Human Molecular Genetics, 2008, Vol. 17, No. 1 **1–14**
doi:10.1093/hmg/ddm280
Advance Access published on September 27, 2007

Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development

Rocío M. Rivera[1†], Paula Stein[1], Jamie R. Weaver[2], Jesse Mager[2‡], Richard M. Schultz[1*] and Marisa S. Bartolomei[2*]

[1]Department of Biology and [2]Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA 19104, USA

Received July 31, 2007; Revised and Accepted September 20, 2007

In vitro **culture of mouse embryos results in loss of imprinting. The aim of the present study was to examine how two of the techniques commonly used during assisted reproduction, namely embryo culture and embryo transfer, affect genomic imprinting after implantation in the mouse. F1 hybrid mouse embryos were subjected to three experimental conditions: control (unmanipulated), embryo transfer and *in-vitro*-culture followed by embryo transfer. Concepti were collected on d9.5 of development and allelic expression determination of ten imprinted genes (*H19*, *Snrpn*, *Igf2*, *Kcnq1ot1*, *Cdkn1c*, *Kcnq1*, *Mknr3*, *Ascl2*, *Zim1*, *Peg3*) was performed. Although control concepti had monoallelic imprinted gene expression in all tissues, both manipulated groups had aberrant expression of one or more imprinted genes in the yolk sac and placenta. Culture further exacerbated the effects of transfer by increasing the number of genes with aberrant allelic expression in extraembryonic, as well as embryonic tissues. Additionally, placentae of both groups of manipulated concepti exhibited reduced levels of *Igf2* mRNA and increased levels of *Ascl2* mRNA when compared with their unmanipulated counterparts. Furthermore, we show that biallelic expression of *Kcnq1ot1* coincided with loss of methylation on the maternal allele of the KvDMR1 locus, a phenotype often associated with the human syndrome Beckwith–Wiedemann. In conclusion, our results show that even the most basic manipulation used during human-assisted reproduction, namely, embryo transfer, can lead to misexpression of several imprinted genes during post-implantation development. Additionally, our results serve as a cautionary tale for gene expression studies in which embryo transfer is used.**

INTRODUCTION

Imprinted genes are a small group of mammalian genes that are subject to silencing in a parent-specific manner. To date, more than 80 imprinted genes have been identified in humans and mice [http://www.mgu.har.mrc.ac.uk/research/imprinting; (1)]. Genomic imprinting is a multi-step process that starts every reproductive cycle with the epigenetic marking of the gametes in a sex-specific manner. The stable transmission of the parent-specific mark to the offspring, and the resulting monoallelic expression of imprinted genes, which render them functionally hemizygous, are necessary for proper regulation of embryonic growth, placental function and neurobehavioral processes. The majority of imprinted genes are found in clusters throughout the genome and it is through epigenetic modifications of the cluster's regulatory region [imprinted control region (ICR)] that the correct allelic expression of the linked genes is achieved (2,3). ICRs have high densities of CG dinucleotides that are differentially methylated in a parent-specific manner, which confers the specificity of the domain by facilitating gene silencing.

*To whom correspondence should be addressed. Tel: +1 2158989063; Fax: +1 2155735434; Email: bartolom@mail.med.upenn.edu (M.S.B.); Tel: +1 2158987869; Fax: +1 2158988780; Email: rschultz@sas.upenn.edu (R.M.S.)
†Present address: Animal Science Research Center, Division of Animal Sciences, University of Missouri-Columbia, Columbia, MO, USA.
‡Present address: Department of Veterinary and Animal Sciences, University of Massachusetts-Amherst, Amherst, MA, USA.

Gene targeting studies in mice have uncovered a theme for imprinted genes that has been coined the kinship (or conflict) theory of genomic imprinting (4,5). This theory proposes that paternally expressed imprinted genes enhance fetal growth, whereas maternally expressed imprinted genes suppress fetal growth. For example, deletion of the *Igf2* gene, which is expressed from the paternal allele and encodes a fetal growth factor, results in concepti that have a marked decrease in fetal and placental weights when compared with wild-type concepti (6,7). In addition, null mice continue to grow at a reduced rate after birth (6). The opposite is true when the maternally expressed gene *Igf2r* (*Igf2* clearance receptor) is deleted. Concepti that are null for *Igf2r* are 140% heavier than their wild-type counterparts and die around birth from a somatic overgrowth phenotype (8). Although the expression status of imprinted genes (monoallelic or biallelic) might vary among tissues, the majority of the identified genes are monoallelically expressed in the placenta (9). These genes are involved in the proper regulation of placental growth and development (e.g. *Igf2, Igf2r, Ascl2, Peg3, Peg1/Mest, Phlda2/Ipl, Cdkn1, Grb10*) and its functional capacity [e.g. *Igf2, Slc22a1, Slc22a2, Slc22a3*; (9,10)].

Epigenetic alterations of imprinted genes occur in a number of clinical syndromes and are linked to tumorigenesis in humans. One of the most well-characterized loss-of-imprinting (LOI) syndromes is the Beckwith–Wiedemann syndrome (BWS) (OMIM 130650). Patients with this congenital overgrowth syndrome are characterized by large body size, large organs and increased incidence of Wilms' tumors (11,12) and demonstrate heterogeneous molecular alterations (13) of at least two imprinted domains on chromosome 11p15 (syntenic region in the mouse=distal chromosome 7). In the first domain, an ICR that is methylated on the paternal allele regulates the reciprocal imprinting of *IGF2* and the non-coding (nc) maternally-expressed RNA, *H19*. A significant number of individuals with BWS have microdeletions and gain of methylation of the ICR on the maternal allele, which is associated with increased *IGF2* expression (13,14). In the second domain, KvDMR1 encompasses the promoter for the paternally expressed *KCNQ1OT1* gene (also known as *LIT1*), which encodes an ncRNA (15). Transcription of the *KCNQ1OT1* gene silences several maternally expressed genes on the paternal chromosome, including *KCNQ1*, *CDKN1C*, *SLC22A18* and *PHLDA2* (16–18). On the maternal chromosome, however, a methylated KvDMR1 silences the *KCNQ1OT1* gene, thus allowing for the correct monoallelic expression of the maternal genes. The most common (~50%) epigenetic phenotype of BWS patients is the loss of methylation of the KvDMR1 on the maternal allele (12,13), which is often accompanied by biallelic expression of *KCNQ1OT1* (19).

Children born from assisted reproductive technologies (ART) can account for as high as 3.9% of all births in developed countries (20). In recent years, it has become apparent that children born from ART have a higher probability of epimutations that can result in LOI syndromes, such as BWS (21–25) and Angelman syndrome (AS) (OMIM 105830) (25–27). In addition, those studies showed that the majority (>90%) of BWS patients conceived through ART manifest a loss of methylation of the KvDMR1, when compared with ~50% in the naturally conceived population. Collectively, ART procedures can involve ovarian hyperstimulation as well as minimal (i.e culture) or invasive (i.e. intracytoplasmic sperm injection and preimplantation genetic diagnosis) procedures to eggs and embryos. These procedures may interfere with the acquisition and/or maintenance of methylation imprints. However, how the manipulations involved in human ART contribute to the mechanisms of LOI remains an enigma.

Previous studies from our laboratory demonstrated that culturing mouse embryos from the two-cell to the blastocyst stage resulted in misexpression of several imprinted genes during mid-gestation (28). In that study, embryos were cultured in either KSOM supplemented with amino acids (KSOM+AA) or Whitten's medium. Embryos cultured in Whitten's showed severe misexpression of imprinted genes on embryonic day 9.5. On the other hand, the expression pattern of embryos cultured in KSOM+AA was comparable with control and only showed some LOI that was confined to tissues derived from the trophectoderm (i.e. placenta). Although this and other previous studies (29,30) suggest that culture itself is detrimental, additional manipulations were employed in those reports. For example, embryo transfer is a technique inherent to any study in which the end-point is to determine the effects of culture on gene expression in the post-implantation embryo. This technique is regarded as safe and is not considered a manipulation with adverse outcomes to normal gene expression. Indeed, the current study was originally designed to examine effects of embryo culture on subsequent post-implantation development. As a control for these studies, we analyzed d9.5 concepti that had been *in vivo*-produced, collected at the blastocyst stage, and subsequently transferred to pseudopregnant females. However, during the course of determining allelic expression of imprinted genes, we observed that many of the concepti from this 'control' group had aberrant expression of imprinted genes. Accordingly, we examined the contribution of embryo transfer to the LOI previously reported by us (28) and others (30). In the present study, we set to expand our knowledge on how two manipulations associated with assisted reproduction in humans, namely embryo culture and embryo transfer, affect genomic imprinting during post-implantation development in the mouse. We were especially interested in determining whether manipulations of mouse embryos prior to implantation would result in similar epimutations and loss of imprinted gene expression as has been identified for BWS patients. Here we show that the embryo transfer procedure itself can result in misexpression of imprinted genes that persists during subsequent post-implantation development. Additionally, culturing embryos during the first few cleavage divisions further exacerbates the LOI observed after embryo transfer.

