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Differential Effects of Culture on Imprinted *H19* Expression in the Preimplantation Mouse Embryo[1]

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ABSTRACT

The *H19* gene is imprinted with preferential expression from the maternal allele. The putative imprinting control region for this locus is hypermethylated on the repressed paternal allele. Although maternal-specific expression of *H19* is observed in mouse blastocysts that develop in vivo, biallelic expression has been documented in embryos and embryonic stem cells experimentally manipulated by in vitro culture conditions. In this study the effect of culture on imprinted *H19* expression and methylation was determined. After culture of 2-cell embryos to the blastocyst stage in Whitten's medium, the normally silent paternal *H19* allele was aberrantly expressed, whereas little paternal expression was observed following culture in KSOM containing amino acids (KSOM+AA). Analysis of the methylation status of a CpG dinucleotide located in the upstream imprinting control region revealed a loss in methylation in embryos cultured in Whitten's medium but not in embryos cultured in KSOM+AA. Thus, *H19* expression and methylation were adversely affected by culture in Whitten's medium, while the response of *H19* to culture in KSOM+AA approximated more closely the in vivo situation. It is unlikely that biallelic expression of *H19* following culture in Whitten's medium is a generalized effect of lower methylation levels, since the amount of DNA methyltransferase activity and the spatial distribution of Dnmt1 protein were similar in in vivo-derived and cultured embryos. Moreover, imprinted expression of *Snrpn* was maintained following culture in either medium, indicating that not all imprinted genes are under the same stringent imprinting controls. The finding that culture conditions can dramatically, but selectively, affect the expression of imprinted genes provides a model system for further study of the linkage between DNA methylation and gene expression.

INTRODUCTION

Over 30 genes in the mammalian genome are preferentially expressed from a single parental allele. These genes are controlled by the process of genomic imprinting that marks the parental alleles and governs this unusual expression pattern [1, 2]. Although how the alleles are designated with their parental identity is the focus of intense investigation, the molecular marking mechanism that underlies the differential gene expression remains to be elucidated. What is clear, however, is that errors that result in loss-of-imprinting are associated with diseases such as Prader-Willi and Angelman syndromes and tumors such as Wilms' tumor [3–7].

The mouse *H19* gene is one of the most highly characterized and studied imprinted genes. This gene, which does not encode a protein product and is transcribed exclusively from the maternal allele [8, 9], is located in a large cluster of imprinted genes on the distal end of mouse chromosome 7 [10, 11]. *H19* and the oppositely imprinted *Igf2* gene are located 90 kilobases (kb) apart and share regulatory elements crucial to the imprinting of both genes [12–14]. *H19* exhibits all the properties that thus far have been hypothesized as central to the imprinting mechanism, including differential chromatin structure, asynchronously replicating alleles, and locus-specific repetitive elements [15–19]. Perhaps the most compelling and best-characterized candidate for the *H19* imprinting mark is differential DNA methylation. The repressed paternal allele of *H19* is hypermethylated over a 7-kb region that includes 4 kb of upstream flanking sequence and the transcription unit [20–22] (Fig. 1). A 2-kb segment of this region located from −2 kb to −4 kb relative to the start of transcription is hypermethylated in sperm and on the paternal allele in somatic tissues throughout development, including the period of genome-wide demethylation that occurs during preimplantation, and is the candidate for harboring the allele-specific imprinting mark [23]. Deletion of this region from the endogenous locus leads to loss-of-imprinting of *H19* and *Igf2* on both parental alleles [14]. In addition to stably marking an allele, DNA methylation can also serve to inhibit gene activity, since hypermethylated DNA is typically transcriptionally repressed. This linkage between DNA methylation and repression of transcription appears to be mediated through the methyl-CpG-binding protein MeCP2 that interacts with a histone deacetylase complex [24, 25].

Definitive proof that allele-specific methylation confers imprinting to the *H19* gene requires the ability to manipulate methylation at this locus. Genome-wide demethylation has been achieved through the use of DNA methyltransferase 1 (*Dnmt1*) mutant mice. Prior to their death at approximately 11 days of gestation, homozygous mutant mice have dramatically reduced levels of overall DNA methylation and, consistent with a role for DNA methylation in imprinting, exhibit perturbations in imprinted expression of *H19*, as well as other imprinted genes [26]. This experiment, however, indirectly tested the effect of methylation at the *H19* locus, since the methylation status of the entire genome is affected.

The in vitro culture of mouse embryos provides another opportunity to test the role of epigenetic marking on im-

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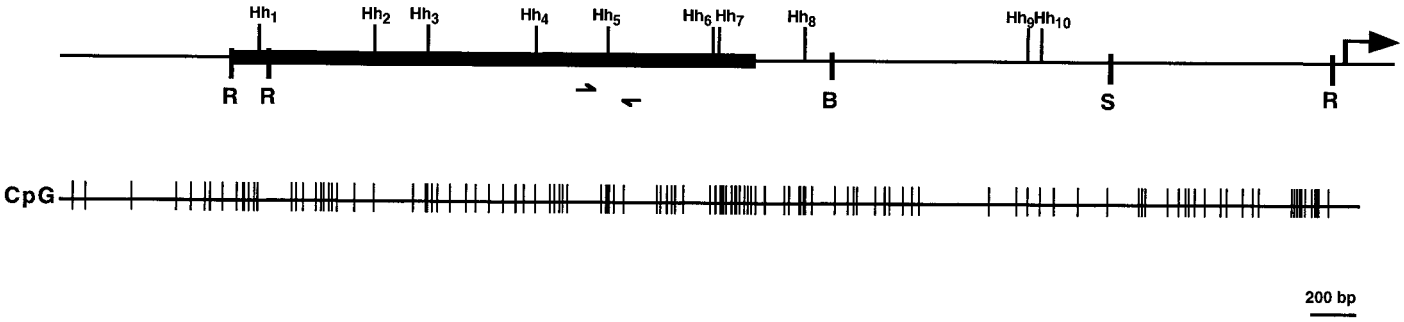

FIG. 1. Location of CpG dinucleotides in the upstream region of the *H19* gene. A 4-kb region upstream of the transcription start site (arrow) is depicted on the top line. CpG dinucleotides cleaved by the methylation-sensitive restriction endonuclease *Hha*I (Hh, vertical line above gene line) are indicated. Other restriction endonuclease sites include *Eco*RI (R), *Bam*HI (B), and *Sac*I (S). The bottom line shows the location of all CpG dinucleotides found in the upstream region (accession no. U19619 [19]). The differentially methylated domain (filled box) is hypermethylated on the paternal allele. The PCR primers used to assay methylation of the *Hh5* site are indicated beneath the top line.

printed gene expression. Although monoallelic expression of *H19* from the maternal genome is observed in mouse blastocysts that develop in vivo and at subsequent stages of development [23], there are a few reports that describe the biallelic expression of *H19* in embryos and embryonic stem cells. For example, embryos generated by fertilization in vitro and then cultured to the early blastocyst stage were reported to experience variability in imprinting with a subset of blastocysts showing biallelic expression of *H19* [27]. Therefore, repression of the paternal allele may be unstable during extended culture.

