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Role of ART in Imprinting Disorders

Ali Eroglu, Ph.D.[1,2,3,4] and **Lawrence C. Layman, M.D.**[1,3,5]

[1]Institute of Molecular Medicine and Genetics, Medical College of Georgia, Georgia Health Sciences University, Augusta, Georgia

[2]Department of Medicine, Medical College of Georgia, Georgia Health Sciences University, Augusta, Georgia

[3]Section of Reproductive Endocrinology, Infertility, and Genetics, Department of Obstetrics and Gynecology, Medical College of Georgia, Georgia Health Sciences University, Augusta, Georgia

[4]Cancer Center, Medical College of Georgia, Georgia Health Sciences University, Augusta, Georgia

[5]Neuroscience Program, Medical College of Georgia, Georgia Health Sciences University, Augusta, Georgia

Abstract

Assisted reproductive technologies (ART) offer revolutionary infertility treatments for millions of childless couples around the world. Currently, ART accounts for 1 to 3% of annual births in industrialized countries and continues to expand rapidly. Except for an increased incidence of premature births, these technologies are considered safe. However, new evidence published during the past decade has suggested an increased incidence of imprinting disorders in children conceived by ART. Specifically, an increased risk was reported for Beckwith-Wiedemann syndrome (BWS), Angelman syndrome (AS), Silver-Russell syndrome, and retinoblastoma. In contrast, some studies have found no association between ART and BWS, AS, Prader-Willi syndrome, transient neonatal diabetes mellitus, and retinoblastoma. The variability in ART protocols and the rarity of imprinting disorders complicate determining the causative relationship between ART and an increased incidence of imprinting disorders. Nevertheless, compelling experimental data from animal studies also suggest a link between increased imprinting disorders and ART. Further comprehensive, appropriately powered studies are needed to better address the magnitude of the risk for ART-associated imprinting disorders. Large longitudinal studies are particularly critical to evaluate long-term effects of ART not only during the perinatal period but also into adulthood. An important consideration is to determine if the implicated association between ART and imprinting disorders is actually related to the procedures or to infertility itself.

Keywords

Epigenetics; genomic Imprinting; methylation; imprinting disorders; ART

Infertility was classified as a disease by the World Health Organization in 2009.[1] According to a recent estimation, 1 in 10 people of reproductive age are involuntarily infertile.[2] Consequently, there is a great demand for infertility treatments suchas assisted reproductive

Address for correspondence and reprint requests Ali Eroglu, Ph.D., Institute of Molecular Medicine and Genetics, Medical College of Georgia, Georgia Health Sciences University, 1120 15th Street, CA-2004, Augusta, GA 30912 (aeroglu@georgiahealth.edu).

technologies (ART). Since the birth of the first test tube baby, Louise Brown in 1978, ART procedures such as in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), embryo culture, and embryo cryopreservation have rapidly evolved and opened a new era in the treatment of infertility for millions of childless couples around the world. Currently, ART accounts for 1 to 3% of annual births in industrialized countries[3] and continues to expand rapidly. Except for an increased incidence of premature births, these technologies are considered safe.[4] However, studies published since 2002 suggest a relatively high incidence of birth defects such as Beckwith-Wiedemann syndrome (BWS), Angelman syndrome (AS), and Silver-Russell syndrome (SRS) among children conceived by ART.[5–15] Similarly, experimental evidence obtained using different animal models suggests that ART may induce aberrant epigenetic changes potentially leading to various disorders.[16–23] This article first briefly explains the overall mechanisms of epigenetic gene regulation and then reviews the current status of imprinting disorders linked to ART.

Epigenetic Gene Regulation

It has long been recognized that phenotype is a result of genotype and environment. However, the molecular mechanism(s) beyond some of the environmental effects has remained poorly understood. Nevertheless, studies published over the last 2 decades revealed that environment may contribute to the regulation of gene expression by inducing various chemical modifications to DNA (e.g., DNA methylation) and histones (e.g., acetylation, methylation, phosphorylation, sumoylation, and ubiquitination of histone tails) without changing DNA sequence.[24–28] Such chemical modifications represent another layer of gene regulation in addition to a given gene sequence and are defined by the term *epigenetics*, which literally means on top of genetics (epi: “over, above”). Originally, the word *epigenetics* was introduced by Waddington in the 1940s to refer to a new discipline known today as developmental biology.[29] Nowadays, the term *epigenetics* is typically used more narrowly to define heritable changes in gene expression without altering the underlying DNA sequence, although different definitions have also been proposed.[30–32] The principal mechanisms of epigenetic gene regulation include DNA methylation, histone modifications, and non-coding RNAs, all of which are involved in chromatin remodeling and thus in the regulation of a transcriptionally permissive/nonpermissive state.

DNA Methylation

Among the epigenetic modifications just mentioned, DNA methylation is best characterized and involves the addition of a methyl group from S-adenosylmethionine (SAM) to the carbon-5 position of a cytosine base (Fig. 1A). This covalent chemical modification is catalyzed by DNA methyltransferases (DNMTs) that include DNMT1, DNMT3A, and DNMT3B.[33] Whereas DNMT3A and DNMT3B primarily establish methylation marks by de novo methylation during early development, DNMT1 maintains methylation patterns during DNA replication by adding methyl groups to hemimethylated sites.[34–37]

In contrast to DNA methylation, demethylation of the established marks is less well understood and seems to occur through both passive and active mechanisms.[38] Passive DNA demethylation may occur by suppression of maintenance methyltransferase activity (i.e., addition of methyl groups to cytosine bases during DNA replication). According to recent studies, active demethylation seems to occur through successive oxidation of 5-methylcytosine (5mC) by ten eleven translocation (Tet) proteins into first 5-hydroxymethylcytosine (5hmC), then 5-formylcytosine (5fC), and finally 5-carboxylcytosine (5caC).[39–41] The latter appears to be excised by thymine-DNA glycosylase (TDG) and then repaired by the base excision repair (BER) pathway into an unmethylated cytosine.[41] In mammals, DNA methylation overwhelmingly occurs on cytosines (C) preceding guanines (G). These CpG dinucleotides, where “p” indicates a phosphate group,

are nonuniformly distributed in the mammalian genome and usually enriched in the promoter region of ~60% of genes.[42–44] The CpG-rich regions consisting of 1000 to 2000 base pairs with a CG content >55% are also known as CpG islands and generally remain unmethylated with some exceptions.[44–46] DNA methylation is typically associated with silencing of the targeted genes.[44,46,47] Therefore, aberrant de novo methylation of CpG islands may lead to developmental disorders.