RESULTS

Allelic determination of imprinted genes

C7×B6 F1 hybrid embryos were subjected to one of three experimental conditions (Fig. 1): control (unmanipulated d9.5 concepti), transfer [collection of *in vivo*-produced blastocysts followed by immediate transfer (embryos were held in KSOM+AA prior to transfer for <1.5 h) to pseudopregnant

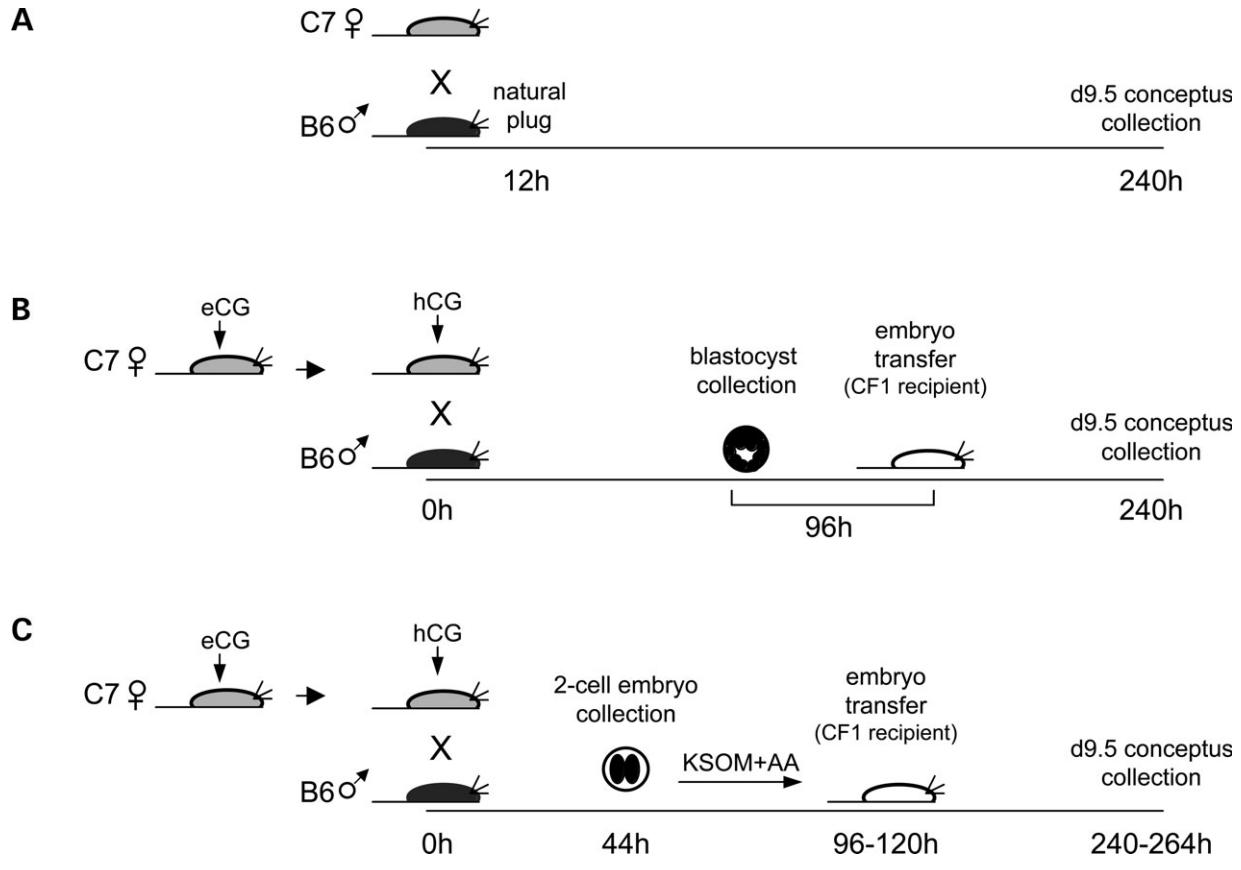

Figure 1. Experimental design. (**A**) Control group; (**B**) Transfer group; (**C**) Culture+transfer group. The timeline in hours (h) is relative to the presence of a natural plug (12 h) in the control group (A) and the hCG injection (0 h) in the manipulated groups (B and C). For embryo transfer experiments, the recipients were pseudopregnant d3.5 CF1 females. B6, C57BL/6; C7, B6(CAST7).

recipients and subsequent collection on embryonic d9.5] and culture+transfer (collection of two-cell embryos followed by culture in KSOM+AA to the blastocyst stage and embryo transfer with subsequent collection on embryonic d9.5). At embryonic d9.5, concepti were dissected in three tissues: fetus, yolk sac, and placenta, and imprinted gene expression in each was analyzed (Fig. 2).

We determined allelic expression of 10 imprinted genes located throughout chromosome 7. Five of these genes, namely *H19*, *Cdkn1c*, *Kcnq1*, *Ascl2* and *Zim1*, are expressed from the maternal allele, whereas *Snrpn*, *Kcnq1ot1*, *Peg3*, *Igf2* and *Mkrn3* are expressed from the paternal chromosome. Our results show that the extraembryonic tissues from the control (unmanipulated) d9.5 concepti had monoallelic expression of all genes analyzed with the exception of a few samples that had only one misexpressed gene (Supplementary Material, Table S1). The paternally expressed gene *Mkrn3* had a differential pattern of allelic expression between the embryonic and extraembryonic tissues; therefore this gene was independently analyzed and the results are presented below.

Both the culture and transfer procedures affected genomic imprinting in the extraembryonic tissues during post-implantation development, as evidenced by the increased percent of yolk sacs and placentae with biallelic expression of several imprinted genes when compared with controls ($P < 0.001$; Supplementary Material, Table S1). To ascertain whether or not embryo culture exacerbated the LOI effects observed as a result of embryo transfer, we first determined the percentage of extraembryonic tissues in each experimental group that misexpressed at least one (≥ 1), two (≥ 2), three (≥ 3) or four (≥ 4) of the imprinted genes analyzed [Fig. 3A (placentae) and 3B (yolk sacs)]. The data showed that for the transfer-only group 27% of yolk sacs and 61% of placentae had misexpression of at least one imprinted gene, whereas 83% of the yolk sacs and 82% of the placentae of the culture+transfer group had misexpression of at least one of the genes analyzed (≥ 1; Fig. 3, leftmost set of bars). In addition, a greater proportion of yolk sacs and placentae of the culture+transfer group misexpressed at least two imprinted genes when compared with extraembryonic tissues of the transfer-only or the control concepti (≥ 2; Fig. 3, second set of bars). Even though several of the samples from both manipulated groups had extraembryonic tissues that misexpressed three or more imprinted genes (≥ 3 and ≥ 4; Fig. 3 and Supplementary Material, Table S1), this was not statistically different from controls.