In the present study, we report that the expression of *H19* can be experimentally manipulated by in vitro culture conditions. Culture of 2-cell embryos to the blastocyst stage in Whitten's medium results in biallelic *H19* expression in these blastocysts, whereas monoallelic maternal *H19* expression is maintained following culture of 2-cell embryos to the blastocyst stage in KSOM containing amino acids (KSOM+AA). Moreover, a reduction in methylation of upstream *H19* sequences occurs after culture in Whitten's medium, but not after culture in KSOM+AA. It is unlikely that the biallelic expression of *H19* after culture in Whitten's medium is a generalized effect of lower methylation patterns, since DNA methyltransferase activity is maintained in Whitten's medium, and the imprinting of another gene, *Snrpn,* is also maintained.

MATERIALS AND METHODS

Generation of Congenic B6(CAST 7$^{27–71}$) Mice

For allele-specific expression studies, F_1 hybrid mice were derived from crosses with C57BL/6J (B6) (The Jackson Laboratory, Bar Harbor, ME) and B6(CAST-*H19*) mice [19]. The latter served as a source of the *Mus musculus castaneus* (CAST) *H19* allele. For allele-specific expression studies involving the *Snrpn* gene, we produced a new strain of mice (B6[CAST 7$^{27–71}$]) that is CAST for a region encompassing two imprinted domains on chromosome 7 (*Snrpn* [27.6 cM] and *H19* [69 cM]) on a B6 background. CAST mice were crossed with B6 mice, and the F_1 hybrid progeny were subsequently mated to B6 mice. Progeny inheriting a CAST *H19* and *Snrpn* allele were again bred to B6 mice. Mice that were heterozygous at the *H19* and *Snrpn* loci were then intercrossed, and progeny were selected that were homozygous for the CAST *H19* and *Snrpn* alleles. Backcross and intercross progeny were genotyped with the microsatellite markers (Research Genetics, Huntsville, AL) D7Mit198 (27 cM), D7Mit211 (27 cM), D7Mit222 (52 cM), D7Mit207 (65.5 cM), and D7Mit140 (71 cM) and the *p57^{KIP2}* gene (69 cM) to select for animals that were CAST for a region extending from 27 to 71 cM. The *p57^{KIP2}* gene was used to determine the genotype of the *H19* imprinted domain. The amplification reaction contained 100 ng tail DNA, single-strength polymerase chain reaction (PCR) buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl [Perkin Elmer, Foster City, CA]), 1.5 mM $MgCl_2$, 100 μM dNTP each, 0.2 μM forward and reverse primer, and 0.5 U AmpliTaq DNA polymerase (Perkin Elmer). The *p57^{KIP2}* forward and reverse primers were 5′TACACCTTGGGACCAGCGTACTCC′3 (MMU20553; position 1304–1281) and 5′CGGACGATGGAAGAACTCTGG3′ (MMU20553; position 1138–1158), respectively. PCR amplification consisted of 32 cycles, 94°C for 45 sec, 55°C for 45 sec, 72°C for 60 sec with an elongation cycle 72°C for 7 min. The resulting amplicons were subjected to gel electrophoresis in a 12% polyacrylamide gel using single-strength TBE (89 mM Tris-borate, 4 mM EDTA) to resolve the strain-specific PCR fragments. The *p57^{KIP2}* primers amplify a product of 167 base pairs (bp). Restriction digestion with *Taq*I allows for identification of a polymorphism in CAST (cytosine) at position 1256. Cleavage products are 118 bp and 49 bp in length. The B6 PCR amplification product is undigested since a thymine is present at this site.

Oocyte and Embryo Collection and Embryo Culture

Fully grown, germinal vesicle-intact oocytes were collected from mice 44–48 h after injection with 5 IU eCG [28]; metaphase II-arrested ovulated eggs were collected from mice injected with 5 IU eCG and then 5 IU hCG 44–48 h later [29]. One-cell, two-cell, eight-cell, morula, and blastocyst stage embryos were flushed with MEM/Hepes (bicarbonate-free minimal essential medium [Earle's salt] supplemented with pyruvate [100 μg/ml], gentamicin [10 μg/ml], polyvinylpyrrolidone [PVP; 3 mg/ml], and 25 mM Hepes, pH 7.3) from the oviducts/uteri of superovulated and mated mice 21 h, 48 h, 72 h, 80 h, and 96 h post-hCG, respectively, as previously described [30]. The embryos were then transferred to 50-μl drops of either Whitten's medium [31] or KSOM containing amino acids (KSOM+AA) [32] and cultured to the blastocyst stage at 37°C in an atmosphere of 5% CO_2, 5% O_2, 90% N_2. Inner cell masses (ICM) were obtained by immunosurgery as previously described [33]. Mice were used according to a protocol approved by the Institutional Animal Care and Use Committee.

Isolation of RNA and Reverse Transcription-PCR for Analysis of the Temporal and Spatial Pattern of H19 Expression

RNA was isolated from oocytes/embryos recovered from CF-1 females (Harlan Sprague-Dawley; Harlan Industries, Indianapolis, IN) that were mated with B6D2/F1 males (The Jackson Laboratory) and prepared for reverse transcription (RT)-PCR using gene-specific primers as previously described [34]. In these experiments, β-globin mRNA was added prior to RNA isolation; the β-globin mRNA served as an internal standard for the efficiency of the RNA isolation and RT-PCR reactions [34]. The amount of cDNA product was measured in the linear region of semi-log plots of the amount of PCR product as a function of cycle number. This method permits the comparison of relative changes in the abundance of a particular transcript [34].

