Ovulation induction and epigenetic anomalies

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In this systematic review of ovulation induction and epigenetic control, studies mainly done in the mouse model highlight how hormone treatments may be prejudicial to the epigenetic reprogramming of gametes as well as early embryos. Moreover, the hormone protocols used in assisted reproduction may also modify the physiologic environment of the uterus, a potential link to endometrial epigenetic disturbances. At present, the few available data in humans are insufficient to allow us to independently determine the impact of a woman's age and infertility problems and treatment protocols and hormone doses on such processes as genomic imprinting. (Fertil Steril® 2013;99:616–23. ©2013 by American Society for Reproductive Medicine.)

Key Words: Epigenetics, ovulation induction, DNA methylation, genomic imprinting, ovarian stimulation

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Millions of children, comprising roughly 1% to 2% of all births in the United States and Europe, have been born through the use of assisted reproductive techniques (ART) to couples experiencing fertility problems (1). Although these techniques are considered generally safe, evidence suggests that an increased risk of adverse perinatal outcomes and morbid congenital problems are associated with their use (2). Furthermore, even if contradictory findings exist (3–7), several reports have pointed to an increased risk of diseases caused by abnormal genomic imprinting such as Beckwith-Wiedemann syndrome (BWS-OMIM #130650) (8–12) and Angelman syndrome (AS-OMIM #105830) (11, 13, 14).

Genomic imprinting is a phenomenon specific to mammals that results from imprinting marks acquired in a sex-specific manner in the female and male gametes on regulatory sequences (differentially methylated regions, DMRs, also called imprinting control regions, ICRs) of a subset of key developmental genes, referred to as imprinted genes (15). Indeed, these genes have been discovered to play crucial roles in embryonic growth and development, placental functions (16), postnatal metabolic pathways, and behavior associated with the control of resources (17). Moreover, oncogenesis may also be associated with altered epigenetic alterations (18).

These genes are generally located in clusters, epigenetically marked mainly by DNA methylation—the addition of a methyl group ($-CH3$) on the fifth carbon of a cytosine base on a cytosine guanine (CG) dinucleotide (19), by histone modifications (acetylation/deacetylation and methylation) and sometimes associated with antisense RNAs (20, 21). Genomic imprinting generally operates to silence the maternal or paternal alleles of imprinted genes and permits the expression from only one allele. To date, it is the DNA methylation that has been studied most extensively. At present, about 150 imprinted genes have been identified in mice and humans (http://igc.otago.ac.nz; www.geneimprint.com; www.mousebook.org/catalog.php?catalog=imprinting).

The allele-specific methylation of primary DMRs (also called germline DMRs) occurs in germ cells in a sex-specific manner during gametogenesis and provides a heritable "memory" that is maintained throughout fertilization and embryo development. Indeed, this differential methylation is preserved during preimplantation development, despite genomewide changes in global DNA methylation occurring at these early stages for the purpose of allele-specific expression (17). These germline DMRs act in *cis* to control the parental allele-specific expression of several imprinted genes.

Because of their functional haploidy and their epigenetic determinism, abnormal expression of imprinted genes can result from genetic disorders (deletion or duplication, mutation or uniparental disomy) but also epimutations (methylation anomalies). Observations that BWS patients were approximately nine times more likely

to have been conceived by ART than patients without BWS (10) and that the defect observed after ART is systematically an epimutation strongly suggest that ART could perturb the epigenetic control of the genome during gametogenesis and/or early embryogenesis. It is also notable that the epigenetic defect found for all BWS and AS children born after ART is hypomethylation on the maternal allele of the relevant DMR. Thus, deleterious effects inherent to ART may primarily occur during the female epigenetic reprogramming, which takes place during the maturation process of oocytes.

Assisted reproduction, which includes primarily in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), encompasses several steps of manipulation of the gametic and early embryonic material, generally after hormone stimulation protocols. Intrauterine insemination (IUI), another assisted reproductive technology often performed in combination with ovulation induction, also includes sperm preparation.

