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Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes

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SUMMARY

In vitro manipulation of preimplantation mammalian embryos can influence differentiation and growth at later stages of development. In the mouse, culture of embryonic stem (ES) cells affects their totipotency and may give rise to fetal abnormalities. To investigate whether this is associated with epigenetic alterations in imprinted genes, we analysed two maternally expressed genes (*Igf2r, H19*) and two paternally expressed genes (*Igf2, U2af1-rs1*) in ES cells and in completely ES cell-derived fetuses. Altered allelic methylation patterns were detected in all four genes, and these were consistently associated with allelic changes in gene expression. All the methylation changes that had arisen in the ES cells persisted on in vivo differentiation to fetal stages. Alterations included loss of methylation with biallelic expression of *U2af1-rs1*, maternal methylation and predominantly maternal expression of *Igf2*, and biallelic methylation and expression of *Igf2r*. In many of the ES fetuses, the levels of *H19* expression were strongly reduced, and this biallelic repression was associated with biallellic methylation of the *H19* upstream region. Surprisingly, biallelic *H19* repression was not associated with equal levels of *Igf2* expression from both parental chromosomes, but rather with a strong activation of the maternal *Igf2* allele. ES fetuses derived from two of the four ES lines appeared developmentally compromised, with polyhydramnios, poor mandible development and interstitial bleeding and, in chimeric fetuses, the degree of chimerism correlated with increased fetal mass. Our study establishes a model for how early embryonic epigenetic alterations in imprinted genes persist to later developmental stages, and are associated with aberrant phenotypes.

Key words: Embryonic stem cells, Epigenetic, Genomic imprinting, Methylation, *Igf2*, *Igf2r*, *H19*, *U2af1-rs1*, Mouse

INTRODUCTION

Culture and manipulation of mammalian preimplantation embryos can affect their subsequent development at fetal and perinatal stages. Pronounced effects have been observed in sheep and cattle where culture, nuclear transfer and asynchronous embryo transfer frequently result in the birth of larger than normal offspring with increased mortality and malformations (i.e. the 'large calf syndrome', reviewed in Walker et al., 1996). In the mouse, culture of preimplantation embryos may influence growth during fetal development (Bowman and McLaren, 1970), and also micromanipulation can have long-term influences on growth and gene expression (Römer et al., 1997). Phenotypic effects have also been observed in murine embryonic stem (ES) cells, which are derived from the inner cell mass of the blastocyst. Early-passage ES cells can be used to produce completely ES cell-derived fetuses (Nagy et al., 1990). However, upon prolonged culturing the potential of many ES cell lines becomes impaired, resulting in ES-derived fetuses with abnormalities such as increased size and mass, polydactyly, swollen edematous skin and perinatal death (Nagy et al., 1990, 1993; Wang et al., 1997). High passage stem cells may give rise to abnormalities even in chimeras, and frequently result in postnatal death of chimeras with high ES contributions (Nagy et al., 1990). It has been hypothesized that this loss of developmental potential results from the accumulation of epigenetic alterations in the ES cells and that these in particular affect imprinted genes (Nagy et al., 1993). Such 'epimutations' could also potentially explain the abnormalities observed in the large calf syndrome (Moore and Reik, 1996; Walker et al., 1996).

DNA methylation is one of the epigenetic features involved in the regulation of gene expression (Bird, 1995). One reason for believing that methylation may play a role in the changes observed on culture of stem cells and embryos, is that the genome is undergoing extensive changes in both global and gene-specific methylation patterns at precisely the stages when ES cells are being derived and manipulated. During preimplantation development, the bulk of the DNA in the genome becomes demethylated. As a consequence, only low

levels of methylation persist at the blastocyst stage and hence in ES cells. Most genes acquire their fetal levels of methylation after implantation through de novo methylation (Monk et al., 1987; Frank et al., 1991; Kafri et al., 1992). For imprinted genes, genes whose expression is parent-of-origin-dependent, the developmental regulation of methylation seems to be rather different. All analysed imprinted genes show parental allele-specific methylation (John and Surani, 1996), and studies on embryos deficient in methyltransferase have established that this methylation is involved in at least the somatic maintenance of allele-specific gene expression (Li et al., 1993). In contrast to non-imprinted genes, imprinted genes already have considerable levels of methylation at the blastocyst stage which, for the insulin-like growth factor 2/cation-independent mannose-6-phosphate-receptor gene (*Igf2r*), (a region upstream of) the *H19* gene, the *U2af1-rs1* gene and the *Snrpn* gene, is at least partly derived from the gametes (Stöger et al., 1993; Tremblay et al., 1995; Feil et al., 1997; Shibata et al., 1997; Shemer et al., 1997). In other imprinted genes, such as the insulin-like growth factor 2 (*Igf2)* gene, these allelic patterns become established during or shortly after implantation (Brandeis et al., 1993). Although the complex regulation of imprinted gene methylation in the early embryo needs further elucidation, these data suggest that imprinted genes might be particularly prone to epigenetic mutation on derivation and culturing of stem cells. In order to investigate this, we selected a number of imprinted genes to characterise in vitro-induced alterations in DNA methylation, and determined the effects of such alterations on gene expression. For our analysis we chose two maternally expressed genes, *H19* and *Igf2r*, and two paternally expressed genes, *Igf2* and *U2af1-rs1*. The *Igf2*, *H19* and *Igf2r* genes are known to be involved in the regulation of fetal and postnatal growth through the IGF signalling pathway, whereas *U2af1-rs1* encodes a splicing factor, the biological function of which is presently unknown (John and Surani, 1996).

The methylation and expression patterns of these four imprinted genes were examined in interspecific hybrid ES cell lines and completely ES cell-derived fetuses, using sequence polymorphisms that allowed us to distinguish the parental alleles. This study confirmed that, after derivation and subsequent passaging, stem cell lines can give rise to developmentally compromised fetuses. The main finding is that, in all ES lines analysed, allelic methylation changes had occurred in the four imprinted marker genes. These epigenetic alterations persisted in ES fetuses in which they were associated with aberrant imprinted gene expression.

