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Are there subtle, genome-wide epigenetic alterations in normal offspring conceived from Assisted Reproductive Technologies?

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Abstract

Objective—To review recent data regarding subtle, but widespread epigenetic alterations in phenotypically normal offspring conceived of ART compared to offspring conceived in vivo.

Design—A PubMed computer search was performed to identify relevant articles.

Setting—Research institution.

Intervention(s)—None.

Result(s)—Studies in animals indicate that *in vitro* culture may be associated with widespread alterations in imprinted genes, compared to *in vivo*-conceived offspring. Recently, studies in humans have likewise demonstrated widespread changes in DNA methylation, including genes linked to adipocyte development, insulin signaling, and obesity in offspring conceived by ART, compared to in vivo-conceived children. Changes in multiple imprinted genes following ART were also noted in additional studies, which suggested that the diagnosis of infertility may explain the differences between *in vivo*-conceived and ART offspring.

Conclusion(s)—These data suggest that ART is associated with widespread epigenetic modifications in phenotypically normal children, and that these modifications may increase risk of adverse cardiometabolic outcomes. Further research is needed to elucidate the possible relationship between ART, genome-wide alterations in imprinted genes, and their potential relevance to subtle cardiometabolic consequences reported in ART offspring.

Keywords

genome-wide epigenetic; imprinting; epigenetics; assisted reproductive technology; cardiometabolic

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Introduction

In 2009, assisted reproductive technologies (ART) resulted in 54,656 infants, contributing to over 1% of annual births in the United States (1). There is evidence that children born through *in vitro* fertilization (IVF) are at increased risk of cardiometabolic disorders, specifically elevated systolic and diastolic blood pressure, higher fasting glucose, elevated triglycerides, increased body fat composition, and increased incidence of subclinical primary hypothyroidism. These changes are certainly subtle, but have been detected in several studies (2–6). This raises concern, given the well-established association between environmental influences and cardiometabolic disorders in the developmental origins of adult disease (DOHaD), widely known as the Barker hypothesis (7–10).

In addition, ART has been associated with changes in DNA methylation, leading to an increased likelihood of rare genetic disorders (11, 12). More recent evidence suggests that ART is associated with widespread epigenetic alterations. It is unclear whether the altered methylation is directly associated with ovarian stimulation, ART, or the diagnosis of infertility itself. Here we review the existing evidence for global epigenetic changes associated with ART, which might contribute to the increased risk of cardiometabolic disturbances in phenotypically normal ART conceived children.

Epigenetics refers to heritable modifications of DNA that do not alter the underlying sequence. Imprinting is an epigenetic modification that is reprogrammed in the germ line and results in mono-allelic expression of genes (13, 14). DNA methylation and histone modification are examples of epigenetic modifications that lead to imprinting. The majority of evidence regarding the effect of ART on imprinting involves DNA methylation. DNA methylation is a stable, inheritable covalent addition of a methyl group to the fifth carbon of cytosine (13), and occurs most commonly in CpG sequences (15).

Gametogenesis is a critical time in the imprinting process. Normally, paternal imprints are established in the spermatogonium prior to the potential changing effects of ART (16). In contrast, oocyte imprints are established later in gametogenesis, and are completed just prior to ovulation (17). Around the time of implantation, the majority of male and female germ line-derived methylation patterns are erased, followed by *de novo* re-methylation of the genome into somatic patterns (18, 19). The methylation patterns of imprinted genes are not altered during this wave of methylation, so that parent-specific expression of these genes, many of which are important in fetal growth and development, is faithfully preserved (20, 21). Both oocyte and pre-implantation imprinting processes could potentially be influenced by the ART process (22, 23).

Methods

A systematic literature review was performed in August 2011. Pubmed was thoroughly searched for any pertinent publications through 2011 using key words “epigenetic, methylation, imprinting, assisted reproductive technologies, assisted reproduction, in vitro, and metabolic.” Relevance was evaluated from the titles and abstracts, and bibliographies of relevant publications were cross-referenced for additional pertinent citations.

Animal Studies

ART-associated errors in imprinting have been documented in animals. Very briefly, several studies have shown that superovulation is associated with methylation changes in maternal and paternal alleles in both oocytes and blastocysts (24–27). Methylation errors have also been demonstrated following bovine *in vitro* oocyte maturation (IVM) protocols with associated fetal overgrowth and pathologic endocrine changes (28). Aberrant methylation

has likewise been demonstrated in human oocytes following IVM (29). Embryo transfer alone has been shown to result in imprinting errors in the yolk sac and placenta (30). Culture media has been implicated in imprinting alterations (24, 31). Epigenetic alterations of the imprinted, maternally expressed H19 and IGR2 have been noted following *in vitro* fertilization (IVF) (24, 32), with a more profound loss of methylation in vitrified embryos (33).

