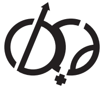

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Is ART changing the epigenetic landscape of imprinting?

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Abstract

Imprinted genes, which are epigenetically modified such that only a single parental allele is expressed, are often regulated by imprinting control regions (ICRs). ICRs typically are DNA methylated in the male or female germline and this DNA methylation is subsequently maintained, even when the genome is reprogrammed after fertilization. Many of the manipulations associated with Assisted Reproductive Technologies (ART) occur during periods of epigenetic reprogramming and it should, therefore, not be surprising that animal data show that loss of imprinting and loss of DNA methylation of ICRs is associated with procedures such as superovulation, *in vitro* fertilization and embryos culture and transfer. Less clear is whether there is an increase in loss of imprinting disorders associated with ART. Here we review the human and animal literature and discuss what it is necessary to address current controversies.

Keywords: assisted reproductive technologies (ART), DNA methylation, imprinting.

Introduction

A subset of genes in mammals is epigenetically regulated such that only a single parental allele is expressed (Bartolomei, 2009). These genes, which are termed imprinted, are mostly located in 1-2 Mb clusters. A typical cluster contains both maternally and paternally expressed genes that are regulated by a cis-acting imprinting control region (ICR). ICRs are operationally defined as a short segment of DNA that has allele-specific DNA methylation; when the ICR is deleted, the imprinting of genes in cis is disrupted. ICRs have been shown to act as promoters for long non-coding RNAs (*Airn* or *Kcnq1ot1*, as examples) or as CTCF-dependent insulators, as has been described for the *H19*/*Igf2* locus.

Imprinted genes are uniquely marked with their parental origin in the germline and this marking is maintained through the life of the organism, even when the genome is reprogrammed after fertilization. These processes, however, are poorly understood. DNA methylation at ICRs is established at distinct times in the male and female germlines (i.e., during prenatal and postnatal development, respectively). In the male germline, DNA methylation is initially placed on the *H19* ICR around embryonic day (E) 15.5 (Davis *et al*., 2000). In contrast, maternally methylated ICRs undergo DNA methylation during oocyte growth in the postnatal ovary, the timing of which varies by the ICR (Lucifero *et al*., 2004). The mechanism that targets the DNA methylation to ICRs is unclear but most likely involves the recognition of a unique sequence combined with a facilitating chromatin signature. The establishment of DNA methylation patterns during gametogenesis partially depends on DNMT3L, a regulatory factor related in sequence to DNMT3a and DNMT3b that is required for the *de novo* methylation of ICRs in the female germline (Kaneda *et al*., 2004). Enzymatically inactive, DNMT3L stimulates the DNA methylation activity of DNMT3a and DNMT3b but does not directly bind to DNA.

After the initial marking of imprinted genes in the germline, the parental origin must be maintained so that the appropriate allelic expression patterns are assumed in the developing organism. Maintenance is especially complex after fertilization because most of the genome is demethylated in the preimplantation embryos but imprinted genes (specifically ICRs) maintain their parental identity and differential DNA methylation (Weaver *et al*., 2009). Some factors have been described, which when mutated, cause defects in maintenance methylation right after fertilization, including STELLA, ZFP57, NLRP7 and MBD3 (Weaver *et al*., 2009). Nevertheless, the process that specifically protects ICRs from demethylation is poorly understood.

It is likely that the periods of major reprogramming that occur in the germline and preimplantation embryos are sensitive times for embryonic development and may represent the time when environmental exposures are most potentially detrimental. Assisted Reproductive Technologies (ART) employ methods that manipulate germ cells and embryos at these times. As such, there is considerable debate as to whether ART is associated with increased susceptibility to errors in imprinting.

Are imprinting disorders caused by ART?

Infertility affects 10-15% of couples and worldwide, millions of couples have turned to ART to conceive a child. The use of ART started with fertilization *in vitro* and the birth of Louise Brown (Steptoe and Edwards, 1978). At present, it is estimated that 1-3% of children born in developed countries are conceived through some form of ART (Manipalviratn *et*

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al., 2009). Since then, *in vitro* fertilization (IVF) has been optimized with the use of ovarian hyperstimulation with exogenous gonadotropins to obtain larger numbers of oocytes, improvements in embryo culture parameters, extended culturing of embryos to blastocyst and use of cryopreservation of embryos (see Fig. 1). In addition, the repertoire of ART techniques has expanded. For example, couples with male factor infertility have been helped by intracytoplasmic sperm injection (ICSI; Palermo *et al*., 1992). Other more invasive ART procedures include preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS), which entails removal of a single cell from a developing embryo, usually at the cleavage stage, and performing single gene analysis or karyotyping (Handyside *et al*., 1990).

