Gonadotropin stimulation contributes to an increased incidence of epimutations in ICSI-derived mice

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We previously demonstrated that intracytoplasmic sperm injection (ICSI), a type of assisted reproductive technology (ART), can induce epimutations and/or epimutant phenotypes in somatic tissues of adult mice produced by this method. In the present study, we compared the occurrence of epimutations in mice produced by natural conception, ICSI and somatic cell nuclear transfer. Surprisingly, we observed the highest frequency of epimutations in somatic tissues from ICSI-derived mice. We also observed a delay in reprogramming of the maternal allele of the imprinted H19 gene in spermatogonia from juvenile ICSI-derived male mice.

These observations led us to hypothesize that the exposure of the maternal gametic genome to exogenous gonadotropins during the endocrine stimulation of folliculogenesis (superovulation) may contribute to the disruption of the normal epigenetic programming of imprinted loci in somatic tissues and/or epigenetic reprogramming in the germ line of ensuing offspring. To test this hypothesis, we uncoupled superovulation from ICSI by subjecting female mice to gonadotropin stimulation and then allowing them to produce offspring by natural mating. We found that mice produced in this way also exhibited epimutations and/or epimutant phenotypes in somatic tissues and delayed epigenetic reprogramming in spermatogenic cells, providing evidence that gonadotropin stimulation contributes to the induction of epimutations during ART procedures. Our results suggest that gonadotropin stimulation protocols used in conjunction with ART procedures should be optimized to minimize the occurrence of epimutations in offspring produced by these methods.

INTRODUCTION

The use of assisted reproductive technologies (ARTs) has become increasingly prevalent worldwide, and many developed countries now offer a wide range of ART methodologies to treat various types of infertility or subfertility. It is estimated that over 4 million children have been born worldwide through some form of ART, illustrating the rising utilization of these fertility treatments (1). The widespread use of ART procedures is a testament to the acceptance and perceived safety of these complex techniques by the general public. However, the long-term effects of manipulating germ cells and early embryos have not been fully evaluated, both because most individuals produced by ART methods are still under 35 years of age and because studies of potential effects in humans or animal models are still ongoing.

Recent studies have shown that the early stages of development are sensitive to environmental influences (2–4), and this has contributed to a growing concern that the use of ART may increase the risk of certain developmental abnormalities or disease phenotypes in offspring. Indeed, several reports have indicated that children conceived through ART are at an increased risk for premature birth, low birth weight or genomic imprinting disorders (5, 6). These findings warrant investigation into the different aspects of ART to identify those that may contribute to the incidence of any abnormalities, such

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that ART protocols can be optimized to enhance the health of the offspring produced.

Epigenetic mechanisms are critical for facilitating normal development and cellular differentiation in mammals. During gametogenesis, male and female germ cells undergo extensive epigenetic reprogramming to ensure that the next generation will inherit appropriate maternal- and paternal-specific epigenetic marks necessary to regulate the monoallelic expression of imprinted genes (7). Upon fertilization, maternal and paternal gametic genomes undergo further genome-wide epigenetic reprogramming to restore totipotency in the zygote (8). During this period, many of the epigenetic marks inherited from the gametes are erased and are subsequently replaced by new epigenetic profiles that activate the expression of genes needed for normal embryogenesis. However, differential DNA methylation patterns at these loci, although the precise mechanism by which these factors function remains to be elucidated (10–12). Interestingly, the epigenetic machinery responsible for preserving differential methylation at imprinted genes during preimplantation development appears to be particularly susceptible to disruption by ex vivo manipulations associated with ART methodologies (13,14).

Epigenetic defects or imprinting errors encompass abnormalities in epigenetic parameters and are commonly referred to as epimutations (15). Because many genes are controlled by epigenetic mechanisms such as DNA methylation and histone modifications, alterations of these marks can influence gene expression profiles and induce phenotypes, including diseases. Epimutations are generally classified into two types, primary or secondary (15). Primary epimutations involve an alteration in an epigenetic parameter without any change in the DNA sequence. If primary epimutations occur shortly after fertilization, they can be propagated to multiple tissues, but it has been suggested that the maintenance of these defects may be unstable resulting in somatic mosaicism (15).

Secondary epimutations arise from genetic mutations including abnormalities in genes encoding products involved in maintaining normal epigenetic profiles (e.g. a DNA methyltransferase or a histone modifying enzyme), or abnormalities in sequences of DNA that are targets for epigenetic modifications. Secondary epimutations result in abnormalities in the epigenetic parameter normally established or maintained by the affected gene product or at the target gene sequence (16). Therefore, the frequency of secondary epimutations is expected to be comparable with that of other genetic mutations, and propagation of these epimutations will be dependent on normal genetic mechanisms and Mendelian laws (16).

Because primary epimutations are not contingent upon a genetic mutation and impact the epigenome directly, it has been postulated that they may occur at a much higher frequency than secondary epimutations. In addition, their propagation may be less stable and is not likely to follow Mendelian patterns (15,17). Thus, the contribution of epimutations to phenotypic variation and the etiology of diseases may have been significantly underestimated. Epimutations that influence the phenotype of an organism are commonly manifest as an alteration in gene expression. In the case of imprinted genes, this can be detected as a skewing in the extent of allele-specific expression of the gene. We refer to this effect as an epimutant phenotype.