Histone Modifications

The role of histone modifications in epigenetic gene regulation was recognized early on.[48] In eukaryotic cells, core histone proteins (i.e., H2A, H2B, H3, and H4) serve as scaffolds for the packaging of DNA into chromatin by assembling into octamers (Fig. 1A). The nucleosomes, the basic packaging unit of chromatin, are formed by the wrapping of 147 base pairs of DNA around each histone octamer. The resulting nucleosomes are then organized into chromatin, the building block of a chromosome. The extent of chromatin compaction is dynamic and can be influenced by modification of histone tails along with DNA methylation/demethylation.[49] In general, highly compacted chromatin is inaccessible to transcription factors, and thus genes are “off” while the open form of chromatin allows binding of transcription factors and hence gene expression (Fig. 1B).[50]

The NH2-terminal tail of each core histone may undergo numerous covalent posttranslational modifications including acetylation of lysines, methylation of lysines and arginines, ubiquitination and sumoylation of lysines, and phosphorylation of serines and threonines.[50] Acetylation of histone tails is catalyzed by histone acetyltransferases (HATs) and generally associated with open chromatin domains and active gene transcription.[48] The removal of acetyl groups from lysine residues by histone deacetylases (HDACs) results in condensed chromatin and the silencing of gene transcription.[51] Compared with histone acetylation, methylation of histone tails is more complex and can occur in different forms. Lysine residues can be modified with mono-, di-, and trimethyl groups, whereas arginine residues can be either mono- or dimethylated by histone methyltranferases. Depending on modified residues and timing, methylation of histone tails may activate (e.g., H3K4, H3K36, and H3K79) or repress (e.g., H3K9, H3K27, and H4K20) gene expression.[50] Considering numerous other histone modifications and cross talk between them, the so-called histone code gets highly complex and is beyond the scope of this review. For a detailed discussion on this subject, readers are referred to specific review articles.[50,52,53]

Noncoding RNAs

According to high-throughput transcriptomic analyses, up to 90% of the eukaryotic genome seems to be transcribed.[54] Whereas ~1 to 2% of these transcripts encode proteins, the overwhelming portion of the genomic DNA is transcribed into functional noncoding RNAs (ncRNAs) that are not further translated. In addition to well-known transfer RNAs and ribosomal RNAs, ncRNAs include micro RNAs (miRNAs), Piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), long noncoding RNAs (lncRNAs), enhancer RNAs (eRNAs), and promoter-associated RNAs (PARs).[55,56]

In recent years, the involvement of several noncoding RNAs in epigenetic gene regulation was demonstrated.[56–60] For example, miRNAs are small single-stranded molecules (~22 nucleotides long) that downregulate gene expression mostly by binding to the 3′ untranslated region (UTR) of messenger RNAs and subsequently causing their destabilization and degradation.[61] By targeting enzymes responsible for histone modifications and DNA methylation, miRNAs contribute to epigenetic gene regulation.[57–59] SiRNAs are also small but double-stranded RNA molecules (20 to 24 nucleotides long) that are involved in both posttranscriptional and direct sequence-specific transcriptional gene silencing by increasing

epigenetic marks.[62,63] LncRNAs (>200 nucleotides long) represent most of the non-protein-coding transcripts (~80%)[55,64] and seem to regulate gene expression through modulation of the chromatin state. Recent studies suggest that a subgroup of lncRNAs, called large intergenic noncoding RNAs (lincRNAs), guide chromatin-modifying complexes to specific genomic loci to establish cell type–specific epigenetic states.[65,66] The inactivation of one of the X chromosomes in female cells is a good example of the involvement of lncRNAs in epigenetic gene regulation. Experimental data suggest that X-inactive specific transcript (Xist) RNA coats the X-chromosome *in cis* and recruits histone modifications and DNA methylation, which lead to a transcriptionally inactive chromatin state.[67,68] In recent years, the diverse functions of ncRNAs in epigenetic gene regulation have been increasingly recognized as discussed in detail in several recent reviews.[56,69,70] Taken together, epigenetic gene regulation consists of highly complex processes and requires coordinated interactions of DNA methylation/demethylation, histone modifications, ncRNAs, and various nonhistone proteins to ensure timely expression/repression of genes. Therefore, any disturbance to this complex system may result in developmental disorders.

Genomic Imprinting

Genomic imprinting is an epigenetic form of gene regulation that results in expression of certain genes depending on their parental origin. In mammals, genomic imprinting was uncovered in the early 1980s as a result of pronuclear transplantation and chromosome translocation (uniparental disomy) experiments and led to the revision of classical Mendelian genetics.[71–75] Until then, it was assumed that both maternal and paternal alleles of each gene were expressed (Fig. 2), although observations through the centuries provided hints on differential behavior of paternal and maternal genomes.[76] As opposed to diploid mouse embryos containing one maternal and one paternal pronucleus, the combination of two maternal (gynogenote) or two paternal (androgenote) pronuclei in a mouse embryo by micromanipulation resulted in developmental failure despite diploidy and clearly indicated that maternal and paternal genomes are not functionally equivalent and both are required for normal development (Fig. 3). The maternal genome seems to be more important for fetal development, whereas the paternal genome is necessary for placental development. In humans, this is exemplified by two conditions: a molar pregnancy (placental overgrowth and underdeveloped fetal structures with two copies of the paternal genome) and a benign ovarian teratoma (an ovarian cyst manifested by an attempt at fetal development—hair, teeth, cartilage, bone, etc., without placental components, with two copies of the maternal genome). The discovery of the first imprinted genes in the early 1990s further substantiated the impact of genomic imprinting on human health and diseases.[77–81] It has been postulated that genomic imprinting has evolved in mammals to regulate the dosage of developmentally important genes. Several other hypotheses including avoidance of parthenogenesis, the parental conflict hypothesis, the complementation hypothesis, and the ovarian time bomb hypothesis have also been proposed, as discussed in a recent review.[82]

The mechanism of imprinting is complex and not fully understood. However, methylation of CpG-rich domains is a key part of this phenomenon. To date, at least 100 imprinted genes have been identified, and it has been estimated that there could be several hundred.[83] The vast majority of imprinted genes are found in clusters, probably to share epigenetic regulatory elements.[26] Such clusters are similarly organized in humans and mice, and they may include both paternally and maternally imprinted genes, nonimprinted genes, and ncRNAs.[83–85] They also contain CpG-rich regions up to several kilobase pairs, which are differentially methylated in paternal and maternal alleles and known as differentially methylated regions (DMRs).[26] DMRs differ in their function. Some DMRs acquire their methylation marks early in germ cells and serve as imprinting control regions (ICRs) to regulate monoallelic expression *in cis*. Deletion of ICRs (also known as imprinting centers

or imprinting control elements in the literature) results in loss of monoallelic expression in the linked genes, indicating the critical role of ICRs in the imprinting mechanism.[86–89] Whereas methylation marks in some DMRs remain stable throughout development and are maintained in all tissues, other DMRs experience considerable changes during development and acquire tissue-specific methylation marks.[26,90]

Imprinted genes play a critical role in regulating fetal and placental growth and development, as well as in neurological pathways and behavior.[91] Therefore, consequences of aberrant genomic imprinting include phenotypic developmental abnormalities and neurological disorders such as Prader-Willi syndrome, BWS, AS, SRS, Albright hereditary dystrophy, and transient neonatal diabetes mellitus. It has also been recognized that aberrant genomic imprinting is involved in the development of both childhood (e.g., Wilms' tumor, retinoblastoma, and neuroblastoma) and adult (e.g., bladder, breast, cervical, colorectal, ovarian, prostate, uterine) tumors.[92] As explained later, genomic imprinting occurs during gametogenesis and embryogenesis, and thus its timing coincides with the use of ART. Consequently, it is possible that techniques used in ART could cause aberrant genomic imprinting and thus imprinting disorders.