There is accumulating evidence that the use of ART procedures has an impact on pregnancy outcomes and these include problems with the fetus, such as congenital malformations (Bonduelle *et al*., 2005; Reefhuis *et al*., 2009) and loss-of-imprinting (LOI) disorders (Cox *et al*., 2002; DeBaun *et al*., 2003), and problems with the placenta such as intrauterine growth restriction (IUGR) leading to low birth-weight (Schieve *et al*., 2002; Jackson *et al*., 2004; Poikkeus *et al*., 2007), preeclampsia and placental abruption and previa (Tan *et al*., 1992; Shevell *et al*., 2005). In addition, preterm labor and preterm birth, even in singleton pregnancies, may be associated with ART pregnancies (Poikkeus *et al*., 2007). However, it is unclear if subfertility/infertility or the ART procedures themselves play a causative role in these outcomes.

In humans, the association of ART and imprinting disorders is extrapolated from epidemiological studies as molecular studies on ART manipulated human gametes and embryos are rarely possible. These epidemiological studies are subject to confounding factors such as infertility and variability of ART protocols, to ascertainment and recall bias, and inappropriate sample size leading to low statistical power. In contrast, animal models of ART circumvent many of these problems posed by human studies. We will review the current literature regarding the association of imprinting disorders and ART in humans and then review the evidence for the biological plausibility of this association from animal models for ART.

Imprinting disorders and ART in humans

The era of ART started over 30 years ago and in the last decade, multiple studies have questioned the safety of these procedures with respect to the health of the children born from ART. A causal link between ART and imprinting disorders was first suggested in 2002 when two cases of Angelman syndrome (AS) in children conceived by ICSI were reported (Cox *et al*., 2002). This report led to numerous international epidemiological studies investigating whether imprinting disorders are more prevalent in children conceived by ART. Since then, ART has been suggested to be associated with AS, Beckwith-Wiedemann Syndrome (BWS) and maternal hypomethylation syndrome.

Angelman syndrome

AS is a rare neurodevelopmental syndrome characterized by severe mental retardation, delayed motor development, unsteady gait with jerky movements, absence of speech, and a happy disposition (Nicholls and Knepper, 2001). The prevalence of this syndrome is estimated to be between 1 in 10,000-20,000 (Williams, 2005). The underlying genetic cause for AS is loss of function of the maternal allele of the gene *UBE3A* located at chromosome 15q11-13, which is imprinted in the brain (Kishino *et al*., 1997; Matsuura *et al*., 1997). This loss of function can be a result of deletion (68% of cases), point mutation (13% of cases), uniparental paternal disomy (3% of cases) or an imprinting defect where there is a paternal imprint on a maternal chromosome (i.e. loss of methylation of the *SNRPN* ICR, which is located in the imprinting cluster with *UBE3A*, 6% of cases; Williams, 2005).

The imprinting defect as the cause of AS equates to an incidence of approximately 1:300,000 newborns. Surprisingly, the molecular lesion found in both children with AS described by Cox and colleagues was a decrease in maternal methylation at the *SNRPN* ICR (Cox *et al*., 2002). Of note, these children had fathers with known infertility. Subsequently another child, conceived by ICSI, was reported with AS, also having maternal hypomethylation (Orstavik *et al*., 2003). In this case, the father had normal sperm, although the mother had difficulty achieving a successful full term pregnancy.

In 2005, Ludwig *et al*. performed a cohort study investigating the association between infertility treatment and imprinting defects leading to AS. They found that of the 79 children with AS, the parents of 16 of these children identified themselves as having subfertility (20%). The investigators found that the relative risk of having an AS child with an imprinting defect was increased by the same factor, 6.2% (95% CI, 0.70-22.57), in both untreated couples with subfertility and also for couples who were treated with either ICSI or hormone therapy alone, though not statistically significant. However, subfertile couples that also received therapy had a significantly higher relative risk, 12.5% (95% CI, 1.40-45.13). Of the three AS patients who had been conceived with ICSI, only one had an imprinting defect. The authors concluded that the increase in AS is linked to subfertility and that superovulation, instead of ICSI, further amplifies this risk.

