Selective loss of imprinting in the placenta following preimplantation development in culture

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Summary

Preimplantation development is a period of dynamic epigenetic change that begins with remodeling of egg and sperm genomes, and ends with implantation. During this time, parental-specific imprinting marks are maintained to direct appropriate imprinted gene expression. We previously demonstrated that *H19* imprinting could be lost during preimplantation development under certain culture conditions. To define the lability of genomic imprints during this dynamic period and to determine whether loss of imprinting continues at later stages of development, imprinted gene expression and methylation were examined after in vitro preimplantation culture. Following culture in Whitten's medium, the normally silent paternal *H19* allele was aberrantly expressed and undermethylated. However, only a subset of individual cultured blastocysts (~65%) exhibited biallelic expression, while others maintained imprinted *H19* expression. Loss of *H19* imprinting persisted in mid-gestation conceptuses. Placental tissues displayed activation of the normally silent allele for *H19*, *Ascl2*, *Snrpn*, *Peg3* and *Xist* while in the embryo proper imprinted expression for the most part was preserved. Loss of imprinted expression was associated with a decrease in methylation at the *H19* and *Snrpn* imprinting control regions. These results indicate that tissues of trophectoderm origin are unable to restore genomic imprints and suggest that mechanisms that safeguard imprinting might be more robust in the embryo than in the placenta.

Key words: Imprinting, DNA methylation, Placenta, *H19*, *Snrpn*, *Peg3*, *Ascl2*, *Xist*, Mouse

Introduction

Genomic imprinting is defined as an epigenetic mechanism of transcriptional regulation that results in one of the two parental alleles being expressed (Verona et al., 2003). Disruptions in imprinted expression can have severe consequences for growth and development of the mammalian embryo and placenta. Loss of imprinted gene expression has been extensively studied in mice and humans with genetic and epigenetic defects. In humans, such defects result in Prader-Willi, Angelman, and Beckwith-Wiedemann syndromes (Bartolomei and Tilghman, 1997; Maher and Reik, 2000; Nicholls and Knepper, 2001).

Imprinting may be envisaged as a multi-step process that begins in the parental gametes, where epigenetic modifications differentially mark the parental alleles. These parental-specific marks must then be stably maintained during cellular division and differentiation, including during preimplantation development, and finally they must be translated into parental-specific monoallelic expression (Pfeifer, 2000). Disruptions in any of these steps may lead to loss of parental-specific expression.

We and others have previously demonstrated that imprinting can be disrupted during preimplantation development; in vitro preimplantation culture of embryos resulted in biallelic expression of the *H19* gene (Doherty et al., 2000; Sasaki et al., 1995). These results support the hypothesis that gametic imprints are labile, for at least one imprinted gene, during this dynamic period of development. However, a comprehensive analysis of loss of imprinting arising during preimplantation development has not been conducted in any species. Many questions remain unanswered, such as: whether all blastocysts or only a subset lose *H19* imprinting; whether blastocysts that lose imprinted expression are able to restore imprinting of the *H19* gene during postimplantation development; whether other imprinted genes and epigenetic processes display long-term effects of epigenetic errors; and finally, whether loss of imprinting depends on tissue type.

To address these questions we undertook a detailed analysis of allele-specific expression and DNA methylation of imprinted genes after in vitro preimplantation culture of mouse embryos. At the single embryo level, only a subset of individual, Whitten's cultured blastocysts (~65%) displayed biallelic expression, while others maintained allele-specific *H19* expression. Analysis of mid-gestation conceptuses revealed that loss of *H19* imprinting persisted postimplantation. Placental tissues displayed biallelic expression for multiple imprinted genes, including *H19* and *Snrpn*, while in the embryo proper, imprinted expression was mainly preserved, suggesting that there may be tissue-specific

epigenetic disruptions that occurred during preimplantation development. Loss of imprinted expression was associated with reduced methylation at the *H19* and *Snrpn* imprinting control regions (ICRs). These results indicate that genomic imprints are labile in tissues of trophectoderm origin and may be perturbed during preimplantation development.

Materials and methods

Mice

For allele-specific expression studies, embryos were obtained from crosses with C57BL/6(CAST7) or C57BL/6(CAST$^{27\text{-t}}$) females and C57BL/6 (B6) males and from the reciprocal cross with B6 females and B6(CAST7) males. B6(CAST7) mice bear *Mus musculus castaneus* (CAST; The Jackson Laboratory) chromosome 7s on a B6 background, while B6(CAST$^{27\text{-t}}$) are CAST for the central and distal portions of chromosome 7 (27 cM to terminus) and B6 for the proximal region. These mice served as a source of CAST alleles (Mann et al., 2003). No difference was observed in the expression patterns of embryos derived from B6(CAST7) or B6(CAST$^{27\text{-t}}$) females by Fisher's exact test.

Embryos were recovered at the 2-cell stage and cultured in Whitten's medium or in KSOM augmented with amino acids as described (Doherty et al., 2000). For postimplantation analysis, cultured blastocysts were transferred to stage-matched pseudopregnant recipients, and embryos and placentas were recovered at embryonic day (E) 9.5.

Allele-specific expression analysis of blastocyst-stage embryos

Individual blastocysts or pools of blastocysts (~10) were placed in 100 μl Dynal Lysis Buffer, vortexed and stored at –80°C. Dynabeads Oligo $(dT)_{25}$ (Dynal) were equilibrated with 100 μl Dynal Lysis Buffer according to the manufacturer's instructions. Isolation of mRNA and reverse transcription, or generation of Dynabead Oligo $(dT)_{25}$ covalently-linked cDNA libraries and second strand synthesis were performed as described (Mann et al., 2003).

