Anim. Reprod, v.7, n.3, p.154-164, Jul./Sept. 2010

Effects of exogenous endocrine stimulation on epigenetic programming of the female germline genome

E. de Waal[1], J.R. McCarrey[1]

Department of Biology, University of Texas, San Antonio, TX 78249, USA.

Abstract

Although assisted reproductive technologies (ARTs) have allowed millions of otherwise infertile couples to conceive children of their own, concerns remain about the safety of these procedures due to an increased incidence of epigenetic disorders in children born following the use of ART. Specifically, abnormal genomic imprinting and/or diseases caused by abnormal imprinting have been reported. While the frequencies of these defects among all ART offspring remain very low, studies have shown that children born using ARTs can be up to six times more likely to develop certain imprinting disorders than those who are naturally conceived. In addition, studies of animals produced from ART-derived embryos and/or superovulated oocytes have revealed abnormal allele-specific expression and DNA methylation profiles at imprinted genes. Many different aspects of ART procedures have been implicated in the etiology of imprinting disorders. However, it remains difficult to distinguish between abnormalities that develop as a result of inherent consequences of infertility and those induced directly by ART procedures. In support of the latter, there is a growing body of evidence suggesting that the use of exogenous gonadotropins to stimulate folliculogenesis (superovulation) in females undergoing ARTs may contribute to the induction of abnormal genomic imprinting. The association between superovulation and imprinting disorders is difficult to fully assess because of the high variability in ART protocols, especially those applied to human patients, and the small number of animal studies published to date. However, because the use of ARTs is becoming increasingly prevalent in developed countries, and ovarian stimulation is typically an indispensable part of these procedures, further investigation into the potential for these procedures to induce epigenetic defects is highly warranted. Here, we review the existing literature suggesting a potential causal relationship between endocrine stimulation and the induction of imprinting abnormalities. In addition, we suggest directions for future research in this area.

Keywords: Assisted reproductive technology, DNA methylation, epigenetic reprogramming, genomic imprinting, imprinting disorders.

Introduction

Over the last three decades, assisted reproductive technologies (ARTs) have become an invaluable tool for couples that want to have a biological child but lack fecundity. The birth of Louise Brown in 1978 through the use of *in vitro* fertilization (IVF) initiated an era of sophisticated medical procedures to overcome infertility. Since then, more advanced technologies have been developed, such as intracytoplasmic sperm injection (ICSI) and *in vitro* oocyte maturation (IVM), which have allowed even more otherwise infertile couples to become parents (Fig. 1). Many developed countries now offer a wide range of ARTs that can treat several different causes of subfertility or infertility. Looking forward, it may eventually become common to derive mature gametes from stem cells (Zhou *et al*., 2010), foreshadowing a time when any couple, including those who are otherwise naturally subfertile, will be able to conceive a biological child. As ARTs have become more sophisticated and widespread over the years, the popularity of these techniques has increased as well. During the last 30 years, well over 3 million children have been born using some form of ART (Horsey, 2006). In the US alone, the number of ART births increased from 4,000 in 1990 to 40,000 in 2001 (American Society for Reproductive Medicine/Society for Assisted Reproductive Technology, 2007). The increased use of these highly complex techniques worldwide is a testament to their acceptance and perceived safety by the public. Interestingly, because treatments for infertility circumvent natural barriers to reproduction, their use enhances propagation of preexisting infertility phenotypes to offspring in the human population. In addition, however, it is also possible that the use of ARTs may introduce new defects de novo, thus potentially further increasing the incidence of genetic or epigenetic defects in offspring produced by these methods.

The possibility that ex vivo manipulations associated with ARTs may induce genetic and/or epigenetic abnormalities in offspring represents a persistent and even growing concern in the field. That such defects are more likely to be epigenetic than genetic is supported by the study of Caperton *et al*. (2007) that showed that the use of ARTs does not increase the frequency of spontaneous point mutations in ART offspring in mice. In contrast, there are several reports that children conceived through various forms of ART are at an increased risk of developing epigenetic imprinting disorders such as Beckwith-Wiedemann Angelman Syndrome (Cox *et al*., 2002; Orstavik *et al*., 2003), and Silver Russell Syndrome (Svensson *et al*.,

[1]Corresponding authors: John.McCarrey@utsa; abrickindewaal@hotmail.com

2005; Kagami *et al*., 2007). While the absolute risk of ART children developing these rare epigenetic disorders is quite low, the increased incidence of imprinting errors associated with ART births remains troubling. In addition, studies in animal models have directly demonstrated that certain aspects of the ART procedure may induce imprinting errors. For instance, certain culture conditions in which ART embryos are maintained have been found to alter allele-specific expression and DNA methylation profiles at imprinted genes (Doherty *et al*., 2000; Mann *et al*., 2004), as well as influencing the performance of rodents in certain behavioral tests (Ecker *et al*., 2004). Interestingly, the stage at which embryos are normally maintained in culture as part of the ART methodology correlates with the timing of genome-wide epigenetic reprogramming that occurs during the early stages of embryogenesis, suggesting that this developmental period is particularly sensitive to perturbations of environmental conditions that can induce epigenetic defects in the offspring. This view is further supported by the observation that mouse embryos derived from naturally fertilized eggs and subsequently transferred to surrogate females also displayed abnormal genomic imprinting (Rivera *et al*., 2008). Since the embryos in this study were maintained only transiently in culture, it may be that other aspects of the ART process also contribute to the induction of epigenetic abnormalities.