Another survey also concluded that children with AS were more likely to have parents with fertility

problems (19%) and that ovulation induction alone increased the risk of having an AS child (RR = 12.3; Doornbos *et al*., 2007). The molecular cause of two of four AS cases born from ART was due to a deletion of the maternal *UBE3A* gene. No data were available for the other two cases. These authors found no cases of AS after IVF/ICSI. More recently, a British survey of 2,493 children conceived after IVF showed that among the 1,524 responders, there were no reported cases of AS (Bowdin *et al*., 2007). In summary, the increased risk of an imprinting defect causing AS due to ICSI proposed by earlier case reports may be partly explained by the subfertility of these couples. However, this risk is heightened by ART treatment, although whether this additional risk is due to IVF, ICSI or just ovulation induction remains unclear. Further studies are needed to address this issue.

Beckwith-Wiedemann syndrome

Beckwith-Wiedemann Syndrome (BWS) is an imprinting disorder affecting growth regulation and is characterized by somatic overgrowth leading to macrosomia, macroglossia, visceramegaly, congenital anomalies such as abdominal wall defects, ear anomalies, renal abnormalities and tumor predisposition such as Wilm's tumor, rhabdomyosarcoma and hepatoblastoma (Weksberg *et al*., 2005). The reported incidence of BWS is 1 in 13,700 live births (Weksberg *et al*., 2005). The molecular basis of BWS involves a region on chromosome 11p15, where there are two imprinting clusters regulated by two independent ICRs; DMR1, which controls the *H19/IGF2* genes and DMR2, which controls *CDKN1C* /*KCNQ1* genes. In BWS, the affected genes are the paternally expressed *IGF2* and *KCNQ1OT1* and the maternally expressed genes *H19, CDKN1C*, and *KCNQ1*. The most common molecular cause of BWS is due to an epimutation characterized by a loss of methylation at maternal DMR2 leading to LOI of *KCNQ1OT1* (50% of cases; Weksberg *et al*., 2005). 25-50% of BWS cases are due to expression of the normally silent maternal *IGF2*, of which 2-7% are a result of silencing of the normally expressed maternal *H19* by hypermethylation of the promoter. 10-20% of BWS cases are due to paternal uniparental disomy and rare cases are due to paternal duplications, chromosome 11 inversion and translocation and mutation in the *CDKN1C* gene.

Subsequent to the case series proposing a link between AS and ART, three independent case series were published in 2003, suggesting an association of BWS and ART (DeBaun *et al*., 2003; Gicquel *et al*., 2003; Maher *et al*., 2003). In the case series from the United States, the prevalence of ART in the BWS cohort was 4.6% (3/65 cases) compared with 0.8% prevalence rate of ART in the general population in the United States in 1999 (DeBaun *et al*., 2003). Similarly, the United Kingdom case series reported a prevalence of ART in their BWS cohort of 4% (6/149 cases) compared to 1.2% prevalence rate of ART in their general population and the French case series reported an ART prevalence rate of 4% (6/149 cases) in their BWS cases compared to 1.3% in their general population (Gicquel *et al*., 2003; Maher *et al*., 2003).

There have been four additional studies (1 case-control, 1 case series, 2 surveys) that also show an increase in prevalence of ART in BWS children, varying from 2.9-10.8% compared to the general population (Halliday *et al*., 2004; Chang *et al*., 2005; Sutcliffe *et al*., 2006; Doornbos *et al*., 2007). However, of three national cohort studies published, where a cohort of ART children was compared with a cohort of naturally conceived children, only one study had a case of BWS in the ART cohort (1,524 children; Bowdin *et al*., 2007), whereas no cases of BWS were identified in the ART cohort in the other two studies (6,052 and 16,280 children; Kallen *et al*., 2005; Lidegaard *et al*., 2005). In summary, there is some evidence for an increased risk of having a child with BWS after undergoing ART.

