sparc and Afp mRNA levels increased, indicating differentiation of parietal and visceral endoderm lineages respectively (Holland et al., 1987; Dziadek and Adamson, 1978; Wilkinson et al., 1988). Oct-3 mRNA levels decreased, indicating gradual loss of undifferentiated ES cells (Rosner et al., 1990). mRNA levels of Mhox also increased, indicating differentiation of mesoderm. Whilst Mhox RNA has been observed only in early mesoderm and its derivatives (Cserjesi et al., 1992; de Jong and Meijlink, 1993), substantial mRNA levels of this gene were present in ES cells before differentiation.

mRNA levels of parthenogenetic and androgenetic cell lines relative to wild-type levels were calculated as described in the legend to Fig. 1. In many instances, the relative levels were close to 1.0, indicating no difference from wild-type levels. In other cases, the relative levels were above 1.0, indicating increased expression, or below 1.0, indicating decreased expression. The dashed line in the figure indicates that the value for that line was not calculated due to lack of expression at the initial day of suspension of ES cells. Numbers under the bands are the mRNA levels relative to the Wt level in the equivalent lane; values were not calculated for that lane. Approximately 2 µg of poly(A)+ RNA was loaded for each lane.
instances where the relative levels differed substantially from 1.0, e.g., >1.3 or <0.7, parthenogenetic and androgenetic EBs differed in the same direction. These comparisons indicate that the parthenogenetic and androgenetic EBs followed similar pathways of differentiation, and thereby offered a satisfactory framework in which to compare the mRNA levels of imprinted genes.

**mRNA levels of imprinted genes in embryoid bodies**

**Early passage**

Hybridizations for both sets of cell lines are shown in Fig. 2. For wild-type EBs (lanes 5-8, Fig. 2A,B), mRNA levels changed over the 14-day culture period in accordance with previous observations of the developmental expression pattern in embryos. H19 and Igf-2 mRNA levels increased dramatically over the 14-day culture period. RNA of both genes is detected in the extraembryonic lineages of trophoblast and endoderm at the egg cylinder stage, appears in mesoderm during gastrulation, and from then on is present in mesoderm derivatives throughout gestation. H19 RNA is detectable in the endoderm layer of EBs by in situ hybridization (Lee et al., 1990; Poirier et al., 1991). Igf2r mRNA levels also increased over the 14-day culture period. IGF2R protein is present in at least some of the tissues that express Igf-2 during early embryogenesis. Also, at fetal stages, tissues with high levels of IGF2R also possess high levels of Igf-2 RNA (Lee et al., 1990). Snrpn mRNA levels decreased during EB differentiation as previously observed (Sharpe et al., 1990). The tissue-specific expression pattern during early embryonic development has not been characterized.

mRNA levels of imprinted genes in parthenogenetic (lanes 1-4, Fig. 2A,B) and androgenetic (lanes 9-12, Fig. 2A,B) EBs were quantified and related to the mRNA level in wild-type EBs as was described for cell lineage indicator genes. In contrast to the results obtained for indicator genes, in most cases the relative values differed substantially from 1.0. Moreover, if present, the direction of difference was as would be expected from previously determined parental-specific expression patterns in midgestation embryos and at later stage stages of development. Thus, for Igf2r and H19, the paternal alleles of which are repressed in fetuses and postnatal mice respectively (Barlow et al., 1991; Bartolomei et al., 1991), mRNA levels in androgenetic EBs were consistently less than wild type (lanes 11 and 12, Fig. 2A,B). Similarly, for Snrpn, the maternal allele of which is repressed in neonatal brain (Leff et al., 1992; Cattanach et al., 1992), mRNA levels in parthenogenetic EBs were also consistently less than wild type (lanes 3 and 4, Fig. 2A,B). However, this was not the case for the other maternally repressed gene, Igf-2, for which mRNA levels could be regarded as similar to wild type (lanes 3 and 4, Fig. 2A,B). In terms of potential overexpression, mRNA levels of H19 in parthenogenetic EBs (lanes 1-4, Fig. 2A,B), and of Igf-2 and Snrpn in androgenetic EBs (lanes 9-12, Fig. 2A,B), were consistently more than wild type. However, this was not the case for Igf2r, the mRNA levels of which could be regarded as being less than wild type in parthenogenetic EBs (lanes 1-4, Fig. 2A,B). It is worth noting that H19 and Igf-2 exhibit the same temporal and lineage-specific pattern of expression during development (Lee et al., 1990; Poirier et al., 1991; Ohlsson et al., 1994). If this was
I double digest,

also the case in EBs, the differences observed in mRNA levels between parthenogenetic and androgenetic EBs for each of these genes were not likely to be due to dissimilar pathways of differentiation.