To date, it is unclear which ART procedures are involved in epigenetic anomalies. However, one of the important issues regarding possible epigenetic alterations is the artificial induction of ovulation. The observation of epigenetic disorders in children conceived after the IVF and ICSI techniques as well as IUI, with/without donor sperm, supports this notion. Moreover, in some studies, BWS and AS patients have been identified in whose mother the only ART procedure used was ovarian stimulation (11, 22, 23). Another argument in favor of hormonal epigenetic disruption is that the DNA methylation marks are laid down asynchronously in both gametogeneses. DNA methylation acquisition at DMRs occurs during prenatal stages of spermatogenesis and is completed at postnatal stage, whereas in oogenesis it begins later after puberty in growing oocytes from primordial to antral follicles. Ovarian stimulation administered using exogenous hormones during this period may disrupt the acquisition of imprints in oocyte maturation. Moreover, in IVF or ICSI treatments, the dosage of ovarian stimulation is higher to produce an increased number of ovulated oocytes when compared with spontaneous ovulation or even with IUI treatments. This forced oocyte maturation may lead to the loss of maternal-specific expression and the development of imprinting disorders in some oocytes normally nonovulated. In addition, in cases of female infertility with low ovarian reserve or in advanced maternal age, higher dosages of hormones are typically used. It is extremely difficult in humans to distinguish the effects of ovarian stimulation from other infertility-contributing factors on genomic imprinting.

Thus, deleterious effects of hormone treatments may alter the epigenetic reprogramming during the maturation process of oocytes. However, as developed in this review, ovarian induction may also modify the physiologic environment of the uterus as well as the implantation and development of the embryo. In IVF cycles, it has been shown that birth weights of singletons are lower after fresh embryo transfers than after frozen-thawed cycles (4, 24–27). This finding led to the suggestion that by epigenetic processes gonadotropin-stimulated multifollicular development and production of supraphysiologic levels of sex steroid hormones may play a role at the time of embryo implantation. They may modulate the endometrial receptivity and/or trophoblast invasive properties and represent an epigenetic contributing factor to the increased risk of low birth weights and other disorders of abnormal placentation.

EPIGENETIC REPROGRAMMING IN MOUSE AND HUMAN OOCYTES

In mice, epigenetic reprogramming starts with germ cell development from epiblast cells around embryonic day 7.25 (E7.25), continues after the primordial germ cells (PGCs) have reached the genital ridge at E10.5, and lasts until E13.5 (28) (42 to 44 days in humans). After their subsequent migration into the developing gonads, PGCs exhibit a marked genomewide DNA demethylation. Regarding the genomewide view of methylation, the levels are high (73.2% to 85%) in embryonic stem cells whereas those in female PGCs at E13.5 are less than 10% (29). This DNA demethylation also concerns imprinted loci (30, 31). Only a specific subset of repetitive elements, called the intracisternal A-particle (IAP) elements, remains refractory to reprogramming in PGCs (31, 32). Likely due to the restricted cell numbers, studies of oocyte chromatin status have been limited. However, chromatin changes also occur during this period, accompanied by an extensive erasure of several histone modifications (loss of repressive histone H3K9me3 and H3K27me3 as well as loss of the active histone mark H3K9ac) and exchange of histone variants (31). Unlike the chromatin rearrangements, which are very transient (from E11.5 to E12.5) (31), the erasure of differential DNA demethylation of imprinted genes persists until new imprints are imposed later in the embryo in a sex-specific manner, which occurs at different times in the male and female germlines.

As found in the mouse model during the neonatal phase, in the female germ line the establishment of imprint marks occurs in a gene-specific manner at specific times throughout the primary to antral follicle stage while oocytes are arrested at prophase I (33–35). Methylation acquisition increases up to the antral follicle stage in oocytes; it has been found to be fully established in the antral follicle stage. The de novo methylase Dnmt3a in collaboration with Dnmt3L is responsible for establishing a new DNA methylation state at repeat sequences and developmental genes (36, 37) and for resetting the sex-specific germline DMR imprint (38, 39). During mouse neonatal oocyte development, the acquisition of the methylation imprint of an imprinted gene Peg1 appears to be the latest (33, 40). However, during adult mouse follicle growth, the maternal imprint appears to be established at the same time for all the analyzed imprinted genes, and methylation dynamics seem to be more progressive as compared with those in the neonatal period (40).