MATERIALS AND METHODS

Mice and ES cell lines

For the derivation of ES lines SF1-1, SF1-3 and SF1-8, (C57BL/6 × CBA/Ca) F1 eggs were *in vitro* fertilized with *M. spretus* sperm and cultured to the blastocyst stage, in KSOM medium with bovine serum albumin (Lawitts and Biggers, 1993). ES cells were derived as described before and cultured in ES medium (Allen et al., 1994). For ES cell line SF1-G, C57BL/6 female mice were mated with *M. spretus* males. Morula-stage embryos were flushed and ES cell derivation was performed as for the other lines. During the first passages (< passage 4), ES cells were cultured on STO feeder cells. Subsequent culturing was without feeders on gelatinized plates. All lines were karyotyped and chromosomes were counted before being used for the derivation of fetuses. Parthenogenetic ES lines PR4, 5, 9, 10, 13 and 14 were derived as before (Feil et al., 1997) and were grown in ES medium in the absence of feeder cells.

ES cell-derived fetuses

ES fetuses were produced by microinjection of 10-15 ES cells into tetraploid blastocysts, a modification of a methodology developed by Nagy et al. (1990). Initially, tetraploid blastocysts of the mouse line ROSA26 (which has a ubiquitously expressed *lacZ* reporter gene; a gift of P. Soriano) were used to determine the efficiency of the injection method. 28 day-11 fetuses were produced in which all the cells of the embryo proper were non-LacZ staining and hence ES cell-derived. The percentage contribution of diploid cells in all subsequently derived ES fetuses was studied by Southern blotting. In total, 40 day-11 ES fetuses (out of 125 transferred blastocysts), 58 day-13 to -14 fetuses (out of 255 blastocysts), and 3 day-15 ES fetuses (out of 40 blastocysts) were produced (only live animals with a beating heart were scored). Chimeric fetuses were made by injection of hybrid ES cells into naturally fertilized diploid blastocysts. Statistical comparisons between groups of chimeric fetuses were performed using ANOVA.

Methylation, gene expression and protein analyses

DNA extraction, endonuclease digestion, electrophoresis and Southern blotting were performed as described before (Feil et al., 1994). RNA isolation, electrophoresis on 1% formaldehyde gels, transfer to Hybond N^+ filters (Amersham) and northern hybridisation were also as described before (Feil et al., 1994). The following probes were used: *U2af1-rs1* probe 1 (550 bp *Eco*RI fragment; Feil et al., 1997); a 1094 bp *Sfu*I-*Mlu*I fragment from region 2 of *Igf2r* (Stöger et al., 1993); a 1.8 kb *Igf2r* cDNA probe spanning the middle part of the cDNA; *Igf2* probe 6 (0.9 kb *Kpn*I-*Bam*HI fragment; Feil et al., 1994); a 0.5 kb *H19* fragment from the 3′ part of exon 1, and probe pY353/8, corresponding to a Y-chromosome repeat sequence (Bishop et al., 1983). Qualitative expression analysis was performed by RT-PCR amplification; primers used were: *Igf2*, 5′-GGCCCCGGAGAGACTCTGTGC-3′ and 5′-TGGGGGTGGGTAAGGAGAAAG-3′ (annealing at 60°C, 30 cycles); *Igf2r*, 5′-ATGATGACAGCGACGAAGACC-3′ and 5′-GAATTCGGCGCCACATGGTGTTCAAGAAG-3′ (57.1°C, 30 cycles); *U2af1-rs1*, 5′-TGTGGTACGGCCAGCCTATG-3′ and 5′-GATCAGACATACTGCGGATA-3′ (60°C, 35 cycles); *H19*, 5′-TGCTCCAAGGTGAAGCTGAAAG-3′ and 5′-GTAGGGCATGTTGAACACTTTATG-3′ (65°C, 30 cycles).

RESULTS

Altered imprinting in ES cells is maintained in ES fetuses

We derived (*M. musculus* × *M. spretus*) F1 ES cell lines of which four (SF1-1, SF1-3, SF1-8 and SF1-G) were used to produce ES cell-derived fetuses (ES fetuses). The four ES cell lines became stable in culture after an initial 6-8 passages, when most of the cells appeared morphologically undifferentiated. They were analysed for their chromosomes and found to have 40 chromosomes in >80% of chromosome spreads analysed. Southern hybridisation with a Y chromosome-specific probe showed that lines SF1-1, SF1-3 and SF1-8 were male, whereas line SF1-G was female (data not shown). ES fetuses, produced by injection of ES cells (passage 9-12) into tetraploid blastocysts, were analysed on day 13-14 of development, at which stage all fetal tissues were completely ES cell-derived (see Materials and methods).

