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Epigenetics and Assisted Reproductive Technology: A Call for Investigation

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A surprising set of recent observations suggests a link between assisted reproductive technology (ART) and epigenetic errors—that is, errors involving information other than DNA sequence that is heritable during cell division. An apparent association with ART was found in registries of children with Beckwith-Wiedemann syndrome, Angelman syndrome, and retinoblastoma. Here, we review the epidemiology and molecular biology behind these studies and those of relevant model systems, and we highlight the need for investigation of two major questions: (1) large-scale case-control studies of ART outcomes, including long-term assessment of the incidence of birth defects and cancer, and (2) investigation of the relationship between epigenetic errors in both offspring and parents, the specific methods of ART used, and the underlying infertility diagnoses. In addition, the components of proprietary commercial media used in ART procedures must be fully and publicly disclosed, so that factors such as methionine content can be assessed, given the relationship in animal studies between methionine exposure and epigenetic changes.

Introduction

The will to bear children is a categorical imperative for many couples. In the United States alone, the drive to overcome reproductive barriers has led to the development of a multibillion-dollar medical industry that is now supported by insurance laws in some states. Furthermore, the United States Supreme Court (1977) has ruled that the decision to bear children is constitutionally protected, and thus, from a societal standpoint, assisted reproductive technology (ART) differs from other areas of medicine. Indeed, the practice of ART has evolved largely outside the walls of academic medical research institutions, although there are certainly outstanding exceptions to this rule. Nevertheless, this largely extra-academic setting has consequences for ART research. For example, although outcomes affecting the success of pregnancy are closely studied, there has been a general lack of measures of long-term outcomes affecting the offspring themselves, related to the fact that ART and obstetrics care are performed by different people, often in different hospitals or cities. This history has important implications for understanding the consequences of this technology, and it is most important in any discussion of reproduction and disease to be clear about how little is known about these consequences. Recently, several confluent streams of evidence from human and animal studies have suggested that ART may lead to epigenetic defects in the offspring—that is, genetic changes not involving DNA sequence per se. The field of epigenetics is itself new, and our purpose in this review is to summarize that evidence and to suggest what must be learned in this scientifically intriguing—as well as emotional, costly, and legally complex—area of medical practice.

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Birth Defects and ART

ART is defined as those fertility treatments in which both egg and sperm are manipulated in the laboratory (i.e., in vitro fertilization [IVF] and related procedures) (Wright et al. 2003). Although the use of ART has become a widely accepted and implemented therapy for some forms of infertility, there have always been concerns about the long-term safety of removing and handling the germline. Some groups have studied short-term outcomes of ART, consistently describing an increase in multiple births and low birth weight (LBW) but not an increase in major malformations (Wennerholm et al. 1991; Rufat et al. 1994; Saunders et al. 1996; Dhont et al. 1997; D'Souza et al. 1997; Olivennes et al. 1997). There are significant limitations to these studies, however, involving either a small sample size, a short-term period of follow-up, or a lack of matched controls. Recent studies include those of Schieve et al. (2002), who studied 42,000 offspring of ART, comparing them with the rest of the U.S. population born in that year, and found an increase in LBW, even in singletons. They did not examine rates of birth defects in their sample. In a small recent series, Place and Englert (2003) compared 66 children conceived by intracytoplasmic sperm injec-

tion (ICSI) with 52 conceived by IVF and 59 conceived naturally, through the age of 5 years, and found no developmental differences. In a recent large series with a control group from the same population, Hansen et al. (2002) compared 1,138 offspring of ART in Australia and also found LBW, as well as a twofold increase in major birth defects. It is impossible to clearly stratify the offspring with birth defects, given that the authors were restricted by confidentiality rules and could not indicate the specific mode of conception for or the number of birth defects in a given child. One case of Prader-Willi syndrome was reported, when none would be expected in a population of this size. Although the observation is intriguing, statistical inferences cannot be made on only one observation. Anthony et al. (2002) compared 4,224 IVF-conceived children with 314,000 naturally conceived children in a Dutch national database, observing a small (odds ratio 1.20) but statistically significant increase in malformations, including a significant increase in cardiovascular malformations. Given that birth weight and Prader-Willi syndrome are both in part epigenetically controlled, an epigenetic connection seems plausible for the increase in malformations seen in the ART population.