Transgenerational Cycle of Imprinting Marks

Genomic imprinting requires resetting sex-specific imprints in every generation. This process, which has been characterized by studying methylation marks mostly in the mouse and is also referred to as epigenetic reprogramming, goes through cyclic events consisting of erasure, establishment, and maintenance stages of imprinting marks[26] (Fig. 4).

The erasure of the existing imprints occurs in primordial germ cells (PGCs). In the mouse embryo, PGCs first appear in the proximal epiblast on embryonic day 7 (E7) and then migrate along the genital ridge on E8. They colonize the developing gonads around E10.5. The erasure stage starts with a decrease in DNA methylation during migration of PGCs around E8[93,94] and is completed after migration by E13.5D with a possibly active wave of genome-wide demethylation.[95–97] This epigenetic reprogramming event is probably required to restore totipotency.[98,99]

In the next cycle stage, imprinting marks are reestablished during development of germ cells into sperm or oocytes through de novo methylation.[100,101] In the male germ cells, de novo methylation of DMRs starts in mitotically arrested prospermatogonia (gonocytes) around E14.5, and the paternal methylation imprints are entirely established in perinatal prospermatogonia.[100,102,103] In the female germ line, imprinting marks are asynchronously acquired after birth during the growth phase of oocytes.[104,105] Some germ-line DMRs including *Snrpn, Peg3, Igf2r, and p57*^{KIP2} are methylated during the primary and secondary follicle stages; others such as *peg1/mest* acquired their methylation marks later during the tertiary and antral follicle stages.[105,106]

The third stage of the cycle basically refers to maintenance of the imprinting marks during embryonic and fetal development, as well as during adulthood in somatic cells. Further epigenetic reprogramming occurs during embryonic development. Following fertilization, the second wave of demethylation takes place. First, the male pronucleus undergoes active demethylation.[107,108] This is followed by passive demethylation of the maternal genome during successive cleavages. Both parental genomes are equally demethylated by the 16-cell stage. However, methylation marks on imprinted genes remain protected from this demethylation wave to maintain parental imprints.[109] At the time of implantation, another de novo methylation occurs in a lineage-specific pattern.[110] These waves of methylation/demethylation events regulate timely expression of embryonic and tissue-specific genes, and thus they ensure normal development.

The timing of epigenetic reprogramming in humans is not well characterized. Unlike the mouse, methylation marks in the human male germ line seem to be established later during spermatogonial differentiation in the adult testis,[111] whereas human female germ cells appear to progressively acquire methylation marks before[112] and during oocyte maturation[113,114] or after fertilization.[115] Taken together, major imprinting events take place in gametes and embryos when ART is used. Consequently, imprinting defects in the course of assisted reproduction may occur during the establishment of genomic imprinting (ovarian hyperstimulation, in vitro maturation of gametes, cryopreservation of gametes) as well as its maintenance (embryo culture, embryo cryopreservation).

Experimental Evidence Supporting Link Between ART and Imprinting Disorders

Because the establishment and maintenance of genomic imprints occur during both gametogenesis and embryogenesis, it has been suggested that in vitro culture of oocytes and embryos may alter methylation status and thus expression of imprinted genes. Indeed, studies in ruminants such as sheep and cattle have documented a particular overgrowth syndrome (known as large offspring syndrome [LOS]) after in vitro culture of embryos. It was shown that sheep with LOS displayed both lack of expression and abnormal methylation of the *Igf2r* gene.[16] Furthermore, studies using mouse models have clearly shown that embryo culture is associated with altered methylation and expression of imprinted genes.[17–19] Doherty et al[18] investigated the effects of two culture media on the expression of *H19* and *Snrpn* after culturing two-cell embryos to the blastocyst stage. The culture of mouse embryos in Whitten's medium resulted in demethylation of the paternal allele and biallelic expression of the *H19* gene, which is normally expressed from the maternal allele, while embryo culture in KSOM containing amino acids showed normal methylation and expression of the *H19* gene. Expression of the *Snrpn* gene remained unaffected in both media. Using a mouse model, Khosla et al[17] showed that the addition of fetal bovine serum to M16 medium can alter methylation and expression of imprinted genes (i.e.,*H19,Igf2, Grb7,*and *Grb10*) leading to reduced fetal weight. Moreover, a recent study showed that culturing mouse embryos in a commonly used human embryo culture medium (i.e., human tubal fluid [HTF]) also results in aberrant imprinting of *H19*.[20] Other recent studies further support the view that suboptimal culture of embryos may have serious consequences later on.[19,21,116] Furthermore, experimental studies on ovarian stimulation in the human and mouse revealed that hormonal induction of superovulation affects expression and DNA methylation of imprinted genes.[22,23]

Incidence of ART-Related Congenital Anomalies

In general, ART is considered safe.[4] However, recent studies suggest that there may be links between ART and increased risks for congenital anomalies.[5–8,11,12,117–121] It is known that ART is associated with high rates of low birthweight. This can be partially attributable to the increased rates of multiple gestations due to transfer of two or more embryos. However, Schieve et al[117] reported that the rate of low birthweight in singletons conceived with ART is 2.6 times higher than that in the general population. Furthermore, the risks remained high after restricting analyses to subgroups conceived with presumably healthy gametes (e.g., oocytes from egg donors and sperm from a partner without male-factor infertility).[122] Because imprinted genes are involved in the regulation of embryonic and fetal growth, as well in placental growth and function,[123] these findings might be related to inappropriate imprinting, although the role of parental factors also needs to be clarified.

Another retrospective study attempted to determine the risk for major birth defects when IVF and ICSI were used to achieve a pregnancy.[118] The authors evaluated data from

registries in Western Australia for 1993 to 1997 and found that 26 of the 301 infants conceived through ICSI (8.6%) and 75 of the 837 infants conceived through IVF (9%) had major birth defects (musculoskeletal, metabolic, cardiovascular, urogenital, gastrointestinal, central nervous system) diagnosed by 1 year of age, compared with 4.2% reported for natural conceptions. However, findings from this study also did not clarify the role of preexisting infertility in the increased prevalence of birth defects.

Incidence of ART-Related Imprinting Disorders

Normal genomic imprinting is characterized by the expression of either the maternal or paternal allele (but not both) Disruption of normal imprinting results in clinically identifiable disorders in humans and animals. In general, three mechanisms could cause imprinting disorders[124]: (1) deletion or mutation in known imprinted genes or imprinting control regions, (2) large deletions or duplications of chromosomal regions containing imprinted genes, or (3) uniparental disomy (both members of a chromosome pair for a particular chromosome come from one parent). Imprinting disorders that result in clinically recognized syndromes in humans occur principally on chromosomes 6,7,11, 14, 15, and 20 (Table 1). As more small deletions/duplications are identified throughout the genome (referred to as copy number variations, or CNVs), it is likely that additional phenotypes will be added to this list. Most of the data regarding imprinting disorders and ART come from studies of BWS and AS. BWS is the imprinting disorder studied more completely than any others.