RT reactions were carried out as previously described using 28 oocyte/embryo equivalents, i.e., 28 oocytes or embryos per reaction [34]. An appropriate volume of RT reaction containing 4 oocyte/embryo equivalents was used in PCR reactions conducted as described previously [34], but according to the following PCR program: 94°C for 1 min, followed by 29 cycles of a 2-step program of 94°C for 10 sec and 60°C for 15 sec. The last cycle was followed by a 6-min extension at 60°C. The PCR reactions were conducted in the presence of [α-^{32}P]dCTP (3000 Ci/mmol [NEN Research Products, Boston, MA]). The 5′ and 3′ *H19* gene-specific primers were 5′GCACTAAGTCGATTGCACTGG3′ and 5′CTCGCCTAGTCTGGAAGCAG3′, respectively; these primers generate a 195-bp product.

Isolation of RNA, RT-PCR, and Single-Stranded Conformation Polymorphism Gel Electrophoresis

When the parental origin of the transcript was to be determined, single-stranded conformation polymorphism (SSCP) gel electrophoresis was conducted. In these experiments, RNA was isolated and subjected to RT as described above except that β-globin mRNA was not added. After RT, PCR was performed on 4 embryo equivalents of reverse-transcribed product in a 25-μl reaction mixture containing single-strength PCR buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl [Perkin Elmer]), 1.5 mM $MgCl_2$, 125 μM dNTP each, 0.3 μM primer each, 0.1 μl [α-^{32}P]dCTP (3000 Ci/mmol [NEN Research Products]), 1 unit AmpliTaq DNA polymerase (Perkin Elmer).

The *H19* primers were 5′GCACTAAGTCGATTGCACTGG3′ (U19613; position 2658–2678) and 5′TGGGTACTGGGGCAGCATTG3′ (U19613; position 2768–2749); these primers generate a 110-bp amplicon that contains polymorphisms at nucleotides 2701, 2736, and 2739 such that B6 possess thymine, thymine, and adenine, while CAST mice have guanine, cytosine, and guanine at these positions (unpublished results). The *Snrpn* primers were 5′CTCCACCAGGAATTAGAGGC3′ (MMSMN; position 822–841) and 5′GCAGTAAGAGGGGTCAAAAGC3′ (MMSMN; position 966–946); these primers generate a 145-bp amplicon that contains a polymorphism at nucleotide 915 such that B6 possess a cytosine while CAST have a thymine at this position [35].

PCR amplification for both *H19* and *Snrpn* cDNAs consisted of 34 cycles, 94°C for 1 min, 55°C for 2 min, 72°C for 2 min with an elongation cycle 60°C for 7 min. For SSCP analysis, 1–5 μl of PCR reaction product was added to 10 μl loading dye (95% formamide, 0.0125% bromophenol blue, 0.0125% xylene cyanol, 0.75% Ficoll, 50 μM EDTA), and the sample was heated to 95°C for 10 min and immediately placed on ice. Samples were electrophoresed through an SSCP gel (0.5-strength MDE [J.T. Baker, Phillipsburg, NJ]) in 0.6-strength TBE at 500 V, 4°C. For *H19* product analysis, the gels either contained no glycerol and were run for 5 h (see Fig. 3) or contained 5% glycerol and were run for 7 h (see Fig. 7). *Snrpn* PCR products were analyzed on 10% glycerol SSCP gels for 15 h. The relative band intensities were quantitated using ImageQuant (Molecular Dynamics, Sunnyvale, CA), allowing the parental allele-specific contributions to be assessed.

PCR Methylation Analysis

The analysis of the methylation status of the *Hha*I site 5 was conducted essentially as previously described [19]. Briefly, after culture and isolation of DNA, samples were digested with *Pvu*II alone (to reduce the size of the DNA) or *Pvu*II plus the methylation-sensitive enzyme *Hha*I. The 5′ and 3′ primers surrounding the *Hha*I site 5 were 5′TATGCCTCAGTGGTCGATATG3′ and 5′GGTTCAGTGTGTAAGGGAACC3′, respectively, and PCR amplification was conducted as previously described [19]. The PCR products were separated by electrophoresis either under conditions that detect the amplified product or under SSCP conditions that resolve the parental allele-specific DNA strands.

Assay for DNA Methyltransferase Activity

The activity of endogenous DNA methyltransferase was assayed with minor modifications to the method of Monk et al. [36]. Briefly, oocytes/embryos recovered from either CF-1 females mated with B6D2/F1 males or B6(CAST-*H19*) females mated with B6 males (10 per group and 4 groups per assay) were transferred in 2–17 μl of assay buffer (50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1% Tween 80, 100 μg/ml RNase A, and 130 μCi/ml [^{3}H-methyl]S-adenosylmethionine [77 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ]). Oocytes/embryos were then lysed by 4 freeze/thaw cycles using dry ice in methanol. After the addition of water (1 μl) to one tube and 1 μl poly d(I-C) (500 μg/ml [Boehringer Mannheim, Indianapolis, IN]) to the remaining 3 tubes in each embryo group, the samples were incubated for 2 h at 37°C. The reaction was terminated by the addition of 2.5 μl 10% SDS and 3 μl proteinase K (10 mg/ml) and incubated for 30 min at 37°C. The poly d(I-C) substrate was recovered by adding 72 μl of resuspension solution (40 mM Tris-HCl, pH 7.5, 10 mM NaCl, 6 mM $MgCl_2$) and then extracting the samples with 100 μl phenol:chloroform:isoamyl alcohol (25:24:1). After a brief centrifugation, the aqueous phase was removed and transferred to another tube containing 30 μl 1 M NaOH. The sample was incubated for 1 h at 37°C to degrade any RNA. After the incubation, the sample was acidified by addition of 50 μl 1 M HCl. BSA (10 μg) was added as carrier, and the poly d(I-C) was precipitated overnight at 4°C after addition of 200 μl 10% trichloroacetic acid. The precipitate was collected by centrifugation for 15 min at 10 000 × *g* and 4°C, and the resulting pellets were washed twice with 100 μl cold 10% trichloroacetic acid. After washing, the pellets were resuspended in 30 μl 1 N NaOH, then mildly acidified with 40 μl 1 N HCl. The samples were then subjected to liquid scintillation counting using a Beckman (Palo Alto, CA) LS 6500. Background values (no poly d[I-C]) were subtracted from corresponding experimental samples.