Further molecular evidence to strengthen the association between ART and imprinting disorders is that most of the post-ART BWS cases share a common molecular etiology, which is a loss of maternal methylation at DMR2. Combining seven studies with data on molecular analysis of the BWS patients born from ART, there were 60 children in total, of which 51 were tested for loss of methylation at DMR2; 49 of these children tested positive for this epimutation (96%) compared with an estimated 50% of general BWS population having this epimutation (DeBaun *et al*., 2003; Gicquel *et al*., 2003; Maher *et al*., 2003; Halliday *et al*., 2004; Sutcliffe *et al*., 2006; Bowdin *et al*., 2007; Lim *et al*., 2009). In a recent study, Gomes *et al*. compared the level of methylation at DMR2 in clinically normal naturally conceived children, in clinically normal ART conceived children and naturally conceived children with BWS (Gomes *et al*., 2009). They found the level of methylation at the DMR2 to be 36.2% ± 3.6 for the naturally conceived normal children, 13.7% ± 0.6 for the ART conceived normal children and 1.2% ± 0.7 in the BWS children. Notably the difference in methylation between the naturally conceived and ART conceived children was not statistically different though there was a trend towards a decrease in methylation in the ART children. The authors conclude that there is aberrant methylation of the DMR2 in clinically normal ART born children. In conclusion, there is an increase in prevalence of ART in BWS cases and it seems that many of these cases are due to the loss of methylation of the maternal DMR2.

Maternal Hypomethylation syndrome

The maternal hypomethylation syndrome was

described as a consequence of the investigation into the molecular etiology of a cohort of patients with transient neonatal diabetes (TNDM). It was shown that six TNDM patients not only had loss of methylation at the TNDM locus but also at several maternally methylated imprinted loci (Mackay *et al*., 2006). A link with ART was not established in this study but the authors mentioned that two mothers experienced a period of infertility. There have been only two studies addressing the association between ART and maternal hypomethylation syndrome and neither has proved this link exists. In a cohort of BWS born with and without ART, maternal hypomethylation in other imprinted loci occurred in some BWS patients regardless of whether ART was involved (27%) or not (24%; Rossignol *et al*., 2006). Additionally Bliek and colleagues described a cohort of 149 BWS patients in which 17 patients had hypomethylation at multiple maternally-methylated loci (Bliek *et al*., 2009). Only 1 of the 17 patients was born after IVF. The authors concluded that a causal link between ART and maternal hypomethylation could not be excluded but together with the previous study, they did not find an increase in prevalence of ART associated cases. At present, with the limited studies available an association between maternal hypomethylation syndrome and ART cannot be established.

Mouse models of ART and imprinting disorders

Due to the practical and ethical limitations on studies of ART in humans, animal studies have been invaluable for determining whether ART procedures affect embryonic growth, development and imprinted gene expression. The first indication that *in vitro* culture might affect imprinting came from a study by Sasaki and colleagues, in which the authors used IVF (which also involves embryo culture) to characterize the expression of *H19* in preimplantation embryos (Sasaki *et al*., 1995). They found that some blastocysts exhibited biallelic expression of *H19*. In contrast, another study showed monoallelic expression of *H19* in blastocysts that developed *in vivo* (Tremblay *et al*., 1995). This difference suggested that embryo culture could cause sporadic LOI. Confirmation was later provided in a study that compared the expression of *H19* in blastocysts that developed *in vivo* to those cultured from the 2-cell stage in either Whitten's medium or Potassium Simplex Optimized Medium (KSOM) supplemented with amino acids (KSOM+aa; Doherty *et al*., 2000). Loss of *H19* imprinting and loss of methylation (LOM) at the *H19* ICR were observed in embryos cultured in Whitten's, but not those cultured in optimized KSOM+aa. The effect was specific to *H19*, as monoallelic expression of *Snrpn* was retained, suggesting that *H19* is particularly sensitive to the adverse effects of culture. It should be noted, however, that later studies established that culture in any medium was detrimental (Mann *et al*., 2004; Rivera *et al*., 2008).

