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Gametes and Embryo Epigenetic Reprogramming Affect Developmental Outcome: Implication for Assisted Reproductive Technologies

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ABSTRACT

There is concern about the health of children who are conceived with the use assisted reproductive technologies (ART). In addition to reports of low birth weight and chromosomal anomalies, there is evidence that ART may be associated with increased epigenetic disorders in the infants who are conceived using these procedures. Epigenetic reprogramming is critical during gametogenesis and at preimplantation stage and involves DNA methylation, imprinting, RNA silencing, covalent modifications of histones, and remodeling by other chromatin-associated complexes. Epigenetic regulation is involved in early embryo development, fetal growth, and birth weight. Disturbances in epigenetic reprogramming may lead to developmental problems and early mortality. Recent reports suggest the increased incidence of imprinting disorders such as Beckwith-Wiedemann syndrome, Angelman syndrome, and retinoblastoma in children who are conceived with the use of ART. These may result from an accumulation of epigenetic alterations during embryo culture and/or by altered embryonic developmental timing. Further research is urgently needed to determine whether a causal relationship between ART and epigenetic disorders exists. Until then, cautious review of both short-term and long-term ART outcomes at a national level is recommended. **(*Pediatr Res* 58: 437–446, 2005)**

Abbreviations
ART, assisted reproductive technologies
AS, Angelman syndrome
ATRX, X-linked α-thalassemia/mental retardation
BWS, Beckwith-Wiedemann syndrome
DNMT, DNA methyltransferase
FMR1, fragile X mental retardation gene 1
HDAC, histone deacetylase
ICF, immunodeficiency, centromeric instability and facial anomalies
IVF, *in vitro* fertilization
LBW, low birth weight
LOI, loss of imprinting
LOS, large offspring syndrome
MBD, methyl-binding domain
RB, retinoblastoma
RTS, Rubinstein-Taybi syndrome
RTT, Rett syndrome
UPD, uniparental disomy
VLBW, very low birth weight

Recent reports of birth defects and health problems in children who are conceived with assisted reproductive technologies (ART) has led to the initiation of prospective and retrospective follow-up studies in these children to evaluate the safety of these techniques. These investigations have determined that development is normal and that malformation rates are either similar or only slightly greater than those in the general population (1,2). These studies, however, have also revealed a lower birth weight in the *in vitro* fertilization (IVF) and ICSI singletons as compared with naturally conceived children (3–5). It has been postulated that embryos in culture may acquire epigenetic defects as a result of the abnormal environmental conditions that may lead to these aberrant phenotypes. Several lines of evidence support this hypothesis. The accumulation of severe epigenetic disturbances above a certain threshold may lead to early mortality. Alternatively, embryos that develop to term may still have epigenetic defects, and these may result in obvious aberrant phenotypes or in subtle changes in gene expression that can be easily overlooked. These epigenetic reprogramming disturbances can occur at gametogenesis or at preimplantation embryo stage and could be influenced by *in vitro* culture conditions. This review explores epigenetic reprogramming in the gametes and embryo

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and how normal epigenetic modification may be influenced by ART.

INTRODUCTION TO EPIGENETICS

Epigenetic changes are defined as chemical alterations to the DNA or to the histone proteins associated with it that change the structure without altering the nucleotide sequence. The DNA, complexed together with the histones, make up chromatin. These epigenetic changes modify the structure of the chromatin by making it more condensed or more open. These changes in turn affect gene expression within the DNA by either allowing or preventing accessibility to the DNA of the factors involved with transcription.

Unintended gene silencing caused by epigenetic modifications has been linked to several human diseases. Specifically, DNA methylation and histone deacetylation are the major forms of epigenetic modifications that occur in tumors. DNA methylation is a chemical modification of the DNA molecule itself, which is regulated by an enzyme called DNA methyltransferase (DNMT). Methylation can directly switch off gene expression by preventing the ability of transcription factors to access DNA and binding to promoters. Another chemical modification of DNA is histone deacetylation. In this process, enzymes called histone deacetylases (HDACs) are associated with methyl-binding domain (MBD) proteins and are attracted to DNA. Activation of these HDACs leads to alteration of the histones and chromatin structure, thereby causing the chromatin condensation, inaccessibility of the DNA promoters, and gene silencing (see Fig. 1).

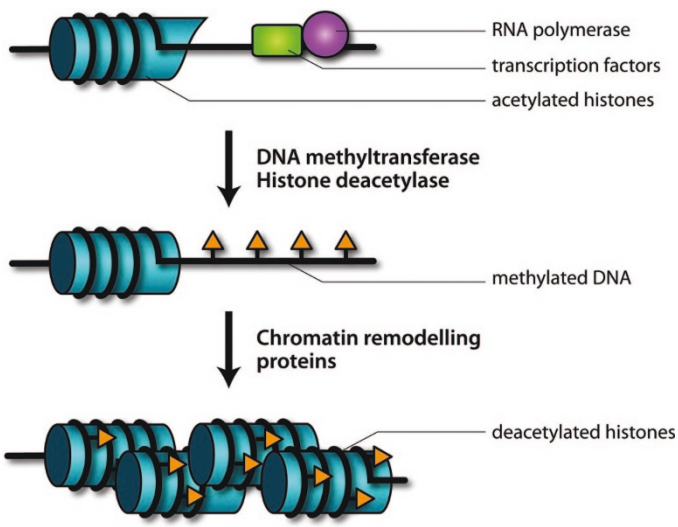

Figure 1. Epigenetic modifications that silence gene expression. (*Top*) Open chromatin is characterized by nonmethylated DNA and histones with acetylated tails. This permits accessibility of the DNA promoters with assembly of transcription factors and transcription by RNA polymerase. (*Middle*) Activity of DNMT leads to methylation of DNA. This event may directly block binding by transcription factors and prevent transcription. In addition, this event may attract and recruit MBD proteins that have associated HDACs. (*Bottom*) DNA methylation and histone deacetylation result in the condensation of chromatin into a compact state that is inaccessible by transcription factors.