Beckwith-Wiedemann Syndrome

BWS is an overgrowth disorder characterized by pre- and postnatal overgrowth, neonatal hypoglycemia, macroglossia, macrosomia, and an increased risk of embryonal tumors such as Wilms' tumor, rhabdomyosarcoma, and hepatoblastoma.[125,126] BWS affects ~1 in 13,700 children[126] with most of the patients having an imprinting defect at the maternal allele of one of two DMRs on chromosome 11p15. Imprinting Center 1 (IC1), also known as DMR1, regulates the genes *H19* and *IGF2;* and IC2, also known as DMR2 and in some studies KvDMR1, regulates the genes *CDKN1C, KCNQ1OT1*, and *KCNQ1*.[124,126]

In seven different studies out of nine, BWS cases were identified and the number having ART was ascertained (Table 2A). When these are combined, 53 of 656 (5.9%) BWS cases were the product of an ART procedure compared with ~0.007% in general population in most countries that perform IVF. Investigators then tried to determine if IVF or ICSI were used (some also were ovulation stimulation only or intrauterine insemination). Through the comparison of the percentage of BWS through ART versus the general population, these authors suggested that BWS was significantly increased in mothers undergoing ART.

Furthermore, some investigators have studied the molecular basis of BWS who had ART to determine if BWS was due to an imprinting defect. For interpretation of the results of these data, it is important to know the prevalence of each mechanism for all cases of BWS. As can be seen in Table 2A, hypomethylation of IC2 accounts for ~50% of all BWS cases, whereas hypermethylation of IC1 occurs in ~5% of BWS. Paternal uniparental disomy of 11p15, annotated as upd (11)pat, is found in 20% of BWS, followed by unknown causes (20%), mutation of the maternally imprinted *CDKN1C* gene in IC2 (5%), and rarely structural changes or CNVs (3%). Therefore, ~75% of all BWS patients have an epigenetic etiology. In studies done to date, the epigenetic status was analyzed by testing for hypomethylation of IC2 or hypermethylation of IC1. No upd(11)pat has been reported, but it has not been addressed in most studies. Given this information, ~55% of BWS cases would be expected to demonstrate epigenetic changes. However, 46 of 50 of BWS patients (92%) exposed to

ART had an imprinting defect of IC1 or IC2, which is considerably more frequent than the estimated 55%.

However, there are some problems determining the prevalence of ART in BWS patients versus the 1 to 3% prevalence of ART in the general population. Ascertainment bias is very likely to be involved in families who have a child with a congenital anomaly. In addition, the appropriate control group should be infertile couples because these disorders may be increased in patients with infertility, regardless of whether they have IVF or ICSI. In fact, cases of BWS with methylation abnormalities have been described in patients who underwent ovulation induction or intrauterine insemination without IVF/ICSI (Table 2A). In addition, in all of the studies cited here, the BWS patient cohort unexposed to ART was not studied for methylation abnormalities. Also, several additional studies reported a very low incidence (either 0 or 1 case) of BWS among the children conceived by ART.[127–129] However, even with these methodological problems, the prevalence of imprinting abnormalities in 92% of BWS patients exposed to ART is concerning and requires further study.

Angelman Syndrome

AS, characterized by severe mental retardation, absence of speech, ataxia, seizures, and hyperactivity, was first recognized in 1965. Maternal deletions of 15q11.2-q13 were found to cause AS, and interestingly, paternal deletions of the same region result in Prader-Willi syndrome. This region contains the *SNRPN* and *UBE3A* genes, which appear to be imprinted. Three decades later, it was found that single gene mutations in *UBE3A* could cause AS.[130] Interestingly, the *UBE3A* gene is imprinted in the human brain and only expressed from the maternal copy while the paternal copy of the gene is silent. There are five major genetic causes of AS: (1) maternal chromosome 15q11.2-q13 deletions (68%), (2) paternal uniparental disomy 15 (7%), (3) *UBE3A* gene mutations (11%), (4) imprinting defects (3%), and (5) partial or whole gene deletions of *UBE3A* (rare). It is important to emphasize that imprinting defects account for ~3% of AS patients.[131]

To date, a total of six ART-conceived AS patients with imprinting defects have been reported.[11–13,120] However, the evidence is less compelling for AS than BWS. Initially, several case reports of AS who had ICSI were reported.[11,12] In the first, two unrelated patients had affected AS children with hypomethylation of the maternal *SNRPN/EBEA3* region on 15q11.2-q13.[11] Another single case was also reported with hypomethylation of the maternal allele.[12] In all three cases, upd(15)pat was excluded. Because AS with imprinting defects is very rare, these authors suggested the possible involvement of ART in AS.

Of three studies that ascertained AS patients exposed to ART, 23 of 252 AS parents (9.1%) had infertility. As shown in Table 2B, 4 of 23 (17.4%) had hypomethylation at the AS IC. Importantly, only three IVF patients were studied, and one of the three had an imprinting etiology. The remaining cases were all infertility, and 3 of 20 (15%) had hypomethylation. The frequency of imprinting in all AS patients approximates 3%, but the numbers with ART are too low to really characterize the effects of ART on imprinting disorders. Nevertheless, the rarity of AS in the general population has prompted some investigators to express concern that ART could be causative. From the data presented in Table 2B, it seems that infertility with or without treatment, rather than strictly ART, could be involved, but this requires future study. As reviewed by Amor and Halliday,[124] considering (1) the rarity of AS in the general population (~1 in 15,000), (2) its occurrence as a result of imprinting defects (~3% of cases), and (3) the overall contribution of ARTs to annual births (1 to 3%), the coincidental occurrence of these three events by chance is ~20 million births, which has prompted the suggestion of a link between AS and ART.

Silver-Russell Syndrome

SRS is a disorder characterized by intrauterine and postnatal growth retardation, craniofacial abnormalities in addition to variable learning disabilities. It affects ~1 in 100,000 children. Up to 60% of SRS patients have an imprinting defect (hypomethylation) on the paternal allele of DMR1 at 11p15.[132,133] Recently, several studies reported a total of nine SRS patients conceived with ART.[14,15,119,134] Of nine patients, six (67%) were confirmed to have an imprinting defect (hypomethylation) in DMR1 on 11p15,[14,15] which is not sufficient to provide evidence for or against an association between ART and imprinting defects in SRS.