Immunocytochemical Localization of Dnmt1

The zona pellucida was removed from embryos at different stages of development by a brief treatment (15–20 sec) in acid Tyrode's (pH 2.5), followed by several washes in PBS containing 3 mg/ml BSA. The embryos were fixed in freshly prepared 3.7% paraformaldehyde for 1 h at 4°C. After fixation, embryos were washed twice in PBS containing 3 mg/ml PVP and then permeabilized in 20 μl 0.1% Triton X-100/PBS for 15 min at room temperature in a humidified container. All subsequent steps were performed at room temperature in a humidified container. The embryos were transferred through two 20-μl drops of PBS/PVP and then transferred into a 20-μl drop of blocking solution (BS: 0.1% BSA, 0.01% Tween 20 in PBS) for 15 min. The fixed and permeabilized embryos were incubated with 20 μl of the anti-DNA methyltransferase antibody (1:1000) for 1 h; the anti-DNA methyltransferase antibody was a generous gift of Dr. T. Bestor (Department of Genetics and Development, Columbia University, New York, NY). Embryos were washed in 20-μl drops BS (4 times, 15 min each) and incubated for 1 h with Texas Red-conjugated goat anti-rabbit IgG (1:500) (Jackson Immunoresearch Laboratories, West Grove, PA). Embryos were washed 3 times in BS (15 min per wash) and mounted on slides in VectaShield (Vector Laboratories, Burlingame, CA). Fluorescence was visualized with a Leica (Milton Keynes, Bucks, UK) TCS NT laser-scanning confocal microscope using an ArKr laser.

RESULTS

Temporal and Spatial Pattern of H19 Expression in Preimplantation Mouse Embryos

H19 is expressed from the maternal allele during all stages of embryonic development, including the blastocyst stage [19, 23]. Biallelic expression, however, has been reported in embryos [27] and embryonic stem cells experimentally manipulated by in vitro culture conditions, suggesting that the *H19* imprint may be unstable during early stages of development. To determine the effect of in vitro culture on imprinting, *H19* expression and methylation were assessed after culture of embryos to the blastocyst stage. Before initiating studies on the regulation of *H19* expression in the preimplantation mouse embryo, however, it was first important to define the temporal and spatial pattern of *H19* expression, since previous analyses have been ambiguous. Several studies employing RT-PCR methods failed to detect *H19* expression in either fully grown germinal vesicle-intact oocytes or blastocysts [37, 38]. In contrast, others have detected *H19* expression in the 4.5-day blastocyst by either in situ hybridization [39] or RT-PCR [19]. One possible explanation for these discordant results is that *H19* is poorly expressed during preimplantation development. To resolve the ambiguity of these data and to determine the temporal and spatial pattern of *H19* expression in the preimplantation embryo, we used an RT-PCR assay that permits quantification of the relative changes in transcript abundance [34]. Very little, if any, maternal *H19* transcripts were contained in oocytes, ovulated eggs, or 1-cell embryos (Fig. 2A). Concomitant with the activation of the embryonic genome at the 2-cell stage [40], a small increase in *H19* transcript abundance was observed. The major increase, however, occurred between the 8-cell and blastocyst stages.

To determine the spatial pattern of *H19* expression at the blastocyst stage, an RT-PCR assay on immunodissected embryos was performed (Fig. 2B). Since essentially no signal was obtained with immunosurgically isolated ICM cells whereas a signal was detected from intact blastocysts, we deduced that *H19* expression is likely limited to the trophectoderm of mid-blastocyst embryos (32–40 cells). The absence of a signal for *H19* in the ICM could not be attributed to RT-PCR failure, since as part of the RT-PCR assay [34] exogenously added β-globin mRNA was included and served as a control for the RT and PCR reaction efficiencies. In addition, cell numbers for ICM and intact blastocysts were equalized prior to RT as described in Brison and Schultz [41]. These results are in agreement with in situ hybridization studies showing that *H19* is preferentially expressed in the trophectoderm of the blastocyst [39]. Moreover, the much higher degree of sensitivity of the RT-PCR method, when compared to in situ hybridization, indicates that *H19* expression in the blastocyst is exclusively restricted to the trophectoderm. Thus, the temporal and spatial expression analyses indicate that *H19* expression initiates by the mid-blastocyst stage and is trophectoderm specific.

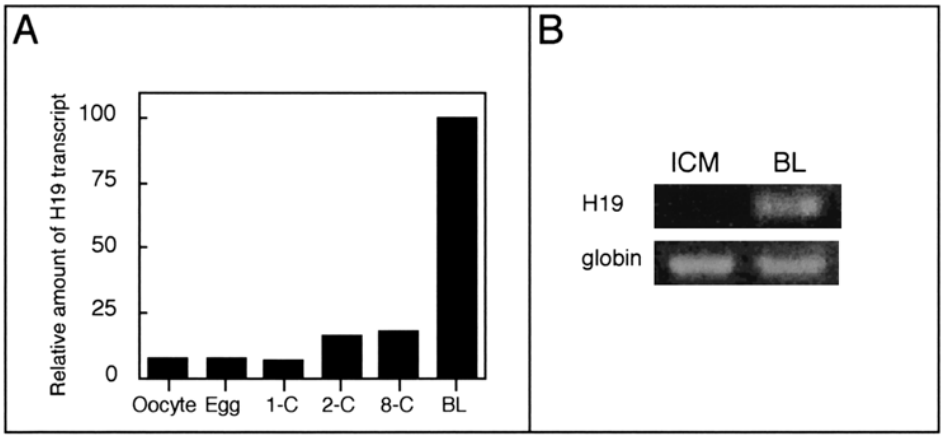

FIG. 2. Temporal and spatial pattern of *H19* expression during preimplantation development in vivo. **A**) *H19* was expressed maximally at the blastocyst stage during preimplantation development. Data are expressed relative to the blastocyst stage (BL). Signals obtained for the oocyte, egg, and 1-cell (1-C) embryo were essentially at the limits of detection. **B**) *H19* expression was trophoblast specific. ICM were obtained by immunosurgery of blastocysts that developed in vivo. RT-PCR analysis failed to detect expression of *H19* in ICM in contrast to blastocysts (BL), where an amplification product was obtained. By deduction, *H19* expression is restricted to the trophectoderm. 2-C, 2-Cell embryo; 8-C, 8-cell embryo. The oocytes, ovulated eggs, or embryos were collected directly from the female mice and immediately transferred to lysis buffer.