Epigenetics and Human Genetic Disorders

Epigenetic alterations cause changes in gene expression that are heritable during cell division and that do not involve DNA sequence per se. These include DNA methylation, a covalent modification of cytosine at CpG dinucleotides, and chromatin modifications such as histone acetylation, methylation, and phosphorylation. In general, cytosine methylation and histone deacetylation are associated with condensed chromatin states and silenced gene expression. Genomic imprinting is an example of epigenetic inheritance in eutherian mammals in which differences in gene expression depend on the parental origin of the allele. Imprinted genes frequently harbor or lie near CpG islands or GC-rich sequences that have differential methylation between the maternal and paternal alleles. Imprinting may involve strict monoallelic expression but can occur with preferential but not absolute expression of a specific parental allele, and imprinting-directed gene expression is often tissue specific (Lee et al. 1997). In addition, there is variation in imprinting between individuals (Giannoukakis et al. 1996; Sakatani et al. 2001) and familial aggregation of methylation patterns (Sandovici et al. 2003) at imprinted genes.

Historically, epigenetics was of interest as the basis of non-Mendelian inheritance of several phenomena in model organisms that could not be explained by conventional genetic variation, such as position effect variegation in *Drosophila melanogaster* and telomere silencing in yeast. However, in the past few years, epigenetics has become central to our understanding of normal mammalian development for several reasons. First, normal embryogenesis cannot proceed without the machinery of epigenetic regulation. Experiments in mice show that knockouts of DNA methyltransferases and histone modifiers are embryonic lethal (Li et al. 1992; Okano et al. 1999; Bourc'his et al. 2001; Peters et al. 2001; Hata et al. 2002; Lagger et al. 2002). Experiments in mice have show that an equal contribution of both maternal and paternal chromosomes is required for normal development (McGrath and Solter 1984; Surani et al. 1984). Conceptuses with paternal disomy have poor embryonic development with relatively normal development of the extraembryonic membranes, whereas conceptuses with maternal disomy have poor extraembryonic membrane development with relatively less impaired embryonic development. In humans, embryos possessing two paternal genomes form trophoblastic tumors, whereas embryos possessing two maternal genomes form teratomas. Second, a substantial fraction of the human genome includes species-conserved nonexonic elements that are rich in CpG dinucleotides and are frequently the target of epigenetic modification (Elgar 1996; Hardison et al. 1997; Onyango et al. 2000). Third, one of the principal lessons of nuclear transfer cloning is that the information that stably maintains a differentiated tissue-specific state in a cell is, under the right conditions, reprogrammable and therefore largely epigenetic. The genome of the new pluripotent embryo is identical to the genome of the differentiated somatic cell from which it was derived; clearly, the information content, epigenetic by definition, is altered in the new cellular environment of the egg.

Epigenetic marks, including genomic imprinting, are reprogrammed during normal gametogenesis (fig. 1). Primordial germ cells undergo demethylation as they migrate along the genital ridge, both genomewide (Hajkova et al. 2002) and within imprinted loci (Hajkova et al. 2002; Lee et al. 2002; Yamazaki et al. 2003). Following this erasure, CpG methylation of imprinted genes is reestablished during gametogenesis through de novo methylation, in both eggs (Obata and Kono 2002) and sperm (Davis et al. 2000; Ueda et al. 2000). After fertilization, there is a phase of genomewide demethylation that occurs in two stages. First, the male pronucleus undergoes immediate active demethylation following fertilization (Mayer et al. 2000; Oswald et al. 2000); this is followed by passive demethylation of the zygote genome (Howlett and Reik 1991; Rougier et al. 1998; Santos et al. 2002). However, methylation marks on imprinted genes are protected from demethylation (Tremblay et al. 1997; Shibata et al. 1998; Warnecke et al. 1998), so that parental imprints are preserved.