In adult human oocytogenesis, the timing of maternal imprinting appears to be identical to the mouse model (40). The maternally methylated DMRs present DNA methylation of around 50% in the adult oocytes at the early follicle stages (primordial and primary follicle stages) and are completely methylated in late antral follicle stage oocytes (Fig. 1). The paternally methylated DMRs remain unmethylated at all stages.

Khoueiry et al. (41) observed also that de novo methylation of KCNQ1OT1 DMR (KvDMR1) occurred gradually with meiosis II progression. About two-thirds of alleles were methylated on this DMR in fully grown germinal vesicle (GV) oocytes up to all alleles in most MII oocytes in stimulated cycles. However, Geuns et al. (42), who analyzed another region within the KvDMR1, reported an overall methylated pattern for this imprinted gene as early as the GV stage. The discrepancy between both groups could be explained by a methylation acquisition dissociated between two regions of this DMR.

It is interesting that most of the ICRs are methylated in the oocyte (17 maternal ICRs from 20 identified gDMRs/ICRs) whereas only three are methylated in sperm (paternal gDMRs). Thus, due to this high number of maternal ICRs, the frequency of imprinting errors during maternal epigenetic reprogramming is statistically higher than in sperm. In addition, maternal ICRs are CpG island promoters, whereas paternal ICRs are relatively CpG poor and intergenic. As has been previously suggested, the evolutionary reasons for these sexual discrepancies may be linked to the different developmental kinetics of male and female gametogenesis (43, 44). Moreover, the results reported by the researchers highlight the crucial role of maternal reprogramming: maternal ICRs have a dominant role in early development, regulating the biologic pathways related to the establishment of the fetomaternal interface (44).

EPIGENETIC EFFECTS OF OVULATION INDUCTION
Preimplantation Period

Animal model. The animal model system is interesting because subfertility is not a confounding issue. To date, animal studies of superovulation effects on imprinting have produced conflicting results (Table 1). Indeed, negative effects of superovulation on genomic imprinting were found by some research groups. Mann's team found loss of *Snrpn*, *Kcnq1ot1*, and *Peg3* maternal methylation in blastocyst-stage embryos recovered from induced ovulating females as compared with spontaneously ovulating females (45). These imprinting errors occurred in a dose-dependent manner, with more frequent disturbances after high than low hormone doses. In addition to the loss of maternal methylation, the investigators observed a gain of maternal methylation for the normally unmethylated maternal *H19* allele in blastocyst-stage embryos. Even though the differences between groups were not statistically significant, El Hajj et al. (46) observed slightly higher levels of imprinting errors in individual mouse 16-cell embryos produced in vivo from superovulated oocytes than in embryos obtained from unstimulated matings. Moreover, Shi and Haaf (47) reported abnormal methylation patterns in embryos from superovulated as compared with nonsuperovulated females using methylcytosine immunofluorescence. Later, in identical superovulation conditions used in their first work, Mann's team found normal imprinted methylation patterns of the same imprinted genes in individual mature oocytes (48). For *Snrpn*, *Peg3*, and *Kcnq1ot1*, the percentage of methylation was close to 100%, and *H19* was found to be unmethylated as expected in mature metaphase II (MII) oocytes. Consistent with these findings, a previous study by another group also found normal methylation patterns of several of these same imprinted genes (*Snrpn*, *Peg3*, and *H19*) as well as for *Igf2r* in pooled mature oocytes from superovulated female mice (49).

Conversely, Sato et al. (40) demonstrated a gain of *H19* methylation in pooled superovulated MII oocytes in two strains of mice (ICR and BDF). However, the differing results