EPIGENETIC REPROGRAMMING

Epigenetics is commonly defined as the study of heritable changes in gene function that occur without a change in the DNA sequence. Epigenetic modifications work in concert with the genetic information of a DNA sequence. These modifications include DNA methylation, imprinting, RNA silencing, covalent modifications of histones, and remodeling by other chromatin-associated complexes. All of these alterations regulate gene expression. These are referred to as epigenetic patterns, and they are normally built into the genome during differentiation through genetically determined programs. Additional epigenetic alterations to the DNA allow cells to alter the expression level of the different genes in response to environmental factors without having to change the DNA code itself.

Inheritance or persistence of these epigenetic modifications is referred to as epigenetic reprogramming. The most critical periods at which epigenetic reprogramming occurs are those during gametogenesis and the preimplantation embryonic stage (6). Reprogramming during gametogenesis is essential for the imprinting mechanism that regulates the differential expression of paternally and maternally derived genes. Primordial germ cells undergo demethylation, both globally (7) and within imprinted loci as they migrate along the genital ridge (7,8). After this demethylation or erasure, which ensures genetic totipotency, CpG methylation of imprinted genes is reestablished during gametogenesis through *de novo* methylation, in both eggs (9) and sperm (10). Imprints are established differentially in sperm and oocyte and are maintained in the embryo and further through all somatic cell divisions. Methylation and chromatin remodeling take place with each cell division and provide the germ cell genome with molecular blueprints for oocyte activation and embryonic development. There are significant differences between mature oocytes and sperm in epigenetic organization. The sperm genome is more methylated than the oocyte genome. The chromatin is well compacted with protamines in sperm but with histones in oocytes. The oocyte chromatin structure is more "closed" than that of sperm cells. Finally, imprinted loci are differentially methylated among sperm and oocytes. At fertilization, the oocyte and sperm genomes are transcriptionally silent. After fertilization, chromosomes from the sperm decondense and are remodeled. Protamines are exchanged for maternal histones as rapid demethylation takes place (11). The oocyte genome is demethylated more slowly by a passive mechanism (12). Genes that are imprinted with methylation marks, however, are protected from demethylation (13), so parental imprints are maintained. At the time of implantation, *de novo* methylation occurs genome-wide in a lineage-specific pattern (14). Epigenetic reprogramming during the preimplantation period is essential for correct development because this phenomenon regulates expression of early embryonic genes, cell cleavage, and cell determination. As the organism ages, the early embryonic genes are silenced and tissue-specific genes are expressed. Imprinted genes maintain their methylation marks from the germ cell stage, and they avoid these general erasure processes of the preimplantation stage.

Consequence of disturbances in epigenetic programming. Aberrant or incomplete epigenetic reprogramming at the preimplantation embryo stage or earlier may result in developmental delays and embryonic lethality. The epigenetic disturbances that are tolerated may result in phenotype changes that will manifest at a later stage. Epigenetic modifications are normally erased in the germ line, whereas genetic mutations or modifications are not. Partial erasure results in epigenetic inheritance as demonstrated by the epigenetic inheritance of the Avy allele in mice (15). Epigenetic changes that occur shortly after fertilization, before specification of the germ line, will involve both somatic cells and germ-line cells and thus be transmitted to the next generation (16). Currently, only a few examples of epigenetic inheritance in mammals are known. Methylation blueprints in humans have been shown to be inherited to some degree (17). Somatic cell nuclear transplantation experiments with early mouse embryos have resulted in altered patterns of gene expression that have resulted in phenotype changes (reduced body weight) later in development. These epigenetic changes were transmitted to the majority of offspring of the manipulated parent mice (18). Another example is the unexpectedly increased perinatal mortality and low birth weight observed in the children of women who were malnourished in the first and second trimesters of their own fetal development during the Dutch famine of 1944–1945 (19). Despite that these women were not underweight at birth, their offspring manifested abnormalities. It was concluded that in response to the malnutrition, epigenetic deregulation in the fetuses occurred at the level of germ cells. This phenomenon may represent a lack of epigenetic erasure that may not give rise to phenotype changes in the affected offspring but could be transmitted to the next generation. Given these examples, it is possible that incomplete epigenetic modifications in the embryos conceived by IVF and ICSI may be responsible for the reported birth weight difference in these children (3–5). If this is true, then it is also possible that both somatic and germ cells will be affected and that these changes would be reflected not only in the ART-conceived infants but also in their progeny.