The *H19* and *Snrpn* expression assays were conducted on cDNA using the LightCycler Real Time PCR System (Roche Molecular Biochemicals) as described (Mann et al., 2003) except for the *Snrpn* assay, for which Genset hybridization probes were used, DMSO was omitted, and amplification and melting curve analysis was performed as follows. After an initial denaturation step at 95°C for 2 minutes, amplification was performed for 45 cycles at 95°C for 1 second, 50°C for 15 seconds and 72°C for 6 seconds. After amplification, a final denaturation and annealing step was conducted (95°C for 0 seconds, 45°C for 15 seconds) then the temperature was increased from 45 to 85°C in 0.2°C increments. Alternatively, Idaho Technologies probes were employed, DMSO was omitted, amplification was performed for 45 cycles and the melting curve analysis was performed as follows. A final denaturation step was conducted at 95°C for 4 minutes, followed by annealing at 35°C for 3 minutes, 40°C for 1 minute and 45°C for 1 minute, and melting curve analysis with fluorescence acquisition occurred continuously as the temperature was increased from 45 to 85°C in 0.5°C increments.

For the *Peg3* analysis, *Peg3* primers (final concentration 0.3 μM), Peg11 (5′AAGGCTCTGGTTGACAGTCGTG3′) and Peg12 (5′TTCTCCTTGGTCTCACGGGC3′), amplified a 239 bp fragment (95°C for 2 minutes followed by 34 cycles at 95°C for 15 seconds, 52°C for 10 seconds and 72°C for 20 seconds) containing a polymorphism between B6 (A) and CAST (G) (position 3451, AF038939). Restriction digestion with *Taa*I resulted in 224 bp and 16 bp fragments in B6 and 148, 76 and 16 bp fragments in CAST. Parental allele-specific expression patterns for all genes were calculated as the percentage expression of the B6 or CAST allele relative to the total expression of both alleles.

E9.5 embryo and placental RNA isolation and expression assays

Embryos and placentas were recovered at E9.5 and RNA was isolated using the HighPure RNA Tissue Kit (Roche Molecular Biochemicals), with minor modifications to the manufacturer's recommendations. cDNA synthesis was performed as described (Percec et al., 2002).

Allele-specific *H19* and *Snrpn* expression assays were conducted on E9.5 embryo and placental cDNA using the LightCycler Real Time PCR System as described above. For *Peg3* and *Ascl2*, PCR amplification was conducted on cDNA under conditions specific for each primer set. To a Ready-To-Go PCR Bead, 0.3 μM of each primer and [α-^{32}P]dCTP (1 μCi) were added. PCR amplification was performed for 30 cycles as described above (Mann et al., 2003). Products were resolved on a 7% polyacrylamide gel. After exposure (approximately 15 hours), the relative band intensities were quantified using ImageQuant (Molecular Dynamics). The *Xist* expression assay was conducted on E9.5 embryo and placental cDNA using the LightCycler Real Time PCR System as described (Percec et al., 2002).

Genotyping the sex of E9.5 conceptuses

DNA was extracted from E9.5 yolk sacs and amplified using primers for *Zfy* to determine embryo sex (i.e. the presence of the Y chromosome) and for *Mkrn3* to control for DNA extraction as described (Yamazaki et al., 2003).

Allele-specific DNA methylation analysis

DNA was isolated from pools of 25-30 blastocysts and from individual embryos and placentas obtained at E9.5, subjected to bisulfite modification, PCR amplification, subcloning and sequencing as previously described for the *H19* differentially methylated domain (DMD) (1304-1726 bp, U19619) and *Snurf-Snrpn* (herein referred to as *Snrpn*) promoter-exon 1 region (2073-2601 bp, AF081460) (Davis et al., 1999; Mann et al., 2003). Alternatively, bisulfite mutagenesis sequencing with agarose embedding was conducted on whole blastocysts (Olek et al., 1996; Schoenherr et al., 2003). At least two independent PCRs were performed on each sample. *H19* and *Snrpn* parental alleles were distinguished by single nucleotide polymorphisms as previously reported (Lucifero et al., 2002; Mann et al., 2003; Tremblay et al., 1997).

Results

Allele-specific expression of imprinted genes in F1 hybrid blastocysts

We have previously demonstrated that *H19* imprinted expression in blastocysts was lost following culture in Whitten's medium (Doherty et al., 2000). Similarly to this previous report, pools of Whitten's cultured B6(CAST7)XB6 and B6(CAST$^{27\text{-t}}$)XB6 blastocysts exhibited biallelic expression of the *H19* gene, while those cultured in KSOM augmented with amino acids (KSOMaa) maintained maternal monoallelic expression (Table 1). To determine whether all embryos cultured in Whitten's medium experienced a relaxation of imprinted gene expression or if only a subset of embryos in the original pool activated expression of the normally silent paternal allele, we assayed single B6(CAST7)XB6 and B6(CAST$^{27\text{-t}}$)XB6 hybrid embryos that were cultured from the 2-cell to the blastocyst stage in Whitten's medium or in KSOMaa. We found that a significant number of Whitten's cultured embryos expressed *H19* from both parental alleles (63%), although a proportion of blastocysts (32%) maintained monoallelic (defined as <10% expression from the normally silent allele) *H19* expression (Fig. 1). A small number of embryos also

Table 1. Expression of *H19*, *Snrpn* and *Peg3* in pooled in vivo-derived, Whitten's and KSOMaa cultured blastocysts

	H19		*Snrpn*		Peg3	
Pool	Maternal	Paternal	Maternal	Paternal	Maternal	Paternal
V1*	100	0	0	100	0	100
V2	97	3	0	100	0	100
V3	100	0	0	100	0	100
V4	100	0	0	100	0	100
W1	56	44	0	100	0	100
W2	35	65	0	100	0	100
W3	43	57	0	100	0	100
W4	50	50	0	100	0	100
W5†	49	51	0	100	N/A	N/A
K1	90	10	0	100	0	100
K2	85	15	0	100	0	100
K3	91	9	0	100	0	100

V, in vivo-derived; W, Whitten's; K, KSOMaa; N/A, not assayed (alleles are not distinguishable).