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FIGURE 1
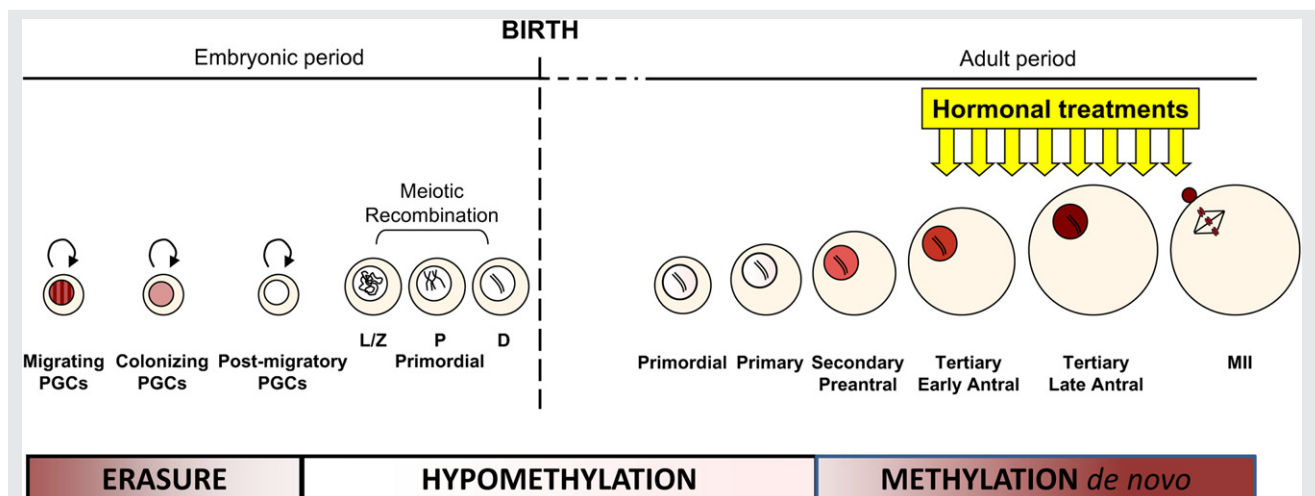
Methylation dynamics during the follicle growth. The epigenetic reprogramming starts with the germ cell development and continues after the primordial germ cells (PGCs) have reached the genital ridge. After their subsequent migration into the developing gonads, PGCs exhibit a marked genomewide DNA demethylation. This methylation erasure phase is followed by a meiotic arrest of gametes (arrested at diplotene of prophase I) within follicles at the primordial stage. Maternal methylation imprints are then established during the postnatal growing phase from the primary to antral follicle stage, the period in which hormone treatments are administered. The various stages of development of the female gametes are shown: D = diplotene; L = leptotene; MII = metaphase II; P = pachytene; Z = zygotene [adapted from Bourc'his and Proudhon (77)].

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TABLE 1

Epigenetic effects after ovarian induction in the mouse model.

Study	Superovulation	Method	Samples	Gene	Results
Sato et al., 2007 (40)	3 × 7.5 IU PMSG, 5 IU hCG	Combined bisulfite PCR restriction analysis	Pool of 30–50 MII oocytes	Peg1, Lit1, Zac, H19	Gain of methylation.
Fauque et al., 2007 (51)	5 IU PMSG, 5 IU hCG	Bisulfite mutagenesis sequencing	20 individual blastocysts	H19	Normal methylation profiles.
		RT-qPCR (TaqMan technology)			Altered expression levels.
Fortier et al., 2008 (58)	5 IU PMSG, 5 IU hCG	Bisulfite mutagenesis sequencing	36 individual embryos and placentae (9.5 dpc)	Snrpn, H19, Igf2, Kcnq1ot1	Normal methylation profiles.
		RT-PCR			Loss of imprinted expression for Snrpn, H19, Igf2, Kcnq1ot1 in principally placentae.
Anckaert et al., 2009 (49)	5 IU PMSG, 5 IU hCG	Bisulfite mutagenesis sequencing	3 pools of 100–150 MII oocytes	Snrpn, H19, Igf2, Peg3	Normal methylation profiles.
Market-Velker et al., 2010 (53)	6 or 10 IU PMSG, 10 IU hCG	Bisulfite mutagenesis sequencing	10 individual blastocysts	Snrpn, H19, Peg3, Kcnq1ot1	Loss of methylation for Snrpn, Peg3, Kcnq1ot1, H19mat and gain of methylation for H19pat.
El Hajj et al., 2011 (46)	7.5 IU PMSG, 7.5 IU hCG	Bisulfite mutagenesis sequencing	10 individual 16-cell embryos	Snrpn, H19	Loss of methylation for Snrpn, H19pat, and gain of methylation for H19mat. Abnormal methylation patterns observed also in embryos produced without superovulation.
Denomme et al., 2011 (48)	6.25 or 10 IU PMSG, 10 IU hCG	Bisulfite mutagenesis sequencing	125 individual MII oocytes	Snrpn, H19, Peg3, Kcnq1ot1	Normal methylation profiles with 6.25 IU PMSG.
de Waal et al., 2012 (69)	5 PMSG, 5 IU hCG	Bisulfite mutagenesis sequencing	4 individual male and 4 individual female juvenile offspring: brain and liver tissue	Snrpn, H19, Peg3	Loss of methylation for H19 in 1/8 juvenile offspring. Loss of methylation for Peg3 in 2/8 juvenile offspring.
		RT-PCR			Altered expression levels for H19, Snrpn, and Peg3.
		Bisulfite mutagenesis sequencing	Spermatogonia from superovulation-derived mice	H19	Delayed remethylation of maternal DMR H19 (8% to 25% methylation) as compared with those from the naturally conceived male pups (46% to 49%).