**Later passage**

Results for both sets of cell lines are shown in Fig. 3. Later passage EBs were studied at 1 and 9 days in culture. In wild-type EBs, changes in mRNA levels of imprinted genes over the 9-day culture period followed the trend observed at early passage. mRNA levels of Igf2r, H19, and Igf-2 had increased by 9 days, whereas mRNA levels of Snrpn decreased (lanes 5-8, Fig. 3). However, for parthenogenetic (lanes 1-4, Fig. 3) and androgenetic (lanes 9-12, Fig. 3) EBs, substantial differences from the situation at early passage were apparent. Igf2r mRNA levels in parthenogenetic EBs had changed from less than wild type, to much less than wild type. H19 and Snrpn mRNA levels in parthenogenetic and androgenetic EBs were now both similar to wild type, indicating derepression with passage of the paternal H19 and maternal Snrpn alleles in androgenetic and parthenogenetic EBs respectively. Igf-2 mRNA levels in androgenetic EBs had changed from more than wild type, to similar to wild type. mRNA levels in parthenogenetic EBs had changed from similar to wild type, to much less than wild type. This latter observation is intriguing, as repression of the maternal alleles of Igf-2 in parthenogenetic EBs was expected at early rather than at later passage.

**DNA methylation of imprinted genes**

**H19 promoter region**

This region was examined as promoter methylation can repress gene activity (Antequera and Bird, 1993), and has been shown to be associated with repression of the paternal H19 allele in fetuses (Bartolomei et al., 1993; Ferguson-Smith et al., 1993). DNA was digested with the methyl insensitive enzyme BamHI to release a 2.3 kb fragment, and smaller fragments produced by cleavage at internal methyl sensitive HhaI or Smal sites and recognized by the probe are described in the legend to Fig. 4. Results from ES cells and EBs were equivalent, thus no differentiation is made in the following discussion. In BamHI/HhaI double digest, parthenogenetic DNA at early passage possessed hypomethylation at the two HhaI sites (lanes 7 and 8, Fig. 4A, C), and lost much of the existing methylation by later passage (lanes 7 and 8, Fig. 4B, D). In contrast, the two HhaI sites in androgenetic DNA were hypermethylated at early passage (lanes 11 and 12, Fig. 4A, C), and by later passage, partial loss of this methylation had occurred (lanes 11 and 12, Fig. 4B, D). The absence of a 1.8 kb band indicates that methylation of the 3′ HhaI site (the site closest to the TATA box) was lost before methylation of the 5′ HhaI site in a cell, but not vice versa (see legend to Fig. 4). In the BamHI/HmaI double digest, in parthenogenetic DNA, the Smal site was hypomethylated at early and at later passage (lanes 13 and 14, Fig. 4A, D). In marked contrast, in androgenetic DNA, the Smal site was hypermethylated at early and at later passage (lanes 17 and 18, Fig. 4A, C), although some of this methylation was lost by later passage (lanes 17 and 18, Fig. 4B, D).

**Igf2r intron II region**

Methylation of this region was investigated because of its possible role in determining monoallelic expression of Igf2r. The active maternal Igf2r allele is methylated at this region, and the inactive paternal allele nonmethylated. Also, some of the differential methylation is inherited from gametes and retained during preimplantation development, consistent with the possibility that it is the imprinting responsible for monoa llelic expression of this gene (Stöger et al., 1993; Brandeis et al., 1993). Results are shown in Fig. 5. Digestion with the methyl insensitive enzyme PvuII released a 3.2 kb fragment, and smaller fragments produced by digestion of internal methyl sensitive HpaII sites and recognized by the probe are described in the legend to Fig. 5. Results in ES cells and EBs were equivalent, thus no differentiation is made in the following discussion. In examining the 3.2 kb band at early passage (lanes 1-6, Fig. 5A, B), HpaII digestion revealed that the wild-type cell line was the most methylated, followed by the parthenogenetic and androgenetic lines in descending order. Thus, in the three types of EBs, there was a positive correlation between the degree of methylation of the HpaII sites with the mRNA level of Igf2r (Fig. 2A, B). This positive correlation was still evident at later passage except for the Pg-1 cell line (lane 7, Fig. 5A, B), which had a similar degree of methylation as the wild-type cell lines (lanes 9 and 10, Fig. 5A, B), but much lower levels of mRNA (lane 2, Fig. 3).