The high level of misexpression observed in the extraembryonic tissues of manipulated concepti was mostly due to disturbances in the imprinted expression of the *H19* gene. The epigenetic mechanisms required for maintaining the paternal

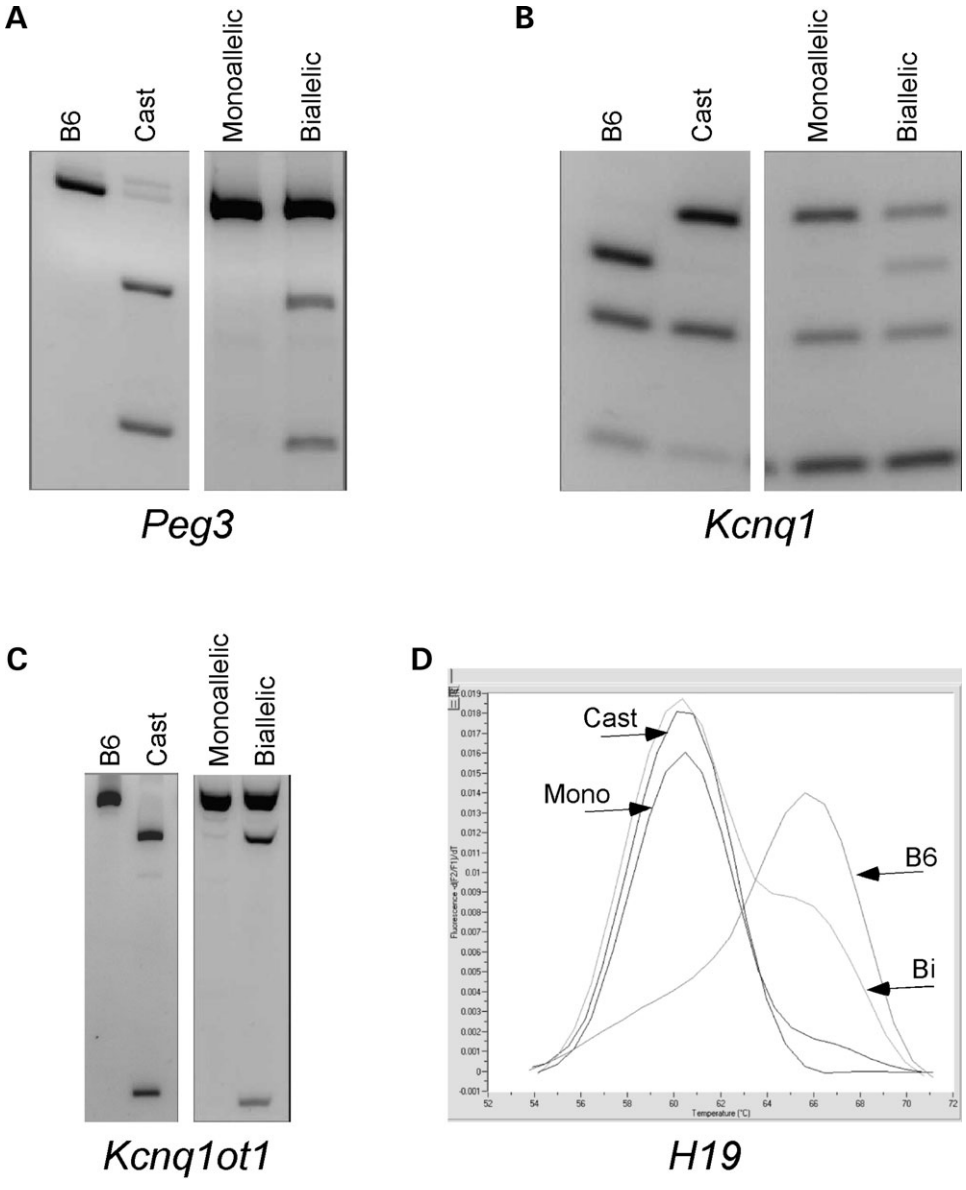

Figure 2. Allele-specific expression of C7×B6 F1 hybrid embryos. Shown are examples of allelic determination by RT–PCR followed by restriction digest and acrylamide gel electrophoresis (**A**–**C**) or real-time RT–PCR followed by a dissociation step that uses fluorescence resonance energy transfer hybridization probes to discriminate between parental alleles (**D**). For (A–C) the left portion of the panel shows the restriction digest pattern of C57BL/6 (B6) and Castaneus (Cast) control tissues (liver for *Kcnq1ot1* and *Peg3*, heart for *Kcnq1*). The digest pattern of control tissues was used as reference to determine parental expression of imprinted genes in tissues from C7×B6 F1 hybrid embryos. The right panels show examples of monoallellic and biallelic expressions of several imprinted genes in d9.5 concepti. (D) is an example of the real-time PCR allelic discrimination assay for *H19*. cDNAs from B6 and Cast neonatal liver were used as control to determine the dissociation temperature of the fluorescent probe and the PCR product. The dissociation temperature, as well as the shape of the curve (single=monoallelic and double=biallelic) were used to determine parental-specific expression. *H19* and *Kcnq1* are expressed from the maternal allele. *Peg3* and *Kcnq1ot1* are expressed from the paternal allele. Mono, monoallelic; bi, biallelic.

allele of *H19* in a repressed form seem to be particularly vulnerable to environmental influences during mouse preimplantation development, because half of the yolk sacs and placentae (60/121; Supplementary Material, Table S1) from the manipulated concepti expressed *H19* from both parental alleles. For this reason, we performed an independent analysis to determine the proportion of tissues that expressed *H19* from both parental alleles. The analysis showed that 21% of yolk sacs and 45% of placentae from the transfer-only group and 69% of yolk sacs and 68% of placentae from the culture+transfer group had expression from the normally silent paternal allele (*H19*, Fig. 3, fifth set of bars). These results showed that LOI of *H19* appeared to account for the majority of the misexpression observed in extraembryonic tissues of manipulated concepti (e.g. $\geq$1).

To ascertain whether or not the two procedures differed in their adverse effects to other imprinted genes, we determined the percent of extraembryonic tissues that misexpressed at