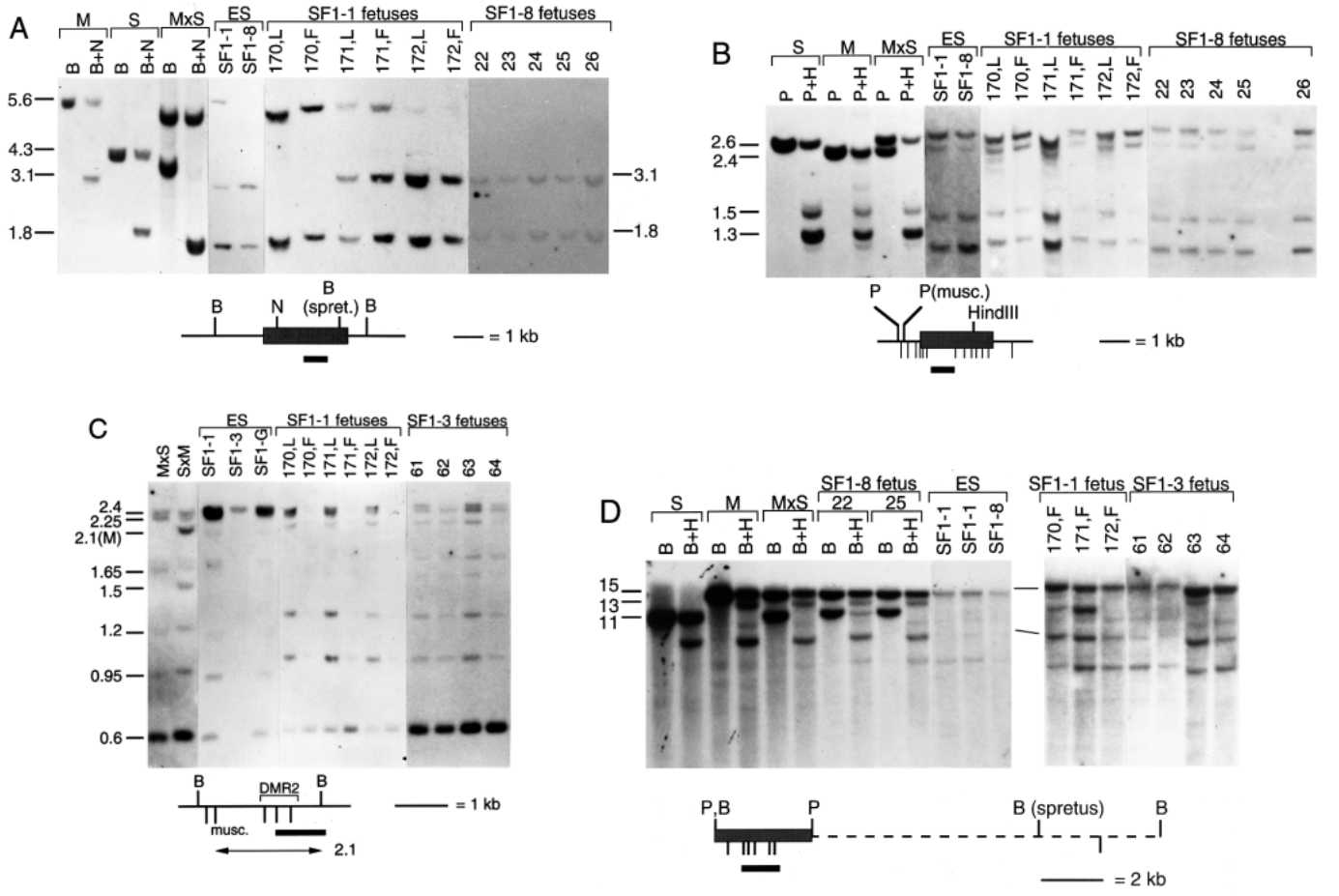

Fig. 1. Imprinted gene methylation in ES cells and ES fetuses. (A) Methylation in *U2af1-rs1*. A *Bgl*II (B) polymorphism between *M. musculus* (M) and *M. spretus* (S) was used to analyse methylation of a *Not*I (N) restriction site. Shown in lanes 1-6 from left to right are B and B+N digested DNAs of day-14 control fetuses. Lanes 7-19 show B+N digested DNAs of ES cell lines and ES derived fetuses (all of the (M×S)F1 genotype). For fetuses 170-172, methylation levels were determined both in the liver (L) and in the remainder of the fetus (F). Fragment sizes are indicated in kb. The map shows the *Bgl*II (spret., in *M. spretus* only) and *Not*I sites, the gene (box), and the probe used for hybridisation (horizontal bar). (B) A *Pvu*II polymorphism was used to study *H19* methylation, and eight *Hpa*II sites in the gene and the promoter were analysed. Lanes 1-6 from left to right show control fetal DNAs digested with *Pvu*II+*Hind*III (P) and *Pvu*II+*Hind*III+*Hpa*II (P+H). Lanes 7-20 show P+H digested genomic DNAs. The map shows the gene (box), the *Pvu*II (musc., in *M. musculus* only), *Hind*III and *Hpa*II (thin vertical bars) restriction sites, and the probe used (horizontal bar). (C) Methylation in the DMR2 of *Igf2*. Shown in lanes 1 and 2, from left to right, are (M×S)F1 and ((M×S)×M)F2 fetal DNAs digested with *Bam*HI+*Hpa*II. Lanes 3-15 contain *Bam*HI+*Hpa*II digested DNAs of ES cells and ES fetuses. The map indicates the DMR2, *Bam*HI and *Hpa*II (vertical bars) restriction sites, and the probe used (horizontal bar). The 2.1 kb partial digestion product indicates *M. musculus*-specific DMR2 methylation (Feil et al., 1994). (D) *Igf2r*, 'region 2' methylation. A *Bam*HI (B) RFLP between *M. spretus* (S) *and M.musculus* (M), comprising the six *Hpa*II sites of region 2, was used to determine allelic levels of methylation. Lanes 1-10 from left to right show fetal DNAs digested with *Bam*HI (B) and *Bam*HI+*Hpa*II (B+H), and lanes 11-20 show B+H digested DNAs from ES cells and ES fetuses. The map shows region 2 (filled rectangle) and the polymorphic *Bam*HI fragment, and *Hpa*II (vertical bars) and *Pvu*II (P) sites within region 2 are indicated. The dashed line indicates the sequences for which no information was available; the line in bold, the fragment used as probe. All samples were also analysed by *Bam*HI+*Mlu*I digestion to analyse methylation of a *Mlu*I site in region 2. This gave the same results as for the *Hpa*II analysis, and confirmed biallelic methylation in fetus 171 (not shown).

Allelic methylation and expression was determined for the *Igf2*, *Igf2r*, *U2af1-rs1* and *H19* genes in ES cells and ES fetuses (Figs 1 and 2; summarized in Table 1).

The *U2af1-rs1* gene is paternally expressed in fetuses and adult tissues (Hatada et al., 1993), in which the gene and its upstream regulatory sequences are methylated on the maternal chromosome (Feil et al., 1997). Methylation was studied by digestion of a *Not*I site in the 5′ untranslated region (Fig. 1A) and levels of methylation at this restriction site were found to represent those of the entire gene (not shown). The *Not*I site was highly methylated on the maternal allele in ES cell line SF1-1, whereas in lines SF1-3, SF1-8 and SF1-G, it was unmethylated on both parental chromosomes. Most of the SF1-1 derived fetuses showed complete maternal methylation, whereas a minority showed partial maternal methylation, and in one fetus (fetus 172), *U2af1-rs1* was unmethylated. In all fetuses derived from lines SF1-3, SF1-8 and SF1-G, both parental alleles of *U2af1-rs1* were unmethylated. Qualitative expression was analysed by RT-PCR (Fig. 2A). In ES line SF1-1, expression was predominantly paternal, and most derived ES

Table 1. Parental allele-specific methylation and expression in ES cell lines and derived fetuses