*Pools of 10 blastocysts, except V1 (which consisted of 14 blastocysts).

†Genotype of all pools were B6(CAST7)XB6, except W5 [which was B6(CAST$^{27\text{-}t}$)XB6].

displayed an allelic switch in imprinting; the mechanistic defect for such a switch is currently not evident. While in vivo-derived blastocysts displayed lower levels of expression than Whitten's cultured blastocysts (data not shown), this expression was monoallelic; 6% of blastocysts exhibited biallelic expression. By comparison, the number of KSOMaa cultured embryos with biallelic expression (14%) did not differ significantly from that of in vivo-derived blastocysts. These results demonstrate that *H19* imprinting was affected in many, but not all, blastocysts after culture in Whitten's medium.

In our previous study, we had observed an effect of genotype on *H19* imprinting in Whitten's in vitro cultured embryos; embryos of the reciprocal cross, B6XB6(CAST-H19), cultured under identical conditions, maintained imprinted expression of *H19* (Doherty et al., 2000). In this study, all individual B6XB6(CAST7) Whitten's cultured blastocysts also preserved *H19* imprinting, and thus these embryos served as a control in postimplantation studies (Fig. 1).

These genotypic effects led us to hypothesize that strain-specific modifiers might affect maintenance of the *H19* imprint. In *Peromyscus* mice, an imprinting modifier is linked to *Peg3* (Vrana et al., 2000) and in humans, the orthologous region is linked to an imprinting defect that gives rise to recurrent biparental, complete hydatidiform moles (Moglabey et al., 1999). Thus, the proximal portion of *Mus musculus* chromosome 7 containing the *Peg3* gene may harbor a presumptive modifier that offers 'protection' from environmental stress when inherited from a B6 mother. To test this, we compared maintenance of *H19* imprinted expression in B6(CAST7) mice (CAST for entire chromosome 7) and B6(CAST$^{27\text{-}t}$) mice (B6 proximal, CAST central and distal portions) following culture in Whitten's medium. No difference was observed in the number of blastocysts with *H19* biallelic expression, indicating that the putative modifier likely resides elsewhere in the genome.

Parental-specific expression was next assayed in pools and individual B6(CAST7)XB6, B6(CAST$^{27\text{-}t}$)XB6 and B6XB6(CAST7) embryos for two paternally transcribed genes, *Snrpn* and *Peg3*. Similarly to our previous study, embryos cultured in Whitten's medium or in KSOMaa maintained monoallelic expression of *Snrpn* (Table 1; data not shown). Likewise, the paternally expressed *Peg3* gene also maintained imprinted expression after culture in KSOMaa and in Whitten's media, suggesting that expression of this gene is fairly resistant to epigenetic disturbances at the blastocyst stage (Table 1; data not shown).

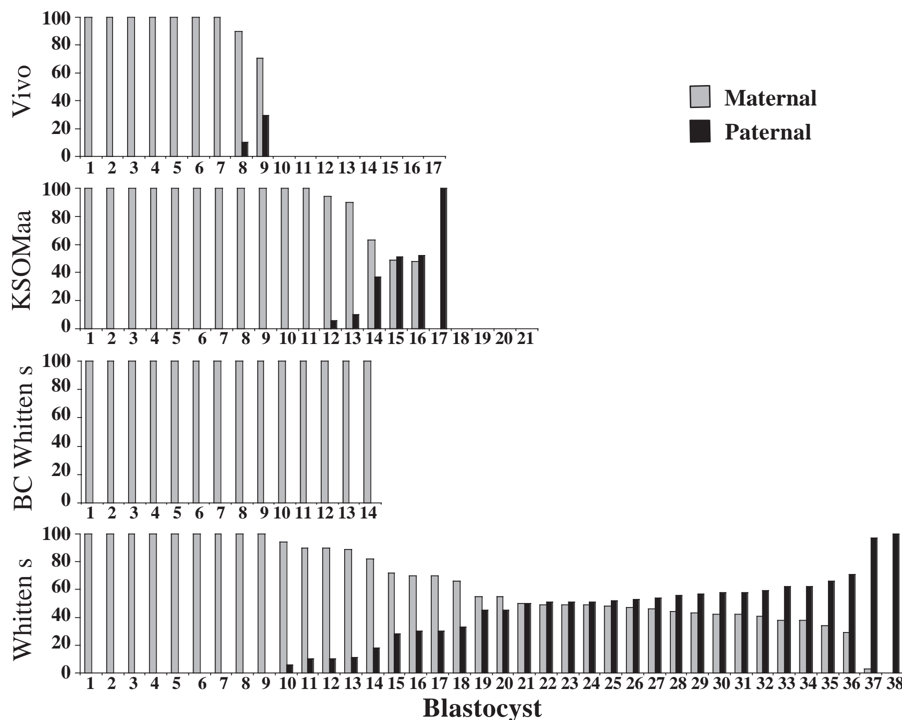

Fig. 1. Allele-specific expression of the *H19* imprinted gene in individual blastocysts. Gray bar height indicates the level of maternal expression, while black bar height represents the level of paternal-specific expression. The number of Whitten's cultured blastocysts with biallelic expression differed significantly from that of KSOMaa cultured (P=0.002), in vivo-derived (P=0.006), and B6XB6(CAST7) Whitten's cultured (P=0.0001) blastocysts as calculated by Fisher's exact test.