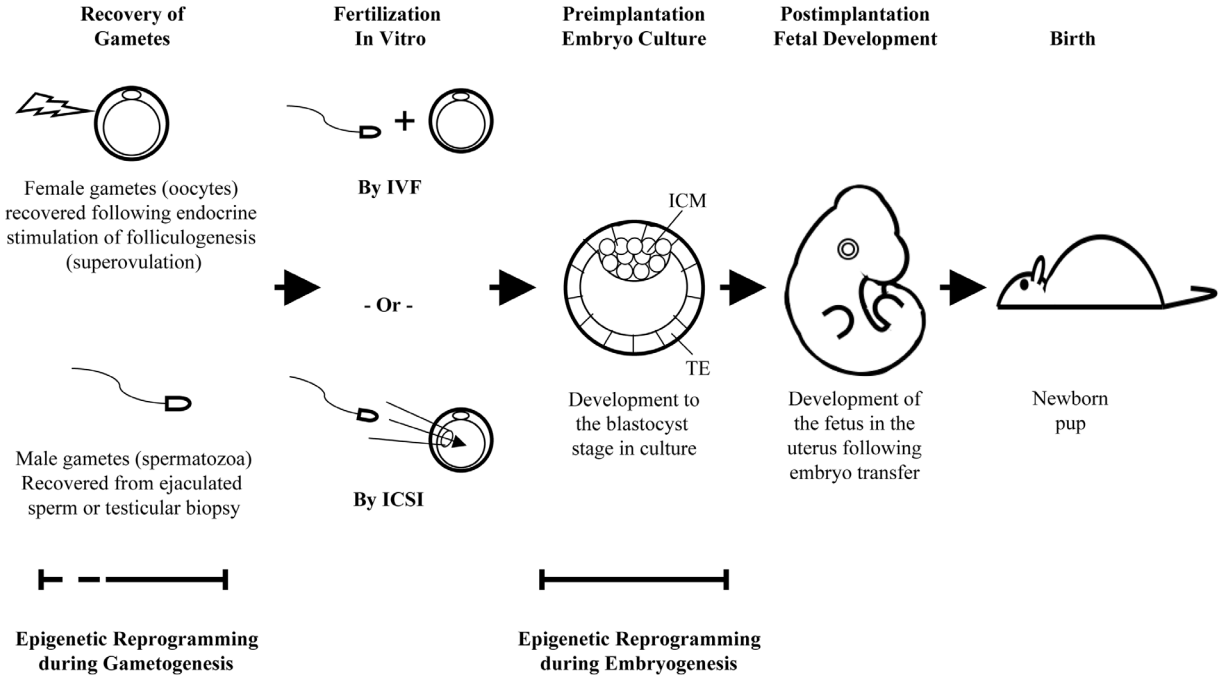

Figure 1. Schematic representation of Assisted Reproductive Technologies (ARTs). Two commonly used ARTs are depicted - *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). For both techniques, female gametes (oocytes) are typically induced to mature by administration of exogenous endocrine stimulation (typically gonadotropins) prior to recovery (indicated by lightning arrow). Male gametes are typically recovered from ejaculated sperm or testicular biopsy. For IVF, sperm and oocytes are mixed in a Petri dish to facilitate fertilization via normal sperm-egg interaction. For ICSI, a single spermatozoon is physically injected into each oocyte. Following fertilization, preimplantation development is allowed to proceed in culture, typically to the blastocyst stage and this is followed by embryo transfer to place the embryo into a female uterus to facilitate postimplantation development of the fetus and subsequent birth of a newborn. Two periods of significant epigenetic reprogramming of the genome are shown – one during gametogenesis and one during embryogenesis.

In addition to culture, another common aspect of the ART process is the use of exogenous endocrine stimulation with gonadotropins to stimulate folliculogenesis to allow retrieval of multiple oocytes that can be fertilized to produce embryos for assisted reproduction. Therefore, this represents another possible source of epigenetic defects observed in ART offspring. Sex-specific imprints are typically not established in oocytes until about the time of ovulation, coinciding with the end of the normal period of folliculogenesis and oocyte maturation (Obata *et al*., 1998). Because the use of exogenous gonadotropins to enhance folliculogenesis could lead to accelerated follicle development in the absence of complete oocyte

maturation, it may be that these oocytes are ovulated prior to completion of epigenetic reprogramming and resetting of biallelic maternal imprints. In this review, we summarize the existing evidence suggesting an association between exogenous endocrine stimulation of folliculogenesis and abnormal imprinting in ensuing offspring. We also suggest additional approaches to determine whether or not a true cause-and-effect relationship exists between exogenous endocrine stimulation and induction of abnormalities in epigenetic programming.

Mechanisms of genomic imprinting

Most autosomal genes are either repressed or expressed simultaneously from both parental alleles (biallelic repression or biallelic expression). In a small subset of genes, however, one allele is selectively silenced in a parent-of-origin specific manner by a process called genomic imprinting. Genomic imprinting refers to the functional asymmetry of parental genomes conferred through epigenetic marks that distinguish maternal and paternal alleles (Reik and Walter, 2001). This phenomenon was first discovered by A. Surani and D. Solter and colleagues (McGrath and Solter, 1984; Surani *et al*., 1984) whose labs each independently demonstrated the non-equivalency of parental genomes by using nuclear transplantation to generate monoparental embryos that exhibited opposing phenotypes. Thus, gynogenetic embryos (produced from two maternal pronuclei) developed fairly normal appearing embryos, but failed to develop normal extra-embryonic membranes and structures, while androgenetic embryos (produced from two paternal pronuclei) were profoundly growth retarded, but developed relatively normal extra-embryonic tissues. These studies demonstrated the surprising concept that maternal and paternal genomes are nonequivalent in their contribution to normal embryogenesis, and the fact that parental nuclei from both sexes are needed for proper development. Experiments based on a variety of different approaches provided a genetic/epigenetic explanation for this phenomenon by revealing specific subregions of the genome that require both maternal and paternal contributions for proper development (Searle and Beechey, 1978; Cattanach and Kirk, 1985). This was followed by the identification of specific (imprinted) genes that required biparental contribution for proper development of ensuing offspring (Surani *et al*., 1990; Bartolomei *et al*., 1991; Barton *et al*., 1991). It was then shown that these genes are normally expressed monoallelically from either the maternal or paternal alleles (but not both), depending on the specific imprinted gene investigated (Barlow *et al*., 1991; Bartolomei *et al*., 1991; DeChiara *et al*., 1991). These genes have since been shown to play important roles in embryonic growth, placental function, and postnatal behavior (Rappolee *et al*., 1992; Guillemot *et al*., 1995; Bartolomei and Tilghman, 1997; Isles and Wilkinson, 2000). To date, approximately 100 imprinted genes have been identified in the mouse, and many of these genes are conserved and imprinted in humans as well (Morison *et al*., 2005).

That imprinted genes are expressed in a monoallelic pattern, even in inbred lines of mice where both alleles are identical at the level of DNA sequence, suggests that maternal and paternal alleles are differentially marked by epigenetic modifications and that such "parent-of-origin-specific" epigenetic reprogramming of these alleles must occur during gametogenesis in each sex. The most prominent differential epigenetic modification observed at or around imprinted genes is DNA methylation (Bartolomei *et al*., 1993; Brandeis *et al*., 1993), and this differential DNA methylation appears to play a critical role in regulating allele-specific expression at imprinted genes (Li *et al*., 1993). Interestingly, the majority of imprinted genes are not isolated, but rather occur in clusters with other imprinted genes (Reik and Walter, 2001). This clustered organization is believed to reflect coordinated regulation that is most often controlled by a single differentially methylated region (DMR) found within the cluster, also known as an imprinting control region (ICR). It is believed that sex-specific, differential methylation at DMRs is established in the germ line during gametogenesis in each sex when the parental genomes are segregated in different compartments and can be modified independently of one another. Because either allele in the germ line has an equal opportunity of contributing to the next generation, resetting of epigenetic programming must involve biallelic methylation, or lack thereof, at each imprinted locus during gametogenesis. Once sex-specific imprints have been established in the gametes, allele-specific methylation at DMRs can then be transmitted to an ensuing zygote following fertilization. If differential methylation is properly maintained throughout development, then monoallelic expression will be tightly regulated, typically with the methylated allele repressed and the unmethylated allele expressed, such that the organism can develop normally.