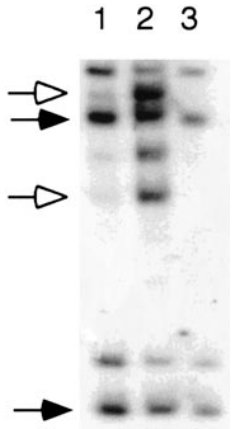

FIG. 3. Effect of culture on *H19* expression. SSCP analysis of RT-PCR products derived from B6(CAST-*H19*) × B6 F1 hybrid embryos that harbor a maternal *H19* CAST allele and paternal B6 allele. Embryos cultured in KSOM+AA (KSOM) primarily expressed the maternal *H19* allele (lane 1), while embryos cultured in Whitten's medium (WM) expressed *H19* biallelically (lane 2). Only the maternal *H19* allele was expressed in embryos that developed in vivo (lane 3). Open arrows, *H19* amplicon derived from the paternal B6 allele; closed arrows, *H19* amplicon derived from the maternal CAST allele.

Effect of Culture Conditions on H19 Expression

The experiments described above established the expression pattern of *H19* in the in vivo preimplantation embryo. To determine the lability of the gametic imprint at this locus we employed a system that has been previously demonstrated to perturb gene expression in the preimplantation mouse embryo, namely in vitro culture conditions. For example, culture of embryos in commonly used media such as Whitten's medium results in developmental rates that are retarded when compared to those of embryos that develop in vivo. These embryos show reduced levels of expression of many genes that have been analyzed [32]. Simplex optimization has led to the development of the medium KSOM containing amino acids that supports preimplantation development in vitro at rates superior to those obtained with commonly used media such as Whitten's medium [42]. Moreover, for every gene analyzed to date, the levels of transcript abundance are not statistically different from those of embryos that develop in vivo [32]. The effect of culture conditions, however, on maintenance of imprinted gene expression has not been studied. Accordingly, we examined the expression of *H19* in embryos cultured in either Whitten's medium or KSOM+AA.

In these experiments, 2-cell embryos derived from either a B6(CAST-*H19*) × B6 or a B6(CAST 7^{27-71}) × B6 cross were flushed from the oviducts and then cultured to the blastocyst stage in either Whitten's medium or KSOM+AA. In contrast to findings for in vivo-derived blastocysts, in which essentially no paternal *H19* expression was detected, culture of embryos in Whitten's medium resulted in biallelic expression of *H19* (Fig. 3). Quantification of results from a series of experiments indicated that paternal expression constituted 40% ± 6% (mean ± SEM, n = 7) of the total *H19* expression after culture in Whitten's medium. In two of these experiments, paternal expression represented only about 20% of total *H19* expression, while in the remaining five, the paternal allele contributed 48% ± 4% of total *H19* expression. *H19* expression was not detected in immunosurgically isolated ICM from blastocysts that had been cultured in Whitten's medium (data not shown), indicating that biallelic *H19* expression was likely restricted to the trophectoderm.

In contrast, after culture in KSOM+AA, only 10% ± 3% (n = 3) of *H19* expression was derived from the paternal allele. Thus, while culture in Whitten's medium resulted in biallelic expression, culture in KSOM+AA more closely approximated the in vivo situation, although a low level of paternal expression was detected.

Effect of Culture on Methylation of the H19 Gene

To ascertain whether the loss-of-imprinting that occurred after culture in Whitten's medium was correlated with loss of methylation within the region hypothesized to confer the imprinting mark [14], we assessed the methylation status of DNA at the *Hha*I site 5 (indicated by Hh_5 of the top line in Fig. 1) that resides in this 2-kb domain. We previously demonstrated that this site is differentially methylated in blastocyst stage embryos [19, 23]. After culture from the 2-cell stage to the blastocyst stage in either Whitten's medium or KSOM+AA, blastocyst DNA was digested with *Pvu*II (cuts outside the region of interest) and the methylation-sensitive restriction endonuclease *Hha*I. If the DNA is methylated, the methylation-sensitive enzyme is unable to digest the DNA, and PCR of this region with flanking primers yields a product. If the cytosine residue is unmethylated, however, the DNA is cleaved and subsequent PCR of this region does not yield an amplification product.

Results of these experiments indicated that little, if any, of the diagnostic PCR amplicon was detected when the embryos were cultured in Whitten's medium, whereas this amplicon was readily detected when the embryos were cultured in KSOM+AA (Fig. 4A). Thus, culture in Whitten's medium resulted in a significant loss of methylation at this site, presumably on the normally methylated paternal allele. To verify this conclusion, SSCP analysis of the DNA was conducted. As anticipated, after restriction with *Hha*I, the paternal allele was not detected for embryos cultured in Whitten's medium (Fig. 4B, lane 2) but was detected for embryos cultured in KSOM+AA (Fig. 4B, lane 4); little if any of the hypomethylated maternal allele was detected, as anticipated. Thus, culture of embryos in Whitten's medium resulted in both loss-of-imprinting and loss of DNA methylation in the putative imprinting control domain on the paternal allele. In contrast, both maternal (essentially) monoallelic *H19* expression and retention of methylation on the paternal allele were observed after culture in KSOM+AA.

Effect of Culture Conditions on Dnmt1 Activity and Localization

To determine whether loss-of-imprinting and changes in methylation resulted from a general effect of culture conditions on DNA methyltransferase, we assayed DNA methyltransferase activity in in vitro-cultured embryos. In vivo, the amount of DNA methyltransferase activity decreases throughout preimplantation development when expressed as the amount of activity per cell [36]; the total amount of DNA methyltransferase activity remains constant until the 8-cell stage, after which it decreases. Since gene expression can be perturbed in in vitro-cultured embryos, a disproportionate decrease in DNA methyltransferase activity in embryos cultured in Whitten's medium when compared to embryos cultured in KSOM+AA could result in the inability of Whitten's-cultured embryos to maintain levels of DNA methylation sufficient to support monoallelic *H19* expression.