Retinoblastoma

Retinoblastoma is a malignant tumor of the retina with an incidence of ~1 per 17,000 live births.[135] Retinoblastoma usually occurs as a result of a mutation of the tumor suppressor gene *RB1;* however, hypermethylation of the *RB1* gene can also cause retinoblastoma by inactivating its tumor suppressor function.[136,137] In 2001, the first case of retinoblastoma was reported in a child born through IVF.[138] An additional five cases of retinoblastoma were reported by a Dutch study in 2003.[121] Of these five cases, one appeared to be caused by a mutation; the imprinting status of the *RB1* gene was not determined in the remaining four children. Based on these cases, the investigators of this study estimated an increased risk of 4.2 to 6.7 for IVF-born infants to develop retinoblastoma. In 2004, another case of retinoblastoma was reported in a child conceived by IVF.[139] In contrast, a survey study found no retinoblastoma cases among 176 children conceived by IVF.[140] When the usual incidence of retinoblastoma (1 per 17,000 live births) is considered, the number of the evaluated children in the latter study is too small to draw any definitive conclusion. Taken together, further studies with the imprinting status of the *RB1* gene are needed to confirm or rule out an association between ART and retinoblastoma.

Conclusions and Future Directions

Until recently, mutations in DNA sequence have mostly been the focus for understanding the genetic background of various diseases, including birth defects. In recent years, compelling evidence has been presented that epigenetic modifications not only regulate gene expression during development but also play a critical role in pathogenesis of complex diseases such as cancer and neurodegenerative disorders.[141]

Considering major epigenetic reprogramming events during gametogenesis/embryonic development and timing of ART, it is possible that suboptimal conditions in ART may induce aberrant epigenetic modifications leading to abnormal development and imprinting disorders. Due to the variability in ART protocols and the rarity of imprinting disorders, it is difficult to determine reliably the causative relationship between an increased risk for imprinting disorders and ART exposure. Nevertheless, despite some conflicting results, both human and animal studies suggest a possible link between ART and imprinting disorders, most convincingly for BWS and less so for AS, but the magnitude of the risk is still unclear. Further comprehensive studies are needed to better address the risk for ART-associated imprinting disorders. Although not always realistically possible, it would be helpful to minimize ascertainment bias by studying all affected individuals, perform similar molecular analyses on both those patients who had affected children from ARTversus those that did not. Perhaps, most importantly, the prevalence of these imprinting disorders in patients experiencing infertility should be compared with those having ART. Evidence from AS in particular suggests that infertility could be a risk factor. Nonhuman primates may serve as excellent experimental models to tackle some questions that cannot be addressed in humans due to ethical reasons. To minimize the risk, further optimization of ART with respect to

imprinting events is of significance. Particularly, large longitudinal studies are critical to evaluate long-term effects of ART during the perinatal period and also into adulthood.

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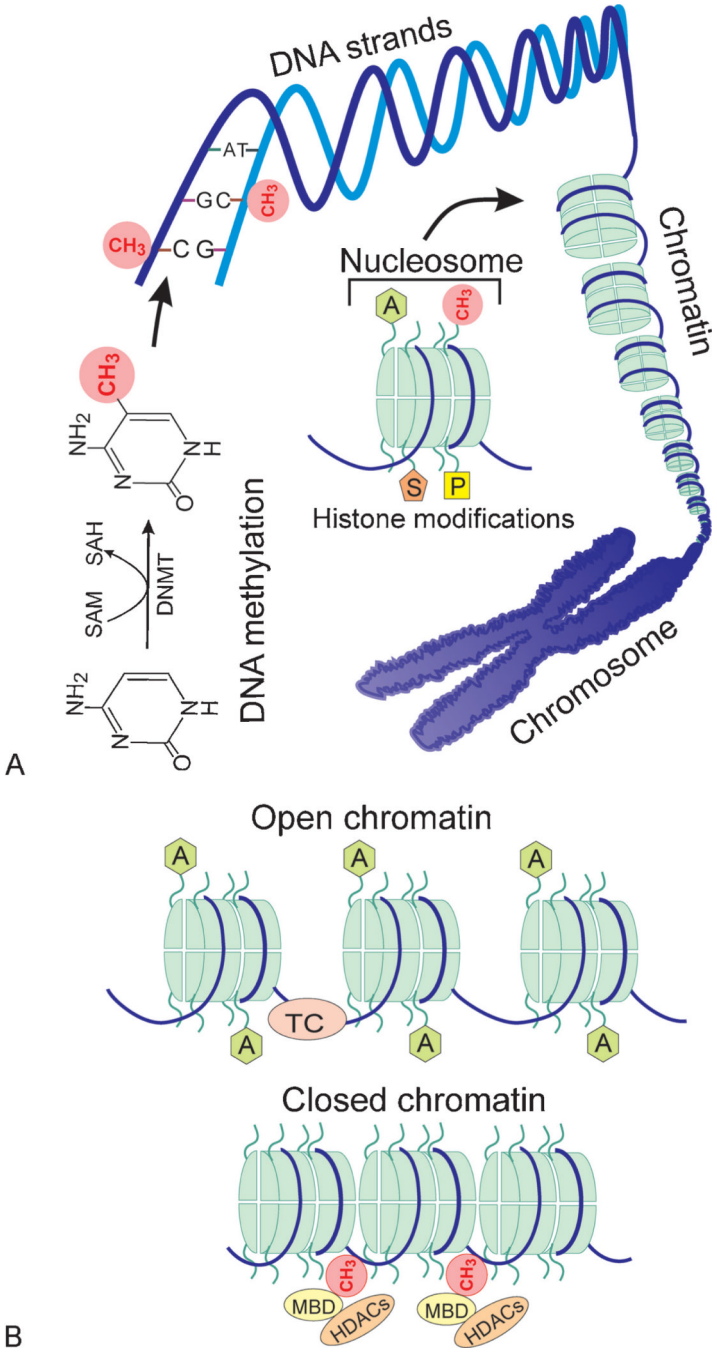

Figure 1.

Epigenetic gene regulation. (**A**) The overall organization of DNA in relation to epigenetic modifications. DNA methylation is mediated through DNA methyltransferases (DNMTs) by transferring a methyl group from S-adenosylmethionine (SAM) to the carbon-5 position of a cytosine base (shown as a red circle). DNA methylation typically occurs on cytosines (C) preceding guanines (G) at both strands of the double helix. DNA strands are wrapped around histone octamers to form nucleosomes that are building blocks of chromatin. The chromatin in turn is organized into a chromosome. The specific structure of nucleosomes facilitates epigenetic gene regulation. Histone tails protruding from each nucleosome can undergo numerous posttranslational modifications such as acetylation (A in a green hexagon),

methylation (CH_3 in red circles), sumoylation (S in a brown pentagon), and phosphorylation (P in a yellow rectangle). Such modifications along with DNA methylation can influence chromatin remodeling. (**B**) The role of chromatin remodeling in gene expression. The open and closed structure of chromatin is regulated by interactions of DNA methylation, numerous histone modifications, and noncoding RNAs. Only shown are DNA methylation (CH_3 in red circles) and acetylation of histone tails (A in green hexagons) for a simplified view. Typically, demethylation of CpG dinucleotides (absence of red circles) and acetylation of histone tails (green hexagons) result in an open chromatin structure, which allows binding of the transcription complex (TC as a pink ellipse) to a specific sequence leading to transcription. In contrast, upon methylation of cytosine residues (red circles) by DNA methyltransferases, methyl binding domain proteins (MBD as yellow ellipses) bind to the methylated DNA and recruit histone deacetylases (HDACs as brown ellipses). These events induce deacetylation of histone tails, compaction of the chromatin, and thus transcriptional silencing due to inability of the TC to bind to DNA.