		U2af1-rs1		*H19*			*Igf2*		*Igf2r*	
ES cell line		Methylation	Expression	Methylation upstream	Methylation gene	Expression	Methylation	Expression	Methylation	Expression
SF1-1		M	P,m	P+M	P,m	M,p	P+M	M,p	M	P+M
fetus (–liver)	170	M	P	P+M	P,m	(M,p)	A	M,p	M,p	M,p
fetus (–liver)	171	M	P+M	P+M	P,m	(M,p)	A	M,p	P+M	P+M
fetus (–liver)	172	A	M,p	P+M	P,m	(M,p)	A	M,p	M	M
liver	170	M	P	--	P,m	(M,p)	M	M,p	M,p	M,p
liver	171	M	P+M	--	P,m	(M,p)	M	M,p	--	P+M
liver	172	A	M,p	--	P,m	(M,p)	M	M,p	M	M
fetus	150	--	M	--	--	M	--	P+M	--	--
fetus	151	--	P	--	--	(M)	--	M	--	--
fetus	152	--	P,m	--	--	(M)	--	M	--	--
fetus	153	--	P,m	--	--	(M,p)	--	M	--	--
fetus	154	--	M,p	--	--	(M)	--	M	--	M
fetus	155	--	P	--	--	(P+M)	--	M	--	--
SF1-3		A	M,p	P+M	P,m	M,p	P+M	M,p	M	P+M
fetuses	61,62	A	M,p	P+M	P,m	(M,p)	M,p	M,p	M	M
fetus	63	A	M,p	P+M	P,m	(M,p)	P+M	M,p	M	M
fetus	64	A	M,p	P,m	P	M	P,m	P+M	M	M
fetus	65	--	M,p	P	--	M	--	P,m	--	M
SF1-8		A	M,p	P+M	P,m	M,p	P+M	M,p	M	P+M
fetuses	22,23	A	M,p	P+M	P,m	(M,p)	P+M	M,p	M,p	M,p
fetus	24	A	M,p	--	P,m	(M,p)	--	M,p	--	M
fetus	25	A	M,p	P+M	P+M	(P,m)	M,p	M,p	M	M
fetus	26	A	M,p	P+M	P,m	(M)	P+M	M,p	M,p	M,p
SF1-G		A	M,p	P	P	M	P+M	P	M	P+M
fetuses (–liver)	176,177	A	M,p	P	P	M	A	P	M	M
fetus (–liver)	178	A	M,p	P	P	M	A	P	--	M
fetuses (–liver)	179,181	A	M,p	P	P	M	A	P	M	M
liver	176,177,178	A	M,p	--	P	M	P	P	--	M
liver	179,181	A	M,p	--	P	M,p	P	P	--	M

P, methylation/expression is detected predominantly (>90%) on/from the paternal allele; M, methylation/expression is detected predominantly (>90%) on/from the maternal allele; P,m, most of the methylation/expression is detected on/from the paternal allele, and some (<35%) on/from the maternal allele; M,p, most of the methylation/expression is detected on/from the maternal allele, and some (<35%) on/from the paternal allele; P+M, comparable levels of maternal (35-65%) and paternal (35-65%) methylation/expression; A, <10% methylation detected on both parental chromosomes; --, analysis could not be performed; parentheses indicate fetuses in which quantitative *H19* expression is strongly reduced or absent on northern analysis. Note that the qualitative, parental allele-specific expression was determined from ethidium bromide-stained gels (Fig. 2), and that measured band intensities were corrected for the relative lengths of the fragments.

fetuses showed (maternal *U2af1-rs1* methylation and) expression from the paternal chromosome (e.g. fetus 170). In other SF1-1-derived fetuses (e.g. fetus 171), in which only a proportion of the maternal chromosomes was methylated, equal expression was observed from both parental chromosomes. One SF1-1 fetus was obtained (fetus 172; with both *U2af1-rs1* alleles unmethylated) which showed biallelic expression, albeit more strongly from the maternal than from the paternal chromosome. Biallelic expression was also observed in SF1-3, SF1-8 and SF1-G ES cells and ES fetuses, in which *U2af1-rs1* was unmethylated on both parental chromosomes. In all instances, therefore, allelic expression of *U2af1-rs1* was inversely correlated with allelic methylation.

Methylation in the *H19* gene was assessed by analysing the allelic digestion of eight *Hpa*II restriction sites located within (the first three exons of) the gene and its promoter (Fig. 1B). These *Hpa*II sites are paternally methylated in normal embryonic and adult tissues (Bartolomei et al., 1993). Allelic *H19* expression was analysed by RT-PCR amplification (Fig. 2B). SF1-G ES cells and ES fetuses displayed the normal (Sasaki et al., 1995) paternal methylation in the *H19* gene and expression was exclusively maternal (Table 1). ES cell lines SF1-1, SF1-3 and SF1-8 were predominantly (~85%) paternally methylated, and these three cell lines and most of the derived fetuses, had predominantly maternal (~85%) expression (Figs 1B, 2B). One SF1-8 derived fetus (fetus 25) had higher levels of maternal *H19* methylation and displayed predominantly paternal expression. Therefore, as with *U2af1-rs1* expression, *H19* expression was inversely correlated with methylation.

Methylation in the paternally expressed *Igf2* gene was assayed in the ‘differentially methylated region 2’ (DMR2), a paternally methylated region in the coding portion of the gene (Feil et al., 1994). We analysed the digestion of three *Hpa*II restriction sites in the DMR2 (Fig. 1C). In all four (*M. musculus* × *M. spretus*)F1 ES cell lines, these *Hpa*II sites were methylated on both parental chromosomes. In the (livers of) SF1-G derived fetuses, the *Hpa*II digestion pattern was as in normal control (*M. musculus* × *M. spretus*)F1 fetuses, suggesting paternal DMR2 methylation (Table 1). In many of the fetuses derived from ES lines SF1-1, SF1-3 and SF1-8, in contrast, the *Hpa*II digestion pattern was similar to that of ((*M. musculus* × *M. spretus)* × *M. musculus*)F2 fetuses, suggesting the presence of maternal methylation. Indeed, in these fetuses,