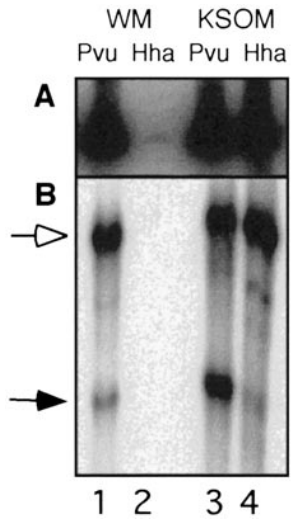

FIG. 4. Effect of culture on methylation of the *Hh5* site located in the upstream region of the *H19* gene. **A**) DNA from B6(CAST-*H19*) × B6 F1 hybrid embryos was digested with *Pvu*II (Pvu) that cuts outside of the region of interest, or with *Pvu*II and *Hha*I (Hha), a methylation-sensitive restriction enzyme. PCR amplification was then performed with primers that flank the *Hh5* site (see Fig. 1). Embryos cultured in Whitten's medium (WM) produced very little amplification product after restriction with *Hha*I (lane 2), indicating that DNA from these embryos was unmethylated. The presence of a PCR product in lane 4 indicates that DNA from KSOM+AA cultured embryos (KSOM) was methylated. **B**) SSCP analysis to determine the parental origin of methylation of samples shown in A. After restriction with *Hha*I, the paternal allele was not detected for embryos cultured in Whitten's medium (lane 2). For embryos cultured in KSOM+AA, the vast majority of methylation occurred on the paternal allele, while the maternal allele was relatively hypomethylated (lane 4). Open arrow, *H19* amplicon derived from the paternal B6 allele; closed arrow, *H19* amplicon derived from the maternal CAST allele.

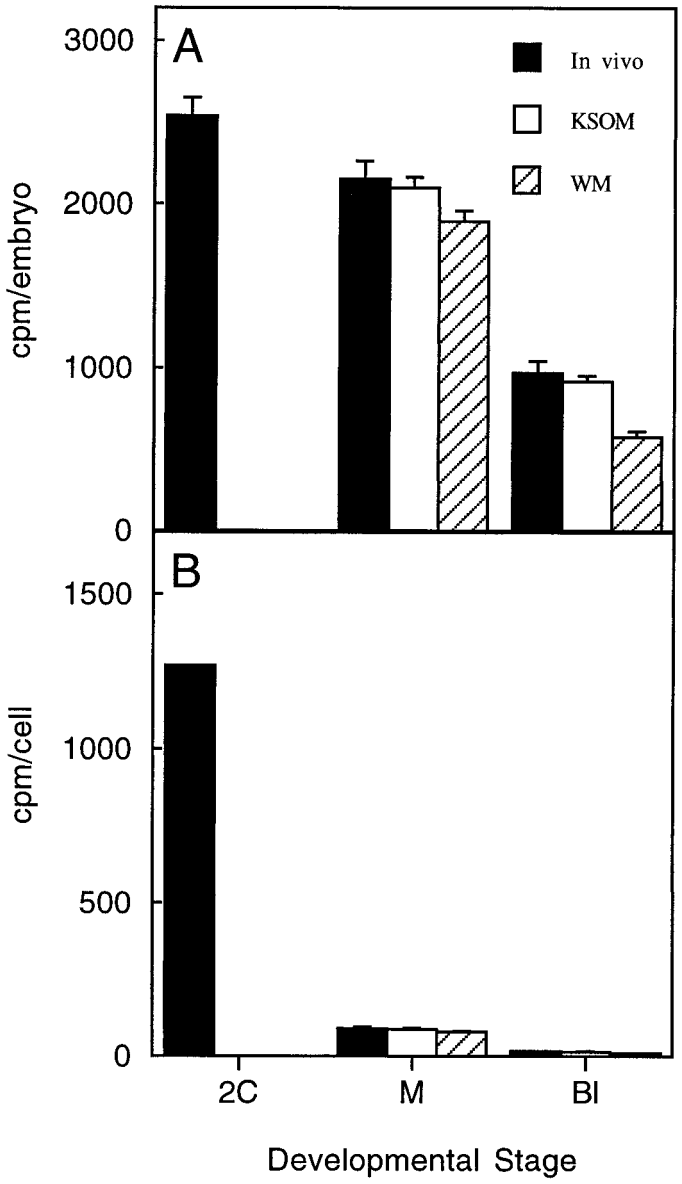

FIG. 5. Developmental changes in DNA methyltransferase activity during preimplantation development. Two-cell to blastocyst stage embryos were collected after development in vivo or in either KSOM+AA (KSOM) or Whitten's medium (WM). Cultured embryos displayed the same developmental pattern of DNA methyltransferase activity as in vivo-derived embryos. Data are expressed as either cpm per embryo (**A**) or cpm per cell (**B**). Morulae possessed 24 cells while blastocysts contained 64.5 cells. Two to three groups of 5 embryos each were assayed per experiment, three times. Data are expressed as the mean ± SEM. In several instances the error bars are too small to be observed. 2-C, 2-Cell embryo; M, morula; BL, blastocyst. Cell number was determined by DAPI staining as previously described [62].

In vivo- and in vitro-derived embryos were isolated and assayed for DNA methyltransferase activity using [^{3}H-methyl]S-adenosylmethionine as the methyl donor. When compared to embryos that develop in vivo, embryos cultured in either KSOM+AA or Whitten's medium displayed similar decreases in DNA methyltransferase activity (Fig. 5). While the difference in activity between embryos cultured in Whitten's medium and KSOM+AA was significant ($P < 0.01$, ANOVA), the magnitude of the decrease was very similar. Moreover, when the data were expressed on a per cell basis, the activity per cell was virtually identical.

The DNA methyltransferase, Dnmt1, that is responsible for the bulk of DNA methylation displays a remarkable stage-specific change in intracellular distribution during preimplantation development [43]. Between the 1-cell and 8-cell stages, the enzyme is predominantly located in the cytoplasm. During the 8-cell stage, the enzyme is preferentially located in the nucleus; but by the morula stage, the enzyme is again located predominantly in the cytoplasm. Although the molecular basis for these changes and the biological *sequelae* are not known, we found similar temporal and spatial changes in Dnmt1 localization in embryos cultured in KSOM+AA or Whitten's medium (Fig. 6). In conclusion, results of these DNA methyltransferase experiments suggest that the loss of *H19* imprinting that occurs following culture in Whitten's medium is unlikely a simple consequence of insufficient levels of DNA methyltransferase activity or inappropriate changes in the spatial localization of Dnmt1 in these cultured embryos.