We recently demonstrated that intracytoplasmic sperm injection (ICSI), a type of ART, can induce primary epimutations in multiple somatic tissues of adult mice produced by this method (18). We detected epimutations in the form of altered allele-specific DNA methylation in the differentially methylated regions (DMRs) of three imprinted genes. Our results indicated that the initial occurrence and subsequent propagation of these epimutations within each affected mouse were stochastic. However, we found that these epigenetic defects were consistently corrected by germline-specific epigenetic reprogramming and were therefore not transmitted to subsequent generations (18). In several cases, we also detected associated epimutant phenotypes in the form of alterations in the allele-specific expression of the imprinted genes in which epimutations were observed, confirming that ART-induced epimutations can impact gene expression and thus potentially lead to phenotypic effects.

Observations by us (18), and others (19,20), that the use of ART appears to predispose the occurrence of epimutations raised the question of which aspect(s) of the ART process directly contribute to the genesis of these defects. In the present study, we first assessed the frequency of epimutations in somatic tissues of ICSI-derived juvenile mice to determine if these defects occur at a frequency similar to that we previously observed in adult ICSI-derived mice. We also examined reprogramming of DNA methylation at the H19 DMR in spermatogonia from juvenile male mice to determine if ICSI affects the early stages of germline-specific epigenetic reprogramming.

Results of these experiments suggest that ex vivo manipulations performed during or prior to the perinatal period were primarily responsible for the induction of epimutations and that some aspects of the ICSI procedure seem to specifically affect the maternal allele of an imprinted gene.

We next compared the occurrence of epimutations in mice produced by natural conception, ICSI and somatic cell nuclear transfer (SCNT). We analyzed SCNT-derived mice in parallel with ICSI-derived mice because while the SCNT process includes many of the same procedures used for ICSI (gonadotropin stimulation, manipulation and injection of oocytes and culture followed by transfer of embryos), it also involves the unique steps of enucleating the oocyte and transferring a donor nucleus into the enucleated oocyte to provide a diploid zygotic genome derived from a somatic cell rather than from gametes. Our initial hypothesis was that SCNT-derived mice would exhibit a significantly higher frequency of epimutations than ICSI-derived mice because of the potential for errors during the extensive epigenetic reprogramming process imposed upon the somatic cell nucleus following transfer into the enucleated oocyte.

Surprisingly, we found that mice produced by ICSI actually showed a slightly greater incidence of epimutations than did mice produced by SCNT. This provided unique insight into one potential source of epimutations in mice produced by ICSI, because a key difference between ICSI and SCNT mice is that the oocyte genome that is exposed to gonadotropin stimulation is retained during the ICSI procedure, but is removed during the SCNT process and replaced by a
somatic cell nucleus that has not been exposed to exogenous hormones. To determine if gonadotropin stimulation contributes to the occurrence of epimutations, we uncoupled this process from the ICSI procedure by subjecting female mice to gonadotropin stimulation and then allowing them to mate naturally to produce offspring. Interestingly, offspring from these females also displayed epimutations in somatic tissues, suggesting that the use of gonadotropin stimulation to enhance folliculogenesis contributes to the occurrence of epimutations.

RESULTS
Epimutations occur at a similar frequency in juvenile and adult mice produced by ICSI

We previously demonstrated that a portion of adult mice produced by ICSI exhibit epimutations in the form of imprinting errors in somatic tissues (18). In the current study, we examined the occurrence of epimutations in juvenile mice produced by ICSI by assessing allele-specific DNA methylation and allele-specific expression of three imprinted genes, H19, Snrpn and Peg3. We examined DNA methylation at the H19 DMR in the liver tissue of naturally conceived and ICSI-derived juvenile male mice using allele-specific bisulfite sequencing as described previously (21). We used B6(CAST7) recombinant inbred mice that carry a chromosome 7 from the Mus musculus castaneus strain on an otherwise C57Bl/6 background (22). This allowed us to definitively distinguish maternal and paternal alleles of each gene based on single-nucleotide polymorphisms (SNPs). The H19 gene is maternally expressed and normally acquires paternal-specific methylation at the DMR during spermatogenesis (23). Therefore, in somatic cells, the repressed paternal allele is normally methylated and the expressed maternal allele is normally unmethylated. As expected, we observed hypermethylated paternal alleles (94–97% of all CpGs methylated) and hypomethylated maternal alleles (3–7% of all CpGs methylated) in liver tissue from all naturally conceived juvenile males (Fig. 1A). Two of the three juvenile male mice produced by ICSI displayed similar allele-specific differential methylation (Fig. 1A). However, the third ICSI-derived juvenile mouse (#1) showed reduced methylation on the paternal allele (70% methylation; Fig. 1A).

To assess epimutant phenotypes, we analyzed the allele-specific expression of each imprinted gene in somatic tissues from the same naturally conceived and ICSI mice using the single nucleotide primer extension (SNuPE) assay (18,24). Because we isolated DNA and RNA simultaneously from each tissue sample, we were able to determine if there was a direct correlation between the occurrence of epimutations manifest as aberrant allele-specific DNA methylation and epimutant phenotypes manifest as the abnormal expression of the affected allele in the same tissue from the same mouse. All of the naturally conceived and two of the three ICSI-derived juvenile males displayed robust H19 gene expression exclusively from the maternal allele, while the paternal allele remained repressed (Fig. 1B). However, the same ICSI-derived juvenile male mouse (#1) that exhibited aberrant hypomethylation of the paternal H19 DMR also showed a low level of ectopic expression from that allele in liver tissue (Fig. 1B). We also analyzed juvenile female mice produced by either natural mating or ICSI. All of the naturally conceived and ICSI-derived juvenile female mice showed normal methylation patterns at the H19 DMR (Fig. 1C). Nevertheless, we detected abnormal expression of the paternal H19 allele in liver tissue from one of the ICSI-derived female mice (#1; Fig. 1D).