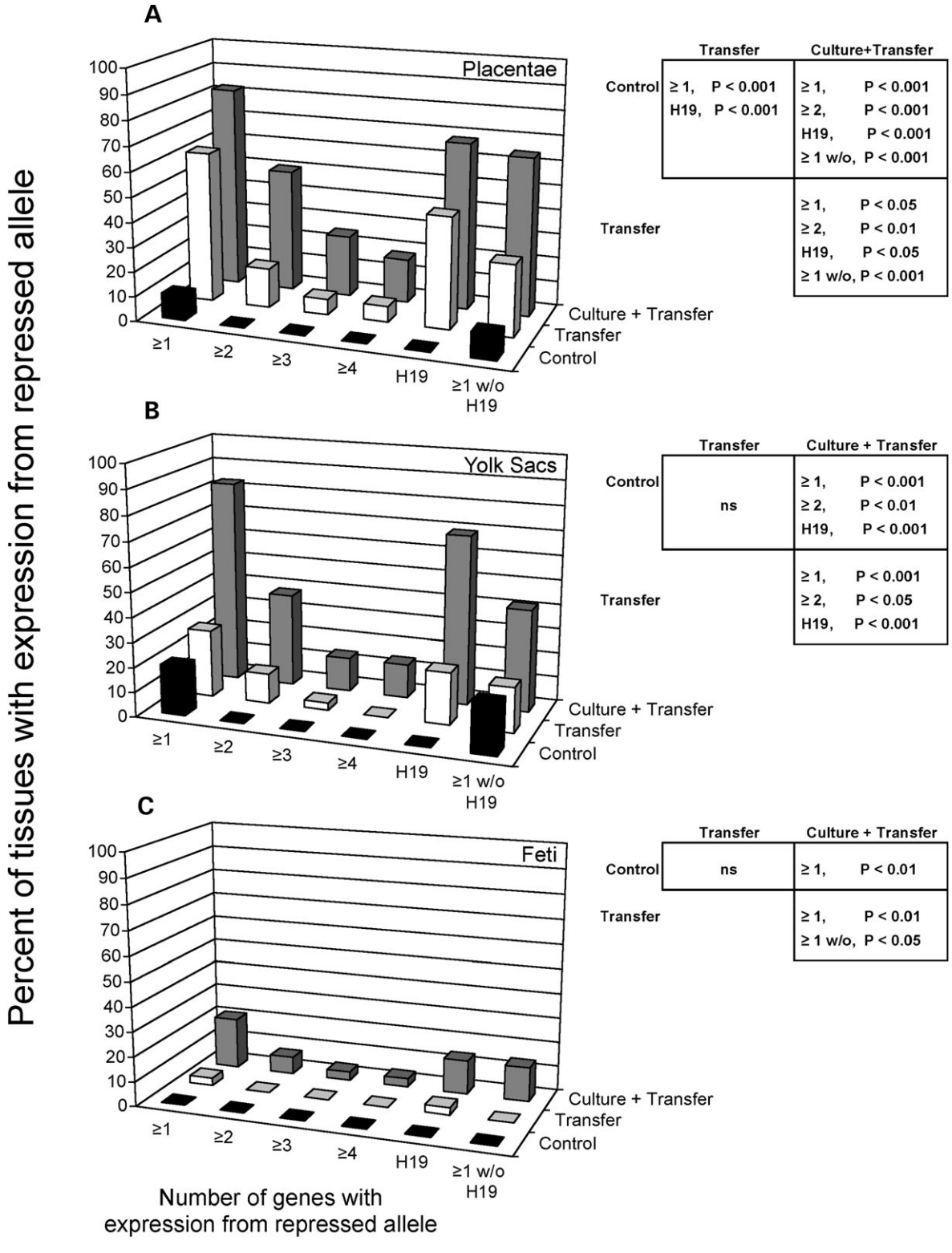

Figure 3. Preimplantation stage manipulations result in aberrant expression of imprinted genes in d9.5 concepti. Data shown in the *Y*-axis are percent of all tissues analyzed [**A**=Placentae (control $n = 19$, transfer $n = 31$, culture+transfer $n = 28$), **B**=Yolk sacs (control $n = 20$, transfer $n = 33$, culture+transfer $n = 30$) and **C** = Feti (control $n = 20$, transfer $n = 33$, culture+transfer $n = 30$); see Supplementary Material, Table S1 for raw data] with expression from the repressed allele ($\geq$10% of total) in any of eight imprinted genes (*H19*, *Kcnq1ot1*, *Cdkn1c*, *Kcnq1*, *Snrpn*, *Peg3*, *Igf2*, *Zim1)*. Allelic expression of *Mkrn3* is not included in this analysis (see Results). The *X*-axis represents individual samples with biallelic expression from at least one ($\geq$1), two ($\geq$2), three ($\geq$3) or four ($\geq$4) imprinted genes. The *H19* group only includes samples that have biallelic *H19*, and the group $\geq$1 w/o *H19* includes samples with biallelic expression from at least one imprinted gene not including *H19*. The *Z*-axis shows the type of manipulation during preimplantation stages; control, transfer and culture+transfer. The tables on the right of the figure show significant differences between treatment groups in the number of genes misexpressed. ns, not statistically significant.

least one imprinted gene as a result of culture or transfer, but this time *H19* was excluded from the analysis ($\geq$1 w/o *H19*, Fig. 3, rightmost set of bars). By analyzing the data in this manner, we unmasked further adverse effects of the embryo culture procedure over the transfer procedure ($P<0.001$ transfer placentae versus culture+transfer placentae). Notably, LOI of the paternally expressed *Peg3* gene correlates with the LOI of *H19*, because 85% (17/20) of extraembryonic tissues that had lost correct allelic expression of *Peg3* also expressed *H19* in a biallelic fashion (Supplementary Material, Table S1).

Feti of unmanipulated concepti had monoallelic expression of all genes analyzed. Imprinted gene expression analyses showed that the fetus appeared to be protected from or resilient to the effects of manipulation during preimplantation. Even though the embryo transfer procedure by itself caused LOI in placentae (and to a lesser extent in yolk sacs) in d9.5 concepti, the feti maintained their correct allelic expression (Fig. 3C and Supplementary Material, Table S1). This was not the case, however, for the culture+transfer group, in which some of the feti had aberrant expression of imprinted genes ($P<0.01$; Fig. 3C and Supplementary Material, Table S1).

Mkrn3 is an imprinted gene expressed from the paternal allele in the majority of mouse tissues. This gene is located on the central portion of mouse chromosome 7 in an imprinted region that is syntenic to the human Prader–Willi syndrome (OMIM 176270) region on chromosome 15q11–q13 (31,32). In our study, this gene showed a differential pattern of expression in the three tissues analyzed (Supplementary Material, Table S1). In the feti of all treatments, restriction digest analyses demonstrated that the *Mkrn3* gene was only expressed from the normally active paternal allele. However, a biallelic pattern of expression was observed in yolk sacs and placentae from all groups regardless of treatment. While both the yolk sac and the placenta had expression from the repressed maternal allele, the level of expression was different between the two tissues. The contribution from the maternal allele was lower in yolk sacs (mean of all experimental samples=21% maternal/total expression) than in placentae (mean of all experimental samples=33% of total expression). Upon closer examination of the data, a clear effect of preimplantation embryo manipulation was observed. Both culture and transfer procedures affected the allelic expression of placental *Mkrn3*, as the maternal transcript accounted for a greater proportion of the total expression when compared with control [mean (%) $\pm$ SEM: 15.96 ± 3.99, 40.00 ± 2.75 and 39.58 ± 3.97 for control ($n=19$), transfer ($n=30$) and culture+transfer ($n=27$) groups, respectively; $P<0.001$].

Effect of embryo manipulations on relative transcript abundance of imprinted genes

Results of experiments described above indicated that embryo manipulations perturb imprinted gene expression. To determine whether biallelic expression was associated with anticipated changes in transcript abundance, we assayed by real-time RT-PCR both maternally and paternally expressed imprinted genes.

H19 and *Igf2* are two imprinted genes that share common regulatory elements (33–36). *H19* is a maternally expressed gene, whereas the linked *Igf2* gene is expressed only from the paternal allele. Although *H19* was biallelically expressed in many of the manipulated extraembryonic samples, allelic expression of *Igf2* was normal in those tissues. Because *H19* and *Igf2* are reciprocally imprinted, it was expected that samples that expressed *H19* from both parental alleles would have lower levels of *Igf2* when compared with monoallelic samples. Quantitative RT–PCR showed that both preimplantation manipulations caused a significant reduction in the total levels of *Igf2* mRNA in placentae of d9.5 concepti ($\sim$50% of control levels; $P<0.001$; Fig. 4) and that the reduction in expression was irrespective of *H19*'s allelic expression. Placentae were then subdivided by the level of expression of paternal *H19*, and those samples in which the paternal contribution accounted for at least 20% of the total amount were compared with the average level of expression of placentae from control concepti. As expected, a more pronounced reduction of *Igf2* transcript levels was observed in placentae that misexpressed *H19* ($\sim$30% of control levels; $P<0.001$; Fig. 4). Given these results, we then determined if the total reduction of *Igf2* mRNA levels observed in placentae was also evident in feti. Even though there was a numerical reduction in *Igf2* amount in manipulated feti (10% reduction for transfer and 40% reduction for culture+transfer), this was not statistically different from control.

Because *H19* and *Igf2* share regulatory elements (i.e. enhancers), we hypothesized that the reduction in total levels of *Igf2 mRNA* seen in placentae from manipulated embryos would be accompanied by a concomitant increase in expression levels of *H19*. Therefore, real-time RT–PCR was performed to determine total *H19* gene expression in the placentae of all concepti. Total levels of *H19* mRNA were not statistically different in the placentae of manipulated concepti when compared with control placentae (data not shown). We then performed a statistical analysis in which we divided all of the manipulated placentae by allelic expression. The first group included those manipulated placentae that expressed *H19* in a monoallelic fashion (also included in this group were placentae in which expression from the paternal allele accounted for $<$20% of the total expression; $n=42$). This analysis revealed that all manipulated placentae that expressed *H19* monoallelically from the maternal allele had a 40% reduction in total levels of *H19* mRNA when compared with control placentae ($P<0.05$; data not shown). The other group included those manipulated placentae that had expression from the normally silent paternal allele at a level corresponding to $\geq$20% of the total expression ($n=16$). For this analysis, we compared the total *H19* mRNA levels of the set of manipulated placentae that expressed biallelic *H19* to the set of manipulated placentae that expressed *H19* monoallelically. The analysis showed that placentae that expressed *H19* from both parental alleles had higher levels of expression of *H19* (increased by 78%; $P<0.001$: data not shown) than the placentae that expressed *H19* in a monoallelic manner.

