Dual effects of superovulation: loss of maternal and paternal imprinted methylation in a dose-dependent manner

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Superovulation or ovarian stimulation is currently an indispensable assisted reproductive technology (ART) for human subfertility/infertility treatment. Recently, increased frequencies of imprinting disorders have been correlated with ARTs. Significantly, for Angelman and Beckwith-Wiedemann Syndromes, patients have been identified where ovarian stimulation was the only procedure used by the couple undergoing ART. In many cases, increased risk of genomic imprinting disorders has been attributed to superovulation in combination with inherent subfertility. To distinguish between these contributing factors, carefully controlled experiments are required on spontaneously ovulated, in vivo-fertilized oocytes and their induced-ovulated counterparts, thereby minimizing effects of in vitro manipulations. To this end, effects of superovulation on genomic imprinting were evaluated in a mouse model, where subfertility is not a confounding issue. This work represents the first comprehensive examination of the overall effects of superovulation on imprinted DNA methylation for four imprinted genes in individual blastocyst stage embryos. We demonstrate that superovulation perturbed genomic imprinting of both maternally and paternally expressed genes; loss of Snrpn, Peg3 and Kcnq1ot1 and gain of H19 imprinted methylation were observed. This perturbation was dose-dependent, with aberrant imprinted methylation more frequent at the high hormone dosage. Superovulation is thought to primarily affect oocyte development; thus, effects were expected to be limited to maternal alleles. Our study revealed that maternal as well as paternal H19 methylation was perturbed by superovulation. We postulate that superovulation has dual effects during oogenesis, disrupting acquisition of imprints in growing oocytes, as well as maternal-effect gene products subsequently required for imprint maintenance during pre-implantation development.

INTRODUCTION

The use of assisted reproductive technologies (ARTs) for the treatment of human subfertility/infertility contributes 1–2% of all children born in developed countries (1,2). However, the safety of these technologies has yet to be fully evaluated. Children conceived through various forms of ART are at an increased risk of low birth weight, intrauterine growth restriction, premature birth and have a higher incidence of genetic and epigenetic disorders, including genomic imprinting disorders such as Beckwith-Wiedemann Syndrome (BWS) and Angelman Syndrome (AS) (3–8). While the absolute risk of developing a genomic imprinting disorder in children born through ART as a result of an epigenetic defect is low, the relative risk when compared with non-ART children is significantly higher (9,10).

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Genomic imprinting is a mechanism of transcriptional regulation that restricts expression to either the maternally or paternally inherited copy of the gene; the opposite parental copy is silent (11). Imprinting may be envisaged as a multi-step process that begins in the gametes, where previous DNA modifications are erased, and sex-specific modifications that differentially mark the parental alleles are acquired (12–14). Imprint acquisition in developing male germ cells is complete by the post-natal stage. Maternal imprints are established in the oocyte, during maturation from primordial to antral follicles. These marks are laid down asynchronously, and in a locus-specific manner (15), correlating with increasing oocyte diameter (16). Imprinting marks are then stably maintained in the developing embryo, amidst genome-wide changes in DNA methylation, where they are translated into parental-specific monoallelic expression (17). Disruptions in any of these steps may lead to loss of parental-specific expression and the development of imprinting disorders.

DNA methylation of CpG dinucleotides is the most widely investigated epigenetic ‘mark’ associated with genomic imprinting. It has generally been linked to transcriptional repression, is both heritable and reversible, and has been shown to interact with, and recruit, chromatin-modifying complexes to silence or activate specific genes (18–20). DNA methylation occurs at regions called differentially methylated regions (DMRs) that display differential methylation of maternal and paternal alleles, or imprinting control regions (ICRs), if it has been ascertained that differential methylation is acquired during gametogenesis and maintained during pre-implantation development. Although the exact mechanisms of imprinted gene regulation have yet to be elucidated, DNA methylation at DMR/ICRs has been correlated with allelic expression of many imprinted genes (11).

Superovulation, or ovarian stimulation, is an ART commonly used to treat subfertility in women, for basic research in animal models, and in the production of livestock to obtain large numbers of offspring. Ovarian stimulation regimens for the treatment of human subfertility/infertility vary between clinics, within clinics between patients, and in dosage and types of hormones. Similarly, in animal models, hormone types and dosages vary between species and between research laboratories for the same species. However, the result of ovarian stimulation is constant: the current maturation of a large cohort of ovarian follicles to produce an increased number of ovulated oocytes when compared with spontaneous ovulation. Recently, increased frequencies of imprinting disorders have been correlated with ARTs, and loss of imprinting is more often the cause of imprinting disorders in affected ART populations than in non-ART children. Significantly, for both AS and BWS, patients have been identified where the only ART procedure used was ovarian stimulation (21–23).

To distinguish between the effects of superovulation and other contributing factors on genomic imprinting, carefully controlled experiments are required on spontaneously ovulated, in vivo-fertilized oocytes and their induced-ovulated counterparts, thereby minimizing effects of in vitro manipulations. Additionally, effects of superovulation on genomic imprinting need to be evaluated in an animal model system, where subfertility is not a confounding issue.