We next analyzed DNA methylation at the Snrpn DMR in brain tissue of the same juvenile male and female mice. The imprinted Snrpn locus is a paternally expressed gene, so the DMR associated with the repressed maternal allele is typically hypermethylated, while that associated with the expressed paternal allele is hypomethylated in somatic cells (25). All of the naturally conceived and ICSI-derived juvenile male and female mice exhibited the normal allele-specific methylation pattern in brain tissue, with hypermethylated maternal alleles (91–98% methylation) and hypomethylated paternal alleles (0–4% methylation; Fig. 1E and G). Allele-specific expression of Snrpn was also analyzed in brain tissue from the same mice, and all displayed normal, monoallelic expression of Snrpn exclusively from the paternal allele, indicating normal epigenetic programming at this locus with no epimutations detected (Fig. 1F and H).

Finally, we analyzed the imprinted Peg3 gene, which is also paternally expressed in brain tissue (26). All of the naturally conceived males and females, plus all three ICSI-derived juvenile females, and two of the three ICSI-derived juvenile males exhibited the expected allele-specific methylation profiles, with hypermethylated maternal alleles (87–97% methylation) and hypomethylated paternal alleles (0–11% methylation; Fig. 1I and K). All of the mice that showed normal allele-specific DNA methylation at the Peg3 DMR also showed normal, exclusive expression of the paternal allele (Fig. 1J and L). However, one ICSI-derived juvenile male mouse (#2) displayed reduced methylation (71% methylation) on the maternal allele (Fig. 1I) and also showed a correlated, low level of abnormal expression from the normally repressed maternal allele (Fig. 1J). Thus, in total, we detected three distinct epigenetic defects manifest as DNA methylation epimutations or allele-specific expression epimutant phenotypes indicative of epimutations, among the six juvenile ICSI mice we investigated, which matched the frequency of epimutations + epimutant phenotypes we previously detected among six adult ICSI mice (Table 1) (18).

Epimutations occur at a slightly higher frequency in mice produced by ICSI than in those produced by SCNT

We next analyzed juvenile and adult mice produced by SCNT in parallel with those produced by ICSI. Procedural aspects unique to the SCNT process, including removing the oocyte nucleus and transferring a donor somatic cell nucleus into the enucleated oocyte followed by extensive epigenetic reprogramming of the donor nucleus, have previously been implicated as a source of epimutations in cloned mice (27,28). Thus, we analyzed allele-specific DNA methylation and expression of imprinted genes in somatic tissues from SCNT-derived mice just as we did in tissues from ICSI-derived mice. All of the SCNT-derived males (two juvenile and three adult) as well as two of the three SCNT juvenile
Figure 1. Allele-specific DNA methylation and expression of H19, Snrpn and Peg3 genes in somatic tissues of naturally conceived and ICSI-derived juvenile mice. Bisulfite sequencing analysis of allele-specific DNA methylation at the H19 DMR in liver tissue from naturally conceived and ICSI-derived juvenile male (A) and female (C) mice. SNuPE analysis of allele-specific expression of the H19 gene in the liver tissue of naturally conceived and ICSI-derived juvenile male (B) and female (D) mice. Bisulfite sequencing analysis of allele-specific DNA methylation at the Snrpn DMR in brain tissue from naturally conceived and ICSI-derived juvenile male (E) and female (G) mice. SNuPE analysis of allele-specific expression of the Snrpn gene in the brain tissue of naturally conceived and ICSI-derived juvenile male (F) and female (H) mice. Bisulfite sequencing analysis of allele-specific DNA methylation at the Peg3 DMR in brain tissue from naturally conceived and ICSI-derived juvenile male (I) and female (K) mice. SNuPE analysis of allele-specific expression of the Peg3 gene in the brain tissue of naturally conceived and ICSI-derived juvenile male (J) and female (L) mice. In each case, bisulfite sequencing data are shown on a per strand basis (rows of circles) and as a bar graph. Each line of circles corresponds to a single strand of DNA, and each circle represents a single CpG dinucleotide. Black circles designate a methylated cytosine in an individual CpG dinucleotide and white circles represent unmethylated cytosines in CpG dinucleotides. The numbers to the left of each set of circles represent different individual mice, and the percent of methylated CpG sites is indicated below each set of DNA strands. For each gene expression analysis, the parental allele is indicated to the left of each gel. The numbers below each lane indicate the relative level of expression compared with the internal controls and are shown as percentage values determined by phosphorimager software. Percentages were adjusted for background and number of nucleotides added to the primer using the values obtained for B6 and Cast control samples. The amount of purified RT–PCR product loaded is indicated to the right of each gel. The 100-ng panel could not be quantified because the internal controls were saturated. Black boxes designate abnormal methylation patterns. Black arrows indicate abnormal allelic expression patterns that correlate with aberrant DNA methylation patterns. White arrows indicate abnormal allelic expression patterns that do not correlate with a detected aberrant methylation pattern. Expression controls: B6, 100% C57BL/6 RNA; Cast, 100% B6(CAST7) RNA; B6:Cast, 50% B6 RNA and 50% Cast RNA.
females and all four SCNT adult females exhibited the expected differential methylation at the *H19* DMR (Fig. 2A and C), as well as maternal allele-specific expression of the *H19* gene (Fig. 2B and D). However, one juvenile SCNT female (#1) showed a reduction in methylation on the paternal allele (74% methylation, Fig. 2A), and this same mouse also displayed aberrant expression from the normally repressed paternal allele (Fig. 2B). Allele-specific DNA methylation and expression profiles were also analyzed for the *Snrpn* gene in brain tissue from the same SCNT-derived mice and were found to be normal in all SCNT-derived juvenile and adult, male and female mice (Fig. 2E–H).