Allele-specific methylation analysis of ICRs in cultured blastocysts

As methylation of distinct CpG-rich regions around imprinted genes plays an important role in the control of monoallelic expression, methylation at the *H19* and *Snrpn* ICRs was assayed by bisulfite mutagenesis analysis in cultured blastocysts. The *H19* ICR (designated the differentially methylated domain, or DMD) is paternally hypermethylated (Tremblay et al., 1997), whereas the *Snrpn* promoter-exon 1 region is maternally

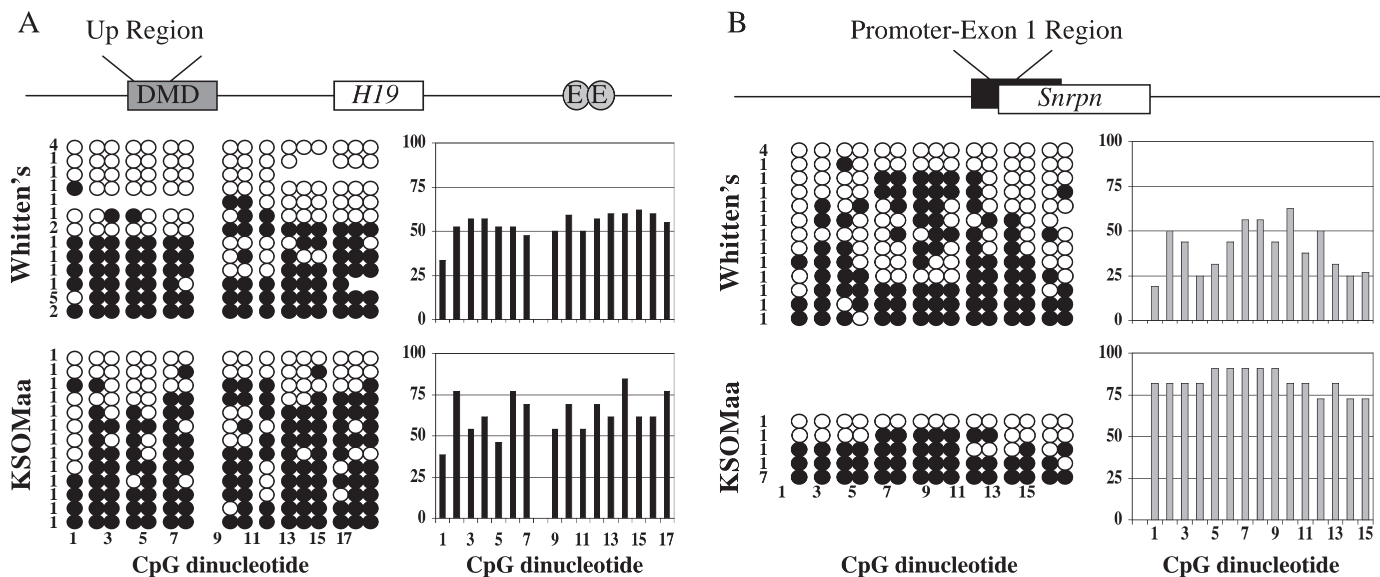

Fig. 2. Methylation status of individual DNA strands in (A) the *H19* upstream differentially methylated domain (DMD) (paternal strands shown) and (B) *Snrpn* promoter-exon 1 region (maternal strands shown) in cultured blastocysts as determined by bisulfite mutagenesis analysis. Unmethylated CpGs are represented as empty circles, while methylated CpGs are depicted as filled circles. Each line denotes an individual strand of DNA with the number of strands showing a given pattern indicated to the left. Bar height indicates the percentage of strands that have a methylated CpG at each specific site. Paternal and maternal alleles are depicted by black and gray bars, respectively. For *H19*, a base pair change in the paternal B6 allele eliminates CpG dinucleotide 8, while for *Snrpn*, CpG dinucleotide 1 is not present in the maternal CAST allele.

hypermethylated (J. Trasler and M. Toppings, personal communication) in in vivo-derived blastocysts. Our analysis revealed that a large proportion of paternal *H19* strands lacked significant methylation in blastocysts cultured in Whitten's medium; only 59% of paternal *H19* strands displayed the expected pattern of hypermethylation (defined as >50% CpGs on a given strand methylated) (Fig. 2A). By comparison, in blastocysts cultured in KSOMaa, 77% of paternal strands were methylated. One explanation for the proportion of paternal hyper- and hypomethylated strands is the composition of blastocysts within the pool; some blastocysts have maintained, while others have lost, *H19* imprinting. To test this hypothesis, we examined methylation of the *Snrpn* ICR with the expectation that *Snrpn* monoallelic expression would correlate with preservation of the methylation imprint. Surprisingly, substantial loss of methylation was observed at the ICR of this gene following Whitten's culture; similarly to *H19*, only 40% of maternal *Snrpn* strands were hypermethylated, while the remaining strands were hypomethylated (Fig. 2B). Blastocysts cultured in KSOMaa exhibited 82% maternal hypermethylation; a loss of methylation comparable to that of *H19*.

Allele-specific expression analyses of E9.5 conceptuses following preimplantation culture

Because *H19* imprinted expression and methylation were disrupted in blastocyst-stage embryos, the question remained as to whether these embryos restored *H19* imprinting during later stages of development. To address this question, F1 hybrid 2-cell embryos were cultured to the blastocyst stage, transferred to recipient mothers, and embryos and placentas were recovered at E9.5. Although many embryos appeared normal (Whitten's 42% normal, KSOMaa 70% normal), we found a proportion of embryos were developmentally delayed or abnormal (not shown) compared with embryos from in vivo-derived and transferred blastocysts (91% normal). Allelic expression was assayed in normal, abnormal (abn) and delayed conceptuses. While imprinted expression was maintained for *H19* in B6(CAST7)XB6 embryos that were subjected to culture in Whitten's medium, the paternal *H19* allele was activated in the corresponding placentas (Fig. 3), indicating that the placenta lacked the ability to restore *H19* imprinting as development proceeded. E9.5 embryos and placentas derived from B6(CAST7)XB6 in vivo blastocysts, B6XB6(CAST7) Whitten's cultured blastocysts, and B6(CAST7)XB6 KSOMaa cultured blastocysts, for the most part, displayed maternal *H19* expression, with the exception of the KSOMaa culture regime, in which some placentas exhibited biallelic expression.