DNA methylation is carried out by a class of enzymes termed DNA methyltransferases (DNMTs). There are multiple genes in mammalian genomes encoding these enzymes, including *Dnmt1, Dnmt3a* and *Dnmt3b* (Bestor *et al*., 1988; Okano *et al*., 1998). Each of the methyltransferases encoded by these genes plays an important role in the establishment and/or maintenance of DNA methylation patterns throughout the genome and at imprinted loci in particular (Bestor *et al*., 2000). *Dnmt1* encodes the most abundant DNMT, which is the primary maintenance methyltransferase responsible for maintenance methylation after DNA replication because it predominantly methylates hemimethylated CpG sites in the mammalian genome (Yoder *et al*., 1997**)**. Thus, because DNMT1 localizes to

the replication fork during DNA replication, any preexisting fully methylated CpG site will be propagated as such by DNMT1-mediated maintenance methylation (Bostick *et al*., 2007; Sharif *et al*., 2007). The other DNMTs, 3a and 3b, are believed to be responsible for de novo methylation in the postimplantation embryo, including mediating the establishment of sex-specific imprints in the female and male germ lines (Okano *et al*., 1999; Kaneda *et al*., 2004). Because DNMT3a and 3b are known to methylate specific loci in the embryo and target only certain DMRs in the female and male germ lines, there must be some mechanism that confers sequence-specific activity of de novo methylation. Another member of the DNMT3 family, DNMT3L, lacks catalytic activity but has been shown to be crucial as a type of cofactor for maternal imprinting in the female germ line, and its disruption in the male germ line causes reactivation of retrotransposons (Bourc'his *et al*., 2001; Bourc'his and Bestor, 2004). In addition to the contribution of Dnmt3L in this process, recent data support a scenario in which both cis- and transacting factors recruit DNMTs to facilitate maintenance methylation at certain DMRs of imprinted genes (Reese *et al*., 2007; Nakamura *et al*., 2007; Li *et al*., 2008). Therefore, it seems plausible that germ cells and the early embryo must carefully regulate not only the expression of *Dnmt* genes, but also that of the genes encoding accessory proteins that control the specificity of DNMTs to establish and preserve the proper DNA methylation patterns (imprints) during gametogenesis. If there is an error in this process (or if this process is prematurely interrupted), the transmission of properly imprinted gametic genomes to the ensuing generation will be deleteriously affected. In turn, this can lead to significant developmental or postnatal consequences resulting from aberrant gene expression patterns that reflect the abnormal DNA methylation pattern and these could predispose developmental or birth defects and/or postnatal or adult onset diseases.

Epigenetic reprogramming in the germ line and early embryo

In mammals, epigenetic reprogramming occurs at stages when the developmental potency of cells must change. The process of reprogramming typically involves erasure of existing epigenetic marks followed by establishment and subsequent maintenance of new epigenetic profiles. Genome-wide reprogramming occurs during two periods of mammalian development - early embryogenesis and development of germ cells. The first phase of epigenetic reprogramming occurs during preimplantation development. At fertilization, two highly differentiated gametic genomes converge in a single cell (the fertilized egg) and must be reprogrammed to facilitate totipotency in the zygote so that embryogenesis may proceed normally. Immediately after fertilization, the paternal pronucleus undergoes rapid genome-wide demethylation (Morgan *et al*., 2005). Since this process takes place in the absence of transcription and DNA replication it is called "active" demethylation. During the subsequent cleavage divisions, passive demethylation takes place on the maternal genome by exclusion of DNMT1, and hence, maintenance methylation activity, from the nucleus (Morgan *et al*., 2005). Thus, as the early embryo divides, the newly replicated strands fail to become methylated and the level of CpG methylation declines. This genome-wide demethylation in the preimplantation embryo is believed to contribute to activation of the expression of pluripotency genes that are needed for embryogenesis, but not all loci undergo reprogramming at this stage. Retrotransposons, certain repeat elements, and the DMRs of imprinted genes all escape the normal genome-wide demethylation event in the preimplantation embryo (Reik and Walter, 2001). Uninterrupted DNA methylation is important for suppression of expression and propagation by transposition of certain repeated elements and retrotransposons. It is also critically important to maintain a heritable distinction between the paternal and maternal alleles of each imprinted gene.

In the mouse, genome-wide demethylation during the zygote and early cleavage stages is followed just a few days later by global de novo methylation in the late blastocyst (Reik *et al*., 2001). The blastocyst is made up of two primary cell lineages - the inner cell mass (ICM) and the trophectoderm (TE; Morgan *et al*., 2005; Fig. 1). The ICM, which gives rise to all the tissues of the embryo proper and eventually the adult organism, becomes hypermethylated, while the TE, which forms the extra-embryonic tissues, remains relatively hypomethylated. The extra-embryonic tissues and placenta exist only transiently and do not contribute to adult tissues, thus long-term epigenetic reprogramming many not be critical for these tissues. However, the ICM gives rise to both the somatic tissues and the germ cells of the organism, so maintaining the inherited parental allele-specific methylation profiles in the ICM is crucial for normal development. Any perturbation of allele-specific, differential methylation at DMRs could cause abnormal expression of imprinted genes that could lead to deleterious developmental consequences. Because the epigenome is naturally very labile (having to undergo massive reprogramming during each generation) it is more susceptible than the genome to disruption by various causes including exposure to certain environmental agents. Thus, any environmental influence that disrupts the epigenetic reprogramming process in germ cells or in the early embryo could potentially alter genomic imprinting during development, and this could induce a disease phenotype manifest either during development or subsequently in the postnatal or adult offspring.

Allele-specific methylation at imprinted loci

inherited from the egg and sperm is faithfully maintained in the somatic lineages of the ensuing generation, but is erased and replaced by sex specific, biallelic methylation or biallelic absence of methylation in the germ line. Thus, during early germ cell development in the mouse, primordial germ cells migrate to the genital ridge and undergo genome-wide demethylation between embryonic day (E) 10.5 and E12.5 in both sexes (Hajkova *et al*., 2002). This leads to transient biallelic hypomethylation and potential expression from both parental alleles at imprinted loci (Szabo and Mann, 1995; Yamazaki *et al*., 2003). During subsequent stages of development, sex-specific biallelic methylation imprints are established in germ cells, but the timing of this imprint acquisition is different in the male and female germ lines. In male germ cells, remethylation of paternally imprinted genes is largely completed during the prenatal stages (Davis *et al*., 1999; Shamanski *et al*., 1999). In contrast, much of the methylation that marks maternally imprinted genes is acquired postnatally in the female germ line, especially during maturation from primordial to antral follicles (Obata *et al*., 1998; although some form of epigenetic memory must persist from the preceding maternal germ line to signal this). Therefore, establishment of maternal imprints is normally not complete until nearly the time of ovulation of each mature oocyte (Lucifero *et al*., 2004). One report has even suggested that some maternal imprints are not fully manifest until after fertilization in humans (El-Maari *et al*., 2001). Although the timing of imprint acquisition is different in the male and female germ lines, at the completion of gametogenesis both sperm and oocytes typically possess sex-specific, biallelic imprints (or signals thereof). This epigenetic reprogramming process during gametogenesis ensures that upon fertilization, the zygote will inherit parent-of-origin-specific, differential methylation at DMRs that will subsequently regulate the critically required monoallelic expression of imprinted genes throughout development.