Finally, we analyzed the *Peg3* gene in brain tissue from the same SCNT-derived mice. In male SCNT mice, we observed normal differential methylation and monoallelic expression of the paternal allele of the *Peg3* gene in all three adults and one of the two juveniles (Fig. 2I–L). However, the remaining SCNT-derived juvenile male (#1) showed a substantial reduction in methylation of the paternal *Peg3* DMR (51% methylation, Fig. 2I) along with abnormal expression from this normally repressed allele (Fig. 2J). We also analyzed SCNT-derived female mice and found that all of the juvenile and adult female SCNT mice displayed normal allele-specific DNA methylation profiles at the *Peg3* DMR (Fig. 2I and K). However, two of the four adult SCNT-derived females (#2 and #3) showed the abnormal expression of the maternal allele (Fig. 2L).

Overall, in this study and our previous study (18), we assessed allele-specific DNA methylation and expression at three imprinted genes, *H19*, *Snrpn* and *Peg3*, in somatic tissues of 36 juvenile and adult mice that were produced by natural conception (12 mice), ICSI (12 mice) or SCNT (12 mice). As expected, we observed no epimutations in the naturally conceived mice, but we did detect DNA methylation epimutations and/or allele-specific expression epimutant phenotypes indicative of epimutations in both the ICSI- and SCNT-derived mice.

Our observation that epimutations occurred slightly more frequently in the ICSI-derived mice than in the SCNT-derived mice (Table 1) suggested that aspects unique to the SCNT process did not contribute significantly to the induction of epimutations at imprinted loci, whereas aspects common to both approaches and/or enhanced in the ICSI process did. This led us to consider key differences between the ICSI and SCNT techniques (Fig. 3). In addition to the unique requirement for reprogramming the donor nuclear genome in the SCNT mice, another significant difference between the ICSI and SCNT procedures was that the oocyte genome that was exposed to gonadotropin stimulation to induce superovulation was retained during ICSI and propagated to all cells in the ensuing embryo, whereas the genome present in stimulated oocytes used for the SCNT process was removed and replaced with a diploid somatic cell nucleus that had not been subjected to gonadotropin stimulation (Fig. 3). Therefore, we wondered if the exposure of the oocyte genome to exogenous gonadotropins might have contributed to the occurrence of epimutations in the ICSI mice.

**Gonadotropin stimulation of females contributes to epimutations in offspring**

To test the hypothesis that gonadotropin stimulation contributes to the occurrence of epimutations in ICSI-derived mice, we uncoupled gonadotropin stimulation from the ICSI process itself. Thus, a set of B6(CAST7) female mice was subjected to gonadotropin stimulation followed by natural mating to determine if gonadotropin stimulation in the absence of ICSI would induce epimutations in the ensuing offspring. This was necessarily followed by an embryo transfer procedure to ensure normal numbers of developing fetuses in each surrogate dam (limited to 10 fetuses/dam in this case). We examined four male and four female juvenile offspring, termed 'superovulation-derived mice', for DNA methylation epimutations or allele-specific expression epimutant phenotypes of the *H19*, *Snrpn* or *Peg3* genes in somatic tissues. We detected an epimutation in the form of reduced methylation at the paternal allele of the *H19* gene in one of the eight superovulation-derived mice (75% methylation in female #2, Fig. 4A), accompanied by significant abnormal expression of this same, normally repressed allele (Fig. 4B). In addition, two other superovulation-derived mice that did not show abnormal allele-specific DNA methylation patterns nonetheless showed epimutant phenotypes in the form of the aberrant, low-level expression of the paternal allele of the *H19* gene (Fig. 4B). All eight superovulation-derived mice exhibited normal, allele-specific DNA methylation of the *Snrpn* gene in brain tissue (Fig. 4C); however, superovulation-derived male #1 showed abnormal expression from the normally repressed maternal allele of *Snrpn* (Fig. 4D), suggesting that an epimutation had occurred at this locus. Finally,

### Table 1. Occurrence of epimutations or epimutant phenotypes in somatic tissues of naturally conceived, ICSI-derived, SCNT-derived and superovulation-derived mice

<table>
<thead>
<tr>
<th>Mice</th>
<th><em>H19</em>-juvenile</th>
<th><em>H19</em>-adult</th>
<th><em>Snrpn</em>-juvenile</th>
<th><em>Snrpn</em>-adult</th>
<th><em>Peg3</em>-juvenile</th>
<th><em>Peg3</em>-adult</th>
<th>Total</th>
<th>Freq. (%)</th>
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<tbody>
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<td>M</td>
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<td>ME</td>
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<tr>
<td>Natural (n = 12)</td>
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<tr>
<td>ICSI (n = 12)</td>
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<tr>
<td>SCNT (n = 12)</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>SuperOv (n = 8)</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
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Epimutations, abnormalities in allele-specific DNA methylation patterns; epimutant phenotypes, abnormalities in allele-specific expression; Freq., frequency of mice bearing mutations; N, not determined; SuperOv, superovulation-derived mice; M, abnormal DNA methylation without abnormal expression; E, abnormal expression without abnormal DNA methylation; ME, abnormal DNA methylation and abnormal expression.