KvDMR1 regulates several maternally expressed (*Cdkn1c, Kcnq1, Ascl2, Slc22a18, Phlda2*) and one paternally expressed (*Kcnq1ot1*) genes in the mouse. In its unmethylated state on the paternally inherited chromosome, the KvDMR1 serves as the promoter for the *Kcnq1ot1* ncRNA. Transcription of *Kcnq1ot1* silences flanking maternally expressed genes on

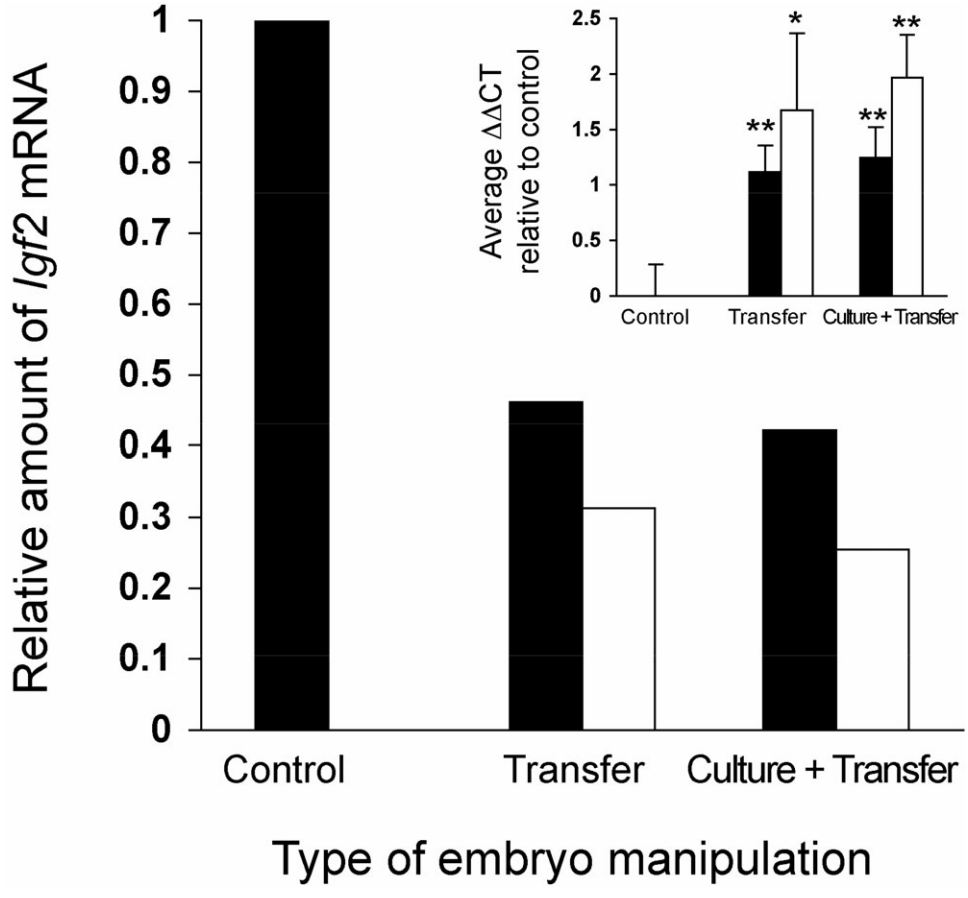

Figure 4. Manipulations during early development result in down-regulation of *Igf2* in placentae from d9.5 concepti. Real-time RT–PCR was used to determine the amount of *Igf2* mRNA relative to the housekeeping gene *Gapdh*. The fold-change was calculated using the comparative C_T method. Black bars represent all placentae (control $n=18$, transfer $n=30$, culture+transfer $n=28$) and white bars represent the subset of placentae that had $\geq$20% of total *H19* expression from the repressed allele (control $n=0$, transfer $n=5$, culture+transfer $n=11$). The inset shows the increase in cycle number ($\Delta\Delta$CT) relative to control. The data are expressed as mean $\pm$ SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ between manipulated groups and control.

the paternal allele. On the maternal allele, where KvDMR1 is normally methylated, the *Kcnq1ot1* gene is silenced, thereby allowing the expression of the flanking genes. We observed that several extraembryonic tissues from the manipulated concepti expressed *Kcnq1ot1* from the normally repressed maternal allele (Supplementary Material, Table S1). Therefore, we expected that samples that expressed *Kcnq1ot1* from the maternal allele would also have a concomitant reduction in expression of *Cdkn1c*. To test this hypothesis, real-time RT–PCR was used to determine *Cdkn1c* mRNA levels in d9.5 placentae. For this analysis, we pooled the placentae from both manipulated groups. We then divided the placentae by allelic expression of *Kcnq1ot1* [i.e. those that expressed *Kcnq1ot1* monoallelically ($n=46$) and those that expressed *Kcnq1ot1* biallelically (samples in which expression from the maternal allele accounted for more than 20% of the total expression; $n=10$)]. Placentae from manipulated concepti that expressed *Kcnq1ot1* in a monoallelic manner had a tendency towards increased total expression of *Cdkn1c* when compared with placentae from control concepti, although this increase only approached statistical significance (56%; $P=0.069$, data not shown). For the next analysis, we compared total placental *Cdkn1c* expression of placentae that expressed *Kcnq1ot1* in a biallelic fashion with those that expressed *Kcnq1ot1* monoallelically. Total *Cdkn1c* expression of placentae that expressed *Kcnq1ot1* from both parental alleles was numerically reduced (36% reduction) when compared with their monoallelic counterparts, however, this difference was not significant.

Ascl2 is a maternally expressed placental gene involved in spongiotrophoblast development. During our restriction enzyme-based allelic determination assay, it was apparent that *Ascl2* was expressed at a higher level in the placentae of the manipulated concepti than in the placentae from control concepti. Consequently, real time RT–PCR was used to determine if the total level of *Ascl2* mRNA increased in d9.5 concepti as a result of embryo culture and/or embryo transfer. Relative quantification analysis showed an increase in the total *Ascl2* mRNA levels of both manipulated groups when compared with control placentae ($P<0.001$; Fig. 5).

Methylation analysis of KvDMR1 and *H19*/*Igf2* DMD

Loss of DNA methylation of KvDMR1 has been reported for ART-associated BWS cases (21,22). The effect of embryo manipulation on KvDMR1 has not been reported in a mouse