ART and imprinting disorders

The use of ART recapitulates many of the normal in vivo processes involved in natural reproduction. The timing of two key techniques commonly used during ART, ovarian stimulation and *in vitro* culture, coincides with important epigenetic programming events that naturally occur during gametogenesis and early embryonic development, in vivo, respectively (Fig. 1). It is, therefore, possible that these ex vivo manipulations or other aspects of the ART procedure could disrupt crucial epigenetic programming events at imprinted loci before and/or after fertilization. In support of this theory, many studies have found an increased incidence of epigenetic disorders in children born following the use of ART. While the association between ARTs and imprinting disorders continues to be debated due to the lack of large, well controlled, multicenter studies of specific cohorts of ART-conceived children, a growing body of evidence supports a causal relationship between ART and an increased incidence of imprinting errors.

Several reports have been published suggesting that there may be an association between ART and Angelman Syndrome (AS). AS is a rare neurogenetic syndrome with an estimated prevalence in the general population of 1 in 15,000 (Williams, 2005). The syndrome is believed to be caused by a loss of function of the *Ube3a* gene in the brain. *Ube3a* is normally monoallelically expressed from the maternal allele in brain tissue, and there is tentative evidence that the paternal allele is silenced by an antisense RNA originating from the *Snrpn* locus (Runte *et al*., 2001; Landers *et al*., 2004). This monoallelic expression of *Ube3a* can be altered through both genetic and epigenetic mechanisms. About 70% of children with AS have either a maternal deletion or a uniparental disomy on chromosome 15, which corresponds to the map position of the *Ube3a* gene (Maher, 2005). Imprinting defects at the *Snrpn* gene have also been identified in the general population as a potential cause of AS, but evidence suggests this may explain less than 5% of cases (Maher, 2005). In 2002, a study by Cox *et al*. (2002) found two children conceived by ICSI who developed AS. They demonstrated that both cases were associated with hypomethylation at the *Snrpn* gene, suggesting a sporadic imprinting defect on the maternal chromosome. However, since the fathers of both patients used ICSI because of male factor infertility, the possibility that these imprinting errors arose from an indirect effect of oligospermia could not be excluded. Alternatively, there is the possibility that an imprinting defect was introduced in the maternal germline genome by the use of ART such that the occurrence of AS was unrelated to the oligospermic phenotype in the fathers. Orstavik *et al*. (2003) reported a third case of a girl with AS following conception by ICSI that also showed hypomethylation at the *Snrpn* locus suggesting an association between abnormal imprinting and the disease phenotype. In contrast to the report by Cox *et al*. (2002), the biological father in this instance had a normal sperm analysis on three different occasions, but the mother was diagnosed with reproductive defects (Orstavik *et al*., 2003). This implies that male infertility is most likely not the sole source of imprinting errors and other factors are involved in the induction of epimutations during ART. The results presented by Cox *et al*. (2002) and Orstavik *et al*. (2003) were quite significant given that the expected incidence of AS caused by an epimutation is about 1 in 300,000, but at the time these authors published their results there were only about 1,000,000 children worldwide that had been born through some form of ART (Schultz and Williams, 2002). Thus, because these studies sampled only a small subset of the total population of ART children at the

time, it appears they detected an increased frequency of AS associated with imprinting errors in ART-conceived children.

Reports of a link between ART and a second classical imprinting disorder, Beckwith-Wiedemann Syndrome (BWS), have reinforced concerns about the safety of ARTs. BWS is a heterogenous congenital overgrowth disorder with an estimated incidence of 1 in 13,000 births (Laprise, 2009). Approximately half of BWS cases are caused by an imprinting defect at one of two imprinted loci, the *Kcnq1ot1* DMR (also called *KvDMR1*) or the *H19/Igf2* DMR (Maher, 2005). The most common etiologic factor (40% of BWS cases) is the loss of maternal methylation at *KvDMR1*, while gain of maternal methylation at the *H19/Igf2* DMR accounts for only about 5% of cases (Maher, 2005). In 2003, DeBaun *et al*. published a case report that found seven children with sporadic BWS born after ART in a BWS registry that was created in 1994. The authors estimated the incidence of BWS in ART children was six-fold greater than that in the general population (DeBaun *et al*., 2003). A molecular analysis was performed on six of the seven affected children to elucidate the cause of their BWS. Interestingly, all six patients demonstrated imprinting errors at one or both of the DMRs. Five of the six children displayed abnormal methylation patterns at the KvDMR1 while one child exhibited imprinting errors at both the *H19/Igf2* DMR and the *KvDMR1*. In 2005, Chang *et al*. re-examined the same BWS registry but expanded the criteria of ART children to include those generated by artificial insemination and/or ovarian stimulation. Using these criteria, the authors identified 19 BWS children born after ART in the BWS registry. Subsequently, records for 12 of the 19 patients with BWS were obtained and it was determined that five of these children had been born using ICSI, five with IVF, and two with superovulation followed by intrauterine insemination. Interestingly, the only common attribute identified in the 12 cases was the use of exogenous drugs to stimulate folliculogenesis (11 gonadotropin, one clomiphine citrate).

That procedures associated with ART may specifically introduce imprinting errors is indicated by the fact that a significantly larger proportion of ART-conceived children with certain imprinting disorders, such as AS and BWS, have alterations in DNA methylation patterns (epimutations), whereas a larger proportion of cases of these same diseases in non-ART offspring show genetic defects (mutations) such as gene deletions (Laprise, 2009). To the extent that there is an association between ART and imprinting disorders, the challenge will be to identify the causative aspect(s) of ART procedures. Currently, exogenous endocrine stimulation of folliculogenesis and/or maintenance of preimplantation embryos in culture appear to be the leading candidates for potentially deleterious aspects of ARTs. However, one study found no association between the incidence of BWS and the culture conditions used to maintain embryos, thus favoring the concept that ovarian stimulation may be the most common source of imprinting errors induced during the use of ARTs (Chang *et al*., 2005). In addition, many of the ART-associated AS and BWS cases involved an epimutation on the maternal allele suggesting that the imprinting defect may originate in the oocyte. Given these observations, and others, it seems plausible that the use of exogenous hormones to promote the production of multiple mature oocytes may indeed induce imprinting errors that can subsequently lead to abnormalities, syndromes or diseases during subsequent development or after birth in children conceived through ART.