Although the *Snrpn* gene displayed paternal-specific expression in blastocysts, loss of methylation at the *Snrpn* ICR in Whitten's cultured blastocysts prompted us to examine *Snrpn* expression in E9.5 embryonic and placental tissues to determine if loss of imprinted expression occurred at a later stage. While imprinting was maintained in the embryo proper, *Snrpn* was biallelically expressed in a subset of B6(CAST7)XB6 placentas derived from Whitten's cultured blastocysts (Fig. 3), indicating that loss of imprinted expression occurred for this gene as well.

To determine whether there were global preimplantation culture effects on imprinting, two additional genes were examined in the E9.5 conceptuses. Similar to *H19* and *Snrpn*, loss of imprinted expression of *Ascl2* and *Peg3* occurred in B6(CAST7)XB6 preimplantation Whitten's cultured placentas. By contrast to expectations, *Peg3* was biallelically expressed in placentas from both crosses, suggesting that this gene is sensitive to preimplantation culture in Whitten's medium regardless of genetic background. Furthermore,

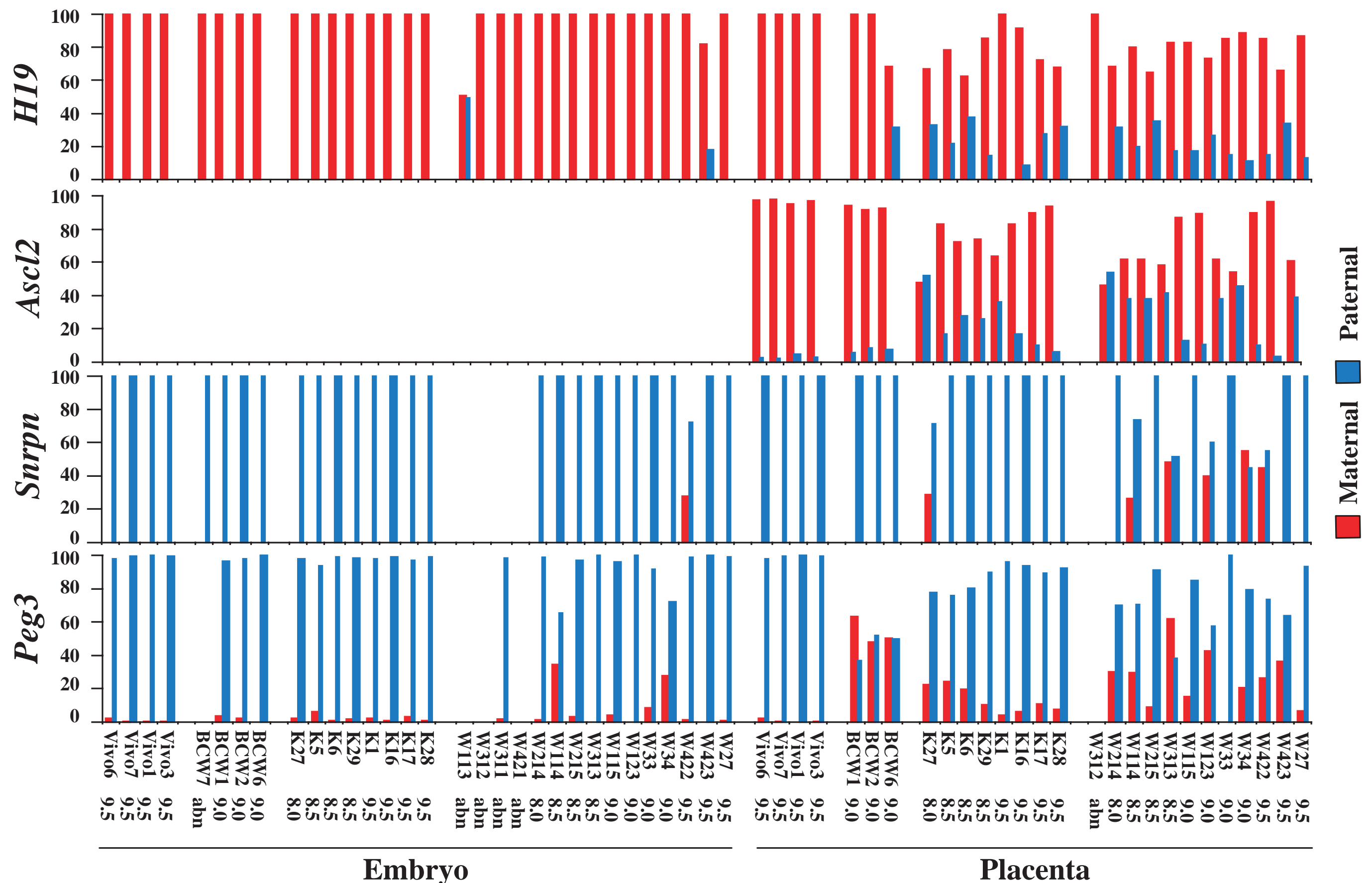

Fig. 3. Loss of imprinted expression in embryonic day (E) 9.5 B6(CAST7)XB6 placentas following preimplantation culture in Whitten's medium. Embryos at the 2-cell stage were cultured to the blastocyst stage then transferred to recipient females. Postimplantation embryo-placental sets were recovered at E9.5. B6(CAST7)XB6 in vivo-derived controls (Vivo), B6XB6(CAST7) Whitten's cultured controls (BCW), the remaining samples are B6(CAST7)XB6 KSOMaa (K) or Whitten's (W) cultured conceptuses. Red bar height indicates the level of maternal expression, while blue bar height represents the level of paternal-specific expression.

expression of *H19* and *Ascl2* was also susceptible to disruption after preimplantation development in KSOMaa. Taken together, these data indicate that the effects of perturbations in preimplantation embryos can be seen long after they have been removed from the culture medium.