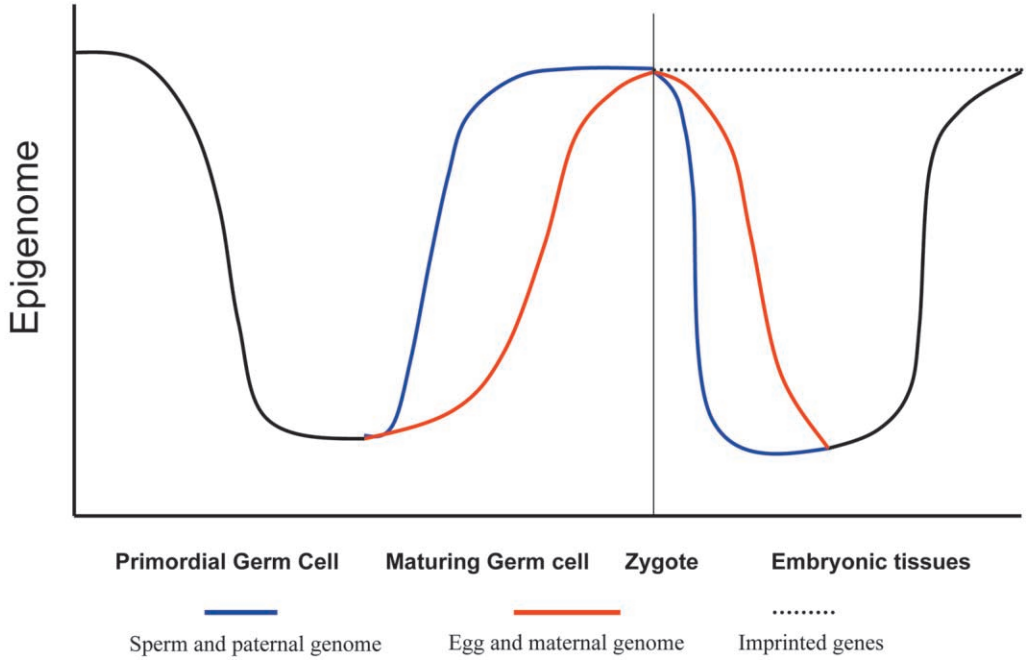

Figure 1 Dynamic reprogramming of the epigenome during development. Epigenetic marks, including DNA methylation and genomic imprinting, are reprogrammed during normal gametogenesis. Primordial germ cells undergo epigenetic erasure as they migrate along the genital ridge, and epigenetic marks are reestablished during gametogenesis, differentially in sperm (*blue*) and egg (*pink*). For example, after fertilization, there is active demethylation of the paternal pronucleus, and then a second wave of passive demethylation of the zygote genome. Imprinted genes (*dotted line*) are protected from this erasure. During development, tissue-specific epigenetic patterns emerge. The drawing is stylized, as details are unknown.

After implantation, embryonic genomewide methylation patterns are established in a lineage-specific pattern by de novo methylation (Rossant et al. 1986; Kafri et al. 1992).

Birth weight appears to be in part epigenetically determined. Studies of mice with engineered uniparental disomy for a region of chromosome 11 show overgrowth where there is paternal duplication and growth retardation where there is maternal duplication (Cattanach and Kirk 1985). Many imprinted genes are known to play important roles in determining fetal growth (reviewed in Young 2001). For example, targeted disruption in mouse of the imprinted *Igf2* gene results in progeny that are growth deficient (DeChiara et al. 1991), whereas overexpression by transgene expression (Sun et al. 1997) or loss of imprinting (Thorvaldsen et al. 1998) results in overgrowth. These observations and others form the basis of the Haig hypothesis, which proposes that genomic imprinting evolved to balance the conflict between paternal evolutionary drive to increase fetal growth and maternal evolutionary drive to limit fetal size (Moore and Haig 1991).

Several human disorders involving birth defects have now been shown to involve epigenetic alterations of genomic imprinting. The cardinal features of Beckwith-Wiedemann syndrome (BWS [MIM #130650]), which is linked to a cluster of imprinted genes on 11p15.5, are prenatal overgrowth, abdominal wall defects (omphalocele or umbilical hernia), neonatal hypoglycemia, and macroglossia. Children with BWS are at an increased risk of developing embryonal tumors, including Wilms tumor and hepatoblastoma. Prader-Willi syndrome (MIM #176270), which is linked to a cluster of imprinted genes on 15p11-13 normally expressed from the paternal allele, is characterized by muscular hypotonia, obesity, mental retardation, and hypogonadotrophic hypogonadism. Another neurological birth defect syndrome, Angelman syndrome (AS [MIM #105830]), is also linked to 15p11-13—in this case, to a gene normally expressed from the maternal allele (discussed in more detail below). The characteristic features of AS include severe developmental delay, absent speech, seizures, ataxia, hyperreflexia, and hypotonia.