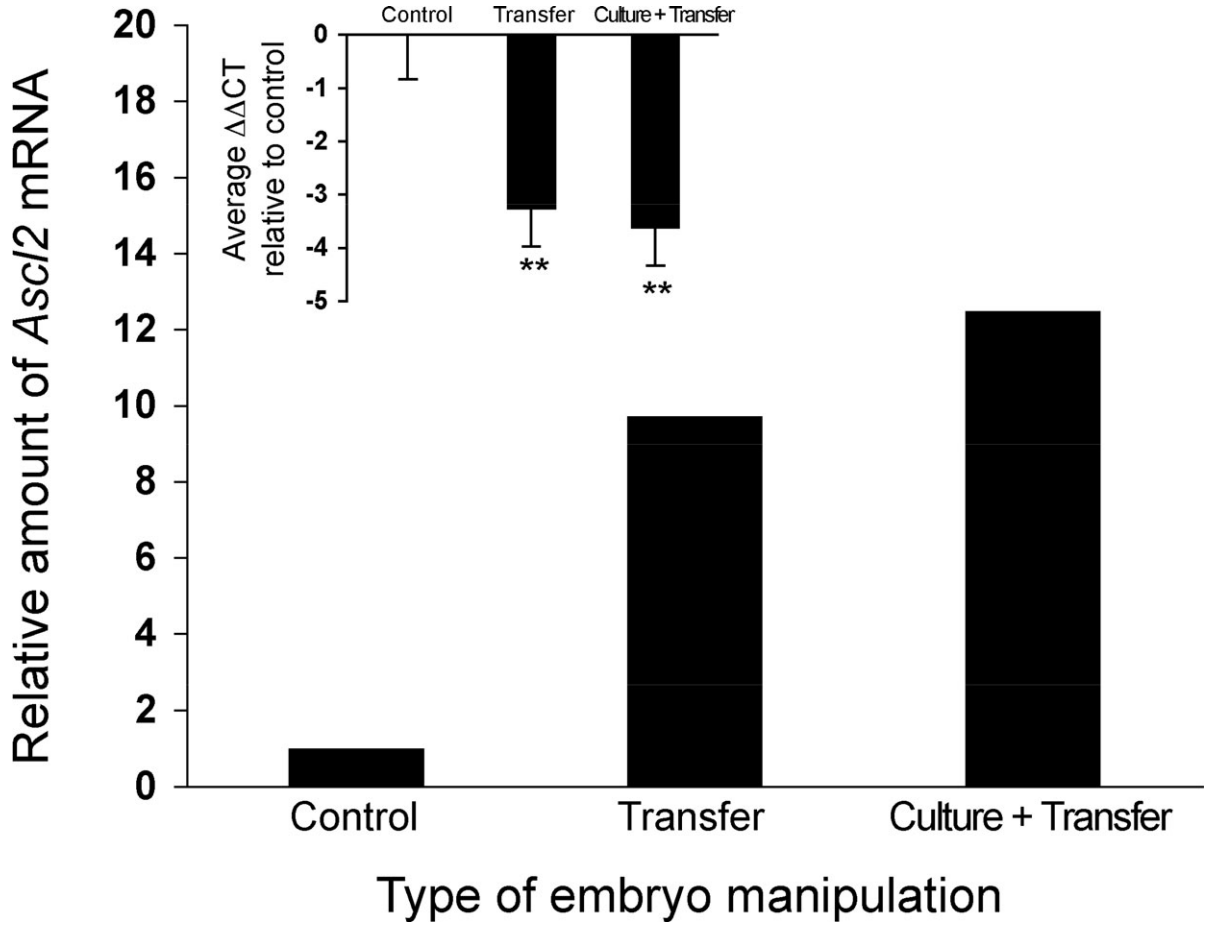

Figure 5. Manipulations during early development result in up-regulation of *Ascl2* in placentae from d9.5 concepti. Real-time RT–PCR was used to determine the amount of *Ascl2* mRNA relative to the housekeeping gene *Gapdh*. The fold-change was calculated using the comparative C_T method. The inset shows the decrease in cycle number (ΔΔCT) relative to control. The data are expressed as mean ± SEM. $^{**}P < 0.01$ between manipulated groups and control. Control $n=17$, transfer $n=31$, culture+transfer $n=28$.

model system. Accordingly, we performed sodium bisulfite analysis of KvDMR1 to determine if biallelic expression of *Kcnq1ot1* in concepti from manipulated groups was accompanied by a loss of methylation on the maternal allele. We determined methylation status of a 606 bp region contained within the differentially methylated CpG island 8b described by Yatsuki *et al*. (37). The sequenced region contains 36 CGs and two informative polymorphisms between B6 and Cast. Figure 6A shows the methylation observed at this locus in tissues from four culture+transfer concepti (KSOM+AA 3-day culture 2-1, 4-2 and 5-2, and KSOM+AA 2-day culture 6-1). Methylation of CGs on the maternal allele was reduced at this locus; this is associated with maternal expression of *Kcnq1ot1* (Fig. 6A). KvDMR1 was unmethylated on the paternal allele in all samples. To assure that the results in these experiments were comparable with our previous studies in which we demonstrated that embryo culture results in loss of DNA methylation in the *H19* and *Snrpn* ICRs (28), we assayed methylation at the *H19* ICR. As previously reported, we show here that loss of methylation of the *H19* ICR coincided with paternal *H19* expression (Fig. 6B).

DISCUSSION

There is a growing consensus that ART-conceived children are at risk for certain LOI syndromes such as BWS and AS (21,22,24,26,38). Embryo transfer is in most instances an integral component of ART and not viewed as a procedure that would have adverse effects on the developing embryo. Nevertheless, results presented here suggest that embryo transfer can result in LOI that persists until mid-gestation and that this LOI is exacerbated when coupled with embryo culture. The two procedures (culture and transfer), however, are not equivalently adverse to the conceptus. Although embryo transfer itself causes misexpression of several imprinted genes in extraembryonic tissues (i.e. yolk sac and placenta), it does not cause LOI in the fetus. In contrast, the group of concepti that were cultured prior to transfer showed a more severe LOI in the extraembryonic tissues, with some of the feti exhibiting misregulation of imprinted gene expression.

The derivatives of the ICM (embryo) and the TE (placenta) responded differently to the manipulations experienced during preimplantation development. Although the fetus maintained monoallelic expression of the imprinted genes analyzed, the