Analysis of individual genes revealed that not all placentas exhibited loss of imprinted expression, consistent with the observation that not all blastocysts expressed *H19* biallelically. However, no single pattern emerged with respect to loss or maintenance of imprinted expression when all genes were considered, suggesting a stochastic response to preimplantation Whitten's culture. Occasionally, biallelic expression was observed in the embryo proper (Fig. 3, see W113 as an example), suggesting that although more resilient, imprinting in tissues arising from inner cell mass (ICM) might also be lost during preimplantation development. Loss of imprinted gene expression was independent of the sex of the embryo. Finally, no correlation was observed between the developmental phenotype of cultured embryos and loss of imprinted expression for the four genes examined.

Methylation analyses of ICRs in E9.5 conceptuses following preimplantation culture

Methylation associated with the ICRs of *H19* and *Snrpn* was assessed in preimplantation cultured and in vivo-derived conceptuses recovered at E9.5. As predicted from the expression data, the paternal *H19* allele was hypermethylated in B6(CAST7)XB6 Whitten's cultured E9.5 embryos (100%, 100% and 83% strands for W35, W36 and W321, respectively) (Fig. 4A). By contrast, one B6(CAST7)XB6 Whitten's cultured E9.5 placenta (W35) exhibited a partial loss of methylation with 63% paternal strands hypermethylated and placentas from the other conceptuses displayed a substantial loss of methylation with 13% (W36) and 10% (W321) paternal hypermethylation. Although different fetuses were analyzed for imprinted methylation and expression, generally paternal methylation loss (37-90%) correlated with the level of paternal activation (~23-75%, to consider only paternal allelic contributions, percentage of paternal expression was multiplied by 2). Allele-specific methylation was preserved in a control B6XB6(CAST7) embryo (WBC5) with 100% paternal strands hypermethylated, while in the placenta lower levels were observed (63%). All paternal strands were hypermethylated in embryos and placentas that were in vivo-derived or subjected to preimplantation KSOMaa culture, consistent with the silent state of this allele.

While not as dramatic as *H19*, B6(CAST7)XB6 placentas derived from cultured blastocysts also experienced a loss of