Genetic mutations of the epigenetic machinery also cause birth defects. Rett syndrome (MIM #312750) is caused by mutations in the X-linked gene encoding MECP2, a methyl-CpG-binding protein (Amir et al. 1999). MECP2 is thought to induce transcriptional silencing by recruiting histone deacetylases to methylated

Table 1

Summary of Studies of Epigenetic Disorders after ART

Syndrome and Molecular Defects	No. in Registry	No. of ART Cases	No. Analyzed Molecularly	ART Technique	Reference
BWS:					
4/6 *LIT1* hypomethylation, 1/6 *LIT1* hypomethylation, and H19 hypermethylation	NA	7	6	IVF and ICSI	DeBaun et al. 2003
1/2 *LIT1* hypomethylation	65	3	3	IVF and ICSI	DeBaun et al. 2003
2/2 *LIT1* hypomethylation	149	6	2	IVF and ICSI	Maher et al. 2003
6/6 *LIT1* hypomethylation	149	6	6	IVF and ICSI	Gicquel et al. 2003
AS:					
2/2 Sporadic imprinting defect at IC	NA	2	2	ICSI	Cox et al. 2002
Sporadic imprinting defect at IC	NA	1	1	ICSI	Orstavik et al. 2003
RB:					
1/5 coding mutation in *RB1*	NA	5	5	IVF and ICSI	Moll et al. 2003

NOTE.—NA = not available.

DNA; this hypothesis is supported by evidence that histones are hyperacetylated in patients with Rett syndrome who have *MECP2* mutations (Wan et al. 2001). Immunodeficiency–centromeric instability–facial anomalies (ICF) syndrome (MIM #242860) is caused by recessive mutations in the *DNMT3B* gene (Hansen et al. 1999; Xu et al. 1999), which encodes a DNA methyltransferase. Repeated sequences that are normally heavily methylated in somatic cells are hypomethylated in patients with ICF syndrome (Jeanpierre et al. 1993). ICF syndrome is characterized by immunodeficiency in association with facial dysmorphism and centromeric instability of chromosomes 1, 9, and 16 in stimulated cells. X-linked α-thalassemia–mental retardation syndrome (ATRX) (MIM #301040) is caused by mutations in *ATRX* (Gibbons et al. 1995), which encodes a putative chromatin-remodeling protein. *ATRX* mutations are associated with abnormalities in DNA methylation in heterochromatic regions (Gibbons et al. 2000), suggesting that ATRX regulates the repressed chromatin state. Rubinstein-Taybi syndrome (RTS [MIM #180849]) is caused by mutations of CREB-binding protein (CBP) (Murata et al. 2001), a histone acetyltransferase. RTS is characterized by mental retardation, short stature, and facial anomalies. CBP enhances transcription of cAMP-responsive promoters by acetylating histones in promoter nucleosomes (Ogryzko et al. 1996).

In addition, birth defects are often caused by uniparental disomy (UPD) for specific human chromosomes that harbor clusters of imprinted genes (reviewed in Engel and DeLozier-Blanchet 1991). UPD occurs when both homologues of a chromosome are inherited from one parent, generating a diploid chromosome number but an imbalance of maternally and paternally inherited genes. It is thought that UPD can arise by a number of mechanisms, including trisomy rescue, in which a lethal trisomy is rescued by the loss of one homologue (one-third of trisomy rescues would result in UPD), and monosomy rescue, in which a lethal monosomy is rescued by the duplication of the monosomic chromosome. In addition, mosaicism for UPD can arise by postzygotic somatic segregation errors (Robinson et al. 2000). A phenotype associated with UPD necessarily implicates an imprinted gene within that region.