We propose that superovulation alone increases the risk of developing imprinting disorders. To address this, we evaluated imprinted methylation of multiple genes from individual mouse pre-implantation embryos. This work represents the first comprehensive examination of the overall effect of ovarian stimulation on genomic DNA methylation imprints at four imprinted loci, Snrpn, Peg3, Kcnq1ot1 and H19, in individual blastocyst stage embryos, and is the first to utilize low and high doses of hormones to assess their effects on genomic imprinting. We report that superovulation resulted in a loss of Snrpn, Peg3 and Kcnq1ot1 imprinted methylation, and a gain of imprinted H19 methylation in pre-implantation embryos and that this perturbation was dose-dependent; dysregulation of imprinted methylation was more frequent at the high hormone dosage. Additionally, we show that maternal as well as paternal specific H19 methylation imprints were perturbed by superovulation, suggesting that superovulation disrupts acquisition of imprints in growing oocytes, as well as maternal-effect gene products subsequently required for imprint maintenance during pre-implantation development.

RESULTS

Methylation levels of Snrpn, Peg3, Kcnq1ot1 and H19 in spontaneously ovulated embryos

Prior to examining the effects of superovulation on genomic imprinting, the methylation status of the Snrpn, Kcnq1ot1 and H19 ICRs, and the Peg3 DMR was first determined in individual blastocysts derived from spontaneously ovulating females. The regions analyzed included 16 CpGs located in the Snrpn ICR (24,25), 24 CpGs located in the Peg3 DMR (15), 20 CpGs located in the Kcnq1ot1 ICR (26) and 17 CpGs located in the H19 ICR (25,27) (Fig. 1). Methylation analyses using bisulfite mutagenesis and sequencing were performed on B6(CAST7)×B6 F1 individual blastocysts. Ten individual embryos were analyzed at the four loci. The Kcnq1ot1 and Snrpn ICRs and the Peg3 DMR acquire maternal-specific methylation during oogenesis, whereas the H19 ICR acquires paternal-specific methylation during spermatogenesis; oocytes are unmethylated at the H19 ICR in mice (15,28). Similar DNA methylation patterns are observed for the human SNRPN and H19 genes (29,30). Therefore, in B6(CAST7)×B6 blastocyst stage embryos, the maternal (CAST) alleles of Kcnq1ot1, Snrpn and Peg3 should be methylated, whereas the paternal (B6) allele of H19 should be unmethylated. As anticipated from previous reports of pools of blastocysts (25,27,31–33), the maternal DNA strands of Snrpn, Peg3 and Kcnq1ot1 were hypermethylated (Supplementary Material, Figs S1–S3), whereas the maternal H19 DNA strands were hypomethylated (Supplementary Material, Fig. S4). Only maternal strands are shown as superovulation is thought to affect genomic imprinting during oocyte development, hence only affecting the maternal allele (Supplementary Material, Figs S1–S4). From the analysis of embryos derived from spontaneously ovulated females, baseline total CpG methylation levels were determined to be greater than 65, 70 and 85% for Snrpn, Peg3 and Kcnq1ot1, respectively, and less than 25% for H19.
Figure 1. Schematic diagram of regions analyzed by bisulfite mutagenesis and sequencing assay. Maternal methylated Snrpn, Peg3 and Kcnq1ot1 alleles and the paternal methylated H19 allele are indicated. ICR, imprinting control region; DMR, differentially methylated region. Open circles, CpGs. Blunt arrow designates transcription start site of non-transcribed allele. Regions analyzed are as follows: Snrpn ICR, 16 CpGs (15 CpGs in CAST) located in the promoter and first exon of the Snrpn gene; Peg3 DMR, 24 CpGs (23 CpGs in B6) located in the promoter and first exon of the Peg3 gene; Kcnq1ot1 ICR, 20 CpGs located in the Kcnq1ot1 ICR; and H19 ICR, 17 CpGs (16 CpGs in B6) in the ICR located 2–4 kb upstream of the transcriptional start site of H19.

The reciprocal B6×CAST cross was also performed to ensure that B6(CAST7)×B6 F1 embryos from spontaneously ovulated females were representative of normal imprinted methylation. Maternal Snrpn strands displayed baseline total CpG methylation levels of 65% (Supplementary Material, Fig. S5). Levels of baseline total CpG methylation on maternal Peg3 and Kcnq1ot1 DNA strands in B6×CAST F1 embryos were 75% and 75%, respectively (Supplementary Material, Figs S6, S7). The maternal H19 DNA strands were hypomethylated (Supplementary Material, Fig. S8), with less than 15% total CpG methylation. As no statistical difference was observed between embryos displaying aberrant methylation from the two crosses as determined by the Fisher’s exact test, these two spontaneously ovulating groups were combined for statistical calculations. We conservatively set the baseline total CpG methylation level to greater than 65, 70 and 75% for Snrpn, Peg3 and Kcnq1ot1, respectively, and less than 25% for H19. These values were used to determine loss or gain of methylation in embryos from superovulated females.