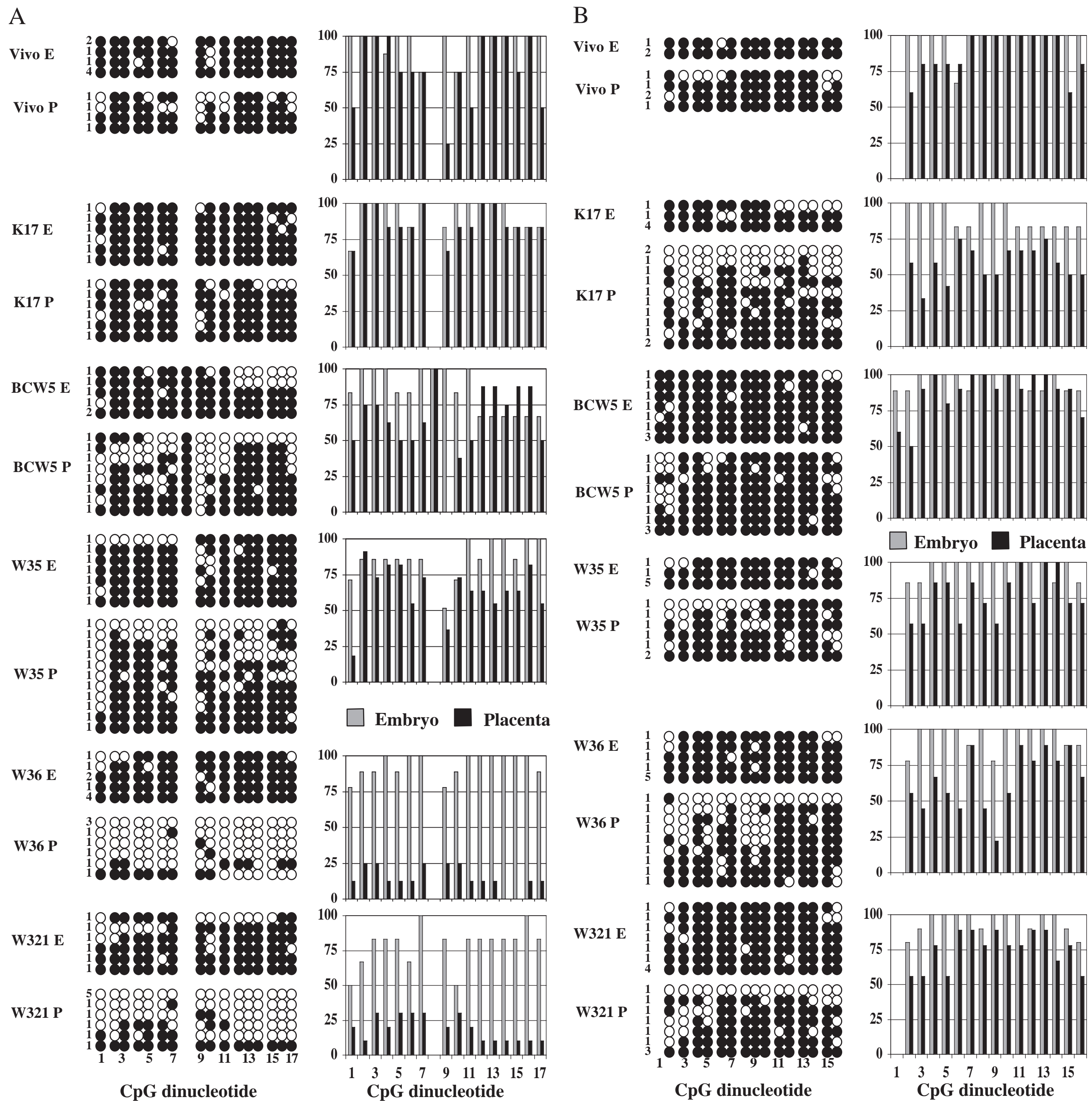

Fig. 4. Methylation status of individual DNA strands in (A) the *H19* upstream DMD and (B) *Snrpn* promoter-exon 1 region in embryos and placentas recovered at embryonic day 9.5 as determined by bisulfite analysis. Details are as described in Fig. 2. A low level of sporadic methylation on the normally unmethylated allele was observed in samples perturbed by preimplantation culture (data not shown).

maternal-specific *Snrpn* methylation with 86% (W35), 89% (W36), 89% (W321) and 67% (K17) hypermethylated strands (Fig. 4B). In this case, the normally silent maternal allele was activated to a greater level (~53-100%, maternal expression multiplied by 2) than would have been predicted from the loss of maternal-specific methylation (11-33%). By comparison, in vivo-derived and control B6XB6(CAST7) placentas, and all embryos, maintained 100% *Snrpn* hypermethylation, which correlated with maternal allele silencing.

Effects of in vitro culture on *Xist* expression

X-inactivation is an epigenetic process whereby one X chromosome is inactivated in female cells. In embryonic tissues, X-inactivation occurs in a random manner, while in extra-embryonic tissues there is preferential inactivation of the

paternal X chromosome (Plath et al., 2002). The X-inactivation process is partly regulated by the X-inactive-specific transcript (*Xist*). *Xist* is expressed from the inactive X chromosome in females but not in males, where the sole X chromosome remains active. To determine whether regulation of another epigenetic process was affected under conditions that resulted in loss of imprinting, *Xist* expression was examined in embryonic and placental tissues of E9.5 conceptuses after preimplantation culture (Table 2). As female B6(CAST7)XB6 mice possess two B6 X chromosomes, effects of preimplantation development in culture on *Xist* expression was determined for males only. Male placental tissues from embryos that were cultured to the blastocyst stage in Whitten's medium, and a proportion that were cultured in KSOMaa, inappropriately expressed the *Xist* gene, with the levels generally falling within the range observed for female tissues, perhaps indicating that the imprinted form of X-inactivation was disrupted. An absence of ectopic *Xist* expression in B6(CAST7)XB6 male embryonic tissues suggests that the machinery regulating the random form of X-inactivation was unaffected during preimplantation development or was corrected as development proceeded. Thus, errors arising during preimplantation can result in general epigenetic dysregulation in trophectoderm lineages.

Table 2. *Xist* expression in XY conceptuses

Conceptus*	Embryo	Placenta
BCW1 M	–	–
K5 M	–	–
K6 M	–	–
K16 M	–	+
K17 M	–	+
K28 M	–	–
W114 M	–	+
W115 M	–	+
W33 M	+	+
W34 M	–	+
W422 M	–	+

*Same conceptuses as in Fig. 3.
M, male.

Discussion

Previous studies in mice have suggested that in vitro culture of embryos and embryonic stem cells can lead to reduced viability and growth, developmental abnormalities and aberrant imprinted gene expression (Bowman and McLaren, 1970; Dean et al., 1998; Doherty et al., 2000; Khosla et al., 2001; Nagy et al., 1993; Reik et al., 1993; Sasaki et al., 1995). With respect to the latter, we and others have observed that culture of preimplantation embryos can result in biallelic or reduced expression of the *H19* gene (Doherty et al., 2000; Khosla et al., 2001; Sasaki et al., 1995), indicating that epigenetic mechanisms that maintain imprinting might be unstable. Sasaki and colleagues were the first to demonstrate that in vitro fertilization and culture of mouse embryos result in loss of *H19* imprinted expression in blastocysts (Sasaki et al., 1995). Postimplantation analysis of these in vitro-derived embryos at E6.5, 7.5 and 8.5 revealed that the paternal *H19* allele continues to be expressed in extra-embryonic but not in embryonic lineages. While we previously demonstrated that in vitro culture alone results in a loss of *H19* imprinted expression in blastocysts (Doherty et al., 2000), we report here that this disruption occurs in only a subset of blastocysts and that preimplantation effects on imprinting persist postimplantation in a tissue-specific manner; placental tissues isolated at E9.5 continue to show loss of allelic expression of *H19* and other imprinted genes. We also demonstrate that activation of the paternal *H19* allele for the most part correlates with loss of paternal-specific methylation at the DMD in both cultured blastocysts and mid-gestation placentas. Together these results demonstrate that appropriate imprinting is not restored during postimplantation development of the placenta.