aData from de Waal et al. (18).
Figure 2. Allele-specific DNA methylation and expression of H19, Snrpn and Peg3 genes in somatic tissues of SCNT-derived juvenile and adult mice. Bisulfite sequencing analysis of allele-specific DNA methylation at the H19 DMR in liver tissue from SCNT-derived juvenile (A) and adult (C) male and female mice. SNuPE analysis of allele-specific expression of the H19 gene in liver tissue of SCNT-derived juvenile mice (B) and in brain tissue of SCNT-derived adult mice (D). Bisulfite sequencing analysis of allele-specific DNA methylation at the Snrpn DMR in brain tissue from SCNT-derived juvenile (E) and adult (G) mice. SNuPE analysis of allele-specific expression of the Snrpn gene in brain tissue of SCNT-derived juvenile (F) and adult (H) mice. Bisulfite sequencing analysis of allele-specific DNA methylation at the Peg3 DMR in brain tissue from SCNT-derived juvenile (I) and adult (K) mice. SNuPE analysis of allele-specific expression of the Peg3 gene in brain tissue of SCNT-derived juvenile (J) and adult (L) mice. Other details are as described in Figure 1.
imprinted genes and biallelic hypomethylation of DMRs associated with maternally imprinted genes (7). In the present study, we first sought to determine when during spermatogenesis this correction is achieved. We analyzed the H19 DMR in purified populations of type A spermatogonia recovered from the testes of the same naturally conceived and ICSI-derived juvenile male mice from which we analyzed somatic cells as described above. We found that the same ICSI-derived male pup (#1) that displayed reduced methylation on the paternal H19 DMR in somatic liver cells (70% methylation, Fig. 1A) also exhibited reduced methylation on the paternal H19 allele in spermatogonia (77% methylation, Fig. 5). This suggests that the germline correction of this epimutation was ongoing but not yet completed by the type A spermatogonium stage.

Previous studies have shown that, following the germline-specific erasure of inherited DNA methylation patterns at the H19 DMR in naturally conceived mice, the paternal allele of the H19 gene becomes fully reprogrammed (methylated) earlier during spermatogenesis than the maternal allele (29,30). Our data confirm this observation in that type A spermatogonia recovered from naturally conceived juvenile males displayed nearly complete remethylation at the paternal DMR (86–97% methylation), while the maternal DMR displayed only partial remethylation (46–49% methylation) (Fig. 5). However, our analysis of DNA methylation profiles at the H19 DMR in spermatogonia from ICSI-derived male pups at the same age revealed an even greater asymmetry in reprogramming of the paternal and maternal alleles, respectively. Thus, while the DMRs on the paternal H19 allele in two of the three ICSI-derived male pups (#2 and #3) were reprogrammed to a similar extent as those in the naturally conceived pups and the H19 DMR in the third ICSI-derived male pup (#1) showed a nearly similar extent of reprogramming, the DMRs associated with the maternal H19 DMR in each ICSI pup showed consistently less methylation (13–19% methylation) than was found associated with the maternal allele of H19 in spermatogonia from the naturally conceived male pups (46–49% methylation; Fig. 5). This suggests that although germline-specific reprogramming of the biallelic hypermethylation of the H19 gene was found to be complete in advanced spermatogenic cells from adult ICSI mice as reported in our previous study (18), reprogramming of the maternal allele appears to occur in a developmentally delayed manner in juvenile mice produced by ICSI. Finally, we also examined reprogramming of the H19 DMR in type A spermatogonia recovered from juvenile superovulation-derived male mice and again observed the normal, complete remethylation of the paternal DMR, but the delayed remethylation of the maternal DMR (8–25% methylation) similar to that seen in the juvenile ICSI-derived males (Fig. 5). This suggests that gonadotropin stimulation of females contributes to a consistent delay in germline reprogramming of the maternal H19 DMR in offspring produced by ICSI.

**DISCUSSION**

The possibility that ex vivo manipulations associated with ART may induce or predispose epimutations during early stages of spermatogenesis in adult males (18). This involves biallelic hypermethylation of DMRs associated with paternally
development is a persistent concern in the field (5,6). Given the intrinsic heritability of epigenetic modifications, any epimutation generated during embryogenesis has the potential to be propagated to a large proportion of somatic cells throughout the lifetime of the organism and thus could cause or predispose developmental defects or disease phenotypes. However, ICSI and related ART procedures involve a wide array of manipulations including gonadotropin stimulation to induce superovulation, injection of a sperm into an oocyte, in vitro culture of the resulting preimplantation embryo and transfer of the preimplantation embryo into a recipient uterus. The different techniques used in ICSI coincide chronologically with different stages of the epigenetic reprogramming process that occurs during gametogenesis and preimplantation development. Therefore, deciphering which aspect(s) of the ICSI procedure is involved in the genesis of epimutations may provide useful mechanistic insights into the normal epigenetic reprogramming process, which could, in turn, facilitate improvements in ART procedures to minimize the occurrence of epimutations in offspring generated by these fertility treatments.