In addition to evidence of specific epigenetic alternations of a single gene or genes associated with ART procedures, several studies in animals have suggested that ART may have a more widespread, or global impact on imprinting. For instance, Zaitseva et al. (34) found a higher overall methylation level in embryos developed *in vitro* than those conceived *in vivo.* Loss of imprinting has been shown to continue into the post implantation period, particularly in the placenta (25, 35, 36). It has been suggested that mechanisms for maintenance of imprinted genes are not as resilient in trophoblastic tissues (25, 37), compared to the developing embryo, raising the possibility that more direct contact with the environment may make the trophoderm and placenta more vulnerable to imprinting errors (25). If true, this finding would be of concern given the established role of environmental influences upon the placenta in the DOHaD or Barker hypothesis (7–9). Stouder et al. (38) demonstrated that imprinting errors occurring after superovulation may have transgenerational effects on offspring. Mahsoudi et al. (39) also described altered postnatal growth and organ size following culture and transfer of mouse embryos. These changes persisted in the second generation, suggestive of epigenetic alterations. The presence of altered fetal growth, methylation alterations in the placenta, and transgenerational inheritance of imprinting errors in animals raise concern for global epigenetic changes following ART procedures.

Human Studies

Recent technologic developments have facilitated the interrogation of many genes simultaneously, and these methods have been used to test for widespread epigenetic changes in phenotypically normal children conceived with ART. Katari et al. (40) examined methylation differences in CpG sites of over 700 genes in samples of placenta and cord blood obtained from *in vitro* and *in vivo* conceived children using an Illumina array platform. Cord blood samples exhibited greater CpG site methylation than placental samples in both ART and non-ART children, suggesting that CpG methylation may play a role in determining cell type in humans. This lower level of methylation in the placenta is consistent with observations for imprinted genes in the placenta of mouse embryos exposed to *in vitro* culture (31, 32). Additionally, methylation differences were observed between *in vivo* and *in vitro* conceived children in a number of genes known or suspected to be imprinted (40). ART conceived children displayed overall lower average methylation levels at specific CpG sites in placenta and higher methylation levels in cord blood (40). This may be related to the response of outer and inner cells of the blastocyst to *in vitro* culture, as in mouse studies (36). Overall, *in vitro* conception was associated with statistically significant differences in CpG methylation which resulted in gene expression differences at both imprinted and non-imprinted loci (40). Of note, several of the genes whose expression differed between the *in vitro* and *in vivo* group are known to impact adipocyte development and differentiation, insulin signaling and obesity (40).

Several other human studies, consistent with animal studies, have shown epigenetic differences at multiple loci between in vitro and in vivo conceived children, as well as increased susceptibility of the placenta to epigenetic alteration. Zechner et al. (41) compared the DNA methylation patterns of seven imprinted genes, one pluripotency, and one tumor suppressor gene between ART and spontaneously conceived chorionic villus samples of human abortions and stillbirths ranging from seven to 42 weeks gestation (41). Karyotype

analysis was done in some, but not all, samples. The authors found “minor but significant” methylation differences between ART and spontaneously conceived children. ART children displayed lower average methylation levels at certain CpG sites in placenta and higher levels in cord blood (41). Significant methylation differences were noted for maternally imprinted LIT1, with a trend toward methylation differences noted in H19. Interestingly, a significant difference in H19 methylation was noted between singletons and multiple gestations in ART population, leading to a secondary trend of methylation differences noted in ART versus non-ART samples (41).

In another study investigating epigenetic changes of several imprinted loci, Katagiri et al. (42) compared the expression of four imprinted genes known to be associated with fetal growth in placental tissue obtained from phenotypically normal singletons conceived using ART with those that were conceived spontaneously. Imprinted gene expression was found to be similar between ART and spontaneously conceived infants with weights appropriate for gestational age. However, both ART and spontaneously conceived infants with fetal growth restriction demonstrated suppression of H19, while spontaneously conceived infants born greater than 3500 grams demonstrated enhanced expression of H19. Expression of CDKN1C, an imprinted growth regulatory gene, was suppressed in ART infants with fetal growth restriction (42). This suggests that hypoexpression of maternally methylated imprinted genes H19 and CDKN1C may be related to growth restriction. The loss of imprinting of genes has been shown to be related to poor fetal growth in humans in previous studies (43). However the differences in gene expression patterns between ART and spontaneously conceived children in this study by Katagiri et al. (42) suggest that ART may modify epigenetics, potentially contributing to fetal growth restriction.