Effect of Culture Conditions on Snrpn Expression

It was also possible that the suboptimal culture conditions confronted by embryos in Whitten's medium could, in principle, result from a global loss-of-imprinting. Accordingly, we examined whether loss-of-imprinting of another imprinted gene, *Snrpn*, occurred after culture in Whitten's medium. The *Snrpn* gene is expressed from the paternal genome starting at the 4-cell stage [35]. Using the same conditions we had previously employed to study in vitro culture effects on *H19* imprinting, we determined that after culture from the 2-cell stage to the blastocyst, *Snrpn*

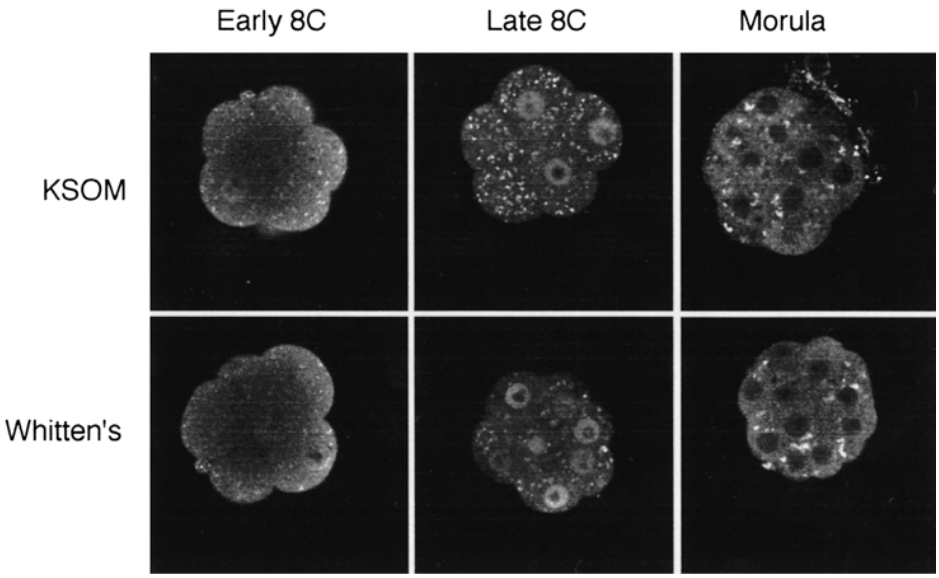

FIG. 6. Stage-specific changes in the intracellular localization of Dnmt1 protein during preimplantation development. After culture, embryos were subjected to immunocytochemistry with antibodies highly specific to the Dnmt1 protein; e.g., omission of the primary antibody results in little staining above background [43]. Whether cultured in Whitten's medium or KSOM+AA, embryos displayed the same transient nuclear localization of Dnmt1 protein during the 8-cell (8C) stage, which was similar to the pattern described for in vivo-derived embryos. Shown are a series of representative laser-scanning confocal images.

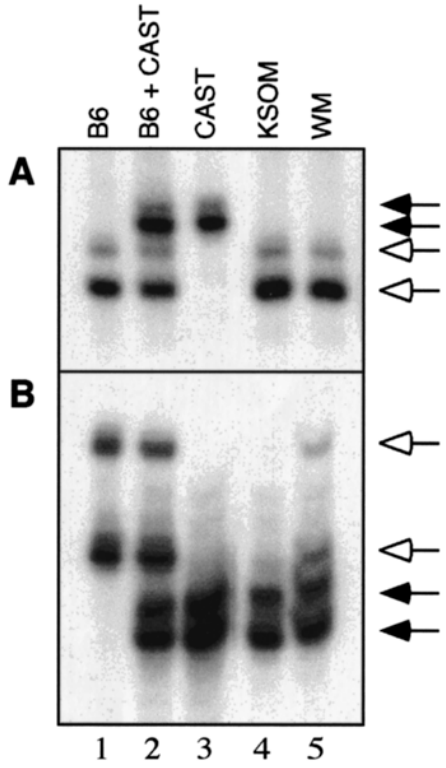

FIG. 7. Effect of culture on *Snrpn* expression. SSCP analysis to distinguish the parental alleles of RT-PCR products derived from B6(CAST 7^{27-71}) × B6 F1 hybrid embryos. Lane 1, B6 parental allele; lane 2, 1:1 mix of B6 and CAST alleles; lane 3, CAST parental allele. (**A**) Strict monoallelic *Snrpn* expression of the paternal B6 allele was observed in embryos cultured in either KSOM+AA (KSOM; lane 4) or Whitten's medium (WM; lane 5); in contrast, (**B**) significant paternal *H19* expression was observed after culture in Whitten's medium, and little if any paternal *H19* expression was observed after culture in KSOM+AA. Open arrow, amplicon derived from the paternal B6 allele; closed arrow, amplicon derived from the maternal CAST allele. Note that in these experiments the gel conditions for *H19* (plus 5% glycerol) resulted in a different electrophoretic mobility than those observed in Figure 3. Also note that the extent of paternal *H19* expression after culture in Whitten's medium was less than that shown in Figure 3.

was expressed exclusively from the paternal allele when the embryos were cultured in either Whitten's medium or KSOM+AA (Fig. 7). As anticipated, loss-of-imprinting of *H19* was observed in the embryos cultured in Whitten's medium, but not when they were cultured in KSOM+AA. Thus, the genomic imprint governing the expression of the *Snrpn* gene does not appear to show the same lability as that controlling the expression of the *H19* gene.

DISCUSSION

The results described here support previous experiments suggesting that methylation of the *H19* gene is crucial for imprinted expression [14, 19, 26]. Embryos cultured in Whitten's medium experience both a loss-of-imprinting and a reduction in the methylation of a CpG dinucleotide in the hypothesized imprinting control domain located upstream from the start of *H19* transcription. In contrast, embryos cultured in KSOM+AA express *H19* predominantly from the maternal allele and are methylated on the paternal allele, thereby approximating in vivo conditions. It is possible, however, that biallelic expression of *H19* after culture in Whitten's medium is a consequence of a reduction in the molecules that recognize the *H19* imprint mark. The loss-of-imprinting observed in Whitten's medium is unlikely attributable to a global decrease in DNA methylation, since DNA methyltransferase activity levels are similar in in vivo-derived embryos and embryos cultured in KSOM+AA or Whitten's medium. Furthermore, the analysis of another imprinted gene, *Snrpn*, shows that imprinting is maintained at this locus during culture in Whitten's medium. We therefore propose that the imprinting of the *H19* gene is hypersensitive to environmental conditions.