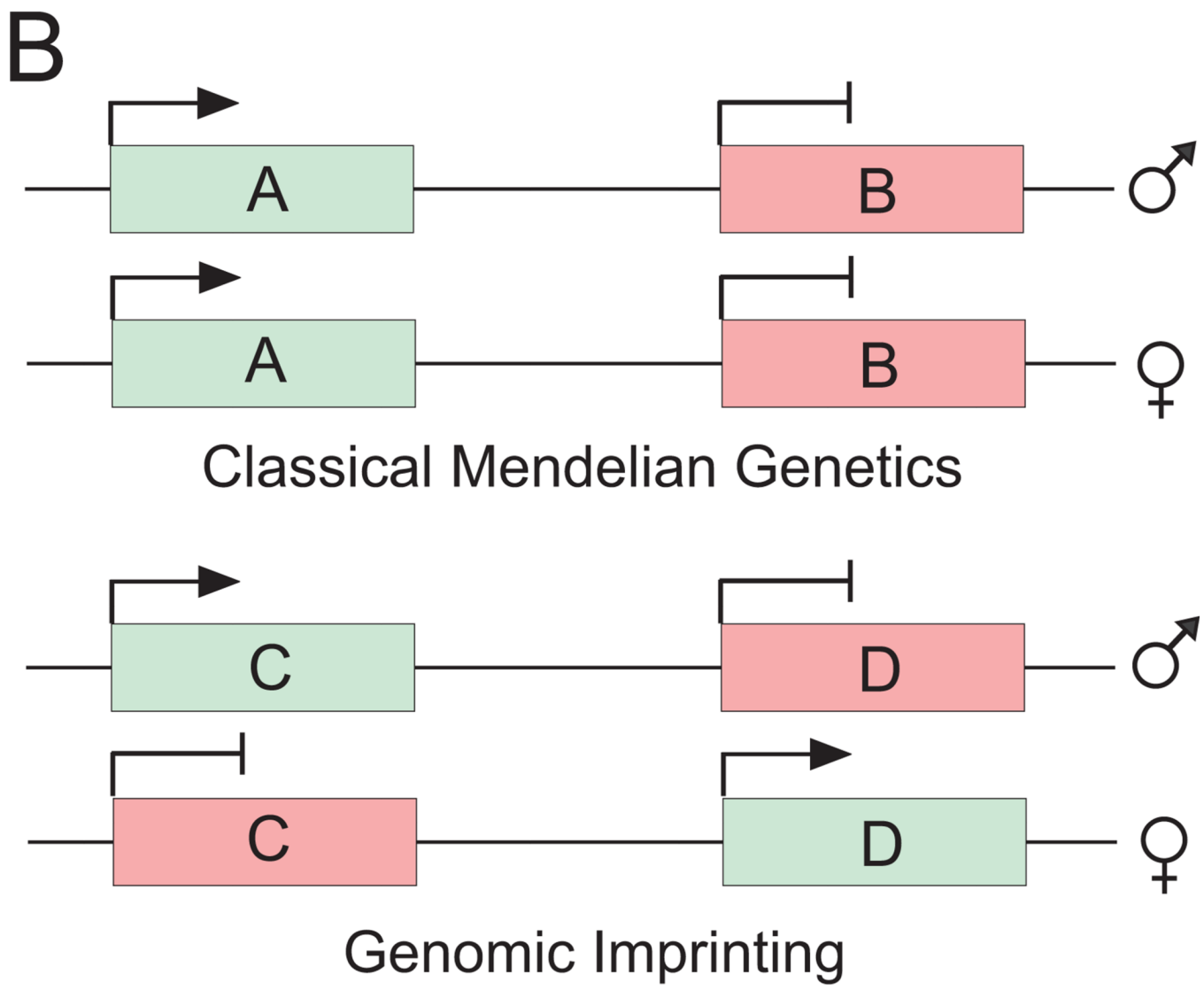

Figure 2.
Expression of imprinted and nonimprinted genes. According to classical genetics, genes are expressed from either both alleles (gene A) or none of them (gene B). This is true for most of the genes that are not imprinted. However, a subset of genes are imprinted; that is, their imprinting control regions are methylated on either the maternal or paternal allele allowing expression of only the paternal allele (gene C) or the maternal allele (gene D), respectively. This sex-specific parent-of-origin allelic methylation mark is inherited from one generation to another. Nonexpressed genes are indicated by red boxes with blunt arrows; expressed genes are depicted with green boxes with an arrowhead.