Note: dpc = days post coitum; DMR = differentially methylated region; hCG = human chorionic gonadotropin; MII = metaphase II; PCR = polymerase chain reaction; PMSG = pregnant mare serum gonadotropin; RT-qPCR = real-time quantitative polymerase chain reaction.

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might be explained by the age of their female mice; also, their superovulation procedures were completely different from the other research groups. Indeed, they collected oocytes from 10-week-old females after superovulation by injection of 7.5 IU of pregnant mare serum gonadotropin (PMSG) for 3 days followed 24 hours later by injection of 5.0 IU of human chorionic gonadotropin (hCG). However, an earlier study reported methylation heterogeneity for the maternal *Peg1/Mest* imprinted gene in matured oocytes obtained after superovulation (50). Fully methylated, mixed, and unmethylated patterns of *Peg1/Mest* were observed in superovulated MII oocytes, suggesting that the methylation imprint of this gene is not established in all oocytes obtained after superovulation (50). Yet another study has shown that superovulation alone does not disturb the DNA methylation status of the *H19*

gene but affects its expression levels in some individual blastocysts (51).

Finally, the majority of results based on animal studies performed in the preimplantation period suggest that imprint acquisition per se may not be affected by superovulation, pointing to its negative role on imprint maintenance. Superovulation may produce modifications in maternal-effect gene products (abnormal maternal factor synthesis or storage in the oocyte) that are later required for imprint maintenance in developing embryos (52). We can hypothesize that the various oocyte and embryo phenotypes observed after hormone-induced superovulation could be involved in efficiency differences in implantation and fetal development (51). In addition, Market-Velker et al. (53) have reported that superovulation may increase the epigenetic aberrant

TABLE 2

Epigenetic effects after ovarian induction in humans.

Study	Superovulation	Method	Samples	Gene	Results
Geuns et al., 2003 (54)	HT	Bisulfite mutagenesis sequencing	7 individual GV, 3 individual MI and MII oocytes	*SNRPN*	Normal methylation profiles
Geuns et al., 2007 (42)	HT	Bisulfite mutagenesis sequencing	6 individual GV and MI and 4 individual MII oocytes	*KCNQ1OT1*	Normal methylation profiles in 15 of 16 oocytes
Sato et al., 2007 (40)	HT	Bisulfite mutagenesis sequencing	10 individual MI oocytes	*PEG1, H19*	Gain of methylation for *H19* and loss of methylation for *PEG1*
Khoueiry et al., 2008 (41)	HT	Bisulfite mutagenesis sequencing	11 pools of 1–3 MII oocytes	*KCNQ1OT1*	Loss of methylation

Note: GV = germinal vesicle; HT = hormone treatment; MI = metaphase I; MII = metaphase II.

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effects of embryo culture, probably through compromising the ability to maintain genomic imprinting.

Human model. Like in the mouse model, Sato et al. (40) found that human oocytes obtained after ovarian stimulation in infertility treatments and individually analyzed by bisulfite mutation sequencing presented imprinted errors for *PEG1* (loss of methylation) and *H19* (gain of methylation) genes (Table 2). However, they could not distinguish whether these defects in DNA methylation were linked to the superovulation protocol, the age of the patients, or delayed oocyte maturation, or whether they were inherent to female infertility. Khoueiry et al. (41) observed that the KCNQ1OT1 DMR (KvDMR1) is more methylated in the GV and metaphase I (MI) oocytes of natural cycles than in those from stimulated cycles (62.5% vs. 67.8% for the GV and 70.3% vs. 63.6% for the MI, respectively), suggesting that gonadotropin stimulation may modify the dynamics of de novo methylation during oocyte maturation or/and may likely recruit too young follicles.