**Igf2r 5′ region**

The repressed maternal allele is hypomethylated in this region, and the active paternal allele hypermethylated (Sasaki et al.,
Digestion with the methyl insensitive enzyme \textit{Pvu} II released an approximately 2.7 kb fragment, and a series of smaller fragments was produced by digestion of internal methyl sensitive \textit{Hpa} II sites. Again, results in ES cells and EBs were similar, and no differentiation is made. At early passage, a similar partial methylation pattern was present in parthenogenetic, wild-type and androgenetic DNA (lanes 1-6, Fig. 6A,B). By later passage, evidently the \textit{Hpa} II sites in parthenogenetic DNA had lost some of this methylation (lanes 7 and 8, Fig. 6A,B), whereas those in wild-type (lanes 9 and 10, Fig. 6A,B) and androgenetic (lanes 11 and 12, Fig. 6A,B) DNA had become further methylated. Thus, the reduction in the level of \textit{Igf-2} mRNA during passage of parthenogenetic ES cells was accompanied by a reduction in the methylation level of this 5′ region.

**DISCUSSION**

The mRNA levels of four imprinted genes during in vitro differentiation of parthenogenetic and androgenetic ES cell lines have been described. Differences in mRNA levels from wild type were similar in two sets of independently derived cell lines, with the direction of difference following expectation based on previously described patterns of parental-specific expression in midgestation embryos and at later stages of development. Thus, it is likely that the aberrant mRNA levels observed are a consistent feature of parthenogenetic and androgenetic cells, and are thereby implicated in the abnormal developmental effects associated with these cell types.

There is evidence, although inconclusive, that aberrant RNA levels of the imprinted genes we have examined could result in abnormal development. Genetic evidence shows that all these genes map to developmentally critical autosome regions as defined by uniparental disomy (Beechey and Cattanach, 1993). Also, \textit{Igf2r} may be the only imprinted gene within the \textit{T\textsubscript{Lub}2} deletion that, when maternally inherited, results in lethality late in gestation (Barlow et al., 1991). Experimental evidence shows that \textit{H19} and \textit{Igf-2} transgenic mice die in utero, one interpretation of which is that excessive expression levels...
of Igf-2 and H19 cannot be tolerated (Brunkow and Tilghman, 1991; Efstratiadis, 1994). Thus, excessive concentrations of the embryonic mitogen IGF-2 (DeChiara et al., 1991; Baker et al., 1993), might be involved in the lethality in utero of androgenetic embryos and chimeras. Also, the mitogenic effects of IGF-2 (Humbel, 1990) suggest that its excess could be involved in the rib cartilage overgrowth syndrome observed in androgenetic chimeras (Mann et al., 1990; Barton et al., 1991; Mann and Stewart, 1991). Excessive Igf-2 in androgenetic chimeras could be exacerbated by the concomitant repression of Igf2r in androgenetic cells, as a function of the IGF2R protein is to sequester IGF-2 (Filson et al., 1993).