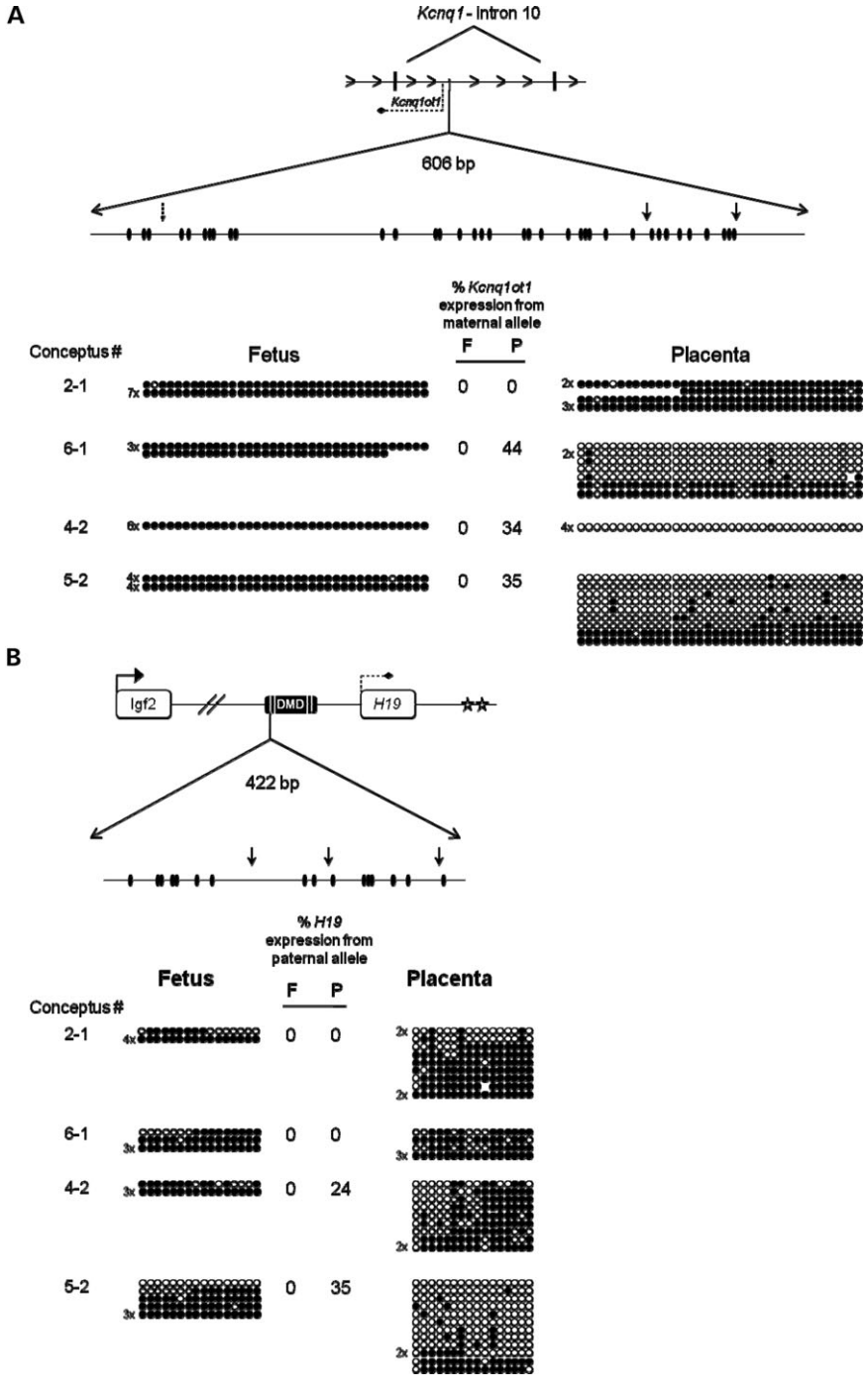

Figure 6. Samples with biallelic *Kcnq1ot1* and *H19* show loss of methylation of the ICRs on the normally methylated alleles. DNA was treated with sodium bisulfite prior to performing nested PCR. The amplicon was cloned prior to sequencing and the information used to determine methylation status of each parental allele. Polymorphisms (vertical solid arrows) between Cast and B6 were used to determine parental allele. Four concepti from the culture+transfer group are shown. Each circle of the figure represents a methylated (filled) or unmethylated (unfilled) CpG dinucleotide. Each line of circles represents an individual strand and the number to the left of the strands represents the number of strands with that phenotype. (**A**) Shown on top is a depiction of the 10th intron of the maternally expressed *Kcnq1* gene. This region encompasses a differentially methylated CpG island (KvDMR1) that resides in the promoter region of the paternally-expressed antisense gene *Kcnq1ot1*. A 606 bp region of the KvDMR1 was used to determine methylation status of 36 CpG's (ovals). There was a third polymorphism that was useful only when the Cast allele was methylated (vertical hatched arrow). The level of maternal *Kcnq1ot1* expression is shown next to the strands. Only maternal strands are shown. Direction of maternal *Kcnq1* transcription is represented by arrowheads, while maternal *Kcnq1ot1* transcription is depicted as a dashed diamond-head arrow. (**B**) Shown on top is a depiction of the *H19*/*Igf2* locus. The stars represent downstream shared enhancers. The ICR, designated as differentially methylated domain or DMD, is represented by a black box. The four white lines within the DMD represent the binding sites for the CTCF insulator protein. The region analyzed encompasses the first CTCF site. A 422 bp region of the DMD was used to determine methylation status of 16 CpG's (ovals). The level of paternal *H19* expression is shown next to the strands. Only paternal strands are shown. Direction of *Igf2* transcription is shown as a solid thick arrow, while paternal *H19* expression is depicted as a dashed arrow with a diamond head.

placentae of many manipulated concepti had substantial LOI. This misregulation of imprinted genes observed in the extra-embryonic tissues could be explained in part by the outer position of the TE in the blastocyst as previously hypothesized by Mann *et al*. (28). In our study, we also showed loss of imprinted gene expression in another extraembryonic tissue, namely the yolk sac. The yolk sac, however, is derived from the primitive endoderm, which in turn is derived from the ICM, and therefore the position hypothesis does not explain why many yolk sacs of manipulated concepti also showed an increased LOI of several genes. A possible explanation for the LOI in the yolk sac and TE of embryos subjected to ART manipulations is that these cell types are epigenetically similar. In fact, DNA sequences from derivatives of the TE and primitive endoderm are substantially undermethylated when compared with DNA sequences from ICM derivatives (39,40). Moreover, the epigenetic modifications required to maintain repression at the *Kcnq1* domain on distal chromosome 7 differ in embryonic and extraembryonic tissues (41,42). In the embryo, repression depends on DNA methylation, whereas the placenta relies on histone modifications.

Two processes occur in gametes and preimplantation embryos that are critical for appropriate imprinting, namely the establishment and subsequent propagation and maintenance of the imprinting mark. Because many of the genes analyzed in this study are differentially methylated in the gametes (43–45), it is likely that the imprinting perturbations reported result from improper maintenance of the imprinting mark during preimplantation development. A likely source of the LOI observed in this study is a stress response to embryo manipulation and culture. Of interest is that embryo culture and even the relatively simple process of embryo pipeting activate the stress kinases MAPK8/9 and SAPK/JNK (46,47). JNK kinases down-regulate Polycomb Group proteins (PcGs), which confer repressive histone modifications at imprinted loci (42,48). Hence, the post-zygotic epigenetic defect observed from the embryo transfer procedure could be the result of an increase in stress kinases and a repression of the PcGs.

In this study, the gene most often misexpressed as a result of embryo manipulation was *H19*, whose biallelic expression we previously demonstrated to be associated with loss of DNA methylation (28). The *H19* gene is linked to the oppositely imprinted gene, *Igf2*, a critical regulator of fetal growth (7,49). The present results demonstrate a reduction in *Igf2*, as would be expected when *H19* is biallelically expressed. Notably, this reduction in *Igf2* is irrespective of *H19* allelic expression, indicating that preimplantation embryo manipulation affects factors that control appropriate levels of *Igf2* expression. Recently, McMinn *et al*. (50) demonstrated that placentae from pregnancies with intrauterine growth retardation exhibit decreased levels of *Igf2*. In addition, children conceived using ART are smaller than naturally conceived children (51). Taken together these reports and our results suggest that the reduced size at birth of children conceived by ART is due, at least in part, to reduced levels of *Igf2*.

Aberrant methylation at ICRs is associated with several human syndromes. For example, patients with the imprinting overgrowth disorder BWS often show loss of methylation at the KvDMR1 (22,52,53). We find that some of the concepti that were manipulated during preimplantation development lost repression of the maternal *Kcnq1ot1* allele. This loss of parent-specific expression was likely preceded by loss of KvDMR1 methylation. The differential methylation of KvDMR1 results in appropriate monoallelic expression of flanking genes. The expression of one of these genes, *Cdkn1c*, is silenced as a result of demethylation of the maternal KvDMR1 in patients with BWS (52). We also observed a decrease, albeit small, in total expression of *Cdkn1c* in those placentae that expressed *Kcnq1ot1* biallelically.

We also noted increased expression of *Ascl2* following embryo manipulation. *Ascl2* is involved in spongiotrophoblast development (54) and inhibits giant cell formation in the mouse (55,56). The spongiotrophoblast, the murine equivalent of human cytotrophoblast, is the middle layer of the rodent placenta and consists of densely packed trophoblast cells that support the underlying villi (57). Similar to our results, Arnold *et al*. (58) showed an increased relative amount of *Ascl2* mRNA in d17 bovine embryos produced by IVF or somatic cell nuclear transfer when compared with control.

Results reported here document that even minimal ART manipulations such as embryo transfer can adversely affect expression of imprinted genes. Our findings provide an impetus to use the mouse as a model system to assess the effect of the full spectrum of ART procedures on imprinted gene expression, e.g. the effect of ovarian hyperstimulation, intracytoplasmic sperm injection and preimplantation genetic diagnosis.

MATERIALS AND METHODS

Animals

To determine parent-specific expression and methylation of imprinted genes, we used the C57BL/6(CAST7) strain [C7; (59)], which possesses chromosome 7 from the *Mus castaneus* (Cast) strain in a C57BL/6 (B6; The Jackson Laboratory, Bar Harbor, ME) background. Chromosome 7 in the mouse is useful for imprinting studies because it contains several clusters of imprinted genes (2). Hence, the F1 progeny generated from the mating of a C7 female to a B6 male can be used to determine allele-specific expression and/or methylation by the use of polymorphisms between the two strains.

Experimental groups.

- *Control*: C7 females of 6–10 weeks of age were mated to B6 males (Fig. 1). The day a vaginal plug was detected was denoted d0.5. On d9.5, females were sacrificed and concepti immediately collected. The placenta, yolk sac and fetus were mechanically separated. The placentae and yolk sacs were cut in half, whereas the fetus was chopped finely with a stainless-steel razor blade before dividing in two equal portions. All tissues were snap-frozen and stored at -80°C until further use. One half of each sample was used for expression analysis and the other half was used for DNA methylation

analysis. Twenty concepti from four control females were used for the experiments.