Interestingly, for all imprinted genes investigated, at least one embryo displayed a drastic loss of methylation at the normally methylated maternal allele. For the B6(CAST7)×B6 F1 embryos, one embryo displayed loss of methylation at the normally methylated maternal allele for Snrpn (E5, 60% methylation), Peg3 (E114, 55%) and Kcnq1ot1 (E112, 23%) (Supplementary Material, Figs S1–S3). For B6×CAST F1 embryos, spontaneous loss of methylation was observed at one embryo at the Snrpn ICR (E80, 50% methylation), the Peg3 DMR (E79, 34%) and at the Kcnq1ot1 ICR (E74, 58%) (Supplementary Material, Figs S5–S7). None of the F1 embryos displayed spontaneous gain of methylation at the H19 ICR. One embryo (E83) was observed to have reversed Kcnq1ot1 methylation; the maternal B6 strand had acquired a paternal imprint pattern, whereas the paternal CAST strand had acquired a maternal imprint pattern (Supplementary Material, Fig. S7). This is a rare event that has been observed previously for H19 imprint expression (25).

**Superovulation results in loss of maternal Snrpn, Peg3 and Kcnq1ot1 methylation in a dose-dependent manner**

To determine the effects of superovulation on imprinted methylation, we examined embryos derived following both low and high dosages of hormonal stimulation. Hormone dosages typically employed for superovulation in the mouse range from 2.5 to 10 IU, with 5 IU being the recommended dose for most mouse strains (34). We chose 6.25 IU to represent the low hormone dose, as lower concentrations were not as effective at inducing superovulation in the B6(CAST7) mice, and 10 IU for the high hormone dose (34). Snrpn, Peg3 and Kcnq1ot1 are normally paternally expressed and maternally methylated. Data were obtained from 10 embryos each in the 6.25 and 10 IU hormone treatment groups for Snrpn, and from nine embryos in each hormone treatment group for Peg3, whereas five embryos from the 6.25 IU group, and nine embryos from the 10 IU group were analyzed for Kcnq1ot1 imprint methylation. Forty to fifty clones were sequenced and analyzed for each gene. Methylation levels were analyzed at individual CpG dinucleotide across each ICR/DMR, as well as for the total number of methylated CpGs for each gene per embryo.

Snrpn displayed a loss of maternal methylation at both hormone dosages (Supplementary Material, Fig. S9), with the loss more frequent at the high hormone dosage (Figs 2 and 3). Analysis of total CpG methylation revealed that Snrpn exhibited a loss of methylation at the low hormone dosage on the maternal allele for four embryos (E29, 31%; E13, 63%; E13, 45% and E33, 54% total CpG methylation of DNA strands) (Fig. 2), and a loss of methylation at the high hormone dosage on the maternal allele for nine embryos (E10, 57%; E8, 55%; E1, 42%; E4, 63%; E23, 53%; E5, 59%; E6, 49%; E13, 63% and E11, 61% total CpG methylation) (Fig. 3), when compared with embryos from spontaneously ovulated females (baseline of 65% methylation). This loss of methylation at the high dosage
was significantly different from control embryos \( (P = 0.001) \) as calculated by the Fisher’s exact test.

A similar pattern of loss of methylation was observed for Peg3 when compared with Snrpn; both hormone dosages displayed a loss of methylation on maternal DNA strands (Supplementary Material, Fig. S10), with a greater frequency of loss in the high hormone dosage group (Figs 4 and 5). Peg3 displayed a loss of maternal methylation for four embryos (E14, 67%; E29, 49%; E18, 67% and E33, 66% total CpG methylation) at the low hormone dosage (Fig. 4), and a loss of methylation for five embryos (E10, 50%; E8, 67%; E1, 64%; E4, 47% and E11, 42% CpG methylation) at the high hormone dosage (Fig. 5), when compared with embryos from spontaneously ovulated females (baseline of 70% total CpG methylation). This loss of imprinted methylation was statistically significant in the higher hormone treatment group when compared with the spontaneous ovulation group \( (P = 0.03) \).

Kcnq1ot1, a third paternally expressed gene, also exhibited a similar loss of methylation on maternal DNA strands at both hormone dosages (Supplementary Material, Fig. S11), with a greater frequency of loss in the high hormone dosage group (Figs 6 and 7). Kcnq1ot1 exhibited a loss of maternal methylation for two embryos (E5, 54% and E33, 52% CpG methylation) at the low hormone dosage (Fig. 6), and five embryos (E2, 64%; E8, 56%; E4, 43%; E5, 62% and E13, 53% total CpG methylation) at the high hormone dosage (Fig. 7), when compared with embryos from spontaneously ovulated females (baseline of 75% CpG methylation). Loss of Kcnq1ot1 imprinted methylation was not statistically significant for either hormone treatment group when compared with controls \( (P = 0.4) \) for the 6.25 IU treatment group and \( P = 0.08 \) for the high hormone dosage), although the high hormone dosage group approached statistical significance. Analysis of additional embryos may be required to achieve significance.
Superovulation results in gain of maternal H19 methylation in a dose-dependent manner