As expected, all of the naturally conceived juvenile control mice displayed normal allele-specific DNA methylation and expression profiles, as did all of the adult mice produced by natural reproduction as reported in our earlier study (18). However, three of the six ICSI-derived juvenile mice showed epimutations or epimutant phenotypes at different imprinted loci in different somatic tissues. Thus, the frequency of epimutations and/or epimutant phenotypes observed in ICSI-derived juvenile mice (50%) was identical to that we previously observed in ICSI-derived adult mice (18) (Table 1). This suggests that ICSI-derived mice accumulate epimutations during early development and that these defects can be maintained indefinitely in somatic tissues.

In at least half of the instances, we observed both epimutations and epimutant phenotypes associated with the same allele in the same tissue of the same mouse; however, in other cases, we observed either an epimutation with no associated epimutant phenotype or vice versa (Table 1). Importantly, our analysis of allele-specific DNA methylation was limited to a portion of the DMR associated with each imprinted gene, such that abnormal hypomethylation or persistent hypermethylation in other parts of the DMR or in other associated regulatory sequences of the gene may have been responsible for cases in which we observed apparent discordance between allele-specific methylation and expression patterns. Alternatively, other types of epimutations (e.g. abnormal histone modifications) could have been responsible for epimutant phenotypes in the absence of epimutations detected at the level of DNA methylation.

Our detection of epimutations in ICSI- and SCNT-derived mice, but not in any of the naturally conceived control mice, suggests that one or more aspects associated with the ICSI and/or SCNT procedures either directly causes or indirectly...
predisposes the occurrence of epimutations. Extensive genome-wide epigenetic reprogramming normally occurs immediately after fertilization in naturally conceived embryos, so we wondered if the ICSI or SCNT procedures might disrupt this process. The ICSI and SCNT procedures differ in this regard, because embryos produced by ICSI are derived from gametes bearing genomes that have undergone normal germline-specific epigenetic programming, whereas embryos produced by SCNT utilize genetic information from a donor somatic cell that has not undergone this programming prior to transfer into the enucleated oocyte. The fact that we observed a slightly lower incidence of epimutations in mice produced by SCNT compared with those produced by ICSI suggests that the disruption of genome-wide epigenetic reprogramming in the zygote was not a primary cause of the epimutations we observed in the ICSI mice.

Embryo culture and embryo transfer were two manipulations common to the procedures we used to generate SCNT and ICSI mice, and both have previously been shown to be a source of abnormal epigenetic effects in mice (3,19,31,32). A key difference between the ICSI and SCNT procedures is that during SCNT, the oocyte genome that was previously exposed to gonadotropin stimulation to induce superovulation was removed and replaced with a somatic cell nucleus that had not been exposed to gonadotropins, whereas during the ICSI process, the oocyte genome that was exposed to gonadotropin stimulation was retained and propagated to all cells in the ensuing embryo. Thus, although gonadotropin stimulation was used to induce the superovulation of oocytes that were subsequently used to produce both the ICSI- and the SCNT-derived offspring, any direct effects of this treatment on epigenetic programming were likely to be retained and potentially more impactful in the ICSI mice than in the SCNT mice.

The small sample size in each category of mice we investigated precluded us from determining if the slightly greater incidence of epimutations observed in ICSI mice compared with that in SCNT mice was statistically significant. Therefore, to further assess the potential contribution of gonadotropin stimulation to the occurrence of epimutations, we subjected female mice to ovarian stimulation followed by natural mating and analyzed epigenetic profiles in the ensuing juvenile offspring. We found that the use of gonadotropin stimulation did indeed lead to the occurrence of epimutations in
somatic tissues in six of the eight superovulation-derived mice. These results support the suggestion that gonadotropin stimulation without subsequent sperm injection can perturb allele-specific methylation and/or expression profiles at multiple imprinted loci in a manner similar to that seen in mice produced by ICSI.

The mechanisms by which epimutations are induced in ART-derived offspring remain largely unknown. Hormonal stimulation may disrupt imprint acquisition during oogenesis by forcing oocytes to develop more rapidly than normal or by ‘rescuing’ lower quality oocytes that might otherwise have never matured (33). Alternatively, exposure to exogenous gonadotropins can promote molecular changes in the oocyte that alter the maintenance of genomic imprints during subsequent embryogenesis (13,14). Our results support the latter hypothesis, because the epimutations we observed were consistently manifest as a loss of methylation and/or a gain of expression of the normally hypermethylated, repressed allele, regardless of whether that was the paternal or maternal allele of the specific imprinted gene investigated. This concept is consistent with a recent report, demonstrating that individual oocytes exposed to gonadotropin stimulation initially exhibited normal methylation profiles at imprinted loci, but nevertheless gave rise to embryos in which epimutations were detected (14).

Our studies provide the first evidence of an additional novel effect of exposure to gonadotropin—delayed reprogramming of the maternal allele of a paternally imprinted gene (H19) during the development of the male germ line. Thus, reprogramming of the maternal DMR of the H19 gene in spermatogonia was delayed in 100% (seven of seven) of the juvenile male mice we investigated that were derived from oocytes exposed to exogenous gonadotropins, indicating that this is a consistent effect of this exposure. In the case of the H19 gene, this represented delayed reprogramming of the maternal DMR while reprogramming of the paternal DMR appeared to proceed normally. Thus, this appears to represent a delay in the acquisition of normal imprinting that is manifest uniquely upon the maternal allele—the same allele that was directly exposed to gonadotropin stimulation during the superovulation process. Importantly, as demonstrated in our previous study (18), complete reprogramming of both alleles was, nevertheless, achieved by the adult stages in spermatogenic cells of ICSI-derived mice such that epimutations induced by the ICSI procedure were not transmitted to the subsequent generation.