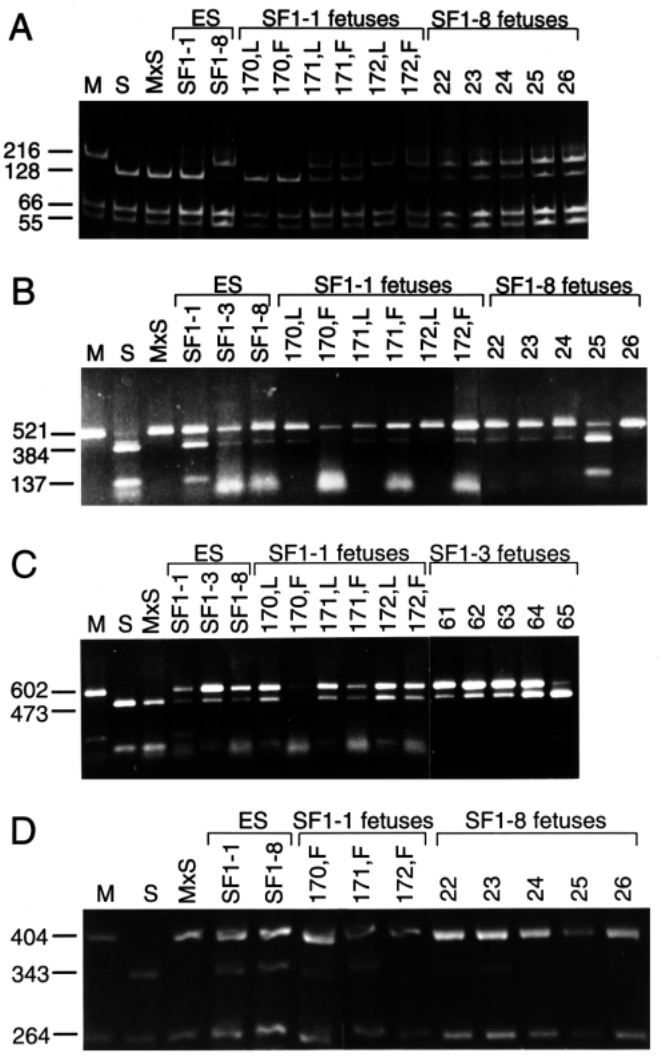

Fig. 2. Imprinted gene expression in ES cells and ES fetuses. Qualitative expression analyses were performed by RT-PCR, followed by restriction endonuclease digestion of amplification products and electrophoresis on 8% polyacrylamide gels (A) or 2% agarose (B-D). RT-PCR analyses were also performed in the absence of reverse transcriptase to control for the occurrence of DNA contamination (not shown). (A) *U2af1-rs1* RT-PCR amplification was performed as described by Hatada et al. (1993), with *M. spretus* displaying the same polymorphic *Rsa*I restriction site in the 5′UTR as published for *M.m. mollosinus.* Fragment sizes are indicated in bp. Note that the *U2af1-rs1* gene is intron-less, and that the primers used amplify equally well from *M. musculus* and *M. spretus* genomic DNA (not shown). (B) For the *H19* analysis, a 521 bp fragment was amplified spanning intron 4, and digested with *Bgl*I, which gives 384 bp and 137 bp digestion products in *M. spretus* only (Sasaki et al., 1995). (C) For allelic *Igf2* analysis, a *Bsa*AI RFLP in the 5′ part of exon 6 was used. Digestion products are 602 bp and 473 bp in *M. musculus* and *M. spretus*, respectively. (D) For *Igf2r* analysis, a 668 bp fragment was amplified (Villar et al., 1995) and digested with *Scr*F1, which gives a 343 bp digestion product in *M. spretus*, and a 404 bp product in *M. musculus*.

a 2.1 kb partial digestion product was apparent, which indicated maternal DMR2 methylation (Fig. 1C). This allelic alteration did not affect the tissue-specificity of DMR2 methylation, which we have previously shown to be present in *Igf2*-expressing tissues, particularly in the liver (Feil et al., 1994). This is shown for SF1-1 derived fetuses 170-172 (Fig. 1C), in which high levels of methylation were detected in liver, whereas the remainder of the fetus displayed much reduced methylation levels. We next analysed *Igf2* expression by RT-PCR amplification (Fig. 2C). In all SF1-G fetuses, expression was exclusively paternal (Table 1). In contrast, the ES fetuses with maternal DMR2 methylation, derived from the three other ES cell lines, showed predominantly maternal *Igf2* expression. In one ES fetus (fetus 64) equal expression was detected by RT-PCR from both parental chromosomes, and in this fetus, the DMR2 methylation analysis suggested biallelic methylation. Although methylation patterns in ES cells (biallelic in all lines) did not persist on differentiation into ES fetuses, the allele-specific expression patterns did. Therefore, allelic *Igf2* expression did not correlate with DMR2 methylation in ES cells, but did so in ES fetuses.

The maternally expressed *Igf2r* gene (Barlow et al., 1991) contains an intronic region (‘region 2’) that is maternally methylated in embryonic and adult tissues (Stöger et al., 1993). We found *Hpa*II restriction sites in region 2 to be maternally methylated in all the ES cell lines, and this was retained by most of the ES fetuses (Fig. 1D). *Igf2r* expression was analysed by RT-PCR (Fig. 2D) according to Villar et al. (1995). In all four ES cell lines, expression was biallelic (albeit more strongly from the maternal allele). In the majority of the ES fetuses only maternal *Igf2r* expression was observed (e.g. fetuses 61-65). Some of the ES fetuses derived from lines SF1-1 and SF1-8 had predominantly maternal expression, but also showed some paternal expression (e.g. fetus 22). In these animals region 2 showed some DNA methylation on the paternal chromosome. Biallelic *Igf2r* expression was observed only in one fetus (171) where region 2 was methylated on both parental chromosomes. Therefore, expression of *Igf2r* did correlate with methylation in the ES fetuses, but not the ES cells.

We did not observe changes in the overall levels of genomic methylation, measured by analysing methylation in the Line 1 repeat element (data not shown). In the imprinted genes analysed, quantitative changes were observed in *U2af1-rs1* only (Fig. 1A), but it was unclear whether these had arisen on derivation or during culture of the ES cells. To address whether culturing on its own can give rise to quantitative methylation changes, we subjected six newly derived parthenogenetic ES cells to prolonged culture, and determined levels of *U2af1-rs1* methylation (Fig. 3). After derivation (passage 2-3) these lines displayed the expected (Shibata et al., 1997) complete (maternal) methylation. On subsequent culture, however, maternal methylation was almost entirely lost in four of the lines (PR5, PR9, PR13, PR14). In the two other lines (PR4 and PR10), quantitative changes were also observed, but these displayed respective decreases and increases in methylation during the culturing up to passage 12.