The same ten embryos analyzed for imprinted methylation of the Snrpn, Peg3 and Kcnqlot1 ICR/DMRs were also used for analysis of the H19 ICR. H19 displayed a gain of maternal methylation at both hormone dosages, particularly for CpG dinucleotides 8–17 (Supplementary Material, Fig. S12), with the gain more frequent at the high hormone dosage (Figs 8 and 9). At the low hormone dose, one of ten embryos displayed a gain of maternal methylation, as seen by the presence of greater than 25% baseline CpG methylation of DNA strands (E14, 32% methylation) (Fig. 8). At the higher hormone dosage (10 IU), four of ten embryos displayed a gain of maternal methylation (E8, 66%; E4, 43%; E13, 67% and E11, 53% CpG methylation) (Fig. 9). This gain of methylation at the higher dosage was significantly different from control embryos (P = 0.003). These embryos acquired a more paternal-like pattern of methylation at the H19 ICR.

Superovulation results in loss of paternal H19 methylation in a dose-dependent manner

Studies of the effects of superovulation on genomic imprinting focused on the maternal allele, as superovulation is thought to affect genomic imprinting during oocyte development. Using our protocol, methylation data were obtained for both maternal and paternal alleles of the four imprinted genes from individual pre-implantation embryos. Surprisingly, not only did we observe significant effects of superovulation on imprinted methylation of maternal alleles as described above, we also observed a loss of methylation on the normally methylated paternal H19 allele at both hormone dosages, especially for CpG dinucleotides 1–7 (Supplementary Material, Fig. S13), with more frequent loss of methylation at the high hormone dosage (Figs 10 and 11). For both B6(CAST7)×B6 (Supplementary Material, Fig. S14), and B6×CAST F1 embryos (Supplementary Material, Fig. S15) from spontaneously ovulated females, H19 displayed 79 and 77% total CpG methyl-
ation on paternal DNA strands. Thus, the baseline level of total CpG methylation on the paternal *H19* allele was set at 75%. Of the above embryos derived from spontaneously ovulating females, two B6(CAST7) × B6 F1 embryo and two B6 × CAST F1 embryo displayed loss of CpG methylation on paternal DNA strands (E10, 71%; E113, 50% and E73, 61%, E74, 56% methylation). By comparison, embryos from induced ovulations exhibited a loss of paternal *H19* methylation. At the low hormone dosage, three embryos (E18, 54%; E20, 69% and E33, 58% methylation) displayed a loss of methylation on paternal DNA strands (Fig. 10), whereas at the high hormone dosage, seven embryos (E10, 71%; E2, 63%; E8, 57%; E23, 68%; E5, 61%; E6, 73% and E11, 47% CpG methylation) showed a loss of paternal methylation (Fig. 11). This loss of imprinted methylation on the paternal *H19* strand was statistically significant in the high hormone treatment group (*P* = 0.02).

For the other imprinted genes analyzed, low levels of total CpG methylation were present on the paternal alleles of *Snrpn*, *Peg3* and *Kcnq1ot1* following spontaneous and induced ovulation (Supplementary Material, Figs S16–S27). After taking baseline levels of total CpG methylation, one embryo from each dosage group showed a gain of paternal-specific *Snrpn* methylation (6.25 IU treatment E14, 35%; and 10 IU treatment group E8, 36%), one embryo from each hormone treatment group displayed a gain of paternal-specific *Peg3* methylation (6.25 IU treatment E31, 51%; and 10 IU treatment groups E11, 36%), and one embryo had a gain in paternal-specific *Kcnq1ot1* methylation in the 6.25 IU treatment (E33, 23%). In contrast to paternal *H19* methylation, these results were not statistically significant, and no effect of dosage was observed.

**Perturbation of imprinted methylation for multiple genes**

To determine the incidence of aberrant methylation (gain or loss) in the various treatment groups, the number of embryos with perturbation in methylation of the maternal *Snrpn*, *Peg3*, *Kcnq1ot1* and *H19* ICR/DMRs and the paternal *H19* ICR were assessed by the Fisher’s exact test (Table 1). At the low hormone dosage, four of 10 embryos (E14, E29, F18 and E33) showed aberrant methylation of two or more genes, which was significantly different than embryos derived from spontaneously ovulated females where only a single embryo (E74) displayed aberrant methylation of more than one gene (*P* = 0.05). At the high dose, 10 of 10 embryos displayed aberrant methylation for two or more genes. When compared with control embryos, this difference was highly significant (*P* = 0.00002). When all four genes were examined, no embryos exhibited aberrant methylation patterns at all loci at the low hormone concentration. However, at the high hormone dosage, one embryo (E8) displayed perturbed methylation at the maternal allele of all four genes, as well as at the paternal *H19* allele. These data clearly demonstrate the dose-dependent effect of superovulation on perturbation of imprinted methylation.
DISCUSSION

In this study, we utilized a mouse model system to investigate the effects of superovulation on genomic imprinting in blastocyst stage embryos. Blastocysts were examined for parental-specific methylation changes to circumvent the chance of cumulus cell contamination that otherwise could be an issue when analyzing oocytes and early cleavage stage embryos. Furthermore, by studying embryos instead of oocytes, we minimized the effects of in vitro manipulations, as well as limited our analysis to those oocytes that were capable of being fertilized and producing embryos. We have demonstrated that superovulation perturbed genomic imprinting of both maternally and paternally expressed genes and that this perturbation was dose-dependent. Previously, superovulation had been postulated to function by affecting oocyte development, and therefore effects were expected to be restricted to the maternal allele. In our study, we have demonstrated that maternal-specific methylation imprints as well as paternal-specific methylation imprints were disrupted by superovulation. Furthermore, we observed that superovulation results in perturbation of genomic imprinting for multiple genes within the same embryo.