Epigenetics and development. Epigenetics plays a major role in many aspects of normal mammalian development. The process of embryogenesis is epigenetically controlled. Knockout studies in mice null for DNMTs and histone modifiers result in embryonic lethality (20–22). In addition, both maternal and paternal chromosomes are required for normal development (23). Fetuses with maternal disomy have poor extraembryonic membrane development with less impaired embryonic development, whereas fetuses with paternal disomy have poor embryonic development with considerably less effect on development of the extraembryonic membranes. Finally, as a result of somatic cell nuclear transfer studies, it now is clear that differentiated, tissue-specific genetic information is reprogrammable under the right conditions and by definition is epigenetic. The genome of the new pluripotent blastocyst is identical to the genome of the differentiated somatic cell from which the DNA is obtained, but the information content is modified as demonstrated by the altered methylation pattern reported in cloned bovine embryos (24).

Some imprinted genes are responsible for regulating fetal size or birth weight (25). Disruption of the imprinted mouse gene *Igf2* results in growth retarded offspring (26), whereas overexpression of the transgene results in fetal overgrowth (27). Similarly, loss of imprinting (LOI) of *Igf2* also results in a mouse model of Beckwith-Wiedemann syndrome (BWS), consistent with macrosomia (28). Uniparental disomy (UPD) for an area of chromosome 11 reflects overgrowth when there is paternal duplication and growth deficiency when there is maternal duplication (24). This finding supports the Haig hypothesis, which proposes that genomic imprinting evolved to match the paternal evolutionary bias to increase fetal growth with the maternal evolutionary bias to restrict fetal size (29).

Genetic disorders caused by (de)methylation and/or (de)acetylation. DNA methylation is caused by chemical modification of the DNA and is regulated by enzymes called DNMTs. Of the five DNMTs described, DNMT1 prefers hemimethylated DNA as a substrate and hence is referred to as "maintenance methyltransferase." Mouse oocytes and preimplantation embryos lack DNMT1 but express a variant of this protein called DNMT1o, which provides maintenance methyltransferase activity specifically at imprinted loci during the fourth embryonic S phase (30). DNMT3A and DNMT3B are referred to as "*de novo* methyltransferases" because they prefer nonmethylated DNA. The fifth protein, DNMT2, has strong sequence and structural affinities with DNMTs but has not shown transmethylase activity in biochemical or genetic tests (31). DNMT1 and DNMT3b gene dysfunction results in midgestation embryo demise. DNMT3a knockout mice live but fail to thrive and die soon after birth (32). DNA methylation attracts methyl-CpG binding proteins, such as MECP2, that have associated HDACs and bind to the methylated regions. The histone deacetylation results in chromatin condensation, thus causing the chromatin to be inaccessible to transcription factors in promoter regions (33). DNA methylation is also transcriptionally regulated. Human CpG-binding protein is a transcriptional activator that binds specifically to unmethylated CpG dinucleotides, modulating the expression of genes located within CpG islands (34). CpG-binding protein plays a crucial role in embryo viability and peri-implantation development. Mouse embryos lacking the *Cgbp* gene are viable only up to the blastocyst stage. These results illustrate loss of methylation control rarely in preimplantation development lead to abnormal development and cell death (35).

Several human genetic diseases have been found to be due to mutations in genes that produce proteins that are known or suspected to be involved in maintaining or modifying DNA methylation (36) (Table 1). The spectrum of mutations identified in patients with immunodeficiency, centromeric instability and facial anomalies (ICF), predominantly missense mutations in the C-terminal catalytic domain of DNMT3B gene located at chromosome 20q11.2, interfere with but not to completely abolish the methyltransferase enzymatic activity (37). The syndrome is a rare autosomal recessive disorder in which patients display immunodeficiency; instability of pericentromeric heterochromatin of chromosomes 1, 9, and 16; and facial anomalies, as well as mental retardation and developmental delay (38). Satellite DNA at pericentromeric heterochromatin

Table 1. *Epigenetic disorders due to methylation and/or acetylation defects*

Defective gene	Effect	Syndrome	Reference
DNMT3B gene	Pericentromeric Hypomethylation	(ICF) Immunodeficiency Centromeric instability Facial anomalies	39
MECP2 gene	Hyperacetylated Histones	Rett syndrome X linked dominant MR, loss of motor Function	112 33
MECP2 gene		Non-specific X linked MR	44
MECP2 gene		PPM-X syndrome Psychosis, Pyramidal signs Macro-orchidism	45
FMRI gene	De novo methylation Histone acetylation Of CGG repeats	Fragile X mental Retardation	46 47
ATRX gene	DNA methylation Abnormalities in Heterochromatic Regions.	ATR-X, X linked alpha thalassemia Mental retardation Syndrome	48
CBP	Acetylation defect In promoter Nucleosomes	Rubinstein-Taybi syndrome (RTS)	113 50