In our initial study (Doherty et al., 2000), we proposed that *H19* is hypersensitive to environmental stress, as analysis of a second imprinted gene, *Snrpn*, revealed that its imprinting is preserved in blastocysts after preimplantation development in culture. However, biallelic expression of several imprinted genes in postimplantation placentas, including *Snrpn*, after culture in Whitten's medium indicates a more global effect on imprinting. This is supported by the partial loss of methylation that was observed at the *Snrpn* ICR in mid-gestation placentas that were derived from cultured blastocysts. The less dramatic loss of methylation at *Snrpn* in comparison with *H19* and the lack of correlation between *Snrpn* imprinted expression and methylation in blastocysts may indicate that disruptions in methylation are not solely responsible for the inability to maintain imprinted expression at this gene.

Loss of imprinted expression is also observed for *Ascl2*, an imprinted gene that is normally biallelically expressed in blastocyst-stage embryos but is monoallelically expressed in placentas. This result suggests that culture in Whitten's medium either disrupted the imprinting mechanism that regulates this gene at later stages or it did not allow the normal imprinting control mechanism to initiate allele-specific expression at the appropriate time in development. Interestingly, imprinted regulation of *Ascl2* operates in a methylation-independent manner (Caspary et al., 1998; Tanaka et al., 1999). The *Xist* gene and its antisense transcript, *Tsix*, also lack germline-derived methylation imprints (McDonald et al., 1998; Prissette et al., 2001). This suggests either that imprinting is disrupted through different mechanisms for distinct genes or that a uniform process upstream of methylation operates at all imprinted loci, resulting in disruptions to both imprinted gene expression and methylation.

In mice, loss of *Tsix* expression results in ectopic activation of *Xist* from the normally silent maternal chromosome in females and males (Lee, 2000; Sado et al., 2001). In our study, aberrant expression of the *Xist* gene in male placentas might indicate that the antisense *Tsix* transcript is inactivated or that transcription is not initiated, thereby resulting in ectopic *Xist* expression. Alternatively, the *Xist* gene itself might be susceptible to culture conditions, independent of the *Tsix* antisense transcript. In either case, these results demonstrate that disturbances arising during preimplantation can result in general epigenetic dysregulation in trophectoderm lineages.

Placental tissues appear to be particularly sensitive to an imbalance of imprinted gene expression. This has been clearly observed in parthenogenetic and androgenetic embryos, in fetuses that underwent round spermatid injection and in interspecific hybrids of *Peromyscus* mice (Barton et al., 1984; McGrath and Solter, 1984; Shamanski et al., 1999; Vrana et

al., 2000; Vrana et al., 1998). We propose that loss of imprinting is a consequence of the failure to maintain imprinting in the preimplantation embryo and that trophectodermal cells might be more sensitive to preimplantation epigenetic upset than ICMs. We can formulate several explanations for the differential response of placental tissues to preimplantation development in culture; trophectoderm cells are in closer contact with the culture medium, are the first cells to differentiate in the embryo and/or have less redundancy in epigenetic modifications that maintain imprints.

Initial studies to determine whether loss of methylation imprints occurs selectively in the trophectoderm revealed that ICMs isolated using immunosurgery and subjected to bisulfite mutagenesis analysis experience a similar loss of methylation to DNA from intact blastocysts (data not shown). While this suggests that loss of methylation might occur randomly in the preimplantation embryo, we cannot rule out the possibility that loss of imprinting within the ICM occurs in cells that have differentiated into or are destined to become primitive endoderm, an extra-embryonic cell-type. Thus, we envision two different scenarios. In the first, extra-embryonic cells are more affected by culture and this translates into loss of imprinting in mid-gestation placentas. In the second, loss of imprinting may also occur in cells destined to become the embryo. Later, these cells are able to restore imprinted expression and methylation. Consistent with this, biallelic expression was occasionally observed in the embryo, suggesting that mechanisms that safeguard imprinting might be more robust in the embryo than in the placenta. Of note is that there is a wave of de novo methylation that is lineage-restricted, occurring in ICM but not in trophectoderm lineages (Monk et al., 1987; Santos et al., 2002). DNA methyltransferases and methyl-binding domain proteins are probable key players in this process and are transcribed in mouse and human blastocysts and embryonic stem (ES) cells (Chen et al., 2003; Huntriss et al., 2004; Okano et al., 1998). While the somatic form of DNMT1 maintains methylation in ES cells and postimplantation embryos, DNMT3a and 3b probably have roles in both de novo-related and maintenance-related methylation of imprinted genes (Chen et al., 2003; Lei et al., 1996; Okano et al., 1998). Interestingly, DNMT3b localizes exclusively to the ICM and its derivates at E4.5 to 7.0 (Watanabe et al., 2002). Therefore, lack of this protein in trophectoderm cells might offer one explanation for their inability to restore methylation imprints in the placenta.

The results reported here might be relevant to the treatment of human infertility by assisted reproductive technologies (ART). Our data indicate that loss of imprinting occurs after the 2-cell stage and prior to the blastocyst stage. As reductions in the level of transcript abundance of non-housekeeping genes following Whitten's culture were present as early as the 8-cell stage (Ho et al., 1995), epigenetic dysregulation in general might be an early event. In humans, ART has been linked to a higher incidence of interuterine growth retardation, premature birth and low birth weight (Maher et al., 2003a), suggesting a loss of epigenetic regulation during preimplantation development. Recently, ART procedures have also become suspect in the generation of sporadic epigenetic errors that result in the development of two imprinting disorders, Angelman and Beckwith-Wiedemann syndromes (Cox et al., 2002; DeBaun et al., 2003; Gicquel et al., 2003; Maher et al., 2003b; Orstavik et al., 2003). Furthermore, an increased incidence of monozygotic twinning occurs in the latter with the affected twin exhibiting loss of imprinting (Weksberg et al., 2002), intimating a period of sensitivity during early embryogenesis. Pinpointing the timing of epigenetic misregulation in mice and humans may reveal a common pathway in mechanisms that maintain imprinting during preimplantation development.

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