Superovulation perturbs genomic imprinting

Assisted reproduction has been linked to the generation of epigenetic errors that result in the development of the human imprinting disorders AS (OMIM #105830) and BWS (OMIM #130650) (3–7,35). Commonality between ART-associated BWS and AS is loss of maternal-specific methylation at the ICRs at 11p15 and 15q11–q13, respectively (3–7).

Multiple studies have examined the association of ARTs and imprinting. Using a survey-based study, the reproductive history of parents with an AS child in Germany was
investigated (22). Molecular studies revealed that 25% of their AS children possessed a sporadic imprinting defect; a significant increase over 3–4% of patients with imprinting defects in the entire Angelman population. Prolonged infertility in combination with infertility treatment, including ovarian stimulation, posed the highest risk for patients in this survey. The use of variable ART procedures in mothers of ART-associated BWS children were reported in France and at the John Hopkins School of Medicine (5,21), although one specific type of procedures was not more likely to cause an epigenetic defect than another. However, in all cases examined in these and other studies, some type of ovarian stimulation regime was consistently employed to facilitate conception (5,21–23,36). Significantly, in both AS and BWS studies, patients were identified where the only ART procedure used was ovarian stimulation.

In the current study, we assessed the effects of superovulation on the ICRs of Snrpn, Kcnq1ot1 and H19 genes that have a causal role in the etiology of BWS and AS. Following superovulation, we observed a loss of maternal methylation in blastocyst stage embryos at the ICRs of the paternally expressed Snrpn and Kcnq1ot1 genes in individual mouse pre-implantation embryos. Although the effects of superovulation have not previously been examined at the blastocyst stage for Snrpn, no effect on Snrpn imprinted methylation was observed following superovulation in midgestation mouse embryo and placents (37). The effects of superovulation on Kcnq1ot1 have not been previously examined at the blastocyst stage; however, a decrease in hypermethylated Kcnq1ot1 alleles from stimulated human oocytes obtained with unstimulated controls has been observed (38). Together these observations show that superovulation is associated with loss of DNA methylation at imprinted loci known to be linked to the development of AS and BWS. This study further provides a mechanistic link between ARTs and imprinting disorders.

The effect of superovulation on maternal methylation of the Peg3 DMR has not been previously evaluated at any stage of development. Similar to the other paternally expressed genes examined, we observed a loss of maternal Peg3 methylation following superovulation. Our results constitute a novel finding and suggest that the effects of ARTs may not be limited to a subset of imprinted genes but may affect multiple imprinted loci. Peg3 is a zinc finger protein thought to interact with p53 and Bax to regulate neuronal apoptosis in response to hypoxia or DNA damage (39–41). Loss of Peg3 expression is associated with aberrant maternal nurturing behavior and an offspring’s ability to thrive (42–44), phenotypes that have been linked to increased neuronal apoptosis during neonatal brain development (45). Furthermore, loss of methylation at the Peg3 DMR has been linked to spontaneous abortion (46). Our data are of interest, in light of the fact that children born through ART are at an increased risk of neonatal mortality and intensive care unit admission (47) and increased risk of low birth weight and premature delivery (8). Our observation that superovulation results in a loss of imprinted methylation at the Peg3 DMR may suggest an additional mechanism contributing to the risks of ART.

In addition to loss of maternal methylation, we observed a gain of maternal methylation for the normally unmethylated maternal H19 allele in blastocyst stage embryos. This is consistent with the report by Sato et al. (48), who observed a gain of maternal H19 methylation following superovulation in mouse and human oocytes and by Borghol et al. (30) who observed methylated H19 alleles in oocytes obtained from women undergoing ovarian stimulation followed by in vitro maturation. In contrast, our data differ from those reported by Fortier et al. (37), who observed that H19 methylation in midgestation mouse embryos and placents derived from superovulated mothers did not reveal a gain of maternal H19 methylation. This discrepancy may be explained by smaller
sample size, single low hormone dosage or technical difficulties with the bisulfite protocol discussed by the authors (37). Another report cited no difference in $H19$ methylation following superovulation in individual blastocysts, however, methylation analyses were not done allelically; therefore, methylated maternal alleles would not have been discriminated from appropriately methylated paternal alleles (49).