is hypomethylated in patients with ICF syndrome (39). Mutations in genes that code for methyl-CpG binding proteins MECP2, MBD1, and MBD2 could induce hyperacetylation of histones (33) and prevent transcriptional repression of methylated DNA (40) as seen in Rett syndrome (RTT), which is caused by mutations in the gene at Xq28 that encodes MECP2 (41). Patients with classical RTT seem to develop normally for 6–18 mo; however, this is followed by a period of regression characterized by a deceleration in head growth, loss of speech and purposeful hand use, and the appearance of repetitive hand movements. Patients can also suffer from autism, apraxia, and severe breathing dysfunction (42). After this initial period of regression, the condition becomes more stable and many survive into adulthood. Because *MECP2* is on the X chromosome, X inactivation patterns play a significant role in determining the severity of symptoms. RTT is now known to be one of the most predominant causes of mental retardation in female individuals, occurring with a frequency of up to 1/10,000 live female births (43). Mutations in male individuals are thought to be lethal, but cases of severe neonatal encephalopathy have been reported in male individuals born in RTT families (44). Mutations at seven different sites on *MECP2* gene have been identified. Mutations in the same gene but distinct from those involved in RTT have been identified as the cause of nonspecific X-linked mental retardation (45). Another A140V mutation on *MECP2* gene is the cause of the X-linked syndrome of psychosis, pyramidal signs, and macro-orchidism in affected male individuals and female carriers (46). *De novo* methylation and histone acetylation of the expanded polymorphic CGG repeats (50 to >200 times) in the 5' region of fragile X mental retardation gene 1 (*FMR1*) located at Xq27.3 cause fragile X mental retardation. *FMR1* gene codes for FMR1 protein, which regulates synaptic plasticity, important in learning and memory (47). The *de novo* methylation spreads to the CpG island in the promoter region and silence the *FMR1* gene and thus FMR1 protein transcription. Children with X-linked α-thalassemia/mental retardation (ATRX) display mental retardation, hypotonia, developmental delays, craniofacial anomalies, α-thalassemia, and urogenital abnormalities. The *ATRX* gene located at X q13 encodes chromatin-remodeling proteins that are critical mediators of cell survival during early neuronal differentiation (48). Mutations in *ATRX* gene cause changes in the methylation patterns of repeated sequences (49). Mutation of the cAMP response element binding (CREB) protein, a histone acetyltransferase, is the cause of Rubinstein-Taybi syndrome (RTS) (50).

CREB protein belongs to a large family of structurally related transcription factors that recognize the cAMP response promoter site (5'-TGACGTCA-3') and enhance transcription by acetylating histones in promoter nucleosomes (51). RTS is characterized by mental retardation, short stature, broad thumbs and toes, and facial anomalies. Mouse RTS model studies suggest that treatment with drugs, such as phosphodiesterase 4, that modulate CREB function by enhancing cAMP signaling could abolish long-term memory defect (52). A common feature of the above diseases is mental retardation. It seems that early neuronal development is transcriptionally regulated by genes involved in DNA epigenetic regulation (53).

Besides mutations in DNMTs, methylation defects may arise from epigenetic alterations of the genes of interest. In addition, altered expression of DNMTs during the cell cycle may lead to methylation defects. DNMT1 and 3b levels were found to be significantly down-regulated in G0/G1 phase, whereas DNMT3a mRNA levels were less sensitive to cell-cycle alterations and were maintained at a slightly higher level in tumor lines compared with normal cells (54). Similarly, changes in embryo or oocyte developmental timing, such as may be caused by *in vitro* culture, may interfere with DNMT activity and result in altered methylation patterns and expression levels.

Imprinting disorders. Most autosomal genes are expressed from both maternal and paternal alleles. However, imprinted genes are an example of non-Mendelian genetics, in which only one member of the gene pair is expressed and expression is determined by the parent of origin (55). Imprinted genes may account for 0.1–1% of all mammalian genes. At least 80 imprinted genes have been identified in humans, and imprinted genes frequently cluster under the control of an imprinting center. Genome imprinting, although observed in species such as *Drosophila melanogaster* (chromatin remodeling proteins), Neurospora Crassa (X chromosome inactivation), and flowering plants (55), have evolved to complex levels to fine-tune growth of the fetus in marsupials (56) and eutherian mammals. Imprinting is epigenetically controlled. Although DNA methylation has a crucial role in the process as evidenced by differential methylation of gametes in highly methylated domains within or near the imprinted genes (57), other processes such as exclusive histone acetylation of expressed allele (IGF2-H19, IGF2r, Snrpn, and U2af1-rs1), antisense transcripts (KCNQ1/KCNQ10T1 and UBE3A/UBE3A-AS), and the presence of repeat elements near or within the differential methylated domains (IGF2r DMD2) are also involved (58). Genes

that are regulated by imprinting have been shown to be essential for fetal growth (IGF2r, Cdkn1c, H19/IGF2, Gnas, Peg 1, Peg 3 Ins2, Kcnq1ot1, Ndn, and Rasgrf1), placental function (Slc22a3, H19/IGF2, Peg1, Peg 3, Ascl2, and Tssc3), neurologic development (Kcnq1, Ube3a, Peg1, Peg 3, Ndn, Snrpn, and Nnat), and carcinogenesis (IGF2, WT1, M6P/IGF2R, and p73) (59). Mutations in these genes are associated with several human syndromes, including Silver-Russell (chromosome 7), Albright hereditary dystrophy (chromosome 20), Prader-Willi/Angelman (chromosome 15), Beckwith-Wiedemann (chromosome 11), and Wilms' tumor. Altered imprinting is also suspected in other neurobehavioral disorders such as autism, epilepsy, Tourette syndrome, late-onset Alzheimer's, schizophrenia, and bipolar affective disorders (60). Mouse knockouts of some imprinted genes show significant neurologic defects ranging from abnormal maternal behavior (*Peg3* and *Peg1*) and impaired memory (*Grf1* and *Gabrb3*) to motor dysfunction with seizures (*Ube3a*).