The lability of the *H19* imprint is not surprising given the results of previous experiments demonstrating that its imprint is more sensitive to changes in DNA methylation levels than other imprinted genes. Mice harboring mutations at the endogenous *Dnmt1* locus have been used to assess the importance of methylation in embryonic growth as well as genomic imprinting [26, 44]. Mice with the less severe $Dnmt1^{n/n}$ mutation have approximately 30% of the wild-type levels of genomic DNA methylation and die later than mice with the more severe $Dnmt1^{s/s}$ mutation. Mice homozygous for the less severe mutation show a loss-of-

imprinting for the *H19* gene but maintain appropriate imprinting of the *Igf2r* and *p57^{Kip2}* genes [26, 45]. In contrast, all three genes are affected in the more severe *Dnmt1$^{s/s}$* background [10, 26]. Like *H19*, the *Kvlqt1* gene is affected in both strains, but a more dramatic change in expression is observed in *Dnmt1$^{s/s}$* mice [10, 45].

H19 imprinting also appears more sensitive to in vitro manipulations than other imprinted genes. The best example of this is seen in the work of Sasaki and colleagues, who were the first to show that in vitro-fertilized and cultured mouse embryos experience a loss in *H19* imprinting when compared to in vivo-derived embryos [27]. In a recent study on the ability of mouse spermatid nuclei to initiate a normal developmental program when injected into mouse oocytes, Shamanski and colleagues [46] determined that five imprinted genes, including *Snrpn* and *Igf2*, are appropriately imprinted in midgestation embryonic and extraembryonic tissues. In contrast, the normally repressed paternal *H19* allele is expressed in extraembryonic but not embryonic tissues. Unfortunately, this study did not examine the allelic methylation patterns of *H19*. It is unlikely, however, that the derepression of the paternal *H19* allele results from incomplete methylation of round spermatid DNA, since we have determined the *H19* methylation patterns of round spermatid DNA to be identical to that of mature spermatozoa [47]. Rather, these results are consistent with a greater sensitivity of *H19* to in vitro manipulation. Interestingly, in this case, the extraembryonic tissues appear more sensitive to perturbations than embryonic tissues. For blastocysts that develop in Whitten's medium, the aberrant paternal *H19* expression appears restricted to the trophectoderm cells where *H19* is normally expressed, since no *H19* expression is detected in the inner mass cells.

Importantly, previous studies in mice have suggested that in vitro culture and manipulation of eggs and preimplantation embryos can lead to reduced viability and growth, as well as developmental abnormalities [48–50]. Where analyzed, these defects are linked to epigenetic changes in the embryo [49, 50]. For example, Dean and colleagues [49] found that developmentally compromised fetuses derived exclusively from the culture of embryonic stem cells exhibited perturbations in the expression and methylation patterns of four imprinted genes, including *H19*. Furthermore, in one case, the phenotypic abnormalities were transmitted to the progeny of the manipulated parental mice, suggesting that some epigenetic changes could be stably propagated through the germline [51].

Epigenetic changes can also occur as a result of genotypic background and can affect such processes as gene expression, methylation, and development. For example, Latham and Solter [52] determined that androgenones produced with fertilized C57BL/6 eggs formed blastocysts much more efficiently than those produced with DBA/2 eggs. Poor development in the latter has been linked to two independently segregating DBA/2 loci [53]. Genotype has also been shown to affect the imprinting and methylation of exogenous DNA in transgenic mice [54–56]. In one study, DBA/2 and 129 genetic backgrounds enhanced expression of a *lacZ* transgene while expression of this same transgene was suppressed and its DNA hypermethylated following the maternal inheritance of a BALB/c strain-specific modifier [54]. We too have noted an effect of genotype on *H19* imprinting in Whitten's in vitro-cultured embryos. While we have observed that embryos derived from a CAST female and B6 male displayed a loss of *H19* imprinting when cultured in Whitten's medium, embryos produced by the reciprocal mating appropriately imprinted *H19* under identical conditions (data not shown). Since the CAST strains that we used in this study have portions of a *M. musculus castaneus* chromosome 7 on a mixed C57BL/6J and *M. musculus castaneus* background, it is possible that strain-specific modifiers are affecting the maintenance of the *H19* imprint.

The results presented here may also have bearing on the strange phenomenon known as large calf syndrome, as well as on the clinical practice of assisted reproduction. Culture of bovine and ovine embryos to the blastocyst stage prior to embryo transfer results in a greater fraction of offspring with a significant increase in birth weight, as well as in higher incidences of fetal and perinatal loss ([57] and references therein). In cattle, these abnormalities are not observed if the embryos are first transferred to the oviducts of sheep and then transferred at the blastocyst stage to the reproductive tracts of recipient heifers [58]. Thus, these abnormalities are likely attributable to embryo culture. Since the culture conditions are most likely suboptimal for these species, loss-of-imprinting of specific genes may occur during the culture period and could, in principle, contribute to the observed differences in birth weight, as well as fetal and postnatal loss. Of note is that certain human syndromes suffering a loss-of-imprinting, such as Beckwith-Wiedemann syndrome, are characterized by pre- and postnatal organ overgrowth [59].

Currently, human embryos are usually cultured for short periods prior to transfer to the mother. It is most likely, however, that with advances in assisted reproductive technologies (ART) (e.g., preimplantation genetic diagnosis), embryos will be cultured for much longer periods prior to transfer to the mother. For example, the incidence of aneuploidy markedly increases in women 40+ yr of age [60]. Thus, embryos derived from women in this age group will likely be scrutinized routinely for aneuploidy to ensure that only embryos containing a normal chromosome complement are returned to the mother. Such genetic diagnosis will entail much longer periods of embryo culture prior to transfer to the mother than are currently employed. Moreover, there is an increasing trend in ART practices to culture embryos for longer periods for later-stage embryo transfer, with the assumption that embryos that have developed further are of ''higher quality'' [61]. Although it is not known whether loss-of-imprinting occurs during culture of human preimplantation embryos or whether such a loss-of-imprinting results in embryos that are developmentally compromised, our results do provide a strong cautionary note for such a practice.

In summary, our finding that culture conditions can dramatically, but selectively, affect the expression of imprinted genes provides a model system to study further the regulation of imprinted expression of *H19*. For example, this system is currently being exploited to assess the linkage between the extent of DNA hypomethylation of the differentially methylated domain and expression of the appropriate *H19* allele. In addition, the results of these experiments raise several obvious questions: is the appropriate monoallelic maternal expression of *H19* reestablished after implantation, and if so, what is the methylation status of the differentially methylated domain? Are blastocysts that display loss-of-imprinting compromised with respect to their ability to implant and develop to term? Questions such as these are also under current investigation.

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