Superovulation perturbs genomic imprinting for multiple genes in the same embryo

Analysis of the incidence of imprinted methylation defects following superovulation revealed that many embryos harbored aberrant methylation for two or more genes, which was significantly different from embryos from spontaneously ovulated females where only a single embryo displayed aberrant methylation for more than one gene. Similar observation were recently reported in ART-conceived children with BWS (50); imprinting defects at multiple imprinted loci other than the $Kcnq1ot1$ ICR were more frequently observed in BWS patients whose parents had undergone some form of ART than in non-ART BWS patients. These data suggest that developmental defects or abnormal growth in ART children might be caused by variable combinations of epigenetic perturbations at imprinted genes, perhaps offering an explanation for a postulated new syndrome characterized by overgrowth and severe developmental delay (51). Developmental and growth abnormalities could also plausibly result from combinations of ART-induced epigenetic perturbations at imprinted and non-imprinted genes. A recent analysis of DNA methylation at more than 700 genes revealed that in vitro conception was associated with significant changes in DNA methylation at both imprinted and non-imprinted genes, which is indicative of broad effects of ART on DNA methylation (52).

Superovulation may lead to perturbation in imprint acquisition as well as imprint maintenance

Loss of imprinted methylation in embryos derived from superovulated mothers, but not in control females, indicates that superovulation disrupts mechanisms that establish imprinting during oogenesis. There are a number of possible explanations for the loss of imprinting following superovulation. Hormonal stimulation may result in the ‘rescue’ of subordinate follicles which may have entered the follicular atresia pathway and that otherwise would not have been ovulated resulting in ovulation of lower quality oocytes (36), it may lead to rapid oocyte maturation that perturbs genomic imprints, or it may induce ovulation of immature oocytes that have not

Figure 7. Methylation of the maternal $Kcnq1ot1$ ICR in B6(CAST7) × B6 F1 embryos derived from high dosage superovulated females. Methylation status of individual DNA strands in the $Kcnq1ot1$ ICR (maternal, CAST strands shown) in blastocysts derived from females superovulated with a 10 IU hormone dosage. Details are as described in Figure 2.
completely acquired their imprints (22,53). In humans, ovarian stimulation has been shown to accelerate the growth rate of ovarian follicles when compared with non-stimulated controls (54). In the case of genomic imprinting, this shortened maturation time may lead to improper or incomplete acquisition of imprinting marks on the maternal alleles. Further investigations are required to distinguish between these possibilities.

As the use of exogenous hormones occurs during oogenesis, effects of superovulation were expected to be restricted to the maternal allele. Surprisingly, we report that H19 displayed a loss of methylation on the paternal, sperm-contributed allele, indicating that events that occur during oocyte maturation regulate imprinting on both the maternal and paternal alleles. At this point, it is not known whether this effect extends to other ICR that are unmethylated on the maternal allele or whether it is limited to the H19 ICR. However, our data support a recent study that observed activated expression of the normally silent, paternal H19 allele following superovulation (37), as well as, a second study that showed aberrant H19 imprinted methylation in F1 and F2 male offspring of superovulated female mice (55). Thus, we postulate that superovulation has dual effects during oogenesis, acting to disrupt the acquisition of imprints in the growing oocyte, as well as causing molecular changes that disrupt maternal-effect gene products subsequently required for genomic imprint maintenance during pre-implantation development.

**Dose-dependent effects of superovulation**

Dose-dependent effects of ovarian stimulation on genomic imprinting have not been previously reported. To evaluate this, we performed experiments using two different dosages of hormones, 6.25 (low) and 10 IU (high). All four imprinted genes investigated displayed a dose-dependent response to superovulation. A greater number of embryos displayed perturbed imprinted methylation on the maternal alleles of Srrpn, Peg3 and Kcnq1tot1, and on both the maternal and paternal allele of H19, at the high hormone dosage compared with the low hormone dosage. Various hormone types and regimens are currently used for the treatment of subfertility. Recently, a mild stimulation regimen was shown to decrease the incidence of aneuploidy in resulting embryos when compared with the standard higher dose regimen (56), and high dosages of exogenous gonadotropins are associated with lower pregnancy rates (57). Our study suggests that increasing hormone dosages in an effort to increase the number of oocytes recovered may have detrimental effects on embryo development. These observations are particularly important...
in light of the movement in the field towards single embryo transfers, where a natural cycling regime would not be detrimental to pregnancy outcome.

A significant finding from these studies is that superovulation results in dysregulation of genomic imprinting in the absence of other confounding factors. This is relevant at the clinical and community-wide level, as ovarian stimulation is currently an indispensable component of the ART protocol to treat human subfertility/infertility. As the genes investigated in this study play an important role in early development, and genetic and epigenetic perturbations lead to imprinting disorders, we propose that superovulation may increase the risk of developing these disorders in the ART population. Our studies and others like it argue for a more conservative use of assisted reproductive technologies, as well as more in-depth investigations of the effects of these technologies on human populations.