Germ cell development and preimplantation embryogenesis are crucial windows in erasure, acquisition, and maintenance of genomic imprints. A complete defect of imprint erasure would result in 50% of the gametes maintaining an inappropriate imprint and carrying the opposite sex's epigenotype at certain imprinted loci. Establishment defects could result in absence of an imprint at a specific locus and again lead to the gametes' harboring the opposite sex's imprint epigenotype. Defects in imprint maintenance could occur at any stage of pre- or postimplantation embryo development. Maintenance defects in postzygotic embryos could lead to mosaicism with a subset of cells affected by the maintenance defect and the remainder of cells unaffected (*i.e.* with normal imprints). The timing of imprinting is also crucial, as disturbances between embryonic clock and imprinting could cause major disturbances and could be the postulated cause for increased methylation defects in infants who were conceived with the use of ART. For example, DNMT1o is essential for the maintenance of methylation on imprinted genes in mouse 8 cell stage embryo, at which time the normally cytoplasmic isoform translocates to the nucleus. Embryos that are absent of DNMT1o implant successfully but die before birth (30).

Angelman syndrome (AS) and Prader-Willi syndrome (PWS) were initially described in the 1950s and 1960s and are associated with deletion at 15 q11–13, which harbors a cluster of imprinted genes including SNRPN, UBE3A, ZNF127, IPW, and NDN (Table 2). The characteristic features of AS include severe developmental delay, absent speech, seizures, ataxia, hyperreflexia, and hypotonia. Approximately 70% of cases of AS arise from a deletion of the maternal homologue of chromosome 15q11–13, including *UBE3A*85, a gene normally expressed from the maternal allele. Ten percent of patients with AS have point mutations involving the maternal allele of *UBE3A* (61). *Ube3a/UBE3A* shows tissue-specific imprinting, being biallelically expressed in most tissues with the paternal allele silenced selectively in hippocampal and cerebellar neurons. AS arises infrequently from paternal UPD for chromosome 15 (62). In addition, ~4% of patients with AS have a maternally inherited microdeletion of an imprinting control center that is normally methylated on 15q (63) and lies proximal to SNRPN or have epigenetic alterations to this locus, leading to abnormal hypomethylation of the chromosome. PWS is associated with loss of function of the paternal allele or maternal duplication of the SNRPN imprinting locus on 15p11–13. It is characterized by muscular hypotonia, obesity, mental retardation, and hypogonadotrophic hypogonadism and characteristic reduced fetal activity *in utero*. In a subset of patients with PWS, paternally inherited microdeletion near exon 1 of *SNRPN* resulting in methylation at the imprinting control center, which causes loss of function of paternally expressed genes in this locus, is seen.

Another disease associated with epigenetic changes is BWS. These alterations occur on the maternal allele at 11p15.5, which contains imprinted genes H19, IGF2, CDKN1C (p57KIP2), KCNQ1, and KCNQ1OT1 (LIT1). BWS is characterized by prenatal overgrowth, abdominal wall defects (exomphalos, omphalocele, or umbilical hernia), neonatal hypoglycemia, visceromegaly, macroglossia, characteristic indentations of the ear, placentomegaly, and placental chorioangioma. Children with BWS are at an increased risk for developing embryonal tumors, including Wilms' tumor and hepatoblastoma. Two different methylation defects or LOI have been described in patients with BWS. LOI at BWS imprinting center 2 (BWSIC2) involving KCNQ1OT1 (LIT1), the antisense transcript of KCNQ1, is seen in 40% of patients with BWS. Expression of KCNQ1OT1 (usually expressed from paternal allele) becomes biallelic, whereas CDKN1C and KCQ1 are silenced (64,65). BWSIC2 LOI seems to be specifically associated with birth defects, including prenatal overgrowth and midline abdominal wall defects, such as omphalocele (66). Another 15% of BWS cases involve imprinting defects of BWSIC1, resulting in gain in methylation of H19 DMD on maternal alleles. As a result, the maternal allele of *H19* is silenced, whereas the maternal allele of *IGF2* is abnor-

Table 2. *Epigenetic disorders due to imprinting defects*

Defective gene	Effect	Syndrome	Reference
15q11-13 deletion	Ubiquitin protein ligase defect	AS (70%)	Knoll et al 1989 (113)
UBE3A point mutation		AS (10%)	Kishino et al 1997 (61)
Microdeletion 15q	ICC defect	AS (4%)	Buiting et al 1995 (63)
Uniparental disomy (chromosome 15)		AS	Malcolm et al 1991 (62)
SNRPN IC	Methylation	PWS	
BWSIC2 (L1T1)	Hypomethylation	BWS (40%)	DeBaun et al 2003 (95)
BWSIC1 (H19/IGF2)	Hypermethylation	BWS (15%)	DeBaun et al 2002 (66)
CDKN1C (P57kip2)	Kinase Inhibitor deactivation	BWS (5%)	
Uniparental disomy (11p15 region)		BWS (10%)	

mally activated, causing biallelic expression. This epigenetic alteration is associated specifically with increased cancer risk in patients with BWS (66). Approximately 5% of BWS cases involve conventional null mutations in the maternal allele of CDKN1C. In addition, approximately 10% of patients with BWS have paternal UPD for the entire 11p15 region, including H19, IGF2, CDKN1C, KCNQ1, and KCNQ1OT1. There is increased frequency of monozygotic twinning among patients with BWS (67), with the affected twin showing LOI and biallelic expression of KCNQ1OT1. Unequal splitting of inner cell mass resulting in mosaicism for the BWS defect or an imprinting defect during preimplantation stage causing a problem with imprint maintenance at KCNQ1OT1 is postulated.