Many developed countries now offer a wide range of ART methodologies for the treatment of various types of subfertility or infertility, and the stimulation of folliculogenesis by exogenous gonadotropins is an integral component of essentially every one of these procedures. Collectively, the data presented here suggest that gonadotropin stimulation used to promote folliculogenesis and oocyte maturation can impact normal epigenetic programming by inducing the formation of epimutations and/or epimutant phenotypes in offspring produced from stimulated oocytes. Thus, it appears that multiple aspects of the ART process, including the gonadotropin stimulation of folliculogenesis, embryo culture and/or embryo transfer, have the potential to induce epimutations in offspring produced by these methods (19,32,33). Taken together, the results presented in this study and several other studies (3,13,19,33) compel further investigation into the occurrence, effects and causative mechanisms of epimutations in offspring produced with the assistance of gonadotropin stimulation of folliculogenesis and other aspects of the ART process.

MATERIALS AND METHODS

Animals

B6(CAST7) mice (22) were used to assess allele-specific DNA methylation and the expression of imprinted genes based on distinguishing SNPs. These mice have a chromosome 7 from the M. musculus castaneus (Cast) strain on a C57BL/6 (B6) background. B6(CAST7) females were mated with DBA males and the resulting F1 progeny carried strain-specific SNPs that were used to identify maternal and paternal alleles of imprinted genes on chromosome 7 (21,31,34). The mice examined in this study were either juveniles (6–8 days old) or adults (>60 days old). Recipient oocytes for SCNT were collected from B6D2F1 (C57BL/6 × DBA/2) females. Different sources of oocytes were used with different methods of ART to ultimately produce offspring with similar genotypes (C57Bl6 × DBA2 F1 hybrids) in all cases. For embryo transfer, surrogate mothers and vasectomized males were CD-1 outbred mice. Protocols for the handling and treatment of all animals were reviewed and approved by the Institutional Animal Care and Use Committees at the University of Hawaii or the University of Texas at San Antonio as appropriate.

Gonadotropin stimulation of folliculogenesis and collection of oocytes

For ICSI- and SCNT-derived mice, oocytes were obtained from 10- to 12-week-old B6(CAST7) or B6D2F1 females, respectively, after superovulation by consecutive injections of equine chorionic gonadotropin (5 IU) and human chorionic gonadotropin (5 IU) administered 48 h apart. At 13–15 h after injection with human chorionic gonadotropin, cumulus–oocyte complexes were collected and treated with 0.1% hyaluronidase to remove the cumulus cells from mature oocytes. For superovulation-derived mice, B6(CAST7) females were treated with the same hormones as above and mated with DBA males. Two days after mating, in vivo fertilized 2-cell embryos were collected and cultured 2 more days to obtain morula/blastocyst stage embryos for transfer.

Intracytoplasmic sperm injection

ICSI was performed as described previously (35) with minor variations (36). In brief, mature oocytes were collected from gonadotropin-stimulated females as described above, and spermatozoa were recovered from the cauda epididymis of DBA males and suspended in HEPES-CZB medium for 20 min. A drop of sperm suspension was then mixed with 12% (w/v) polyvinylpyrrolidone (PVP) in HEPES-CZB and the head of a single sperm was detached from the tail using a Piezo-driven pipette (PrimTech, Japan), and then injected into each oocyte. Sperm-injected oocytes were cultured in
CZB medium for 24 h to allow the first mitotic division to occur. The 2-cell stage embryos were then cultured 2 more days to obtain morula/blastocyst stage embryos that were transferred into surrogate dams.

Somatic cell nuclear transfer

Juvenile and adult SCNT-derived mice were generated using fetal brain cell donor nuclei as described previously (37) with minor modifications (38). Fetal brain donor cells were collected from (B6(CAST7) × DBA) F1 male or female fetuses at 15.5 day post-coitum and mixed with 12% PVP in HEPES-CZB. The recipient B6D2F1 oocytes in HEPES-CZB containing 5 μg/ml of cytochalasin B were enucleated with a glass pipette. After enucleation, a fetal brain cell nucleus was injected into the cytoplasm of an oocyte and the reconstructed oocytes were held in CZB medium for 2 h before activation. For activation, reconstructed oocytes were incubated with 10 mM SrCl2 and cytochalasin B in Ca2+-free CZB medium for 6 h. During the 6-h activation period and the 4-h culture in CZB medium that followed (total of 10 h), 50 mM trichostatin A (TSA) was added to both media as described previously (38). The reconstructed oocytes were then transferred to new CZB medium and cultured without TSA for an additional 14–18 h to obtain cloned embryos at the 2-cell stage and these embryos were then allowed to develop to the morula/blastocyst stage prior to being transferred into surrogate dams.

Embryo transfer

All embryo transfers were performed with embryos at morula/blastocyst stages. Embryos derived from natural mating, ICSI and SCNT were transferred into oviducts of pseudo-pregnant surrogate mothers (day 0.5) that had been mated with vasectomized males of the same strain during the previous night. Newborn pups were obtained on day 19.5 after embryo transfer.

Purification of spermatogenic cells

Germ cells were isolated from individual male juvenile mice using a 50 ml ‘mini’ Sta-Put chamber as described previously (40). Briefly, gonadal tissue was subjected to sequential digestions of 15 min each at 37°C with 0.5 mg/ml of collagenase and then 0.5 mg/ml of trypsin. After the cells were treated with ~200 μg/ml of DNase and resuspended in 0.5% bovine serum albumin (BSA), they were loaded onto a 50-ml gradient of 2–4% BSA in KREBS buffer (39) and allowed to settle at unit gravity for 2 h. Fractions of spermatogenic cells were examined under phase optics to identify the desired cell type at optimal purity, and these fractions were then pooled. Populations of spermatogonia were isolated at purities of >85% from the testes of juvenile mice.