Independently of congenital genetic syndromes, epigenetics plays a critical role in human cancer. Alterations in DNA methylation, including hypomethylation and hypermethylation, are linked to disrupted gene expression in a wide variety of tumors. Loss of imprinting (LOI) is also common in both childhood and adult tumors, and LOI in normal cells has been recently linked to an increased personal and family history of colorectal cancer (Cui et al. 2003). Of particular relevance to this discussion, LOI can play a gatekeeper role in the development of embryonal tumors of childhood, including Wilms tumor of the kidney (reviewed by Feinberg and Tycko [2004]).

BWS and ART

Recently, syndromes involving epigenetic alterations have been reported to occur in individuals conceived by ART techniques, including IVF and ICSI (table 1). These include BWS, AS, and retinoblastoma (RB [MIM #180200]). These disorders share a common etiology, in that they can all involve epigenetic alterations, specifically aberrant imprinting and/or DNA methylation.

BWS is associated with multiple distinct genetic and epigenetic alterations of chromosomal band 11p15 that involve the maternally inherited allele (reviewed in DeBaun and Feinberg 2003). The imprinted gene region on chromosome 11 includes two imprinted subdomains: a more centromeric domain that includes $p57^{KIP2}$, *LIT1*,

TSSC3, and *TSSC5* and a more telomeric subdomain that includes *IGF2* and *H19* (Lee et al. 1999). About 5% of BWS cases involve conventional null mutations in the maternal allele of $p57^{KIP2}$, an imprinted maternally expressed gene encoding a cyclin-dependent kinase inhibitor. Another 15% of BWS cases involve imprinting defects of the maternal alleles of the *H19* and *IGF2* genes. Here, the normally unmethylated maternal allele is hypermethylated at a differentially methylated region upstream of *H19*. As a result, the maternal allele of *H19* is silenced, whereas the maternal allele of *IGF2* is abnormally activated. This epigenetic alteration is associated specifically with increased cancer risk in patients with BWS (DeBaun et al. 2002). In addition, ~40% of patients with BWS show LOI involving the maternal copy of *LIT1,* an antisense RNA normally expressed from the paternal allele (Lee et al. 1999; Smilinich et al. 1999). LOI of *LIT1* in BWS involves activation of the normally silent maternal allele, likely causing epigenetic silencing of $p57^{KIP2}$, because *Lit1* appears to mediate imprinting of $p57^{KIP2}$ in a knockout mouse model (Fitzpatrick et al. 2002) and in vitro experiments (Horike et al. 2000). Loss of imprinting of *LIT1* also appears to be specifically associated with birth defects, including prenatal overgrowth and midline abdominal wall defects, such as omphalocele (DeBaun et al. 2002). Approximately 10% of patients with BWS have paternal uniparental disomy for the entire 11p15 region, including *H19, IGF2, LIT1,* and $p57^{KIP2}$.

DeBaun et al. (2003) identified seven children with BWS who were born after use of ART. Molecular studies of six of these children indicated that four had spontaneous imprinting defects involving the *LIT1* subdomain, and one had imprinting defects at both the *LIT1* and *IGF2-H19* subdomains. In addition, DeBaun et al. (2003) conducted a prospective analysis of the prevalence of ART in a series of patients with BWS, which was 4.6% (3/65), compared with the population rate of 0.76% in the same period in the United States. Similarly, Maher et al. (2003) identified 6 patients with BWS who were born after ART in a cohort of 149 sporadic BWS births (4%). They compare this finding with a background rate of 0.997% births after ICSI or IVF in the United Kingdom. Molecular tests for UPD were negative in all four cases tested, and they also found hypomethylation of *LIT1,* similar to DeBaun et al. (2003), in both patients tested. In a third study, Gicquel et al. (2003) used a registry of 149 patients with BWS to identify 6 patients with BWS born after ART. All six tested positive for hypomethylation at *LIT1*. In addition, it is interesting (although not statistically significant) that one patient with BWS had been identified earlier in a group of 91 children conceived from cryopreserved IVF embryos; molecular studies were not performed (Sutcliffe et al. 1995). It is also interesting to note that these studies identified patients with BWS who were conceived by disparate ART methods, including IVF, ICSI, and embryo cryopreservation, so there does not appear to be an association between BWS and a specific ART technique.