Quantitative gene expression and interactions between *Igf2* and *H19*

Northern analysis did not reveal major differences in *Igf2* expression between fetuses derived from different cell lines (Fig. 4). In contrast, *H19* expression was found to be virtually absent in many of the SF1-1, SF1-3 and SF1-8 derived ES

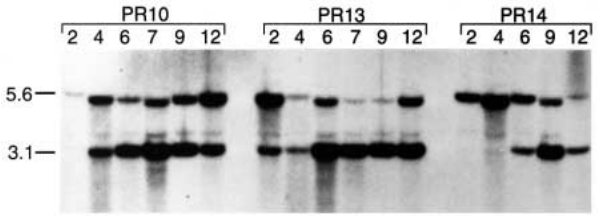

Fig. 3. Culture-induced changes in *U2af1-rs1* methylation. Parthenogenetic ES cell DNAs digested with *Bgl*II+*Not*I, were hybridised with *U2af1-rs1* probe 1. The 5.6 kb band is indicates methylation at the 5′UTR *Not*I site, whereas the 3.1 kb band corresponds to digestion of this methylation sensitive restriction site (see Fig. 1A). Results for three lines (PR10, PR13 and PR14) are shown; passage numbers are indicated above the lanes.

fetuses. This was unexpected since, in most ES fetuses, normal maternal *H19* expression had been detected in the qualitative RT-PCR analysis (Fig. 2B). A systematic comparison revealed that in all ES fetuses with predominantly maternal *Igf2* expression, *H19* was strongly repressed (Table 1). For example, SF1-3 derived ES fetuses 61-65 had comparable levels of *Igf2* expression (Fig. 4), whether expression was predominantly (61-63), biallelic (64) or paternal (65) (Fig. 2C). No *H19* expression was detected in the fetuses with maternal *Igf2* expression (fetuses 61-63), whereas the other two fetuses (64 and 65) displayed high levels of *H19* expression (Fig. 4). We were not able, by northern analysis, to quantify *U2af1-rs1* expression in the fetuses, and the poorer quality of some of the RNAs did not allow comparative expression analysis of the *Igf2r* transcript.

Since the fetuses which showed no expression of *H19* had mostly unaltered paternal methylation in the gene and its promoter (Fig. 1B, Table 1), we were interested in

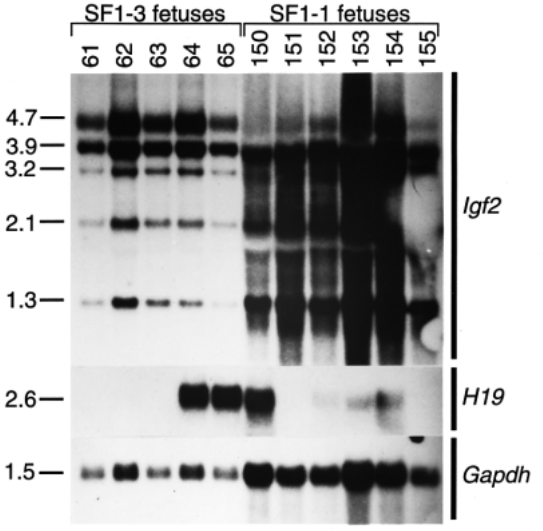

Fig. 4. Repression of *H19* in ES fetuses with maternal *Igf2* expression. Total RNAs from day-14 fetuses were hybridised with *Igf2* (0.9 kb *Kpn*I-*Bam*HI fragment comprising exon 5 and part of exon 6), *H19* (600 bp fragment from 3′ part of exon 1) and *Gapdh* probes. Band intensities for *Igf2* and *H19* were compared with those for *Gapdh*.

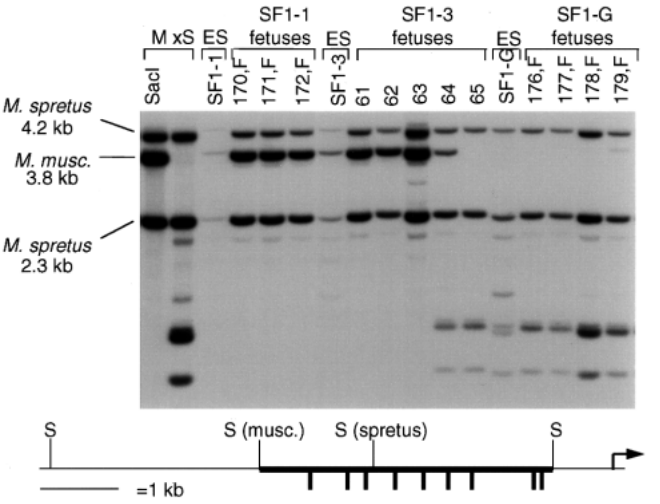

Fig. 5. Biallelic methylation of sequences upstream of *H19*. A *Sac*I polymorphism between *M. musculus* (M) *and M. spretus* (S) was used to determine the allele-specificity of methylation of *Hha*I restriction sites in the 'imprinting element' upstream of *H19*. The first lane shows *Sac*I-digested DNA from a M×S control fetus; all other lanes contain *Sac*I+*Hha*I-digested DNAs. The map shows the position of *Sac*I (S), and *Hha*I (vertical bars) sites located within the 3.8 kb *M. musculus*-specific *Sac*I restriction fragment (thick horizontal bar), which we used as probe for hybridisation of the filter. The arrow indicates the transcription initiation site.

determining whether alterations had occurred further upstream of the gene, in a region which carries a paternal methylation mark that is already detected in the gametes (Tremblay et al., 1995). *Hha*I restriction sites in this upstream region were methylated on both parental chromosomes in all the fetuses which did not express *H19* (Fig. 5). For example, SF1-3 derived fetuses 61-63, which had no *H19* expression and maternal *Igf2* expression, showed biallelic methylation of this upstream region. SF1-3 derived fetus 64, in which *H19* showed high levels of expression (and *Igf2* was expressed biallelically), had full paternal and partial maternal methylation in the *H19* further upstream region. Fetus 65, the only SF1-3 derived fetus with exclusive paternal *Igf2* expression and high levels of *H19* expression, had exclusively paternal methylation in the *H19* upstream region. Importantly, the aberrant methylation in fetuses without *H19* expression was already present in the ES cells, with ES lines SF1-1, SF1-3 and SF1-8 displaying almost complete methylation on both parental chromosomes. Unaltered (Tremblay et al., 1995) exclusively paternal methylation was detected in ES cell line SF1-G and all derived fetuses, and this correlated with unaltered qualitative and quantitative expression of *Igf2* and *H19* (Fig. 4).

We next sought to address which proportion of genes might be expressed at abnormal levels during early fetal development in the ES fetuses. Protein expression was analysed by high-resolution 2-dimensional electrophoresis (2-DE) at day 11 of development, at which developmental abnormalities were not (yet) apparent (Fig. 6A). Of more than 3000 proteins analysed, only seven (0.2%) quantitative differences were apparent, but none of these were consistently altered in ES fetuses derived from the same ES line (data not shown).