DNA and RNA isolation

In order to directly compare allele-specific DNA methylation and allele-specific expression of imprinted genes in the same samples of cells, DNA and RNA were isolated simultaneously from somatic tissues or germ cells using a Trizol-based protocol (18). Briefly, 0.1 g somatic tissue was homogenized in 1 ml Trizol (Invitrogen) and purified populations of spermatogonia were suspended in 200 μl of Trizol and pipetted many times to lyse cells before adding an additional 600 μl of Trizol. After adding chloroform, RNA was isolated from the aqueous phase according to the manufacturer’s instructions (Invitrogen).

DNA was isolated from the interphase/organic phase by adding back extraction buffer (4 M guanidine thiocyanate, 50 mM sodium citrate and 1 M Tris), which transfers the DNA into a new aqueous phase. After centrifugation, the aqueous phase was collected, and the DNA was precipitated using isopropyl alcohol. Samples were then centrifuged and the DNA pellet was washed in 75% ethanol. Following a final centrifugation, the supernatant was removed and the samples were allowed to air dry for 10 min before being resuspended in 0.1× TE buffer.

 Allele-specific bisulfite sequencing

To examine allele-specific DNA methylation patterns at imprinted loci, bisulfite sequencing was performed on genomic DNA isolated from male or female somatic tissues or male germ cells. Genomic DNA (500 ng/sample) was subjected to bisulfite mutagenesis using the EZ Gold DNA methylation kit (Zymo Research). Gene-specific primers for the *H19*, *Snprn* and *Peg3* DMRs were used to amplify the bisulfite-converted DNA using nested polymerase chain reaction (PCR) as described previously (18,21,31). Following amplification by PCR, products were cloned into the pCR2.1-TOPO vector (Invitrogen), transformed into chemically competent Escherichia coli cells (Invitrogen) and plated on LB plates with ampicillin (0.1 mg/ml). Blue/white selection was performed using 50 mg/ml X-gal, and recombinant colonies were picked and cultured overnight. Recombinant plasmids were isolated using the alkaline lysis miniprep method (40) and sequenced with the M13 reverse primer at the University of Texas Health Science Center at San Antonio DNA sequencing facility using an automated ABI 3130xl with BigDye Terminator v3.1 (Applied Biosystems). For each sample and gene analyzed, at least 20 clones were sequenced to obtain a representative sample of DNA strands. The bisulfite sequences were analyzed and filtered using QUMA software (41). Replicate sequences with identical location and number of unconverted non-CpG associated cytosines were excluded from further analysis. Maternal and paternal alleles were distinguished on the basis of SNPs that distinguish B6 and Cast sequences as described previously (21,22,42). Sequences that contained both B6 and Cast polymorphisms were also excluded from further analysis. At least 10 unique bisulfite sequences from each parental allele were obtained from each sample for each imprinted gene analyzed. We previously validated the accuracy of this standard bisulfite genomic sequencing method by performing a pyrosequencing assay in parallel (18).

Reverse transcriptase–polymerase chain reaction

Isolated RNA was treated with DNase (Promega) prior to first-strand synthesis using the iScript cDNA synthesis kit.
(Bio-Rad). Controls without reverse transcriptase (RT) were run for each RNA sample to ensure that genomic DNA was not amplified. PCR was performed by adding 2 μl of cDNA to the PCR mix [1× PCR buffer, 250 μM dNTPs, 0.5 μM forward and reverse primers (18) and 1.25 U reaction Hotmaster Taq DNA Polymerase (Eppendorf)]. The reaction conditions for all genes were as follows: 94°C for 20 s, 55°C for 20 s, 70°C for 30 s for 30 cycles, followed by 70°C for 10 min. The resulting PCR products were run on a 1% agarose gel and gel-purified using the QIAquick nucleotide removal kit (Qiagen).

SNUPE assay

To analyze allele-specific expression of imprinted genes, the SNUPE assay was used as described previously (18,24,43). Briefly, gel-purified RT–PCR products were quantified using a nanodrop 1000 (Thermo Scientific) and adjusted to 25 ng/μl. Either 25 or 100 ng of amplified product were added to the SNUPE reaction [1× PCR buffer, 2 μCi of [32P]-dNTP, 1 μM SNUPE primer (18) and 1 U Hotmaster Taq DNA polymerase] and then subjected to the following reaction conditions: 95°C for 1 min, 55°C for 2 min and 72°C for 1 min. Extension products were electrophoresed through 15% polyacrylamide-urea gels to detect the incorporation of radioactive nucleotides by phosphorimaging. For each imprinted gene, the specific [32P]-dNTP used to identify each parental allele was as follows: H19: B6/DBA allele-dCTP, Cast allele-dGTP, Peg3: B6/DBA allele-dTTP, Cast allele-dATP. After electrophoresis, the relative quantity of each band was determined using Quantity One Analysis software (Bio-Rad). We analyzed brain tissue for the expression of the Snrpn and Peg3 genes in both juvenile and adult mice. We examined H19 expression in liver tissue from juvenile mice and in muscle tissue from adult mice because H19 expression is no longer detectable in the liver in adult mice (44).

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Conflict of Interest statement. None declared.

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