Imprinting in embryonic stem cells of Igf-2 and H19 cannot be tolerated (Brunkow and Tilghman, 1991; Efstratiadis, 1994). Thus, excessive concentrations of the embryonic mitogen IGF-2 (DeChiara et al., 1991; Baker et al., 1993), might be involved in the lethality in utero of androgenetic embryos and chimeras. Also, the mitogenic effects of IGF-2 (Humbel, 1990) suggest that its excess could be involved in the rib cartilage overgrowth syndrome observed in androgenetic chimeras (Mann et al., 1990; Barton et al., 1991; Mann and Stewart, 1991). Excessive Igf-2 in androgenetic chimeras could be exacerbated by the concomitant repression of Igf2r in androgenetic cells, as a function of the IGF2R protein is to sequester IGF-2 (Filson et al., 1993). Igf-2 inactivity in parthenogenetic embryos would presumably be of little consequence, as wild-type embryos lacking Igf-2 are viable (DeChiara et al., 1991). The in utero lethality of H19 transgenic mice raises the possibility that excessive H19 mRNA may be a factor in the abnormal development of parthenogenetic embryos. To date, there is no information on the effect of a dysfunctional H19 gene on development. Snpn mRNA levels are not likely to be involved in developmental anomalies in utero, as uniparental disomic embryos with two

![Image](image_url)

**Fig. 5.** Southern blot analysis of methylation at the Igf2r intron II region. ES, undifferentiated ES cells purified by differential adherence. EB, embryoid bodies after 9 days of suspension culture. DNA was double digested with PvuII (not methyl sensitive) and HpaII (H). The approximately 3.2 kb PvuII fragment contains six internal HpaII sites (sequence in Genbank database deposited by Stöger et al., 1993). The probe used is indicated by the solid bar, and recognised a 3.2 kb band (no digestion; all internal sites methylated) and three smaller bands of 1.2, 0.6 and 0.2 kb (complete digestion; the three most HpaII sites nonmethylated). Intermediate band sizes resulting from partial methylation at an allele (any one of the three most HpaII sites nonmethylated concomitant with any other site methylated) were not recognised or barely detected, demonstrating that the HpaII sites at an allele were usually all methylated or all nonmethylated. The extent of digestion was checked with the C-JUN probe as described in the legend to Fig. 4. For the PvuII/HpaII double digest, a single 0.35 kb band was recognized, demonstrating complete cutting (autoradiogram not shown).

**Fig. 6.** Southern blot analysis of methylation at the Igf-2 5′ region. ES, undifferentiated ES cells purified by differential adherence. EB, embryoid bodies after 9 days of suspension culture. DNA was double digested with PvuII and HpaII (H). PvuII released a 2.7 kb band, which contained at least six internal HpaII sites. The solid bar indicates the region spanned by two overlapping probes, which were mixed together in the hybridizations, and which contain HpaII sites 4 and 5 (Brandeis et al., 1993). This hybridization was carried out with the same filters used in the Igf2r hybridization.
maternal or paternal copies of this gene can survive to birth without incident (Cattanach et al., 1992). Further elucidation of the role of imprinted genes in parthenogenetic and androgenetic development could be achieved by correction through genetic manipulation of their mRNA levels in androgenetic and parthenogenetic ES cell lines and embryos.

Although mRNA levels in early passage parthenogenetic and androgenetic EBs differed from wild type in the directions expected, we did not observe complete repression of alleles as has been described previously in midgestation embryos and at later developmental stages. This ‘leakage’ phenomenon may be peculiar to the ES cell type, involving partial loss or erasure of imprints, as has been interpreted for the biallelic expression of H19 and IGF-2 observed in human tumors (Rainier et al., 1993; Ogawa et al., 1993). However, it is also possible that the mRNA levels of imprinted genes in parthenogenetic and androgenetic EBs may accurately represent the expression status of the maternal and paternal alleles in early embryonic cells. This would imply the existence of a multistep imprinting process, such that postfertilization epigenetic modifications, in addition to those imparted in the germ line, may be required for monoallelic expression. Quantitative assessment of the expression of imprinted genes in parthenogenetic and androgenetic embryos at early stages of development would help to resolve this question.