UPD disorders. UPD that arises spontaneously has been well documented in humans and causes birth defects when imprinted genes are affected (68). It occurs when both homologues of a chromosome are inherited from one parent, generating a diploid chromosome number but an imbalance in maternally and paternally inherited genes. Another cause for UPD is mosaicism as a result of postzygotic somatic segregation errors (69). Embryos that possess two maternal genomes form teratomas, whereas embryos that possess two paternal genomes form trophoblastic tumors. Teratomas may arise from parthenogenetic activation of an unfertilized oocyte within the ovary and may contain several types of differentiated tissues but no extraembryonic tissue. In gestational trophoblastic tumors, including hydatidiform mole, there is hyperplasia of extraembryonic tissue with lack of embryo development. They arise from fertilization of enucleated egg or develop after loss of maternal chromosome from the embryo and develop into completed mole with only two paternal sets of chromosomes (andogenetic) or partial mole with triploidy (two paternal and one maternal set of chromosomes). Recently, completed mole with biparental origin has been reported and is associated with global disruption of maternal imprinting, likely occurring during oocyte development (70). There is LOI in the normal maternally methylated genes (*KCNQ1OT1*, *SNRPN*, *PEG1*, and *PEG3*) and a recessive maternal effect mutation causing the maternal genome to assume the paternal epigenetic appearance. Silver-Russell syndrome is thought to be caused by maternal UPD of a region on chromosome 7. The syndrome is characterized by low birth weight, dwarfism, and lateral asymmetry (71).

Epigenetic disorders and childhood cancers. Recently, multiple pieces of evidence are emerging associating epigenetic disorders with childhood and adult cancers (72). Both methylation and imprinting defects are implicated. DNA methylation is one mechanism by which cancer cells switch off the expression of different tumor suppressor genes, thus allowing the cancer cells to escape normal growth control mechanisms. Wilms' tumor is the most common childhood kidney tumor. Recent studies suggest that in 54% of cases, the tumor suppressor gene *RASSF1A* in the 3p21–3 region is hypermethylated, whereas in 10%, p16 promotor gene is methylated (73). LOI at IGF2/H19 locus (BWSIC2) is noted in Wilms' tumor, occurs very early in development, and is seen in kidney tissue adjacent to the tumor (74). In embryonal rhabdomyosarcoma, silencing of the active copy of imprinted gene *H19* occurs by methylation (75). Hypermethylation of cell regulatory gene p15 (*INK4b*) has been shown in acute myeloid leukemia and myelodysplastic syndrome (76). In retinoblastoma (RB), another early childhood tumor of the retina, the *RB1* gene is epigenetically silenced by hypermethylation of the promoter region and exon 1 (77) and results in reduced *RB1* expression (78). Differential methylation of chromosome 13q around the *RB1* gene (79) and the preferential loss of maternal alleles in certain sporadic cases of RB suggest latent or aberrant imprinting in these individuals. Ohtani-Fujita *et al.* (80) determined that the frequency of hypermethylation in unilateral tumors is 9% (13 of 140), whereas the frequency was only 1% in hereditary bilateral tumors (1 of 101). Neurofibromatosis type 1 (NFI) is a common autosomal dominant disorder characterized by cafe-au-lait spots, neurofibromas, and iris hamartomas. The NF1 gene is involved in the down-regulation of p21 (ras) oncogenes and is hypermethylated in NF1, thus promoting tumorigenesis (81). Epigenetic alterations are also observed in adult cancers. Methylation defects are noted in renal cell carcinoma (*G250* promoter gene and *3p21.3* suppressor gene), personal and familial colorectal cancer including hereditary nonpolyposis colon cancer (*RASSF1A* gene and *GATA-4* and *GATA-5* gene), and breast and gastric cancers (*TMS1* gene).

INTERFERENCE OF EPIGENETIC PROGRAMMING BY ART

Culture media and low birth weight and LOS. There are concerns that epigenetic alterations could be caused by ART involving gamete and embryo culture and manipulation (82,83). Numerous studies suggest that singleton infants who are born out of ART are at increased risk for low birth weight (LBW), very low birth weight (VLBW), preterm delivery, and fetal growth restriction in comparison with naturally conceived infants. These include population-based studies that compared infants who were conceived with the use of ART with expected rates for various outcomes in the countries from which the ART samples were drawn (84) and well-designed analyses from clinical settings in which ART singletons were compared with non-ART control groups (85,86). Schieve *et al.* (3) recently reported that singletons who were born after ART in the United States had nearly twice the risk for LBW and VLBW as expected on the basis of rates for singleton births to nonteen mothers in the general U.S. population during the same period. Although there was some variation, the increased risks for both LBW and VLBW were observed for all infertility diagnoses subsets. In addition, the risks remained elevated after restricting analyses to subgroups that were conceived with presumably healthy gametes (oocytes from an egg donor and sperm from a partner without a diagnosis of male-factor infertility) or carried by a presumably healthy woman (no female infertility diagnoses reported; ART used because of male-factor infertility). Similar reduction in birth weight was observed in mice and was associated with decreased expression of H19 and IGF2 and increased methylation of H19 DMD (87), whereas in cattle and sheep, several reports have described an enhancement in fetal growth referred to as the large offspring syndrome (LOS) (88) with an increase of ~8–50% from mean control weight