The effect of endocrine stimulation on genomic imprinting

Endocrine stimulation of the ovary to induce maturation of multiple oocytes is an integral part of most ART procedures to improve pregnancy success. Unfortunately, many studies in mice have collectively demonstrated that such stimulation can adversely affect oocyte quality, embryonic development, birth weight, and/or DNA methylation profiles at imprinted loci (Ertzeid and Storeng, 2001; Van der Auwera and D'Hooghe, 2001; Sato *et al*., 2007). In regard to the latter, as noted above maternal imprints are established largely postnatally, during maturation of the oocyte, which makes the imprinting process in the female germ line vulnerable to environmental influences during folliculogenesis. Because only a small cohort of follicles/oocytes is induced to mature during each cycle (even following stimulation with exogenous gonadotropins), the opportunity for environmentally-based induction of epigenetic errors (epimutations) in the maternal germ line recurs during each stimulation cycle. Indeed, it may be that the endocrine-induced acceleration of folliculogenesis is actually not accompanied by equally accelerated epigenetic reprogramming of the oocyte genome, such that superovulated oocytes may possess incompletely programmed maternal genomes. In support of this theory, several reports have been published in the last 10 years suggesting a link between superovulation and imprinting errors in oocytes, the early embryo, and/or placental tissue.

The observation that ART-associated AS and BWS most likely involve an imprinting error on the maternal allele gave credence to the notion that the loss of imprinting may originate in the oocyte in children conceived through ART. In addition, Shi and Haaf (2002) demonstrated that abnormal DNA methylation occurred at a higher rate in two-cell mouse embryos when the dams were subjected to hormonal stimulation regimens, compared to embryos produced from natural cycles with no exogenous endocrine stimulation. Importantly, this observation provided direct evidence

suggesting that imprinting defects can be induced solely by exposure to exogenous hormones. Further evidence of this sort was provided by experiments performed by Sato *et al*. (2007), which revealed abnormal DNA methylation profiles at imprinted loci in superovulated oocytes. In this study, DNA methylation was analyzed at the DMRs of four imprinted genes, *Peg1*, *Kcnq1ot1*, *Zac*, and *H19.* Methylation analysis was performed on human and mouse oocytes recovered from females that had undergone endocrine stimulation. Interestingly, these authors observed a gain of methylation at the *H19* DMR following ovarian stimulation in human and mouse oocytes which was surprising since the *H19* DMR is paternally imprinted, and is normally completely unmethylated in oocytes. The authors also detected a loss of methylation at the *Peg1* DMR in superovulated human oocytes, possibly reflecting the late imprint acquisition normally observed at this locus (Lucifero *et al*., 2004; Sato *et al*. 2007). In contrast to this report, Anckaert *et al*. (2009) analyzed DNA methylation at the DMRs of four imprinted genes, *Snrpn*, *Igf2r*, *Peg3* and *H19,* in superovulated mouse oocytes, but did not find any imprinting abnormalities. This discrepancy may be explained by the difficulty of detecting a low incidence of imprinting errors in pooled samples. Sato *et al*. (2007) found imprinting defects in samples containing 30-50 mouse oocytes and in individual human oocytes, whereas Anckaert *et al*. (2009) analyzed samples containing 100-150 mouse oocytes per pool. Since Shi and Haaf (2002) found that only 20% of embryos from endocrine-stimulated females developed imprinting errors, it is possible that the large pools of samples in the Anckaert *et al*. (2009) study may have obscured the low frequency of oocytes containing imprinting errors in their pooled samples. Nevertheless, further studies are needed to clarify this contradiction.

If the use of ovarian stimulation can influence genomic imprinting in the female germ line, then it seems likely that these abnormalities should also be observed in the embryo and extra-embryonic tissues due to the heritability of epigenetic programming. To evaluate the impact of superovulation on embryonic development Faque *et al*. (2007) analyzed DNA methylation at the *H19* DMR as well as expression of the *H19* gene in individual blastocysts generated from superovulated and non-superovulated female mice. They found that superovulation reduced *H19* expression in blastocysts, but this abnormal expression of *H19* was not correlated with aberrant DNA methylation (Faque *et al*., 2007). Nevertheless, the diminished expression of *H19* in early embryos derived from superovulated oocytes suggests that imprinting defects induced in the female germ line by exposure to exogenous hormones are maintained after fertilization during embryonic development (Faque *et al*. 2007). In another study, Market-Velker *et al*. (2010) analyzed allele-specific DNA methylation at the DMRs of four imprinted genes, *Snrpn*, *Peg3*, *Kcnq1ot1*, and *H19*, in individual blastocysts from either spontaneously ovulated or superovulated oocytes. They found an increased incidence of abnormal DNA methylation profiles at all four imprinted genes in blastocysts from superovulated oocytes and there appeared to be a dose-dependent effect, with imprinting errors occurring more frequently in those embryos produced from dams that received a higher dosage of hormone (Market-Velker *et al*., 2010). Loss of methylation at the maternal allele was observed at the *Snrpn*, *Peg3*, and *Kcnq1ot1* DMRs, while a gain of maternal and loss of paternal methylation was observed at the *H19* DMR (Market-Velker *et al*., 2010). This was a surprising result because it was believed that the effects of superovulation were limited to the maternal allele due to the vulnerability of imprint acquisition during oocyte maturation. However, since the paternal alleles of *H19* also demonstrated imprinting abnormalities, superovulation may disrupt the maintenance of differential methylation during preimplantation development as well. In order to study the effects of ovarian stimulation during the postimplantation period of development, Fortier *et al*. (2008) examined allele-specific DNA methylation at the *H19* and *Snrpn* DMRs as well as allele-specific expression of the *Snrpn*, *H19*, and *Kcnq1ot1* genes in the embryo and placenta from offspring of superovulated dams after 9.5 days of gestation. The authors did not observe any epigenetic abnormalities in the embryos (Fortier *et al*., 2008). In contrast, biallelic expression of *H19* and *Snrpn* was detected in the placenta after superovulation and in vivo development, but the abnormal expression was not correlated with aberrant DNA methylation (Fortier *et al*., 2008). This data suggests that extra-embryonic membranes and structures may be more susceptible to imprinting errors than the embryo proper, which likely reflects the lower genome-wide level of DNA methylation indicative of a less stable, more transient form of epigenetic programming in these tissues. Nevertheless, this heightened potential for imprinting errors in the extra-embryonic tissues could be particularly significant because expression of many imprinted genes is critical to normal placental function.