These results are consistent with those previously published by Geuns et al. (42), who found abnormally low methylation patterns for *KCNQ1OT1* in half the spontaneously matured oocytes analyzed (2 of 4 MII oocytes) obtained from ovarian stimulation cycles. These imprint disruptions may be caused by the developmental delay in the oocytes preventing imprint establishment at the right time or by ovarian stimulation or in vitro culture interfering with the imprint acquisition in the oocytes. However, the same research group earlier demonstrated normal methylation profiles for another maternal imprinted gene (*SNRPN* gene) in three spontaneously in vitro matured oocytes (54).

Implantation and Postimplantation Periods

Evidence from superovulated mice demonstrates a high risk of fetal growth retardation and an increased number of resorptions with hormone treatment (55–57). Three key actors may be involved during these postimplantation processes: the embryonic cells, placenta tissue, and endometrium.

Epigenetic disruption after superovulation observed at the blastocyst stage was not found later in gestation of mouse embryos (56). However, abnormal biallelic expression of maternal and paternal imprinted genes (*Snrpn* and *H19* genes) was observed in mouse placenta during that same period of gestation. These imprinting defects were also associated with an expression increase of the *Igf2* gene in placental tissue. These results suggest that superovulation alone may compromise the maintenance of imprinting during the preimplantation period, especially in trophectoderm-derived tissues. One can easily imagine that these imprinting interferences observed in the mouse model may have human clinical implications in assisted reproduction, such as a lack of embryo implantation, spontaneous abortion, or fetal growth retardation by dysfunctional placentas.

Another possible cause of the low implantation rate could be reduced endometrial receptivity in stimulated cycles. It is well known in humans that hormone treatments modify the maturation of the endometrium (58, 59). Ovarian stimulation leads to the advancement of endometrial development with a shift in the window of endometrial receptivity, being on average 1 to 2 days earlier than in natural cycles (59). The endometrium in stimulated cycles is exposed to supraphysiologic steroid hormone levels during the follicular phase; this might be responsible in some cases for an altered steroid receptor expression profile in the early luteal phase (60). Ovarian stimulation may also modulate the expression profile of several genes involved in the complex mechanisms of endometrial receptivity, including endometrial chemokines and growth factors, as shown in deep sequencing approaches (61).

Methylation of DNA could play a significant role in regulating the endometrial changes associated with ovulation induction, as has been recently proved by observations of functional endometrial changes throughout the menstrual cycle. Indeed, DNA methylation has been clearly demonstrated to be involved in endometrial receptivity as well as in decidualization (62, 63). Recently, two studies reported different expression patterns of three DNA methytransferases (Dnmt1, 3a, 3b) that are dependent on periods of the reproductive cycle (64, 65). In the secretory phase of human endometrium, levels of DNMT1, DNMT3a, and DNMT3b mRNA are lower as compared with the proliferative phase. These mRNA expression profiles of Dnmts during the cycle were also observed in the mouse model (66). In one study, mice treated with a DNA demethylating agent, the

nucleoside analog 5-aza-2′-deoxycytidine, had a dose-dependent decrease in the number of implantation sites, associated with altered expression of endometrial Dnmts and genes controlling endometrial changes (67).

All of these findings provide support for a potential role of DNA methylation in endometrial changes during embryo implantation after hormone treatments. Further experiments are needed to clarify the impact of ovarian stimulation on endometrial DNA methylation changes and implantation failure processes, as the respective roles of embryonic or endometrial epigenetic disturbances in implantation failure remain unclear. Yin et al. (68) attempted to address this question in an early pregnancy loss model. The investigators observed that defects in maintenance methylation in the embryo, not in the uterus, could be at the origin of abnormal embryonic implantation and development.

Offspring and Transgenerational Effects

A study by de Waal et al. (69) in mice has reported that gonadotropin stimulation can induce epimutations in the ensuing offspring independent of any procedures used during ART (e.g., ICSI). They found that use of gonadotropin stimulation did indeed lead to DNA methylation disruptions and/or abnormal allelic-specific expression of *H19, Snrpn,* or *Peg3* in the somatic tissues of superovulation-derived females and males (six of the eight mice). The investigators concluded that superovulation may contribute to the induction of epimutations during ART procedures.