Turan et al. (44) likewise examined the effect of ART on imprinted loci by studying intra and inter-individual variation at the differentially methylated regions (DMRs) of IGR2/H19 and IGR2R in peripheral blood, cord blood, and placenta obtained from phenotypically normal children conceived *in vitro* and *in vivo* (44). IGF2/H19 was chosen due to its role as a placental growth factor; the authors reasoned that its imprinted locus might also be susceptible during ART treatments and procedures, contributing toward low birth weight in ART infants. DNA methylation levels were calculated as a maternal/paternal ratio (M/P) as an indicator of imprinting status. Comparing individual *in vitro* versus *in vivo* tissue samples, the M/P ratio mean and variance were greater in the *in vitro* group, although cord blood means and placenta variance did not reach statistical significance (44). Comparing all tissue results together, allele-specific methylation ratio mean and variance were significantly greater in the *in vitro* group, consistent with observations of IGF2/H19 in the mouse (32). Turan also noted that epigenetic variability was greater in extraembryonic tissues that embryonic tissues (44), consistent with evidence from mouse studies (25, 37) that the placenta is particularly susceptible to epigenetic modifications.

An effect of ART on a single maternal imprinting control region has been demonstrated in normal children. Gomes et al. (45) focused on the effect of IVF and ICSI on epigenetic changes of the maternally methylated imprinting control region (ICR) KvDMR1. The study included samples of peripheral blood or umbilical cord blood and placenta obtained from clinically normal children conceived by assisted reproduction technology, by spontaneous conception, and as a positive control, spontaneously conceived children with Beckwith Wiedemann Syndrome. None of the spontaneously conceived children displayed hypomethylation of KvDMR1. In the ART conceived group, three of the twelve children from whom peripheral blood was collected demonstrated KvDMR1 hypomethylation. All three of the children with KvDMR1 hypomethylation had a dizygotic twin who expressed a discordant (normal) KvDMR1 methylation pattern (45). This discordance was hypothesized to result from either different vulnerability to imprinting of the embryos or epigenetic

alterations that occurred during gametogenesis (45). While statistical significance was not achieved, the differences noted between KvDMR1 methylation in spontaneously conceived and ART children, as well as between dizygotic twins, support the vulnerability of maternal imprinting in ART.

The significance and impact of ART on global methylation changes such as those noted by Katari et al. (40) remains controversial. While the aforementioned studies suggest alteration in DNA methylation in ART-conceived, versus naturally conceived children, some studies suggest that epigenetic changes may be linked to the diagnosis of infertility itself, rather than specific ART processes. Kobayashi et al. (46) analyzed DNA methylation at seven autosomal imprinted loci as well as XIST, a gene involved in X chromosome inactivation, in trophoblastic tissue samples obtained from failed pregnancies resulting from both in vitro and in vivo conception. Seventeen of 78 ART-treated fetal samples were found to have imprint methylation errors. In seven of these 17 cases (41%), the precise DNA methylation error was present in the paternal sperm, suggesting that that the methylation was inherited, not a result of ART per se. Two of these seven cases also had sequence variations in the DNMT3L gene, which is involved in DNA methylation and deficiency has been shown in mice to be associated with oligospermia (47). Both the imprinting errors and the DNA sequence variants were more prevalent in patients with oligospermia (46). These data suggest that inherent imprinting errors may be present in men with impaired sperm production, which could then be passed to offspring using ART.

Intrinsic imprinting errors associated with infertility have also been demonstrated in females. Tierling et al. (48) examined the effect of ART on the stability of DNA methylation in imprinted genes. DNA was extracted from maternal blood, umbilical cord blood, and amnion/chorion tissue obtained from phenotypically normal children conceived spontaneously, with IVF, and IVF with intracytoplasmic sperm injection (ICSI). Children conceived by IVF had a higher cord blood MEST DMR methylation index than children conceived spontaneously or with ICSI (48). Women undergoing IVF also had a higher MEST DMR methylation index compared to those women using ICSI or women who spontaneously conceived, suggesting that the hypermethylation was not due to ART procedures, but may be passed down from mothers with infertility. For the other nine DMRs there was no significant difference among the different types of conception; however methylation differences were noted between peripheral and cord blood samples compared to the placental tissue (48), similar to the findings of Katari et al. (40).