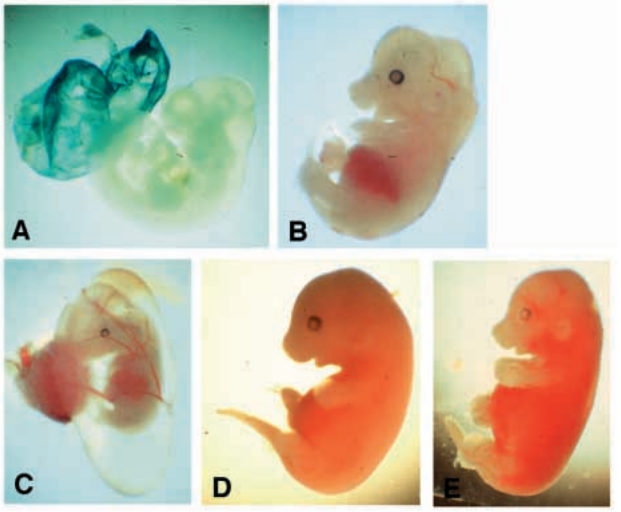

Fig. 6. Developmental abnormalities in ES fetuses. (A) One of the lacZ-stained day-11 ES embryos obtained by microinjection of SF1-8 stem cells into tetraploid blastocysts of mouse strain ROSA26, which has a ubiquitously expressed *LacZ* gene. β-galactosidase activity (blue staining) is present only in tetraploid cells, which are present in the yolk sac but not in the embryo proper. (B) Day-14 SF1-G derived ES fetuses with normal external morphology. (C) A representative day-13 SF1-8-derived ES fetus with pronounced polyhydramnios. (D) A moribund day-13 SF1-3 derived ES fetus. Incomplete development of the mandible and interstitial bleeding are noticeable. (E) A day-14 SF1-3 derived fetus with severe interstitial bleeding, craniofacial abnormalities and axial elongation.

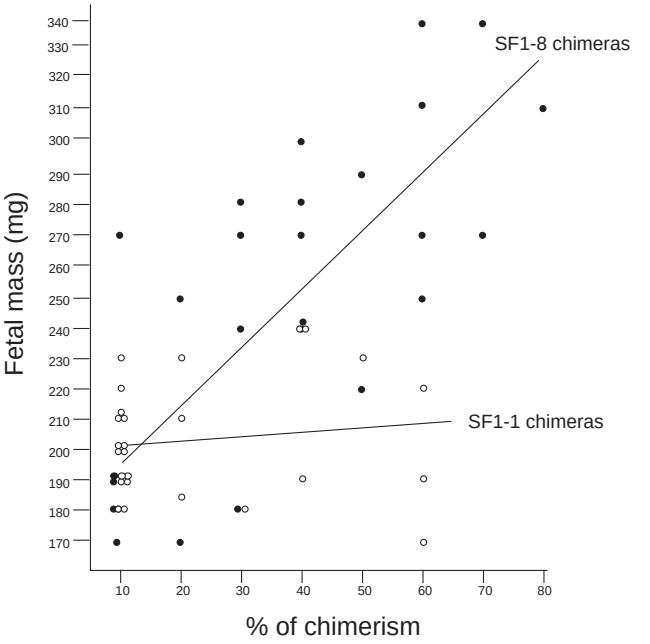

Fig. 7. Enhanced fetal growth in chimeric fetuses. Plotted are the percentage of chimerism against fetal mass (in mg) for SF1-1 chimeras (open circles; n=26) and SF1-8 chimeras (filled circles; n=24), which were concomittantly derived. In SF1-8 chimeras, fetal mass correlated (P<0.001) with the percentage of chimerism (fetal mass in mg = 187+1.72×%chimerism), and these chimeras were approx. 30% heavier than the SF1-1 chimeras (249 versus 189 mg). A significant correlation (P<0.001) was also found between SF1-8 chimerism and crown-rump length, with average lengths of 11.5 mm and 13 mm, for SF1-1 and SF1-8 chimeras, respectively.

Altered imprinting is associated with altered phenotypes of ES derived fetuses

The previous analysis demonstrates that the imprinted genes examined showed alterations of allelic methylation and allelic expression in ES cells, and that these alterations were maintained to fetal stages. A phenotypic characterisation of the ES fetuses was therefore carried out to see whether aberrant imprinting status was associated with altered phenotypes. ES fetuses derived from lines SF1-3 and SF1-8 had external developmental anomalies, the most striking of which was polyhydramnios, observed in all SF1-3 and SF1-8 derived fetuses (Fig. 6C). SF1-3 and SF1-8 derived fetuses also showed poor development of the mandible and in about 80% of these fetuses, interstitial bleeding was apparent (Fig. 6D,E). Lines SF1-1 and SF1-G yielded fetuses in which external morphology and the extraembryonic tissues appeared to be normal (Fig. 6B), although on closer examination we found that about 20% of these did have interstitial bleeding, albeit less severely so than the SF1-3 and SF1-8 derived fetuses.

Given the low recovery (approx. 23%) of live ES fetuses at days 13-14 of development, and therefore the limited and variable number of ES fetuses in each recipient mouse, the ES fetus system did not allow simple comparison of ES lines for their fetal growth potential. To address this important question we derived chimeric fetuses by injection of ES cells into diploid host blastocysts. We compared day-14 chimeras derived from line SF1-1, which had produced externally normal ES fetuses, and ES line SF1-8, which produced fetuses with developmental abnormalities. Chimeras were obtained at frequencies of 90% and 80%, for lines SF1-1 and SF1-8, respectively. For line SF1-1, there was no significant correlation between the percentage of chimerism (measured by Southern blot analysis using RFLPs) and fetal mass or crown-rump (C/R) length. In contrast, the mass of day-14 SF1-8 chimeras (and C/R length) correlated significantly with the percentage of chimerism, and on average these fetuses were about 30% heavier than SF1-1 chimeras (Fig. 7). Interestingly, 18 of the 24 SF1-8 chimeras appeared morphologically normal. This contrasted with the aberrant developmental phenotype of the ES fetuses derived from this stem cell line, and demonstrates rescue of phenotypic abnormalities by the host embryo.