AS and ART

AS (reviewed by Clayton-Smith and Laan [2003]) is caused by loss of function of the *UBE3A* gene on chromosome 15, which is normally expressed from the maternal allele in the brain and encodes a ubiquitin protein ligase (Scheffner et al. 1993). Although 10% of patients with AS have point mutations involving the maternal allele of *UBE3A* (Kishino et al. 1997; Matsuura et al. 1997), ~70% of cases result from an interstitial deletion of the maternal homologue of chromosome 15q11-13, including *UBE3A* (Knoll et al. 1989). AS also arises infrequently from paternal uniparental disomy for chromosome 15 (Malcolm et al. 1991). In addition, ~4% of patients with AS have a maternally inherited microdeletion of an imprinting control center (ICC) on 15q (Buiting et al. 1995) or have epigenetic alterations to this locus, involving aberrant hypomethylation of the maternally inherited chromosome (Buiting et al. 1998). The bipartite ICC lies proximal to the small nuclear ribonucleoprotein polypeptide N (*SNRPN*) gene; this ICC is normally methylated on the maternal allele. A region 35 kb upstream of *SNRPN* exon 1 controls the paternal-to-maternal imprint switch; maternally inherited microdeletions disrupt the establishment of the maternal imprint and lead to AS. Similarly, a region around exon 1 of *SNRPN* controls the maternal-to-paternal imprint switch; paternally inherited microdeletions disrupt the establishment of the paternal imprint and lead to a related neurodevelopmental disorder, Prader-Willi syndrome. In patients with AS who have sporadic imprinting defects, the maternal copy of the ICC is aberrantly hypomethylated (Buiting et al. 1998).

Cox et al. (2002) identified two children with AS who were conceived by ICSI, and Orstavik et al. (2003) identified another child with AS who was conceived by ICSI. All three ICSI-conceived patients with AS showed hypomethylation of the ICC proximal to *SNRPN*. FISH studies and microsatellite analysis ruled out interstitial deletions of chromosome 15q and uniparental disomy, respectively. In addition, microdeletions within the ICC were ruled out by Southern blot analysis. Thus, it was concluded that all three children had sporadic imprinting defects manifested as hypomethylation. This is particularly interesting, considering that the most common cause of AS is deletion within 15q (70% of all cases), whereas imprinting defects account for only 3% (at most) of all cases.

It is striking that most (but not all) ART-associated

defects in imprinting disorders involve the maternal allele—for example, aberrant hypomethylation of the maternal *LIT1* allele in BWS and aberrant hypomethylation of the maternal ICC in AS. Although this inference admittedly is based on very limited data, it would suggest that ART effects are greater on the oocyte than on the sperm and are related either (1) to maturational or environmental changes caused by ART or (2) to the nature of reproductive problems affecting the oocyte and are not caused by the procedure. Either way, a mechanistic understanding would have profound consequences both for reducing ART-related birth defects and for reproductive success.

RB and ART

RB is a tumor of the retina that occurs in early childhood and is the classic embryonal tumor used to confirm to the Knudson two-hit hypothesis of cancer. The *RB1* gene on chromosome 13q14 was the first tumor suppressor identified. In most cases, RB arises from a mutation (either inherited or sporadic) on one *RB1* allele, with loss of the other allele by chromosomal loss or deletion. Children with bilateral disease have generally inherited a germline mutation in *RB1,* whereas, in children with unilateral disease, both *RB1* alleles undergo somatic inactivation. However, in some unilateral cases, the *RB1* gene is epigenetically silenced, as indicated by hypermethylation of the promoter region and exon 1 (Greger et al. 1989; Ohtani-Fujita et al. 1993) and reduced *RB1* expression (Sakai et al. 1991). In addition, Ohtani-Fujita et al. (1997) determined that the frequency of hypermethylation in unilateral tumors is 9% (13/140), whereas the frequency was only 1% in hereditary bilateral tumors (1/101). Thus, epigenetic mechanisms clearly play a role in some patients with RB. Additional evidence for epigenetic mechanisms involved in RB includes the observation of differential methylation of the region of chromosome 13q around the *RB1* gene (Blanquet et al. 1991) and the preferential loss of maternal alleles in sporadic cases, suggesting latent or aberrant imprinting in some individuals (Leach et al. 1990; Naumova et al. 1994; Naumova and Sapienza 1994).