- *Embryo transfer*: C7 females received 0.5 I.U. of eCG (Calbiochem) followed by 0.5 I.U. of hCG (Sigma) 44 h later (Fig. 1). Females were then mated to B6 males. At ~96 h post-hCG, the uteri of the C7 females were flushed with warm bicarbonate-free minimal essential medium (Earle's salt) supplemented with 3 mg/ml of polyvinylpyrrolidone (PVP) and 25 mM Hepes (MEM+PVP), pH 7.3. C7×B6 F1 hybrid embryos were then collected and placed in drops of equilibrated potassium simplex optimized medium supplemented with amino acids [KSOM+AA; Specialty Media; (60)] and the embryos were cultured at 37°C in an atmosphere of 5% CO_2 until transfer (<1.5 h from beginning of collection to completion of transfers). Six blastocysts were transferred to each uterine horn of pseudopregnant CF1 females. The concepti were collected on d9.5 and processed as described for the control. Thirty-three concepti were collected from eight recipients.
- *Embryo culture+transfer*: C7 females received 0.5 I.U. of eCG followed by 0.5 I.U. of hCG 44 h later (Fig. 1). Females were then mated to B6 males and F1 hybrid two-cell embryos harvested from the oviducts ~44 h post-hCG injection by flushing with warm MEM+PVP. Two-cell embryos were washed in KSOM+AA prior to placing them in microdrops of KSOM+AA at a density of 1 embryo/3.5 μl of medium. Embryos were cultured for 2 or 3 days (until 96 or 120 h post-hCG, respectively) at 37°C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 at which time blastocysts were transferred to the uteri of pseudopregnant CF1 females and collected on d9.5 as described earlier. Embryos were cultured for 2 or 3 days to determine if differences existed in the way the embryos responded to length of time in culture. Because no difference was noted (see in what follows), data were combined for further analysis. There were a total of 30 concepti collected from 11 recipients.

Embryo transfer recipients. CF1 females of at least 6 weeks of age were mated to vasectomized B6D2F_1/J males 4 days prior to embryo transfer. The morning after mating, females were checked for the presence of a vaginal plug and this was denoted as d0.5 of pseudopregnancy. Embryos were transferred to the uteri of pseudopregnant females on pseudopregnant d3.5 according to standard procedures (61).

All animal experiments were approved by the Institutional Animal Care and Use Committee and were consistent with National Institutes of Health guidelines.

RNA isolation and quantification

Total RNA was extracted from d9.5 tissues by using High Pure RNA tissue kit (Roche Molecular Biomedicals) according to the manufacturer's instructions. cDNA was prepared by using Superscript II reverse transcriptase and random hexamers. To ensure that RNA samples were free of genomic contamination a minus reverse-transcriptase control was included. For PCR amplifications, the cDNA was added to a Ready-To-Go PCR Bead (General Electric) along with 0.3 μM of each primer and sterile water (refer to Supplementary Material, Table S2 for primers and conditions). For amplification of some genes, 0.38 μl of TaqStart Antibody (Clontech) was added to the PCR reaction (see Supplementary Material, Table S2). Samples were denatured at 95°C for 2 min and then amplified for 35 cycles (feti and yolk sacs) or 37 cycles (placentae) at 94°C for 10 s, 58–64°C for 15 s and 72°C for 20 s. For real-time RT–PCR determination of *H19* and *Snrpn* allelic expression procedures were as described previously [(59,62); Supplementary Material, Table S2].

Allelic determination of imprinted genes

Expression of eight imprinted genes located throughout chromosome 7 was determined by RT–PCR followed by allele-specific restriction digests (see Supplementary Material, Table S2). The digested PCR products were resolved by polyacrylamide gel electrophoresis. The contribution of each parental allele to the total expression was determined by the use of a digital gel documentation system (Bio Rad). The eight genes studied by this method were the maternal genes *Cdkn1c, Kcnq1, Ascl2* and *Zim1* and the paternal genes *Kcnq1ot1, Mkrn3, Peg3* and *Igf2*. Allelic expression of two other imprinted genes (*H19* and *Snrpn*, maternal and paternal genes, respectively) was conducted on cDNA using the LightCycler Real-Time PCR system (Roche Molecular Biochemicals) as described previously (59). Only samples that had at least 10% expression from the repressed allele were considered biallelic.

Quantification of *Igf2*, *H19*, *Cdkn1c* and *Ascl2* gene expression

TaqMan gene expression assays (Applied Biosystems; see Supplementary Material, Table S2) were used to determine if differences existed in the total amount of *H19*, *Cdkn1c* and *Ascl2* mRNA in placentae from control and manipulated groups. The samples were analyzed in at least duplicates and the threshold cycle (C_T) was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) using an ABI Prism 7000 system (Applied Biosystems). The amount of *Gapdh* RNA did not vary between treatments ($P = 0.112$). The mRNA level for each manipulated group relative to the control group was calculated using the comparative C_T method.

Comparison of the relative amount of *Igf2* mRNA from feti and placentae was accomplished by using SybrGreen (Applied Biosystems) and quantified as described earlier. Because binding of SybrGreen to double-stranded DNA is non-specific, the specificity of the PCR amplicon was determined by a dissociation curve step at the end of the amplification. Only samples that showed a single curve were used for analysis.

DNA isolation and methylation analysis of the KvDMR1 and *H19/Igf2* DMD

DNA from C7×B6 F1 hybrid concepti was extracted with phenol–chloroform. Bisulfite mutagenesis of DNA was carried-out in agarose beads as previously described (28,63). Procedures for determination of methylation at the *H19* ICR were previously described (59,64).

A 606 bp region of the sodium bisulfite-converted KvDMR1 (*Kcnq1ot1* promoter differentially methylated region; GenBank accession no. AF119385) was amplified using nested PCR (for primer information see Supplementary Material, Table S2). This region contains 36 CpG's and two useful polymorphisms between Cast and B6. The first polymorphism is at position 2810 (Cast [G] and B6 [A]) and the second, at position 2883 (Cast [G] and B6 [T]). The final concentration of the primers was 0.3 μM and the PCR conditions for both rounds of PCR were as follows: an initial denaturation step at 94°C for 2 min, followed by 35 cycles at 94°C for 15 s, 56° (first round) or 57°C (second round) for 20 s and 72°C for 30 s. Two microliters of the first PCR reaction was used for the second round of amplification. The amplified DNA region was gel isolated and cloned using the CopyControl PCR Cloning Kit (Epicentre Biotechnologies), according to the manufacturer's specifications. Individual clones were sequenced at the University of Pennsylvania DNA sequencing facility using an automated ABI 3730 with BigDye *Taq* FS Terminator V 3.1 (Applied Biosystems). The primers used for sequencing were T7 and the reverse primer for the second round of PCR (Supplementary Material, Table S2).

Statistical analyses

Data were analyzed by Analysis of Variance using the PRISM software. No statistical differences were observed between the two culture groups (2 and 3 days) for any of the parameters measured; therefore results from both treatments were pooled for further analyses. Given that percent data are not normally distributed, percent data were normalized by performing an arcsine transformation, and analysis of transformed data was used to obtain probability values. Analysis of untransformed data was used to obtain mean ± SEM and these values were used for data representation. The post-tests used to determine differences between treatment groups were Bonferroni post-test or Dunnett's multiple comparison test. The Grubbs' test was used to identify outliers that fell ≥3 standard deviations (std dev) from the mean in the analysis of relative levels of *Igf2* expression. Using this criterion, one control placenta (3.36 std dev), one fetus from the transfer group (3.75 std dev) and one fetus from the culture+transfer group (3.45 std dev) were removed from the final analyses. The mRNA levels of *Igf2*, *H19*, *Cdkn1c* and *Ascl2* for each manipulated group relative to the control group was calculated using the comparative cycle threshold (C_T) method. Briefly, the C_T for each sample was normalized to the reference gene *Gapdh*. The average C_T for a given group was calculated by averaging the C_Ts of all the independent samples. These averages were then compared with the average C_T of the control by the comparative C_T method ($\Delta\Delta C_T$). The $\Delta\Delta C_T$s were used for statistical analysis.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank Dr Jaquetta Trasler and Ms. Amanda Fortier from McGill University in Montreal, Quebec, Canada for communication of data prior to publication. We also thank Dr Joanne Thorvaldsen and Dr Francesca Duncan for critically reviewing this manuscript and Ms Toungvi Pham for technical assistance.

Conflict of Interest statement. None declared.

FUNDING

National Institutes of Health (HD42026 to M.S.B. and R.M.S.). J.M. was supported by a post-doctoral fellowship from the Damon Runyon Cancer Research Foundation and J.W. was supported by 5 T32 GMO7229-33.

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