The phenotypic impact of these epigenetic modifications remains uncertain. Turan et al. demonstrated that despite increased variance in methylation patterns in the *in vitro* group, this was poorly correlated with gene expression (44), implying that gene transcription may not be influenced by imprinting alone. Caperton et al. (49) used a mouse model to explore the frequency and spectrum of point mutations in midgestation fetuses resulting from natural reproduction compared to different methods of ART, and found no increase in de-novo point mutations after ART as compared to natural reproduction. This study examined genetic point mutations and not epigenetic alterations, thus it does not refute the evidence for global methylation changes as a result of ART, but as the authors state, may provide evidence that “maintenance of genetic integrity is more stringent that maintenance of epigenetic integrity (49).”

The influence of environment on widespread epigenetic modifications has been demonstrated in humans (50). Fraga et al. used a global methylation DNA fingerprinting technique to compare the genomes of eighty monozygotic twins ages 3–74 and found up to 35% discordance between MZ twin pairs. Those twins with the largest discrepancy in DNA methylation patterns were older, had different lifestyles and medical histories, and had spent

less of their life together (50). Given that this was studied in genetically identical individuals, these results highlight the susceptibility of epigenetics to the environment (50). Since DNA methylation patterns appear to be dynamic, it could be argued that epigenetic changes that occur as a result of ART could potentially self-correct. However this study also provides further evidence for the effect of environment on global DNA methylation patterns (50) and it can be inferred that the artificial environment of *in vitro* fertilization could contribute to global epigenetic changes and altered phenotypic expression.

There is growing evidence that the environment in which an embryo develops can effect its metabolism, epigenetic alterations and developmental potential (51). Culture conditions have been shown in mouse models to impact epigenetic patterns of the embryo (24, 31, 52) and especially the placenta (36, 37). Specifically, culture at atmospheric (20%) oxygen tension as compared to physiologic (5%) oxygen tension resulted in marked differences in global gene expression, in particular genes involved in cell growth and maintenance, relative to embryos developed in vivo (52). It is hypothesized that the inappropriate oxygen exposure results in elevated reactive oxygen species production by the mitochondria, which then alters the normal epigenetic pattern and subsequent gene expression in the embryo (51). Human studies have also indicated altered gene expression after IVF, specifically in the placenta (53). Zhang et al. used proteomic analysis to identify differential protein patterns in human placentas resulting from ART and from natural conception (53). This study found significant downregulation of FTL and ATP5A (involved in energy metabolism) and upregulation of hnRNP C1/C2 (involved in DNA damage response) and ORP150,PDIR and Hsp60 (involved in stress response) in the IVF group, indicating that ART may result in increased environmental stress, insufficient energy production in the placenta and gene metabolism dysfunction (53), and highlighting the vulnerability of the placenta to its environment.

Epigenetic alterations resulting from these environmental exposures during early embryonic development may contribute to long term health consequences, consistent with the developmental origins of adult disease (Barker hypothesis) (7–9). Studies comparing the cardiometabolic profiles of children born from IVF to those of spontaneously conceived controls found higher systolic and diastolic blood pressure (2, 3), higher triglycerides (3), higher fasting glucose (2), increased peripheral adipose tissue mass (5), and increased incidence of subclinical primary hypothyroidism (4) in children conceived by IVF, though no increase in insulin resistance (2, 3), inflammatory markers (3), BMI (2), or incidence of metabolic syndrome (3). These metabolic derangements were found to occur independently of children being born small for gestational age (2, 3, 5), a condition which is common after ART and has been shown to increase risk of adverse cardiometabolic outcomes (10), thus implicating ART as an independent cause (6). Evidence from animal and human studies has shown that epigenetic changes can be transmitted to future generations (54). Nutritional changes have been shown to result in transgenerational obesity in a mouse model (55) and impact longevity of subsequent generations in a human model (56), providing further evidence that environment can have a transgenerational impact, likely due to epigenetic modification (54).

Future research is needed to understand the long term implications of these epigenetic changes on children born from ART procedures as well as subsequent generations.

Summary

Recent studies suggest that changes in methylation following ART may occur throughout the genome in phenotypically normal offspring (40, 41), as well as in multiple imprinted genes (41–45). Ovulation induction as well as *in vitro* oocyte maturation have been shown to

induce changes in DNA methylation in humans (26, 29). Intrinsic imprinting errors have also been found in sperm retrieved from men with impaired sperm production (16, 46, 57), and women undergoing IVF have been found to have higher methylation indices than non-IVF mothers, suggesting that the need for ART may be selecting for a patient group enriched for imprinting errors, even before ovarian stimulation and exposure of the gametes/embryo to the techniques of ART.