Conclusions and future direction

Taken together, the results summarized above document the occurrence of epigenetic abnormalities in the oocyte, embryo, and/or placenta following stimulation of folliculogenesis by administration of exogenous gonadotropins in conjunction with ART procedures. These observations raise the concern that such endocrine stimulation used to induce superovulation has the potential to disrupt normal epigenetic programming in the female germline genome during oogenesis in a manner that can lead to immediate developmental defects or subsequent postnatal or adult

onset of certain disease states. In turn, this raises concerns about the safety of assisted reproductive technologies and suggests that further research is warranted to determine the extent to which endocrine stimulation of folliculogenesis may be deleterious and/or how this effect might be minimized or prevented.

Specifically, these results support the hypothesis that an increased risk of imprinting disorders in ART-conceived children is due, at least in part, to the use of exogenous hormones to stimulate folliculogenesis. Further evidence comes from the observation that a high proportion of these ART-associated imprinting disorders appears to be caused by epimutations that 1) are not commonly found in the general population and 2) a majority of which occur on the maternal allele. It is also noteworthy that the timing of ovarian stimulation to enhance production of oocytes that will give rise to children born using ART coincides with the normal timing of natural imprint acquisition in the female germ line. In addition, because exogenous gonadotropins stimulate maturation of a greater number of follicles than normally mature during any single natural cycle, it may be that this leads to the premature ovulation of oocytes that have not yet completed the epigenetic programming process. Alternatively, hormonal stimulation may actually "rescue" poor quality follicles that would have otherwise undergone atresia, and these may carry incompletely or improperly programmed maternal genomes. In any of these scenarios, exogenous stimulation of folliculogenesis has the potential to promote ovulation of poor quality oocytes that may not have fully established the sex-specific, biallelic maternal imprints needed to ensure normal development of the ensuing embryo.

Despite the several reports that have been published demonstrating an association between ovarian stimulation and the induction of imprinting errors, the actual incidence of imprinting defects in oocytes and embryos from superovulated females appears to be low and stochastic (Sato *et al*., 2007; Anckaert *et al*., 2009; Market-Velker *et al*., 2010). This suggests that the vast majority of oocytes collected after hormonal stimulation have established normal, biallelic maternal imprints, and only a small percentage, if any at all among any particular group of oocytes recovered, will have developed imprinting defects. Interestingly, one study found that an individual mouse blastocyst from a dam subjected to endocrine stimulation exhibited imprinting errors at multiple genes (Market-Velker *et al*., 2010), and this observation correlates with the finding that some children with ART-associated BWS display imprinting defects at both the *H19/Igf2* DMR and the *KvDMR1* (DeBaun *et al*., 2003). These studies imply that if ovarian stimulation disrupts normal epigenetic programming during the maturation of an oocyte, this can affect DNA methylation profiles at multiple imprinted loci in the same oocyte. Since many of the imprinted genes play important roles in prenatal growth and placental function, this deleterious effect on genomic imprinting in the female germ line could explain the reported increased risk of premature delivery and low birth weight in children born through ART (Sunderam *et al*., 2009). Nevertheless, while animal and human studies have provided strong support for the notion that there is an association between the use of endocrine stimulation and an increased incidence of imprinting errors, to the best of our knowledge a complete mechanistic relationship between these effects is yet to be established.

To the extent that a direct association between superovulation and imprinting disorders is confirmed, the next challenge will be to elucidate the details of the mechanism(s) involved, including how exogenous endocrine stimulation disrupts epigenetic programming, which genes are most commonly affected, how disrupting epigenetic programming at these loci affects their expression, and how abnormal programming of these genes leads to developmental or postnatal phenotypes associated with imprinting errors. Much of the data reported to date suggests that abnormal DNA methylation profiles originate in the oocyte following exogenous endocrine stimulation. However, the recent observation that imprinting errors also occur on paternal alleles in individual blastocysts from superovulated oocytes suggests that endocrine stimulation can induce imprinting defects through a process other than disrupting imprint acquisition in the female germ line (Market-Velker *et al*., 2010). The observation that transacting factors are involved in the maintenance of genomic imprints provides an alternative mechanism for induced disruption of genomic imprints (Reese *et al*., 2007). *Stella* and *Zfp57* have been identified as maternal-effect genes that contribute to the maintenance of DNA methylation in both the oocyte and preimplantation embryo (Nakamura *et al*., 2007; Li *et al*., 2008). When the expression of these genes is repressed in oocytes or zygotes, the differential methylation at the DMRs of imprinted genes is altered (Nakamura *et al*., 2007; Li *et al*., 2008). Therefore, it seems plausible that the use of endocrine stimulation could, indirectly or directly, alter the expression of maternal-effect gene products required for imprint maintenance which may, in turn, lead to abnormal DNA methylation profiles at imprinted loci during preimplantation development.

The increasing popularity of ARTs mandates that we continually monitor and optimize the safety of these procedures. Multicenter, longitudinal studies of specific cohorts of ART-conceived children are needed to determine if the use of exogenous endocrine stimulation of folliculogenesis increases the risk of developing rare epigenetic disorders, or if an increased frequency of these disorders in ART offspring is actually the result of a genetic predisposition among infertile couples. To the extent that ART procedures are implicated, it will be critical to decouple the different

aspects of each form of ART to determine the specific step(s) at which disruption of proper epigenetic programming of imprinted genes occurs. Further, because of the low incidence of such defects reported to date, studies designed to examine the effects of specific aspects of ART procedures, such as endocrine stimulation, must necessarily be performed on small sample sizes to reveal individual variation. Finally, it should be noted that recent discoveries of transgenerational propagation of epigenetic defects indicates that newly introduced germline epimutations (such as those caused by any form of ART) may have the potential to be transmitted beyond the immediate offspring to subsequent generations (Anway and Skinner, 2006). Thus, the concern about inducing errors in epigenetic programming through the use of ARTs is even more significant because such errors may become a permanent characteristic of the germline genome.

Acknowledgments

The authors wish to thank Dr. John Eppig for valuable discussions about oogenesis and opportunities during this process for introduction of epigenetic abnormalities.

References

American Society for Reproductive Medicine. Society for Assisted Reproductive Technology. 2007. Assisted reproductive technology in the United States: 2001 results generated from the American Society for Reproductive Medicine/Society for Assisted Reproductive Technology registry. *Fertil Steril*, 87:1253-1266.

Anckaert E, Adriaenssens T, Romero S, Dremier S, Smitz J. 2009. Unaltered imprinting establishment of key imprinted genes in mouse oocytes after *in vitro* follicle culture under variable follicle-stimulating hormone exposure. *Int J Dev Biol*, 53:541-548.

Anway MD, Skinner MK. 2006. Epigenetic transgenerational actions of endocrine disruptors. *Endocrinology*, 147:S43-S49.