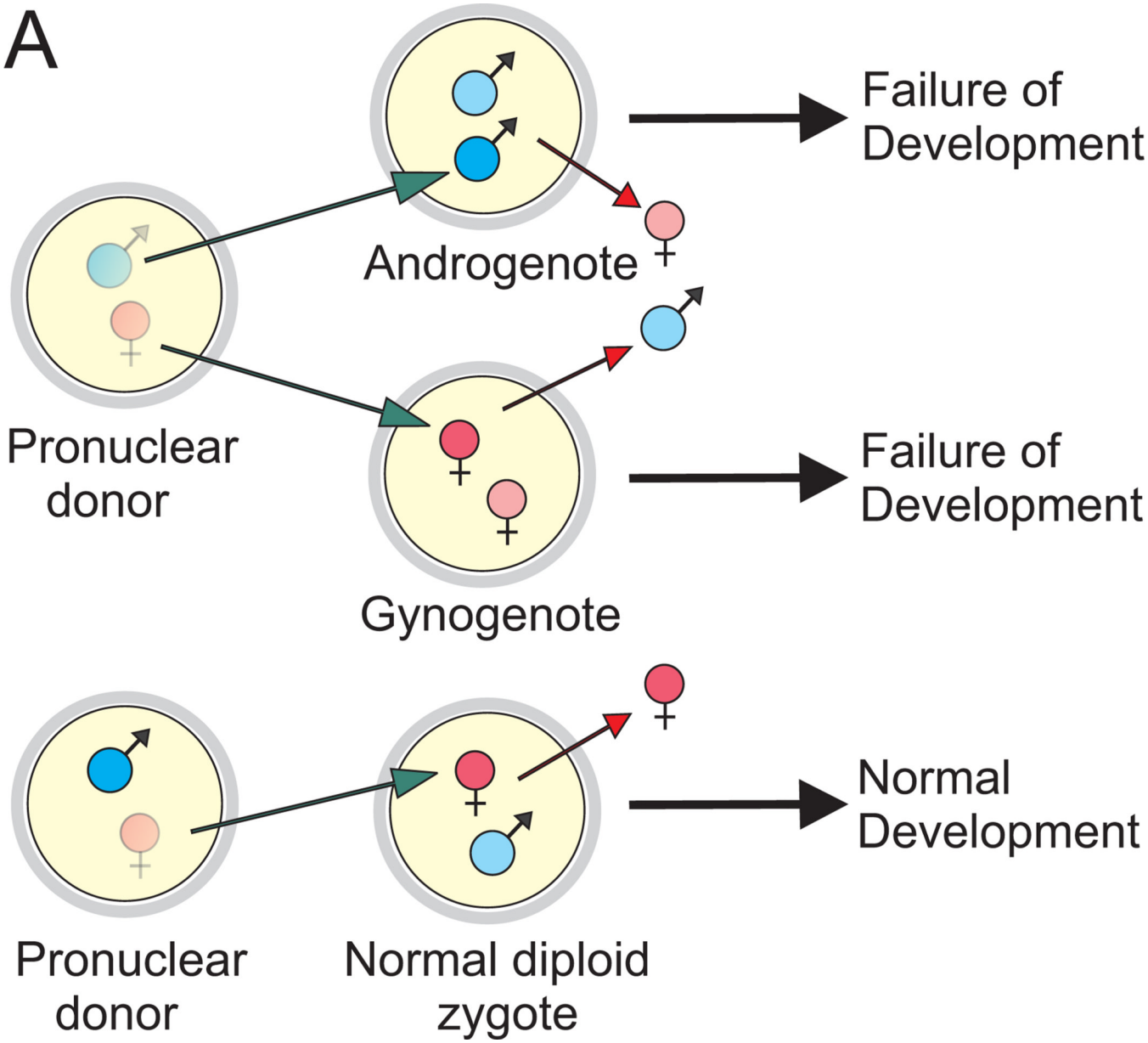

Figure 3.

Pronuclear transplantation studies demonstrating experimental evidence for genomic printing. Through sophisticated micromanipulation techniques, maternal or paternal pronuclei from normal mouse zygotes were removed and replaced with reciprocal ones resulting in diploid zygotes containing either two paternal pronuclei (androgenote) or two maternal pronuclei (gynogenote). Such androgenotes and gynogenotes were transferred to pseudopregnant recipients to study their subsequent development. Neither type of the reconstituted mouse zygotes was viable, whereas mouse zygotes reconstituted with paternal and maternal pronuclei normally developed to term. The gynogenotes displayed some fetal growth but poorly developed placentas. In contrast, androgenetic development was rather extraembryonic. These results suggested that both maternal and paternal genomes are necessary for normal development. Further, these experiments indicated that paternally

expressed genes contribute to placental development, whereas maternally expressed ones are involved in embryonic development.

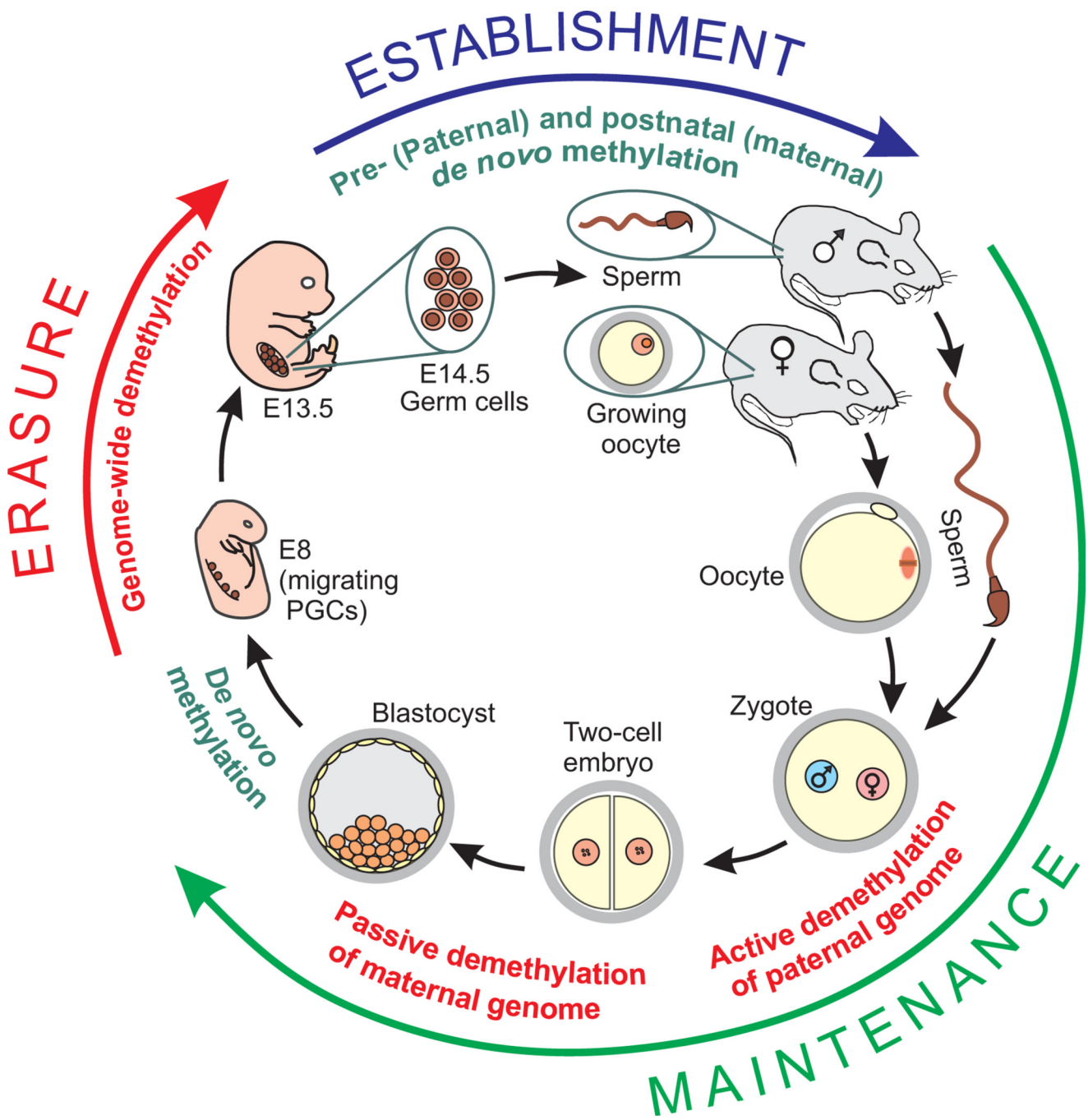

Figure 4.