Moll et al. (2003) reported five cases of RB in children conceived by IVF in the Netherlands. As in the studies reporting BWS and AS in IVF-conceived children, these cases were not ascertained through studies of an IVF population; rather, they were ascertained from an ophthalmology clinic. The authors estimated that the incidence of RB in the Netherlands is 1/17,000 live births. They inferred a relative risk of 4.9 or 7.2 under the alternative assumptions that 1.5% or 1.0% of all births follow ART. Two of the affected children in their series had bilateral disease, and, in one of these cases, a mutation was identified. The remaining three cases had unilateral disease, and no mutation could be identified. Unfortunately, the authors did not report the status of methylation or expression of the *RB1* gene.

Insights from Ruminants

In the animal husbandry industry, there has been a long history of reproductive technology, from sperm cryopreservation in the 1950s to a more recent rapid expansion of the use of ART, paralleling that in humans (Thibier 1998). Studies of ruminants (cattle and sheep) born after ART have documented the frequent appearance of abnormally large calves and lambs, with an increase of 8%–50% from mean control weight, at incidences of up to 100% (reviewed by Young et al. [1998]). This overgrowth is termed "large offspring syndrome" (LOS) (reviewed by Sinclair et al. [2000]) and is characterized by a significant increase in birth weight, increased gestational length, breathing problems at birth, and increased frequency of perinatal death. LOS occurs after embryo culture, transfer of embryos into an asynchronous uterine environment (i.e., one in which gestational age is unmatched between the embryo and the recipient), or a maternal gestational diet high in urea. LOS also occurs after nuclear transfer cloning, in which cloned embryos are cultured in vitro and then are transferred into an asynchronous recipient.

Virtually all culture systems associated with LOS involve exposure to sera or cocultured support cells, such as granulosa cells, oviductal cells, or fibroblasts (reviewed in Catt 1994). Thus, it is thought that growth factors in the sera or secreted by the support cells may be involved in the etiology of LOS. Important steps in epigenetic reprogramming occur in germ cells and in the early embryo.

There could also be an epigenetic explanation for LOS following embryo culture. Young et al. (2001) report a differentially methylated region (DMR) in intron 2 of the insulin-like growth factor II receptor gene (*IGF2R*) in sheep, which is imprinted in mouse (Barlow et al. 1991) and possibly variably imprinted in humans (Xu et al. 1993). The IGF2R protein is a multifunctional transmembrane receptor that binds mannose-6-phosphate residues on lysosomal enzymes and transports them into lysosomes (Wang et al. 1994). IGF2R also has a separate binding site for IGF2 (Kiess et al. 1988) and is thought to act as a sink for IGF2 by transporting it to lysosomes for degradation. Young et al. (2001) used a model for LOS in which in vivo fertilized eggs are cultured in vitro for 5 d. Young et al. recovered fetuses after 125 d of gestation; 25% of cultured fetuses (12/48) were abnormally large. They reported 30%–60% reduction of *IGF2R* mRNA and protein in the fetuses with LOS, and this difference was accompanied by loss of methylation of the maternal *IGF2R* DMR.

In addition, experiments in mice provide direct evidence for alterations of methylation patterns and birth weight in cultured embryos. Khosla et al. (2001) determined that addition of serum to mouse embryos cultured in M16 medium results in changes in expression and methylation of the imprinted genes *H19, Igf2, Grb10,* and *Grb7* and reduction in fetal weight. Also, Doherty et al. (2000) studied the effect of culture on mouse *H19* expression and methylation. They found that the normally silent paternal allele was aberrantly expressed and hypomethylated. These alterations were dependent on the type of culture media used, with specificity for culture in Whitten's medium but not in KSOM with amino acids. Although the authors did not address this point, we hypothesize that the critical difference may be methionine content, which can affect DNA methylation and imprinting (Wolff et al. 1998; Waterland and Jirtle 2003). In this regard, it is particularly worrisome that many IVF clinics purchase media from companies that do not divulge the formulation of the media they market for human embryo culture. This secrecy is particularly worrisome, since embryos adapt to imperfect media with unclear consequences on gene expression and long-term effects (Summers and Biggers 2003). It is remarkable that the U.S. Food and Drug Administration (2001) regulates tissue-culture media for human ex vivo use only for toxicity and sterility and does not consider the effect of varying media on pregnancy outcome or birth defects.