Several other studies have further characterized the impact of embryo culture on imprinting. Khosla and colleagues addressed the effects of including serum in the culture medium (Khosla *et al*., 2001). The investigators examined individual fetuses at E14 that had been transferred to recipient females as blastocysts after development *in vivo* or culturing from the 1- or 2-cell stage in M16 medium with or without fetal bovine serum. Embryos cultured with serum were smaller than those that developed *in vivo* or without serum, and embryos cultured with serum had decreased *H19*, *Igf2* and *Grb7* expression and increased *Grb10* expression. These expression changes appeared to be stochastic, as not all genes were altered in each fetus. It is not known whether the increase in *Grb10* expression was due to biallelic expression. Similarly, Fernandez-Gonzalez *et al*. cultured embryos in KSOM with or without serum from the 1-cell to blastocyst stage but identified different alterations in expression: reduced *Igf2*, *Mest* and *Grb10* (Fernandez-Gonzalez *et al*., 2004). The precise genes and levels of expression are likely to be less important than the fact that imprinted gene expression is affected by embryo culture.

To ascertain the long-term effects of embryo culture on allelic expression and DNA methylation patterns of imprinted genes, Mann and colleagues transferred embryos cultured from the 2-cell stage to the blastocyst to recipient females (Mann *et al*., 2004). When embryos were examined at E9.5 more extensive LOI was apparent: *H19*, *Snrpn*, *Peg3* and *Ascl2* were all biallelically expressed. Similarly, extensive LOM at the *H19* and *Snrpn* ICRs was observed. The LOI and LOM effects were much greater in the placenta, with embryos experiencing less dramatic perturbations in imprinted gene regulation. In sum, embryo culture has long-term effects on both methylation and expression of imprinted genes, especially in extra-embryonic tissues.

While the above experiments suggested that embryo culture was detrimental to imprinting, each step in the ART process could potentially alter gene expression (see Fig.1). For example, multiple studies have indicated that ovarian stimulation can disrupt imprinting. Using immunofluorescence, one study reported abnormal methylation at the 2-cell stage in embryos produced by superovulation and *in vivo* fertilization (Shi and Haaf, 2002). Although these results indicate disruption of the methylation machinery, immunofluorescence mainly reveals repetitive DNA and therefore may not reflect changes in imprinted genes. Fauque and colleagues found variable, lower expression of *H19* in blastocysts derived after superovulation, whether followed by *in vivo* development, culture, or IVF, compared to normal *H19* expression in *in vivo*-derived embryos conceived without superovulation (Fauque *et al*., 2007). Market-Velker *et al*. (2010) examined the effects of various doses of superovulatory hormones on methylation in blastocysts (Market-Velker

et al., 2010). They found a dose dependent effect on LOM at three maternally methylated ICRs (*Snrpn*, KvDMR1 and *Peg3*) and gain of methylation on the maternal allele of the *H19* ICR. Surprisingly, they also found LOM on the paternal allele of the *H19* ICR, indicating that maternal hormones can disrupt methylation after fertilization. Another study also saw gain of methylation at *H19* following superovulation (Sato *et al*., 2007). Finally, Fortier *et al*. (2008) compared embryos conceived either with or without superovulation at E9.5 (Fortier *et al*., 2008). They reported biallelic expression of *H19* and *Snrpn* in placentas conceived with superovulation but no effect on expression of *Igf2* or *Kcnq1ot1*. However, they detected no change in methylation at the *H19* or *Snrpn* ICRs at this stage. In conclusion, ovarian stimulation alone can alter imprinting.

A few studies have examined the effects of IVF on imprinting. Biallelic *H19* expression was reported in blastocysts derived by IVF (Li *et al*., 2005). Fauque and colleagues observed altered expression of *H19* and methylation of the *H19* ICR and promoter in blastocysts conceived by IVF, but these defects were also seen in embryos conceived *in vivo* and then cultured (Fauque *et al*., 2007). In a follow-up study, a survey of imprinted genes at E10.5 revealed aberrant expression in placentas from conceptuses following *in vitro* culture with or without IVF (Fauque *et al*., 2010). However, more genes appeared to be misexpressed in placentas following IVF than culture alone. Unfortunately, these studies were not performed allele-specifically, so one cannot definitively conclude whether imprinted gene regulation or just gene regulation itself was perturbed.

The use of intracytoplasmic sperm injection (ICSI), which entails injection of sperm into an egg and bypasses many of the selective processes involved in natural fertilization, has more recently been assessed. In this case, changes in expression of *Cd81*, *H19* and *Slc38a4*, as well as reduced methylation of *H19*, were identified in blastocysts derived by ICSI using sperm that showed signs of DNA damage (Fernandez-Gonzalez *et al*., 2008). These expression changes were greater than those caused by culture alone. However it was again unclear if the expression changes were due to loss of imprinting. It is also unclear where ICSI itself, or the damaged state of the sperm DNA, was the cause of the defects.