It has been generally acknowledged that, due to reprogramming erasure, epigenetic errors are not transmitted to the next generation (70). This was recently demonstrated by de Waal and colleagues in the mouse model after ICSI (71) and superovulation treatments (69). They reported that epimutations induced by superovulation or ICSI are corrected in the male germline in adult males. However, for *H19* gene they observed delayed reprogramming in spermatogenetic cells for both superovulation and ICSI, likely due to delayed remethylation of the maternal DMR in 100% (seven of seven) of the juvenile superovulation-derived mice (69).

Previously, Stouder et al. (72) reported that transgenerational effects of superovulation may also exist. Altered methylation patterns of paternally and maternally imprinted genes were observed in the offspring sperm of superovulation-derived mice. It also has been suggested that in some cases the epigenetic defects can be transgenerational. Because the exposure of pregnant female rats to fungicide endocrine disruptors during the period of gonadal sex determination results in altered DNA methylation profiles of the male germline for at least three generations (73), one cannot totally exclude that such transgenerational effects also exist after superovulation.

Limited data are available in human offspring concerning the epigenetic effects of hormone treatment. Recently, in a birth cohort, Rancourt et al. (7) found that the methylation levels across the ICRs of *GRB10, MEST, H19, SNRPN,* and *KCNQ1* as well as *IGF2* DMR0 were not disrupted by ovulation induction alone in cord blood and placenta. However,

these reassuring findings do not allow us to assess the impact on genomic imprinting.

CONCLUSION

In assisted reproduction treatments, it is difficult to determine whether epimutations found in ART infants are a consequence of the ART procedures or are inherent in the infertility problems per se. In addition, it is currently unclear at which stage(s) during the ART procedures the epigenetic alterations could arise. However, several factors seem to indicate that ovarian induction may contribute to an increased incidence of epimutations. First, the acquisition of maternal imprints (which are present at most of the imprinting control regions) occurs over a relatively long period of time and thus is potentially more exposed to disturbance. Second, the exogenous gonadotropins used to increase the number of embryos available for transfer may force the growth of nearly all the obtained oocytes normally destined for destruction. Last, the hormone treatments may modify the kinetics of oocyte maturation by inducing accelerated follicular growth in some cases (74). As previously mentioned, it appears that the acquisition of methylation in the oocyte is a complex phenomenon that requires above all proper oocyte growth. Human oocytes may be more prone to epigenetic errors and/or may encounter more stressors—such as multiple hormone administration, advanced maternal age, environmental factors, or inherent infertility (75). Animal studies excluding infertility effects have highlighted the negative impact of ovulation induction per se in this critical period.

The use of gonadotropins in ART may cause the release of some MII oocytes with incomplete or labile imprints. In addition, several studies in humans and mice have suggested that exogenous gonadotropins may induce molecular changes in the oocyte that could have a negative impact on the maintenance of genomic imprints during subsequent embryogenesis. As proposed by Mann's group, considering the frequency of epigenetic perturbations in embryos at the blastocyst stage compared with oocytes, ovarian stimulation may have a greater adverse impact on the maternal factors required for imprint maintenance than on imprint acquisition (76). Thus, hormone treatments may be prejudicial to the epigenetic reprogramming of the gametes as well as the early embryos. However, other epigenetic factors remain totally unexplored in this topic, such as noncoding RNAs or histone modifications. The deleterious effects may also alter the physiologic environment of the uterus.

The observation that epimutations can arise in offspring from superovulated females and that the defect can be transgenerational has raised the concern that hormone treatments may lead to long-lasting detrimental effects. This strongly supports the need for further studies on the health of children born after ART.

Finally, the few data available in humans do not allow us to independently determine the impact on genomic imprinting of the woman's age, infertility problems, or the treatment protocols and doses of hormones, so further human studies are needed. Because superovulation may have deleterious effects on imprinting maintenance, research in

humans needs to be performed not only on oocytes but also on embryos. Better knowledge can improve risk assessment among ART practitioners of the impact of ovarian induction protocols on epigenetic control of the genome.

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