**MATERIALS AND METHODS**

**Ovarian stimulation and embryo collection**

Embryos were obtained from crosses of C57BL/6 (CAST7) females and C57BL/6 (B6) males (Jackson Laboratory or Charles River). B6(CAST7) mice contain *Mus musculus castaneus* chromosome 7s on a B6 background (31). Two hormone regimens were used for ovarian stimulation, 6.25 (low dose) and 10 IU (high dose). Low or high doses of Pregnant Mare’s Serum Gonadotropin (Intervet Canada) were administered to female B6(CAST7) mice, followed by the same dose of Human Serum Chorionic Gonadotropin (hCG, Intervet Canada) 40–44 h later. Females were mated with B6 males, and pregnancy was determined by the presence of a vaginal plug the following morning (day 0.5). F1 hybrid embryos were flushed from the genital tract of females 96 h post-hCG to recover blastocyst stage embryos.
Additionally, females were set up in timed-matings that allow for spontaneous ovulation cycles (untreated controls). B6(CAST7) females were crossed with B6 stud males. As well, B6 females were mated with Mus musculus castaneus (CAST) males (spontaneous ovulation). Embryos were recovered at day 3.5 postcoitum; all analyzed embryos were blastocysts, except for B6(CAST7)/C2 B6 E6 (spontaneous ovulation group), E29 (6.25 IU group) and E23 (10 IU group) which were late stage morulae. Embryos were flushed in pre-warmed M2 media (Sigma), washed three times in 30 ml, and individually snap frozen in 1–5 ml of M2. Individual embryos were stored at −80°C. For each control and experimental group, embryo collections were performed multiple times, and embryos analyzed were recovered from multiple litters. Experiments were performed in compliance with the guidelines set by the Canadian Council for Animal Care, and the policies and procedures approved by the University of Western Ontario Council on Animal Care.

DNA isolation and bisulfite mutagenesis for individual embryos

Bisulfite mutagenesis with agarose embedding was conducted on single embryos as described (58,59), with modification. Individual embryos were lysed with 0.1% IGEPAL (Biochemika), and 2 mg/ml Proteinase K (Sigma) in 10 μl of lysis buffer [100 mM Tris–HCl pH 7.5 (Bioshop), 500 mM LiCl (Sigma), 10 mM EDTA pH 8.0 (Sigma), 1% LiDS (Bioshop), 5 mM DTT (Sigma)] for 1 h at 50°C. Lysed embryos were embedded in 2% low melting point agarose (Sigma) under mineral oil at 95°C. DNA/agarose beads were allowed to solidify for 10 min on ice. Oil was removed and denaturation of DNA was performed in 0.1 M NaOH (Sigma) at 37°C for 15 min with shaking. Agarose beads were placed in 2.5 μl bisulfite solution [0.125 μ hydroquinone (Sigma), 3.8 g sodium hydrogen-sulfite (Sigma), 5.5 ml water, 1 ml 3 M NaOH] at 50°C for 3.5 h to allow bisulfite mutagenesis to occur. Following incubation, agarose beads were washed once in TE pH 7.5, and desulphonated with 0.3 M NaOH at 37°C for 15 min with shaking. Agarose beads were washed twice with TE pH 7.5, and twice with water. Beads were incubated under oil at 65°C and ~60 μl of pre-warmed water was added. Agarose beads were mixed by pipetting and 20 μl of diluted agarose was added to one ready-to-go PCR Bead (GE) containing genespecific primers and 1 μl of 240 ng/ml tRNA as a carrier. PCRs were split in half allowing two independent PCR reactions to be completed for each gene analyzed. Nested primer sequences and associated information for each gene can be found in Table 2. Negative controls (no embryo) were processed alongside each bisulfite reaction.

Figure 10. Methylation of the paternal H19 ICR in B6(CAST7)×B6 F1 embryos derived from low dosage superovulated females. Methylation status of individual DNA strands in the H19 upstream ICR (paternal, B6 strands shown) in blastocysts derived from females superovulated with a 6.25 IU hormone dosage. The region of the paternal B6 H19 allele analyzed contains 16 CpGs due to a polymorphism that eliminates CpG8. Details are as described in Figure 2.
Allele-specific DNA methylation analysis of individual embryos for *Snrpn*, *Peg3*, *Kcnq1ot1* and *H19*

Gene-specific primers used for nested PCR amplification of *Snrpn*, *Peg3*, *Kcnq1ot1* and *H19* as well as melting temperatures for each primer set can be found in Table 2. Five microliter of first round product was seeded into each second round PCR reaction. Second round products were digested with restriction enzymes that cleave methylated bisulfite converted DNA to ensure no bias in the amplification of methylated/unmethylated products, or with restriction enzymes that cleave species-specific SNPs to ensure no allelic bias was introduced during PCR amplification. PCR amplified products were directly cloned without intervening gel extraction steps, as we observed that column purification drastically decreases the variability of DNA strands recovered (data not shown). One microliter of second round PCR product was used for ligation with the pGEMT-EASY DNA ligation kit (Promega). Ligation was performed overnight at 4°C and transformed into competent *Escherichia coli* cells (Invitrogen or Zymo Research). Blue/white selection (100 mg/ml IPTG, 50 mg/ml X-gal) was used to select bacterial colonies with ligated products.

Individual sequences were obtained by colony PCR of individual bacterial colonies. The pGEMT-EASY vector contains M13 primer sites flanking the multiple cloning site, which were used for amplification of inserted DNA fragments. Approximately 2 μl of PCR product was used for agarose gel electrophoresis to verify amplicon size, and the remainder of the PCR reaction was sent to the Nanuq Sequencing Facility located at McGill University (Montreal, QC, Canada) or BioBasic Inc. (Markham, ON, Canada) for sequencing. As *Kcnq1ot1* was the last gene in each set to be analyzed, a proportion of embryos did not produce a sufficient number of DNA strands to be included in the analysis.