Transgenerational cycle of imprinting marks in mouse. Generational inheritance and epigenetic reprogramming occur through a cycle of erasure, establishment, and maintenance of imprinting marks as illustrated here by successive demethylation and methylation events in the paternal and maternal genome. All methylation marks are erased in primordial germ cells (PGCs) by a genome-wide methylation event between embryonic day 8 (E8) and E13.5. Next, the imprinting marks are established by de novo methylation starting first in the male germ cells around E14.5 and then in growing oocytes after birth. The established sex-specific imprints are maintained until the appearance of the next-generation PGCs; however, further epigenetic reprogramming events occur to direct embryonic development and tissue-

specific gene expression in nonimprinted genes. While the paternal genome undergoes active demethylation at fertilization, the maternal genome is passively demethylated during successive cleavages of the early embryo. Finally, another de novo methylation takes place at the time of implantation.

Table 1

Human Imprinting Disorders and Uniparental Disomy

Chromosome	**6**	**7**	**11**	**14**		**15**		**20**	
	Paternal	**Maternal**	**Paternal**	**Maternal**	**Paternal**	**Maternal**	**Paternal**	**Maternal**	**Paternal**
Phenotype	TNDM	SRS	BWS	Pre-postnatal growth restriction, dimorphisms; Early puberty; Developmental delay	Polyhydramnios; Pre-postnatal growth restriction; bell-shaped thorax; muscular hypotonia	PWS	AS	Pre-postnatal growth restriction; microcephaly; psychomotor developmental delay	Pseudo-hypoparathyroidism type 1b
Prevalence UPD	40%	6–10%	20%	50 cases	30 cases	25%	2%	?	?
Gene	*PLAGL, HYMA1*	*GRB10, MEST?*	*IFGF2;* miR483, miR675, *CDKN1C*	*DLK1, RTU*	*DLK1, RTU*	*UBE3A*	*SNRPN, snoR116*	*GNAS* cluster	*GNAS*

The consequences of uniparental disomy and copy number neutral loss-of-heterozygosity during human development and cancer. (Modified from Lapunzina P and Monk D. Biol Cell 2011;**103**:303–317.[142]) TNDM, transient neonatal diabetes mellitus; SRS, Silver-Russell syndrome; BWS, Beckwith-Wiedemann syndrome; PWS, Prader-Willi syndrome; AS, Angelman syndrome.

Table 2

Studies on Beckwith-Wiedemann Syndrome and Angelman's Syndrome with Respect to Assisted Reproduction Techniques and Methylation Analysis Imprinting Disorder

	Locus and Genes	Reference	Study Location	Study Type	Total No. of Cases in Series	No. of Cases and ART Type	Cases Studied for Imprinting	Expected Etiology
A. Studies published regarding BWS with respect to ART and methylation analysis								
BWS	11p15.5 *ICF2* and H19in IC1 (DMR1); *CDKN1C KCNQ10T1* and *KCNQ1* in IC2 (DMR2)	1. DeBaun et al, 2003[5]	USA	Case series	3/65 (4.6%) BWS cases had IVF; 4 BWS cases from another registry (not able to determine prevalence of IVF)	2 IVF/5 ICSI	6 studied; 5 had hypomethylation of IC2; 1 of these had both hypomethylation of IC2 and hypermethylation of IC1	Maternal hypomethylation of IC2 (50%); Maternal gain methylation of IC1 (5%); Paternal UPD (20%); Mutation of maternal *CDKN1C* allele (5%); Unknown (20%) Microdeletion (1%); Translocation/ inversion (1%); Duplication (<1%)
		2. Maher et al, 2003[6]	UK	Case series	6/149 (4%)BWS cases had IVF	3 IVF/3 ICSI	4 studied: 0/4 had UPD; 2/4 hypomethylation of IC2	
		3. Gicquel et al, 2003[7]	France	Case series	6/149 (4%) with IVF	4 IVF/2 ICSI	6/6 studied had hypomethylation of IC2	
		4. Halliday et al, 2004[8]	Australia	Case-control	4/37 (10.8%) of BWS cases had IVF vs. 1/148 controls had IVF	3 IVF/1 ICSI	3/3 had hypomethylation of IC2 (1 BWS in non-IVF was not studied)	
		5. Chang et al, 2005[9]	USA	Case series	19/341 (5.6%) BWS had IVF (12 available)	5 IVF/5 ICSI; 1 CC/IUI; 1 IUI	ND	
		6. Rossignol et al, 2006[10]	France	Case series	40 BWS with demethylation of IC2 (11 had IVF and 29 did not)	NA	3/11 (27%) IVF and 7/29(24%) had abnormal methylation at other loci than IC1 and IC2	
		7. Sutcliffe et al, 2006[120]	UK	Case series	213 BWS; 83 replied (4 familial); 11/79(13.9%) had ART	5 ICSI; 1IVF; 5 OI	8/8 had hypomethylation of IC2 (not specified which had IVForOI)	
		8. Doornbos 2007[143]	Dutch	Case series	138 BWS; 75 responded and 4/76(1.3%)	4 IVF; 1 OI; 1 IUI	6/6 tested had hypomethylation of IC2	
		9. Lim 2009[144]	UK	Case series	25 BWS referred compared with 87 BWS known to have IC2 hypomethylation	12 IVF; 13 ICSI	24/25 had hypomethylation of IC2; Loss of maternal allele methylation in other DMRs in 37.5% of ART and 6.4% of non-ART BWS IC2 defect cases	
					Total: 656 BWS available	Total: 53 (5.9%) IVF	Total: 46/50 (92%)	

	Locus and Genes	Reference	Study Location	Study Type	Total No. of Cases in Series	No. of Cases and ART Type	Cases Studied for Imprinting	Expected Etiology
B. Studies published regarding AS with respect to ART and methylation analysis								
AS	15q11.2-q13 *SNRPN* and *UBE3A*	1. Ludwig et al 2005[13]	German	Case Series	270 AS requested; 79 replied (30%); 16/79(20.2%) had infertility	3 ICSI 5 OI 8 untreated	1 of 3 hypomethylation 1 of 5 hypomethylation 2 of 8 hypomethylation	Maternal 6–7Mb 15q11.2-q13 deletion (~68%) Upd(15)(7%) IC 6- to 200-kb deletions (3%) *UBE3A* mutations (11%) Deletion/ duplication analysis UBE3A (rare)
		2. Sutcliffe et al, 2006[120]	UK	Case series	75 AS cases; 3/75 (4%) had infertility	2 donor IUI 1 IVF	2 had deletions 1 had imprinting defect (donor IUI had hypomethylation)	
		3. Doornbos 2007[143]	Dutch	Case series	135 AS cases; 98 responded and 4/98(4.1%) had infertility	3 OI 1 donor IUI	2 of 4 had deletion 2 others unknown	
					23/252 AS patients (9.1%) had infertility		4/23 (17.4%)	

ART, assisted reproduction techniques; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; OI, ovulation induction; IUI, intrauterine insemination; ND, not determined; NA, not applicable.

Table 2A: Studies 1–5, 7, and 8 were used to calculate frequencies of ART in BWS; study 5 did not examine methylation status (ND = not determined); study 6 could not be used for ART prevalence because it only enrolled 40 BWS patients with hypomethylation of IC2; studies 6 and 9 showed additional loci (other than 11 pi 5.5) that had imprinting abnormalities.