Barlow DP, Stoeger R, Herrmann BG, Saito K, Schweifer N. 1991. The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. *Nature*, 349:84-87.

Bartolomei MS, Zemel S, Tilghman SM. 1991. Parental imprinting of the H19 gene. *Nature*, 351:153-155.

Bartolomei MS, Webber AL, Brunkow ME, Tilghman SM. 1993. Epigenetic mechanisms underlying the imprinting of the mouse H19 gene. *Genes Dev*, 7:1663-1673.

Bartolomei MS, Tilghman SM. 1997. Genomic imprinting in mammals. *Ann Rev Genet*, 31:493-525.

Barton SC, Ferguson-Smith AC, Fundele R, Surani MA. 1991. Influence of paternally imprinted genes on development. *Development*, 113:679-687.

Bestor TH, Laudano A, Mattaliano R, Ingram V. 1988. Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cell. The carboxyl-terminal domain of the mammalian enzyme is related to bacterial restriction methyltransferases. *J Mol Biol*, 203:971-983.

Bestor TH. 2000. The DNA methyltransferases of mammals. *Hum Mol Genet*, 9:2395-2402.

Bostick M, Kim JK, Esteve PO, Clark A, Pradhan S, Jacobsen SE. 2007. UHRF1 plays a role in maintaining DNA methylation in mammalian cells. *Science*, 317:1760-1764.

Bourc'his D, Xu GI, Lin CS, Bollman B, Bestor TH. 2001. Dnmt3L and the establishment of maternal genomic imprints. *Science*, 294:2536-2539.

Bourc'his D, Bestor TH. 2004. Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. *Nature*, 431:96-99.

Brandeis M, Kafri T, Ariel M, Chaillet JR, McCarrey J, Razin, Cedar H. 1993. The ontogeny of allele-specific methylation associated with imprinted genes in the mouse. *EMBO J*, 12:3669-3677.

Caperton L, Murphey P, Yamazaki Y, McMahan CA, Walter CA, Yanagimachi R, McCarrey JR. 2007. Assisted reproductive technologies do alter mutation frequency or spectrum. *Proc Natl Acad Sci USA*, 104:5085-5090.

Cattanach BM, Kirk M. 1985. Differential activity of maternally and paternally derived chromosome regions in mice. *Nature*, 315:496-498

Chang AS, Moley KH, Wangler M, Feinburg AP, DeBaun MR. 2005. Association between Beckwith-Widemann syndrome and assisted reproductive technology: a case series of 19 patients. *Fertil Steril*, 83:349-354.

Cox GF, Burger J, Lip V, Mau UA, Sperling K, Wu BL, Horsthemke B. 2002. Intracytoplasmic sperm injection may increase the risk of imprinting defects. *Am J Hum Genet*, 71:162-164.

Davis TL, Trasler JM, Moss SB, Yang GJ, Bartolomei MS. 1999. Acquisition of the H19 methylation imprint occurs differentially on the parental alleles during spermatogenesis. *Genomics*, 58:18-28.

DeBaun MR, Niemetz EL, Feinberg AP. 2003. Association of *in vitro* fertilization with Beckwith-Wiedemann syndrome and epigenetic alteration of Lit1 and H19. *Am J Hum Genet*, 72:156-160.

DeChiara TM, Robertson EJ, Efstratidiadis A. 1991. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell*, 64:849-859.

Doherty AS, Mann MR, Tremblay KD, Bartolomei MS, Schultz RM. 2000. Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. *Biol Reprod*, 62:1526-1535.

Ecker DJ, Stein P, Xu Z, Williams CJ, Kopf GS, Bilker WB, Abel T, Schultz RM. 2004. Long-term effects of culture of preimplantation mouse embryos on

behavior. *Proc Natl Acad Sci US*A, 101:1595-1600.

El-Maari O, Buiting K, Peery EG, Kroisel PM, Balaban B, Wagner K, Urman B, Heyd J, Lich C, Brannan CI, Walter J, Horsthemke B. 2001. Maternal methylation imprints on human chromosome 15 are established during or after fertilization. *Nat Genet*, 27:341-344.

Ertzeid G, Storeng R. 2001. The impact of ovarian stimulation on implantation and fetal development in mice. *Hum Reprod*, 16:221-225.

Faque P, Jouannet P, Lesaffre C, Ripoche MA, Dandolo L, Vaiman D, Jammes H. 2007. Assisted reproductive technology affects development kinetics, H19 imprinting control region methylation and H19 gene expression in individual mouse embryos. *BMC Dev Biol*, 7:116. (abstract).

Fortier AL, Lopes FL, Darricarrere N, Martel J, Trasler JM. 2008. Superovulation alters the expression of imprinted genes in the midgestation mouse placenta. *Hum Mol Genet*, 17:1653-1665.

Guillemot F, Caspary T, Tilghman SM, Copeland NG, Gilbert DJ, Jenkins NA, Anderson DJ, Joyner AL, Rossant J, Nagy A. 1995. Genomic imprinting of Mash2, a mouse gene required for trophoblast development. *Nat Genet*, 9:235-242.

Hajkova P, Erhardt S, Lane N, Haaf T, El-Maari O, Reik W, Walter J, Surani MA. 2002. Epigenetic reprogramming in mouse primordial germ cells. *Mech Dev*, 117:15-23.

Horsey K. 2006. Three million IVF babies born worldwide since 1978. BioNews, 364. Available on: http://www.bionews.org.uk/page_12772.asp. Accessed on: Aug 13th 2010.

Isles AR, Wilkinson LS. 2000. Imprinted genes, cognition and behavior. *Trends Cogn Sci*, 4:309-318.

Kagami M, Nagai T, Fukami M, Yamazawa K, Ogata T. 2007. Silver-Russel syndrome in a girl born after *in vitro* fertilization: partial hypermethylation at the differentially methylated region of PEG1/MEST. *J Assist Reprod Genet*, 24:131-136.

Kaneda M, Okano M, Hata K, Sado T, Tsujimoto N, Li E, Sasaki H. 2004. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature*, 429:900-903.

Landers M, Bancescu DL, Le Meur E, Rougeulle C, Glatt-Deeley H, Brannan C, Muscatelli F, Lalande M. 2004. Regulation of the large (approximately 1000kb) imprinted murine Ube3a antisense transcript by alternative exons upstream of Snur/Snrpn. *Nucleic Acids Res*, 32:3480-3492.

Laprise S. 2009. Implications of epigenetics and genomic imprinting in assisted reproductive technologies. *Mol Reprod*, 76:1006-1018.

Li E, Beard C, Jaenisch R. 1993. Role for DNA methylation in genomic imprinting. *Nature*, 366:362-365.