Sequence analysis

For each sample and gene analyzed, 40–50 clones were sequenced to obtain a representative number of DNA strands. Chromatograms from each sequence were visualized using FinchTV. Ambiguous base pairs were manually reviewed and assigned a designation (where possible). Each sequence was analyzed for total number and location of CpG associated cytosines, as well as location and number of converted and unconverted non-CpG associated cytosines to obtain conversion rates (number of converted non-CpG cytosines/total number of non-CpG cytosines). Sequences with less than 85% conversion rates were not included. Identical clones (identical location and number of unconverted CpG associated cytosines, and identical location and number of unconverted non-CpG associated cytosines) were not included. Multiple polymorphisms are present between B6

Figure 11. Methylation of the paternal *H19* ICR in B6(CAST7)× B6 F1 embryos derived from high dosage superovulated females. Methylation status of individual DNA strands in the *H19* upstream ICR (paternal, B6 strands shown) in blastocysts derived from females superovulated with a 10 IU hormone dosage. The region of the paternal B6 *H19* allele analyzed contains 16 CpGs due to a polymorphism that eliminates CpG8. Details are as described in Figure 2.
### Table 1. Comparison of hormone dosage and aberrant imprinted methylation

<table>
<thead>
<tr>
<th>Dosage genotype</th>
<th>Embryo</th>
<th>Snrpn Mat loss (% &gt;65%)</th>
<th>Peg3 Mat loss (% &gt;70%)</th>
<th>Kcnq1ot1 Mat loss (% &gt;75%)</th>
<th>H19 Mat gain (% &lt;25%)</th>
<th>H19 Pat loss (% &gt;75%)</th>
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<td>0 IU CAST7 × B6</td>
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<td>ND</td>
<td>ND</td>
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<td>53</td>
<td>ND</td>
<td>63</td>
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<td>E11</td>
<td>ND</td>
<td>ND</td>
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</table>

ND, not determined; R, reversal of imprinted DNA methylation. Dark grey boxes indicate perturbation in maternal methylation patterns while light grey boxes indicate loss of paternal-specific methylation.

### Table 2. Regions and conditions for PCR analysis following bisulfite mutagenesis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession</th>
<th>Position</th>
<th>Primer type</th>
<th>Primer sequence (5’–3’)</th>
<th>Annealing temperature</th>
<th>Reference</th>
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</thead>
<tbody>
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<td>Snrpn</td>
<td>AF081460</td>
<td>2151 -2570</td>
<td>OF OR IF IR</td>
<td>TAT GTA ATA TGA TAT AGT TTA GAA ATT AG AAT AAA CCC AAA TCT AAA ATA TTT TAA TC</td>
<td>52</td>
<td>24,25</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AAT TG TGT GAT TGT TGT AAT TAT TGG G ATA AAA TAC ACT TTC ACT ACT AAA ATC C</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Peg3</td>
<td>NT_039413.7</td>
<td>3683033 -3682588</td>
<td>OF OR IF IR</td>
<td>TTT TGA TAA GGA GGT GTT T ACT CTA ATC CCT ACT ATA ATA A TTT TTA GGT GGT GGT TGA TTA GAT T</td>
<td>50</td>
<td>This study</td>
</tr>
<tr>
<td>Kcnq1ot1</td>
<td>AJ271885</td>
<td>141392 -141598</td>
<td>OF OR IF IR</td>
<td>GTG TGA TTT TAT TTT GAG AG CCA CTC ACT ACC TTA ATA CTA ACC AC GGT TAG AAG TAG AGG TGA TT</td>
<td>52</td>
<td>26</td>
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<tr>
<td>H19</td>
<td>U19619</td>
<td>1304 -1726</td>
<td>OF OR IF IR</td>
<td>GAG TAT TTA GGA GGT AT A AG A ATC AAA AAC TAA CAT AAA CCT CT GGT AGA AGA TTA TGT TTA TTT TGG G</td>
<td>55</td>
<td>25,27</td>
</tr>
</tbody>
</table>

OF, outer forward; OR, outer reverse; IF, inner forward; IR, inner reverse.
and CAST sequences at each gene analyzed, allowing parental alleles to be discriminated. Clones possessing both B6 and CAST polymorphisms were determined to be due to crossover during PCR amplification and were not included. Methylation levels across the region of analysis were determined by calculating the number of methylated CpG/total number of CpG for each individual CpG site as a percentage. Total DNA methylation for each gene was calculated as a percentage of the total number of methylated CpG/total number of CpG dinucleotides.

Statistical analysis

To compute the significance of non-random association between groups of embryos, we used the Fisher’s exact test. As changes in methylation status were anticipated to be in only one direction (increase or decrease), a one-sided test was utilized. P-values were calculated using software provided online (http://www.langsrud.com/fisher.htm) and were considered to be significant at \( P < 0.05 \).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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