A Call for Investigation

Two related questions call for investigation: (1) the relationship, if any, between birth defects and ART and (2) the overlapping but independent question of the relationship between ART and epigenetic defects in the resulting offspring.

Are Children Born after ART More Likely to Develop Birth Defects?

A strong but circumstantial argument has been made for an association between ART and some of the disorders known to result from an epigenetic mechanism. However, large prospective studies have not yet been performed. A beginning step was taken by the Panel to Evaluate the U.S. Standard Certificates and Reports, which recommended that a question relating to fertility treatment should be added to the U.S. Standard Certificate of Live Birth (National Center for Health Statistics 2000). Information collected on standard certificates is used to produce national vital statistics and is therefore a critical source of population-based public health data. Unfortunately, due to limited resources, implementation of the revised certificate will be phased in state by state over a number of years. Therefore, a complete national data set of revised certificate items is not expected to be available until the end of the present decade. Long-term follow-up is also critical. For example, one of the most troubling potential complications suggested by the studies of BWS and RB with ART is malignancy, and ascertainment of cancer would necessitate multiyear follow-up. A comprehensive evaluation of existing data on the health and developmental outcomes of children born after ART is underway, and the results of this study will be valuable in lending support to this call for further research and in suggesting study design and outcome measures (Redfearn 2003).

Is ART Associated with Epigenetic Defects, and, If So, What Is the Mechanism for This Association?

Determining the answer to this question would require additional in-depth investigation, including laboratory studies—in a limited population group in which parents are asked to consent voluntarily to cord blood sampling for epigenetic testing and to provide detailed clinical information. If the association of epigenetic alterations with ART is confirmed, there are two equally important alternative explanations for it:

1. Epigenetic alterations could arise directly from some aspect of ART. The most obvious mechanism is in vitro culture itself, or the media used, but there are other possibilities. For example, medically induced ovarian hyperstimulation that precedes fertilization could be responsible, as could alterations in maturational timing of the gametes that are harvested.
2. Epigenetic alterations could be a significant cause of infertility, rather than a consequence of the procedure used to treat it. Thus, infertile couples using ART could have an increased prevalence of epigenetic defects in their gametes, which ART is simply uncovering. To address this possibility, it is critical to ascertain in any study design the underlying basis of infertility. If, for example, epigenetic alterations are found only among those couples with ovarian failure or sperm maturational defects and not among those with mechanical problems (e.g., tubal disease), then these changes are unlikely to be caused by the procedure. Of course, uncovering an epigenetic cause for infertility would be a major advance in and of itself.

Conclusion

An association between ART and epigenetic defects is credible and is supported by experimental studies of mouse embryo culture and by LOS in ruminants. However, conclusive demonstration of such an association and identification of its cause can only be accomplished

by large-scale and long-term outcome studies, as well as by laboratory research. In addition to their importance for understanding the epigenetics of ART, such studies may reveal common causes of birth defects that are magnified by ART. For example, if the methyl donor content of media is critical to ART-mediated embryo anomalies, this would suggest that dietary factors might play a similar role in commonly occurring birth defects in non-ART pregnancies. Furthermore, it is possible that additional studies may reveal that epigenetic defects are a mechanism for some causes of infertility, rather than a consequence of ART. Such insights would represent an important first step in diagnosing and treating the underlying causes of infertility, reducing the need and cost of surgical treatment. Finally, although pregnancy rates from ART have been reported to the Centers for Disease Control, birth defects and cancer are not routinely assessed, and neither is the relationship between specific outcomes and media or procedural details. In this era of heightened involvement of institutional review boards in even minimally invasive research, it is surprising that there is a relative lack of such surveillance in developing methods for creating and culturing human embryos intended for birth. Design of the studies we propose is a complex question that itself requires further study and discussion—hopefully in an appropriate forum and with broad input from investigators, practitioners, and ethicists to ensure appropriate study design and privacy protection.

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Electronic-Database Information

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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