Li X, Ito M, Zhou F, Youngston N, Zuo X, Leder P, Ferguson-Smith AC. 2008. A maternal-zygotic effect gene, Zfp57, maintains both maternal and paternal imprints. *Dev Cell*, 15:547-557.

Lucifero D, Mann MR, Bartolomei MS, Trasler JM. 2004. Gene-specific timing and epigenetic memory in oocyte imprinting. *Hum Mol Genet*, 13:839-849.

Maher ER. 2005. Imprinting and assisted reproductive technology. *Hum Mol Genet*, 14:R133-R138.

Mann MR, Lee SS, Doherty AS, Verona RI, Nolen LD, Schultz RM, Bartolomei MS. 2004. Selective loss of imprinting in the placenta following preimplantation development in culture. *Development*, 131:3727-3735.

Market-Velker BA, Zhang L, Magri LS, Bonvissuto AC, Mann MR. 2010. Dual effects of superovulation: loss of maternal and paternal imprinted methylation in a dose-dependent manner. *Hum Mol Genet*, 19:36-51.

McGarth J, Solter D. 1984. Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell*, 37:179-183.

Morgan HD, Santos F, Green K, Dean W, Reik W. 2005. Epigenetic reprogramming in mammals. *Hum Mol Genet*, 14:R47-R58.

Morison IM, Ramsay JP, Spencer HG. 2005. A census of mammalian imprinting. *Trends Genet*, 21:457-465.

Nakamura T, Arai Y, Umehara H, Masuhara M, Kimura T, Taniguchi H, Sekimoto T, Ikawa M, Yoneda Y, Okabe M, Tanaka S, Shiota K, Nakano T. 2007. PGC7/Stella protects against DNA methylation in early embryogenesis. *Nat Cell Biol*, 9:64-71.

Obata Y, Kaneko-Ishino T, Koide T, Takai Y, Ueda T, Domeki I, Shiroishi T, Ishino F, Kono T. 1998. Disruption of primary imprinting during oocyte growth leads to the modified expression of imprinted genes during embryogenesis. *Development*, 125:1553-1560.

Okano M, Xie S, Li E. 1998. Cloning and characterization of a family of novel DNA (cytosine-5) methyltransferases. *Nat Genet*, 19:219-220.

Okano M, Bell DW, Haber DA, Li E. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell*, 99:247-257.

Orstavik KH, Eiklid K, Van Der Hagen CB, Spetalen S, Kierulf K, Skjeldal O, Buiting K. 2003. Another case of imprinting defect in a girl who was conceived by intracytoplasmic sperm injection. *Am J Hum Genet*, 72:218-219.

Rappolee DA, Sturm KS, Behrendtsen O, Schultz GA, Pederson RA, Werb Z. 1992. Insulin-like growth factor II acts through an endogenous growth pathway regulated by imprinting in the early mouse embryos. *Genes Dev*, 6:939-952.

Reese KJ, Lin S, Verona RI, Schultz RM, Bartolomei MS. 2007. Maintenance of paternal methylation and repression of the imprinted H19 gene requires MBD3. *PLoS Genet*, 3:1407-1417.

Reik W, Dean W, Walter J. 2001. Epigenetic reprogramming in mammalian development. *Science*, 293:1089-1093.

Reik W, Walter J. 2001. Genomic imprinting: parental influence on the genome. *Nat Rev Genet*, 2:21-32.

Rivera RM, Stein P, Weaver JR, Mager J, Schultz RM, Bartolomei MS. 2008. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. *Hum Mol Genet*, 17:1-14.

Runte M, Huttenhofer A, Gross S, Kiefmann M, Horsthemke B, Buiting K. 2001. The IC-SNURF-SNRPN transcript serves as a host for multiple small nucleolar RNA species and as an antisense RNA for UBE3A. *Hum Mol Genet*, 10:2687-2700.

Sato A, Otsu E, Negishi H, Utsunomiya T, Arima T. 2007. Aberrant DNA methylation of imprinted loci in superovulated oocytes. *Hum Reprod*, 22:26-35.

Schultz RM, Williams CJ. 2002. The science of ART. *Science*, 296:2188-2190.

Searle AG, Beechey CV. 1978. Complementation studies with mouse translocations. *Cytogenet Cell Genet*, 20:282-303.

Shamanski FL, Kimura Y, Lavoir MC, Pederson RA, Yanagimachi R. 1999. Status of genomic imprinting in mouse spermatids. *Hum Reprod*, 14:1050-1060.

Sharif J, Muto M, Takebayashi SI, Suetake I, Iwamatsu A, Endo TA, Shinga J, Mizutani-Koseki Y, Toyoda T, Okamura K, Tajima S, Mitsuya K, Okano M, Koseki H. 2007. The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA. *Nature*, 450:908-912.

Shi W, Haaf T. 2002. Aberrant methylation patterns at the two-cell stage as an indicator of early developmental failure. *Mol Reprod Dev*, 63:329-334.

Sunderam S, Chang J, Flowers L, Kulkarni A, Sentelle G, Jeng G, Macaluso M. 2009. Assisted reproductive technology surveillance-United States, 2006. *MMWR Surveill Summ*, 58:1-25.

Surani MA, Barton SC, Norris ML. 1984. Development of reconstituted mouse eggs suggested imprinting of the genome during gametogenesis. *Nature*, 308:548-550.

Surani MA, Kothary R, Allen ND, Singh PB, Fundele R, Ferguson-Smith AC, Barton SC. 1990. Genome imprinting and development in the mouse. *Development Suppl* :89-98.

Svensson J, Bjornstahl A, Ivarsson S. 2005. Increased risk of Silver-Russel Syndrome after *in vitro* fertilization? *Acta Paediatr*, 94:1163-1165.

Szabo PE, Mann JR. 1995. Biallelic expression of imprinted genes in the mouse germ line: implications for erasure, establishment, and mechanisms of genomic imprinting. *Genes Dev*, 9:1857-1868.

Williams CA. 2005. Neurological aspects of the Angelman syndrome. *Brain Dev*, 27:88-94.

Van der Auwera I, D'Hooghe T. 2001. Superovulation of female mice delays embryonic and fetal development. *Hum Reprod*, 16:1237-1243.

Yamazaki Y, Mann MR, Lee SS, Marh J, McCarrey JR, Yanagimachi R, Bartolomei MS. 2003. Reprogramming of primordial germ cells begins before migration into the genital ridge, making these cells inadequate donors for reproductive cloning. *Proc Natl Acad Sci USA*, 100:12207-12212.

Yoder J, Soman N, Verdine G, Bestor T. 1997. DNA (cytosine-5)-methyltransferases in mouse cells and tissues. *J Mol Biol*, 270:385-395.

Zhou GB, Meng, QG, Li N. 2010. *In vitro* derivation of germ cells from embryonic stem cells in mammals. *Mol Reprod Dev*, 77:586-594.