Several of the genes demonstrating aberrant methylation after ART have been linked to adipocyte development and differentiation, insulin signaling, and obesity (40). Given the suspected association between ART and cardiometabolic derangements (2–6), aberrant CpG methylation associated with ART could have metabolic implications later in life. Indeed, this data raises the possibility that some of the cardiometabolic outcomes seen in ART children, such as elevated systolic and diastolic blood pressure (2, 3), high triglycerides (3), high fasting glucose (2), increased peripheral adipose tissue mass (5), and increased incidence of subclinical primary hypothyroidism (4), could arise as a result of epigenetic modifications induced or selected for by the ART procedures (2–6). Further, these changes could result in a pre-disposition to adult-onset diseases such as type II diabetes, obesity, and cardiovascular disease later in life (8, 9, 58). While transgenerational effect of ART has not yet been studied in humans, there is concern that the transgenerational imprinting effects seen in animals (38, 39) could also be possible in humans.

In conclusion, there is evidence that children born following ART have an increased risk of cardiometabolic abnormalities as well subtle, genome wide changes in DNA methylation. Given demonstrated alterations in fetal and placental methylation status, combined with the established DOHaD hypothesis, additional research is needed to elucidate the relationship between ART, genome-wide alterations in imprinted genes, and their possible relevance to subtle metabolic consequences reported in ART offspring.

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Table 1

Studies comparing imprinted genes between *in vivo* and *in vitro*-conceived normal offspring in humans

Author	Patients tested	Approach	Genes Studied	Tissue Studied	Difference*
Gomes et al (45)	18 ART 30 *in vivo* 3 BWS (control)	MS-PCR	1 (KvDMR1)	Cord Blood Placenta Peripheral Blood	yes
Katagiri et al (42)	65 ART 924 *in vivo*	RT PCR	4 (IGF2, H19, KCNQ1OT1, CDKN1C)	Placenta	yes
Katari et al (40)	10 ART 13 *in vivo*	platform array RT-PCR	736 genes 183 imprinted 23 mono-allelic	Cord Blood Placenta	yes
Kobayshi et al (46)	78 ART 38 *in vivo*	Bisulfite PCR	8 (H19, GTL2, PEG1, KCNQ1OT1, ZAC, PEG3, SNRPN, XIST)	Placenta (CVS)	yes
Tierling et al (48)	77 ICSI 35 IVF 73 *in vivo*	Bisulfite PCR	10 (KvDMR1, H19, SNRPN, MEST, GRB10, MEG3, IG-DMR, GNAS, NESP55, GNAS, NESPas, GNAS XL alpha-s, GNAS Exon1A)	Cord Blood Peripheral Blood Amnion/Chorion	no
Turan et al (44)	45 ART 56 *in vivo*	RT PCR	2 (IGF2/H19, IGF2R)	Cord Blood Placenta Peripheral Blood	yes
Zechner et al (41)	42 ART 29 *in vivo*	Bisulfite PCR	9 (H19, MEG3, LIT1, MEST, NESP55, PEG3, SNRPN, NANOG, APC)	Placenta (CVS)	yes

*Differences noted between ART and *in vivo*

APC: Adenomatous polyposis coli

ART: Assisted reproductive technology

BWS: Beckwith Wiedemann Syndrome

CDKN1C: Cyclin-dependent kinase inhibitor 1C

CVS: Chorionic villus sampling

GNAS=NESP: Guanine nucleotide binding protein alpha stimulating activity polypeptide=neuroendocrine secretory protein

GRB10: Growth factor receptor-bound protein 10

GTL2: Gene trap locus 2

IGF2: Insulin-like growth factor

IGFR: Insulin-like growth factor receptor

KCNQ1OT1=LIT1=KvDMR1: KCNQ1 Overlapping transcript 1=Long QT intronic transcript 1=Kv Differentially methylated region

MEG3: Maternally expressed imprinted gene

MEST: Mesoderm specific transcript

MS: Methylation sensitive

NANOG: Homeobox transcription factor

PCR: Polymerase chain reaction

PEG: Paternally-expressed gene

RT: Real time

SNRPN: Small nuclear ribonucleoprotein-associated protein N

XIST: X (inactive)-specific transcript