In later passage androgenetic and parthenogenetic EBs, H19 and Snrpn were derepressed, suggesting gradual erasure, bypass or override of imprints with cell division. Similar parental-specific changes with passage may also have occurred in wild-type ES cells. Some information regarding whether this derepression in later passage cells represents irreversible loss of imprints might be obtained by placing them into an in vivo environment in chimeras, then determining RNA levels. An intriguing finding was that the opposite effect occurred for Igf-2 in parthenogenetic EBs, with the maternal Igf-2 alleles being repressed at later rather than at early passage numbers. Thus, although early passage parthenogenetic EBs possessed substantial levels of Igf-2 mRNA, it would appear that they still had imprints with the potential to invoke repression of this gene. This finding is consistent with the idea that imprinting could be a multistep process. Alternatively, it is possible that Igf-2 might not be imprinted per se, but that its transcription might be subject in cis to the activity and imprinting of the very closely linked H19 gene, such that it is repressed when H19 is active and vice versa (Bartolomei et al., 1993). This hypothesis, to account for the repression of Igf-2 in later passage parthenogenetic EBs, requires that the H19 transcription rate or mRNA level was concomitantly increased. It would also require that the derepression of H19 in later passage androgenetic EBs be accompanied by repression of Igf-2. Although such changes in mRNA levels cannot be clearly ascertained in the northern blots, it is not possible to conclude that the transcription of Igf-2 and H19 in cis are not in opposition. By later passage, each cell line probably contained significant variability in mRNA levels between clonally derived subpopulations. Yet, within these subpopulations, relationships between H19 and Igf-2 expression as proposed by the hypothesis could hold true.

The correlations we observed in EBs between parental-specific mRNA levels and methylation of H19, Igf2r, and Igf-2 were in general agreement with previous reports. Thus, methylation of the paternal H19 promoter region is correlated with repression of the paternal allele (Bartolomei et al., 1993; Ferguson-Smith et al., 1993), and we observed hypermethylation in androgenetic EBs in which the gene was relatively repressed. Also, methylation of the maternal Igf2r intron II region is correlated with activity of the maternal allele (Stöger et al., 1993), and we observed the greatest degree of methylation in this region in wild-type EBs, which possessed the highest levels of Igf2r mRNA. Furthermore, in instances where methylation and mRNA levels were changing with passage, the correlations persisted. Thus, demethylation of the paternal H19 promoter region with passage was accompanied by an increase in H19 mRNA levels in androgenetic EBs. Also, methylation of the paternal Igf-2 5′ region is correlated with activity of the paternal Igf-2 allele (Sasaki et al., 1992; Brandeis et al., 1993), and in later passage parthenogenetic EBs we observed a demethylation of this region accompanied by a reduction in Igf-2 mRNA levels. These changes in mRNA levels of H19 and Igf-2 that were accompanied by demethylation concur with previous observations of the activity of imprinted genes in mutant mouse embryos with a generalized deficiency in methylation. In these embryos, the normally repressed paternal H19 is active, and the normally active paternal Igf-2 allele is repressed (Li et al., 1993).

The comparisons we have made between the methylation status and mRNA levels of imprinted genes in EBs are complicated by the fact that the methylation status of EBs as a whole may not have been representative of those subsets of cells in which the genes were expressed. However, the methylation status we observed had already been attained in parthenogenetic and androgenetic ES cells, i.e., before differentiation and the high level expression phase of these genes. In no instance did a significant change occur in the degree of methylation of a region during the ES cell to EB transition. Thus, the degree of methylation in EBs did not appear to be dependent on differentiation or the prevailing level of transcription, but in ES cells was predictive of the level of transcription achievable in a more conducive cellular environment. For Igf2r, these observations are consistent with the suggestion that methylation of the intron II region is involved in imprinting of this gene (Stöger et al., 1993). The methylated sites examined in the paternal H19 promoter region are not the initial or primary imprints for H19, as the HpaII sites in this region are nonmethylated in sperm (Bartolomei et al., 1993; Ferguson-Smith et al., 1993). Nevertheless, if promoter methylation does contribute to repression of the paternal H19 allele, as would seem likely from what is known of the effect of methylation at other promoters (Antequera and Bird, 1993), the possibility is raised that it may be a secondary imprinting mechanism of H19, imparted after fertilization and essential for repression of the paternal allele. Confirmation or rejection of this possibility requires determination of the parental-specific expression and promoter methylation status of H19 during preimplantation development.

We have provided evidence for the existence of parental-specific differences in mRNA levels and methylation of imprinted genes in parthenogenetic and androgenetic ES cell lines. Whilst the degree of concurrence of our findings to analogous developmental stages in vivo requires elucidation, the resemblances to previous observations in midgestation embryos and at later stages of development suggest that these
cell lines should prove useful in further analysis of the genomic imprinting phenomenon.

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