DISCUSSION

Using an interspecific ES cell system we considered whether, on derivation and culture of ES cells, epigenetic alterations accumulate in imprinted genes and whether such epimutations are associated with altered gene expression in ES fetuses. Indeed, aberrant methylation patterns were detected in all imprinted genes analysed, and these either corresponded to primary epigenetic alterations or reflected such alterations. Hence, methylation changes in *U2af1-rs1* and *H19* were already present in ES cells and persisted on differentiation into ES fetuses. We assume the same is true of *Igf2r*, but because fetuses with aberrant *Igf2r* imprinting were less frequent,

methylation changes in ES cells would have gone undetected in bulk culture. Alterations in *Igf2* became apparent only in fetuses. Interestingly, the methylation changes we observed were of the three possible types: loss of methylation (*U2af1-rs1*), gain of methylation (*H19* upstream region; region 2 of *Igf2r*) and allelic change in methylation (DMR2 in *Igf2*). Our observation that normal methylation patterns do not become re-established on differentiation into ES fetuses extends earlier findings on ES cells homozygous for a disruption of the DNA methyltransferase gene (Li et al., 1993). Expression of a methyltransferase cDNA in these targeted ES cells restored normal levels of methylation in non-imprinted genes, but not in imprinted genes (Tucker et al., 1996). The stem cells in our study had been cultured for a number of passages before they were used for the production of ES fetuses and it was therefore unclear whether the methylation changes had occurred during the derivation, or the subsequent culturing of the cells. For *U2af1-rs1*, we could address this question further by analysing parthenogenetic ES cell lines, which all had full maternal methylation after derivation. Methylation in *U2af1-rs1* was highly unstable and frequently lost on culturing of these cells. This observation extends a study by Szabó and Mann (1994), who described quantitative methylation changes in *Igf2* and *H19* in monoparental ES cells. How specific these methylation changes are to imprinted genes is not known. However, CpG islands in non-imprinted genes do not become methylated in ES cells (Antequera et al., 1990; Frank et al., 1991), suggesting that methylation patterns in imprinted genes might be particularly unstable in ES cells.

Altered methylation patterns were associated with qualitative and, in the case of *H19*, quantitative changes in gene expression. In the *U2af1-rs1* gene, loss of maternal methylation correlated with biallelic expression. In ES cells and ES-derived fetuses from line SF1-1, *U2af1-rs1* was maternally methylated in a proportion of the cells, which correlated inversely with levels of maternal *U2af1-rs1* expression. Allelic expression of *Igf2r* was mostly unaltered in the ES cells and altered in few of the derived fetuses, and this stability of the region 2 imprint has been reported for 129/Sv ES lines as well (Labosky et al., 1994). Although the maternal region 2 methylation is present throughout preimplantation development (Stöger et al., 1993), it has been shown that repression of the *Igf2r* allele occurs only after implantation (Lerchner and Barlow, 1997) and paternal anti-sense transcription originating in region 2 has been implicated in this repression (Wutz et al., 1997). Our finding of maternal methylation and biallelic *Igf2r* expression in all four ES cell lines agrees with these studies.

In most of the ES fetuses produced from lines SF1-1, SF1-3 and SF1-8, the normally paternally methylated and expressed *Igf2* gene showed maternal DMR2 methylation, which correlated with unequal biallelic expression strongly biased in favour of the maternal allele. Unexpectedly, fetuses with strong activation of the maternal *Igf2* allele showed no expression of *H19*. This biallelic repression of *H19* was not associated with methylation changes in its promoter, but with biallelic methylation of sequences further upstream of the gene. It has been shown that these sequences function as a silencer in *Drosophila* (Lyko et al., 1997). Our study suggests that in the mouse this imprinting element may be involved in long-range chromatin interactions. Based on our findings, we propose that, when on the maternal chromosome and unmethylated, the *H19* upstream element allows downstream enhancers to interact with the *H19* promoter via appropriate looping of chromatin, and thereby insulates the flanking *Igf2* gene from interactions with such regulatory sequences. On the paternal chromosome, in contrast, this region is fully methylated, and its conformation might not allow higher order folding required for *H19* expression, thus leading to *Igf2* expression. Such a mechanistic, methylation-dependent, involvement of the *H19* further upstream element agrees with the enhancer competition model proposed by Bartolomei et al. (1992), in that only one of the two genes can be expressed from a single chromosome. This model does not, however, explain our finding that fetuses with biallelic methylation in the *H19* upstream element expressed *Igf2* predominantly from the maternal chromosome. This strong activation of the maternal gene may have arisen because of additional alterations in methylation and/or chromatin, possibly within the *Igf2* gene itself. It might be relevant that these fetuses had substantial levels of maternal methylation in the DMR2, a normally paternally methylated region that we proposed to be involved in silencing of transcription (Feil et al., 1994).

Our data show that epigenetic alterations arise on derivation and culturing of ES cell lines, do not become corrected during postimplantation development, and are associated with aberrant imprinted gene expression in the fetus. Therefore, this in vivo differentiation system might constitute an experimental model for deregulation of imprinting in developmental disorders and embryonal tumours. In sporadic Wilms' tumours of the kidney, for example, healthy tissue adjacent to the tumour already shows biallelic *IGF2* expression in association with biallelic repression and methylation of *H19*, suggesting that epigenetic alterations occurred during early development (Okamoto et al., 1997). In the human fetal overgrowth syndrome Beckwith Wiedemann Syndrome, germline or early embryonic errors may occur in the *IGF2-H19* domain without DNA mutations (Reik et al., 1995), and also in Angelman Syndrome, imprinting defects have been detected without apparent genetic alteration (Burger et al., 1997). We have previously shown that developmental abnormalities resulting from micro-manipulation can be transmitted to the next generation (Römer et al., 1997). The question that arises is whether altered methylation patterns in imprinted genes are heritable as well. Although we did produce adult chimeric animals for three of the ES lines (not shown), germline transmission could not be achieved because of hybrid sterility, and this intriguing question should be addressed on a different genetic background.

From this and other studies on ES cells one might also anticipate that in in vitro cultured preimplantation embryos, imprinted genes can undergo epigenetic alterations. Indeed, Sasaki et al. (1995) found that culturing of preimplantation mouse embryos may lead to biallelic *H19* expression in extraembryonic tissues. Earlier studies suggested that culture and manipulation of preimplantation mouse embryos may result in aberrant growth and developmental abnormalities (Bowman and McLaren, 1970; Reik et al., 1993). In cattle and sheep, culture and manipulation of preimplantation embryos can lead to enhanced fetal growth and developmental abnormalities such as increases in bone lengths, facial/head abnormalities and polyhydramnios (Walker et al., 1996; Kruip

and den Daas, 1997), and high birthweights, perinatal death and reduced development of the lower jaw have been reported in sheep cloned by transfer of nuclei from cultured fibroblasts (Schnieke et al., 1997). In ES fetuses derived from two of the stem cell lines we observed possibly similar abnormalities, which included polyhydramnios, interstitial bleedings and reduced mandible development, and growth-enhancement was apparent in chimeras. Our observations make it likely that these defects are at least partly caused by aberrant expression of imprinted genes. Future research should aim to link specific components of the aberrant phenotypes with specific epigenetic alterations in gene expression.

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