(89). LOS is characterized by a significant increase in birth weight, increased gestational length, breathing problems at birth, and increased frequency of perinatal death. LOS can arise after embryo culture, asynchronous transfer, and maternal diet rich in urea and after nuclear transfer cloning. All culture systems associated with LOS contain sera or are coculture with support cells, such as granulosa cells, oviductal cells, or fibroblasts, and may play a role in epigenetic alterations (90). In sheep with LOS, lack of expression and aberrant methylation of IGF2R has been reported (91). The IGF2R locus is imprinted in mice, sheep, cows, and pigs but not in humans. Similarly in BWS and AS, both associated with ART, there is loss of function of maternal alleles and duplication of paternal alleles at different imprinted loci. This suggests that maternal genome is more vulnerable than paternal genome to imprinting or methylation defects when exposed to culture media as part of ART. Possible explanation for increased sensitivity of maternal genome is that the majority of imprinted genes are regulated by maternally derived methylation imprint, whereas only a few imprinted genes (*H19*, *RASGRF1*, and *GTL2*) are paternally inherited. Second, there is rapid and active demethylation of paternal genome within hours of fertilization, thus resulting in less exposure of paternally imprinted genes to culture media, compared with maternal genome, which is demethylated by a slow, passive process, thus allowing exposure to components of the culture media for a longer duration.

Components in culture media could also affect the methylation pattern. Addition of FCS to M16 medium caused mouse pups to be lower in weight compared with control and exhibited decreased H19 and IGF2 expression (87). Similarly, mouse blastocysts that were exposed to Whitten's medium led to aberrant expression of the normally silent paternal allele of H19, but those that were cultured in KSOM with amino acids showed no sign of aberrant H19 expression or methylation (92). This suggests that media components could remove or interact with methyl groups on DNA or on histone tails and cause methylation defects. The critical difference between culture media is their methionine content, which can affect DNA methylation and imprinting (93,94). Alternatively the culture media could alter the embryonic developmental timing and could cause epigenetic disturbances, especially when prolonged use of culture media is used as in blastocyst culture for IVF cycles in which preimplantation genetic diagnosis is used. However, there was no difference in birth weight noted after blastocyst transfers compared with cleavage-stage embryo transfers (95).

ART and imprinting disorders. Concerns of epigenetic disturbances caused by culture media in animal studies led to studies that examined similar defects in children who were conceived with ART. The first study by Manning *et al.* (96) to determine abnormal methylation at 15q11–13 (linked to AS and PWS) in ICSI did not show any defect or any clinical syndrome in the children conceived. More recently, however, five studies reported a possible association between ART and BWS (97–99), AS (100,101), and RB (102). DeBaun *et al.* (97) screened a combined National Registry of BWS patients and detected seven children with BWS after ART. When they were examined for imprinting disorders, DeBaun *et al.* determined that four had spontaneous imprinting changes in the LIT1 subdomain and one had changes at both LIT1 and IGF2-H19 subdomains. In this same study, they determined a prevalence of 4.6% (3 of 65) in their registry when compared with the general population of 0.76% during the same period in the United States. Maher *et al.* (98) also examined a chart of 149 sporadic BWS births and detected six (4%) patients whose parents had used one ART to conceive, compared with a background rate of 0.997% in the United Kingdom. Similar to DeBaun *et al.* (97), two of the patients who were examined molecularly had hypomethylation of LIT1. Finally, in a third study, Gicquel *et al.* (99) used a registry of 149 patients with BWS to identify six patients with BWS born after ART. In the total 19 cases of BWS, 13 had KCNQ1OT1 (LIT1) hypomethylation (BWSIC2 defect) and one had H19 hypermethylation (BWSIC1 defect). One patient with BWS had normal methylation for H19 and KCNQ1OT1. On the basis of the above studies, assuming that 1% of all children are born as a result of ART, a 4-fold increase in the incidence of BWS is observed with ART.

In a recent review of the national BWS registry used in the study by DeBaun and colleagues (103), 19 children with BWS were born after ART. Clinical data from the IVF clinics were available for 12 sets of parents. There was no difference between children who had BWS and were born after ART in BWS phenotype, type of ART, ovulation induction protocol, cause of infertility of parents, day of embryo/blastocyst transfer, and the type of culture media used. The only common feature among the group was the use of ovarian stimulation medication. The authors summarized that, with the exception of receiving ovarian stimulation medication either as part of IVF or to facilitate conception, no common factor was identified among the women who gave birth to children with BWS, although the study was limited by small sample size.

Cox *et al.* (100) identified two and Orstavik *et al.* (101) identified a third child with AS who were conceived by ICSI. In all three, analysis of the 15q11–13 locus revealed loss of methylation on the maternal allele for SNRPN. Their parents had normal methylation, and the children had no chromosomal deletion at the locus. A casual relationship between ICSI and AS as a result of imprinting disorder is likely based on the rare one in 15,000 incidence of AS in the general population. Moll *et al.* (102) reported five cases of RB in children who were conceived by IVF in the Netherlands. The authors estimated that the incidence of RB in the Netherlands is 1/17,000 live births. They concluded that, assuming that 1% of all children are born as a result of ART, there was a 4-fold increase in the incidence of RB with ART.