Finally, Rivera and colleagues examined the effects of embryo transfer, with or without prior culture, on imprinted expression and methylation at E9.5 (Rivera *et al.*, 2008). The conclusion from this study was that transfer alone resulted in LOI of one or more genes in the yolk sac and placenta. Embryo culture followed by transfer resulted in the deregulation of more genes.

A critical question is how these ART procedures and prenatal alterations in imprinting affect postnatal health and behavior. Only a limited number of studies have addressed this question. Behavioral testing was performed on adult mice conceived in *vivo*, cultured from the 2-cell to blastocysts stage, then transferred to recipient females, as compared to mice that developed naturally (Ecker *et al*., 2004). Male mice derived by *in vitro* culture had reduced anxiety compared to control and also *in vitro* cultured mice of both sexes had defects in spatial memory. Fernandez-Gonzalez *et al*. (2004) found that mice cultured with serum (as described above) had deficiencies in implicit memory and that males had reduced anxiety early in life followed by increased anxiety later (Fernandez-Gonzalez *et al*., 2004). Mice produced by ICSI using damaged sperm also had a variety of behavioral defects (Fernandez-Gonzalez *et al*., 2008).

In summary, interpreting these animal studies, there are some important issues to consider. These studies have used different experimental designs, culture media, techniques, and mouse strains, making it difficult to compare the results. A number of studies only looked at the total expression level of imprinted genes and not at their allele-specific expression, preventing them from distinguishing between LOI and changes in expression without LOI. One difficulty specific to those studies that used embryo transfer is that *in vitro* development causes developmental delay; therefore, embryos cultured and then transferred will be delayed relative to embryos that developed entirely *in vivo*, preventing the direct comparison of these groups. LOI of some genes could in fact be due to this delay. It is also important to keep in mind that a developmental delay will not necessarily have long-term consequences.

A number of questions remain to be answered. One is the mechanism by which these manipulations cause defects in imprinting. Are they due to metabolic or mechanical stress, altered signaling, or the removal of selective pressures on gametes? Moreover, how do these stresses translate into epigenetic disruption? Another question is how the alterations observed in blastocysts and mid-gestation embryos translate to long-term effects. It will be important to determine if disruptions in methylation and expression persist postnatally, as well as to explore further the behavioral changes that may result, especially as they relate to the cognitive and behavioral alterations in known imprinting disorders.

Figure 1. Assisted reproductive technologies and critical stages in imprinting. Assisted reproduction exposes gametes and embryos to extensive manipulation and conditions they would not encounter *in vivo*. These manipulations include hormonal stimulation, *in vitro* maturation, and freezing and thawing, as well as *in vitro* fertilization, intracytoplasmic sperm injection, and preimplantation genetic diagnosis. During culture, embryos are exposed to artificial media, light, and high oxygen levels before finally being transferred back to the mother. These stresses occur during a time when genomic imprints are established and maintained. Imprints are established in gametes. The genome is demethylated following fertilization, and remethylated after implantation, during which imprints must be maintained so that proper expression can be established. Assisted reproductive technologies therefore have the potential to disrupt imprinting at many points in this process. (Please note, the timing of imprinting events indicated is based on mouse studies, and may be slightly different in humans).

Conclusions

It is clear that more work is required to address the questions raised in human population studies as well as animal studies. Firstly, does ART itself really cause an increase in the prevalence of imprinting disorders or is it the underlying fertility of these couples that contributes the greater risk? Larger population studies are required to address this question. Secondly, for children conceived by ART, are there extensive or limited epigenetic changes? Sapienza and colleagues have begun to address this question by assaying genome-wide DNA methylation in placentas and cord blood in ART-conceived and control children and differences have been observed (Katari *et al.*, 2009). Animal studies have already proven quite valuable and they can be used to assess the consequences of manipulations associated with ART. Moreover, animal studies can address mechanisms. In this vain, genome-wide epigenetic profiling will help to address the extent of epigenetic dysregulation. Furthermore, the long-term consequences of ART, including trans-generational inheritance, remain poorly described. Finally, interventions (dietary supplements, for example) should be investigated to ameliorate the observed and hypothesized effects of ART and infertility.

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