Use of immature gametes for ART. There are concerns about reproductive outcome when immature gametes were used for ART, especially the use of round spermatids obtained after epididymal or testicular aspiration procedures (104,105). Of utmost concern was the transmission of gene mutations related to spermatogenesis (*e.g. AZFc*, *DAZ* gene) into the offspring, which was the primary cause of infertility in the parent. Immature sperm cells have an epigenetically distinct imprinting status. However, by the spermatid phase of spermiogenesis, imprinting is largely completed as shown by

mouse studies confirming imprinting of paternally expressed *Snrpn*, *Igf-2*, and *Peg1* and the maternally expressed *Mash2*, *Igf-2r*, and *H19* by spermatid stage (106). Hence, it is unlikely that ART involving male gametes interferes with either erasure or acquisition of imprints, but it is reported that in men with spermiogenesis anomalies, abnormal DNA packaging is observed as a result of a lack of exchange of histone with protamines, resulting in problems with chromatin remodeling, abnormal DNA methylation, and increased sensitivity of DNA to damage (107). The lack of chromatin condensation in immature sperm cells can cause delayed oocyte activation, which could cause aneuploidy in the embryo. Moreover, the methylation patterns of spermatids and sperm derived from testes and epididymis differ from the hypermethylated patterns found in ejaculated sperm (108). Hence, use of immature sperm for ART could increase the risk for an imprinting disorder or malformation (105), although large-scale studies did not show increased risk for malformations (109). The use of round spermatids in ICSI could interfere with mouse embryo preimplantation epigenetic reprogramming, less efficient genome activation, and a higher rate of developmental arrest compared with ICSI with mature sperm. The increased embryo developmental arrest suggests that mechanisms exist in the embryo to arrest aberrant spermatid transcription. Another potential concern is that disturbances in imprinting could occur during sperm and embryo freezing. Cryopreservation potentially could affect the cytoskeleton and the availability of enzymes such as DNMT, which maintain methylation of imprinted loci during the preimplantation phase.

Oocyte maturation *in vitro* is associated with loss of developmental competence (110), unless the oocyte is near completion of its preovulatory growth phase. This loss of developmental competence is associated with the absence of specific proteins in oocytes cultured to metaphase II *in vitro* and loss of methylation at IGF2R and PEG1 and methylation at H19 (111). Their subsequent embryonic development seems to be severely compromised (78,79) and may be attributable to suboptimal culture conditions, incomplete oocyte growth, or abnormal cytoplasmic maturation (79,112,113).

CONCLUSIONS

There is convincing evidence to show that normal embryogenesis cannot occur without epigenetic regulation, which is also critical during gametogenesis. These processes involve DNA methylation, imprinting, RNA silencing, covalent modifications of histones, and remodeling by other chromatin-associated complexes. Epigenetic reprogramming during gametogenesis is essential for imprinting, which regulates the differential expression of paternally and maternally derived genes in the embryo by escaping the general (de)methylation processes at the preimplantation stage. Reprogramming during the preimplantation period is essential for early embryo development as it controls the expression of early embryonic genes, cell cleavage, and cell determination.

Failure or incomplete epigenetic reprogramming at the gamete and preimplantation stages may lead to developmental problems and early mortality. Unlike genetic modifications, some epigenetic alterations may be tolerated during development and are usually reversible as a result of erasure in the germ line. Incomplete erasure results in epigenetic inheritance. Another way for epigenetic inheritance to occur is when these changes occur after fertilization but before specification of the germ line. This may manifest as increased perinatal mortality and LBW as observed in the children of women who were not underweight at birth but were malnourished in the first and second trimesters of their own fetal development during the Dutch famine of 1944–1945.

DNA methylation is a major mechanism by which epigenetic regulation occurs in gametes and embryos, and the maintenance of methylation patterns on DNA depends on DNMT1, DNMT2, DNMT3a, DNMT3b and the isoform of DNMT1, DNMT1o, which provides maintenance methyltransferase activity specifically at imprinted loci during the fourth embryonic S phase. Several genetic diseases have been associated with DNA methylation defects, including ICF, RTT, X-linked dominant mental retardation, nonspecific X-linked mental retardation, and ATRX (Table 1). Imprinting disorders can cause epigenetic alterations and may be due to gene defects (*LIT 1*, *H19*, *IGF2*, *UBE3A*, and *RB1*), deletions (15q-13), or UPD. Conceptuses with UPD have poor embryonic development. Embryos that possess two paternal genomes form trophoblastic tumors, whereas embryos that possess two maternal genomes form teratomas.

Recent reports suggest the increased incidence of imprinting disorders such as BWS, AS, and RB in children who are conceived with the use of ART and may result from an accumulation of epigenetic alterations during embryo culture and/or by altered embryonic developmental timing. It is interesting to note that most (not all) of these ART-associated imprinting disorders involve the maternal allele, suggesting that ART effects are greater on the oocytes than on the sperm, although the data are limited. On the basis of the available data, although limited, there is a 4-fold increase in the incidence of imprinting disorders with ART. Also, it may be suggested that there is increased relative risk for childhood cancers, such as RB, with ART. Moreover, BWSIC2 defect is associated with Wilms' tumor. The use of immature oocytes and spermatids in ART may induce epigenetic programming defects and cause increased developmental arrest in the embryos.

Numerous studies suggest that singleton infants who are born out of ART are at increased risk for LBW, VLBW, preterm delivery, and fetal growth restriction in comparison with naturally conceived infants, whereas in cattle and sheep, several reports have described an enhancement in fetal growth with an increase of ~8–50% from mean control weight referred to as the LOS. Although the exact mechanism by which ART cause these changes are unknown, culture media components (*e.g.* sera, methionine) that could interact with methyl groups on DNA or histone tails and cause epigenetic modifications are implicated. Another explanation may be that embryonic developmental timing is disturbed by the culture media. Clear evidence of such an association and elucidation of a cause needs to be determined by large, extensive, prospective studies over several years with molecular and biologic evaluations.

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