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Epigenetics in Reproductive Medicine

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ABSTRACT: Imprinted genes comprise a small subset of the genome whose epigenetic reprogramming in the germ line is necessary for subsequent normal embryonic development. This reprogramming and resetting of the imprints, through an erasure/acquisition/maintenance cycle, is a subtle and tightly orchestrated phenomenon, involving specific genomic regions and methylation enzymes. Dysregulation of imprinted genes has indeed been shown to lead to several human disorders as well as to affect placental and fetal growth. There have been numerous and conflicting studies assessing the possible association of imprinting disorders with assisted reproductive techniques. This work analyzes all relevant and available reports with regard to the association between assisted reproductive techniques and imprinting disorders. It also discusses whether this possibly increased risk of imprinting disorders may be linked to specific steps of these reproductive techniques or already present in the gametes of infertile patients. A better understanding of epigenetic reprogramming in the germ line is absolutely necessary both to assess the safety of these methods and of the use of impaired spermatogenesis gametes for assisted reproduction. **(*Pediatr Res* 61: 51R–57R, 2007)**

Imprinting and epigenetic reprogramming involve, for specific genes, a sex-specific differential allele DNA methylation pattern (1), resulting in a parent-of-origin-dependent pattern of gene expression. Imprinted genes have been demonstrated to play key roles in the regulation of embryonic growth and placental function at critical stages of development as well as in numerous other essential biologic pathways (1). Disturbed expression of particular imprinted genes has indeed been linked to fetal growth and development abnormalities as well as to various human diseases (2). They may also play a key role in diseases affecting the placenta, such as HM, and in overgrowth or intrauterine growth retardation (IUGR).

Specific imprinting defects have been described in children conceived by ART. The interpretation of these findings was either that one or the other of the steps of ART might affect the process of imprint reprogramming or that the imprinting defect was preexisting. The latter hypothesis implicates that epimutations in the germinal cells used for ART may be the cause of imprinting defects in the concerned conceptuses. Therefore, the exploration of imprinting in defective spermatogenesis is a prerequisite for guaranteeing that the germ cells used for ART do not carry detrimental epigenetic changes. Large-scale international follow-up studies of children conceived by ART are also essential to assess the safety of these techniques.

IMPRINTING AND REPROGRAMMING

The vast majority of genes possess a bi-allelic pattern of expression. Imprinting corresponds to a specific epigenetic regulation leading to expression of only one parental allele of a gene. Some imprinted genes exhibit paternal expression whether others exhibit maternal expression. The best-characterized mark of gene imprinting is DNA methylation/unmethylation (3,4). Usually, methylated DNA sequences are transcriptionally inactive, whereas unmethylated DNA sequences are transcriptionally active (5). There are two mechanisms by which DNA methylation inhibits gene transcription: the first is interference of the methyl group with the binding of particular transcription factors to the DNA (6). The second involves methyl-binding domain proteins mediating transcriptional repression through binding to the DNA (7).

About 75 imprinted genes have been identified to date in human, although it is estimated that from 100 to 600 imprinted genes might exist in the human genome (8,9). Not all imprinted genes encode proteins. Some of them encode untranslated RNA, antisense RNA, or micro RNA (10) that certainly play an important role in regulating gene expression. Imprinted genes are characterized by specific regions up to several kilobases of length—DMD. At these regions, the levels of DNA methylation differ between the maternal and paternal alleles (11). Methylation has been shown to occur at specific CpG dinucleotide structures within DMD. Within a DMD, one parental allele is methylated on all/the majority of the CpG dinucleotides, while the opposite one is methylated on none/a small percentage of its CpG dinucleotides. Outside the DMD, similar patterns of methylation are present on both parental alleles. A constant feature of imprinted genes is that they are clustered into large chromatin domains, or "imprinted domains," at specific chromosomal regions. Their clustering may allow a coordinated regulation of imprinting, imprinted gene expression, and asynchronous replication timing by imprinting control centers (12). These are CpG rich and methylated all/the majority of the CpG dinucleotides on one parental

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Abbreviations: ART, assisted reproductive techniques; **AS,** Angelman syndrome; **BWS,** Beckwith-Wiedemann syndrome; **DMD,** differentially methylated domains; **Dnmt,** DNA methyltransferase; **HM,** hydatiform mole; **ICSI,** intracytoplasmic sperm injection; **IVF,** *in vitro* fertilization; **LOI,** loss of imprinting; **PW,** Prader-Willi syndrome; **SRS,** Silver-Russell syndrome

allele only (13). Well-characterized imprinted domains have been described in human, such as the 11p15.5 and the 15q11-13 regions.

Parental imprints are erased in the immature primordial germ cells of the developing embryo, subsequently re-established during gametogenesis according to a sex-dependent pattern and maintained through fertilization, pre- and postimplantation embryonic development (14). Imprint re-establishment occurs at late fetal stages in male germ cells and after birth in growing oocytes (13). Imprinting reprogramming refers to this erasure/acquisition/maintenance cycle of DNA methylation, occurring at DMD, which plays a key role at critical stages of embryonic development and fetal growth. Imprinted genes are also thought to play a role in the control of postnatal growth, brain function and specific neurobehavioral traits (15).

METHYLATION ENZYMES

Dnmts are responsible for the methylation of DNA; 3 Dnmt families have been identified so far: Dnmt1, Dnmt2, and Dnmt3. Dnmt1 is the most abundant DNA methyltransferase in mammalian cells. Dnmt1 has 3 known isoforms: a somatic Dnmt1, a splice variant (DNMT1b) and an oocyte-specific isoform (Dnmt1o). It predominantly methylates hemimethylated CpG di-nucleotides in the genome and is considered to be the key maintenance methyltransferase during cell division (7). The biologic function and the role in the methylation processes of Dnmt2 is still elusive (16). Dnmt3 is a family of DNA methyltransferases that could methylate hemimethylated and previously unmethylated CpG di-nucleotides. Dnmt3a and Dnmt3b may mediate gene repression through interactions with transcriptional repressors (17). Also, Dnmt3a adds methyl groups on imprinting centers (13). Dnmt3L is hypothesized to be required for the establishment of maternal imprints in the oocyte. It is expressed during gametogenesis (18).

HUMAN DISEASES INVOLVING IMPRINTED GENES

Abnormal expression of imprinted genes, through genetic or epigenetic alterations, can lead to a number of diseases. These diseases are all characterized by a non-mendelian inheritance and a parent-of-origin effect. They consist in four broad categories, including neuron-developmental, metabolic disorders, and psychiatric/behavioral disorders as well as cancer. The first group includes BWS, PWS, and AS (19,20). The second group includes transient neonatal diabetes mellitus. The third group includes autism, schizophrenia, and bipolar disorder. The fourth group includes retinoblastoma (9) (15). Table 1 provides a selection of human diseases linked to imprinting defects.

DEFECTIVE IMPRINTING IN ART

Various imprinting disorders have been recently reported following conception by ART (IVF or ICSI). These techniques (ART) may by themselves have a deleterious effect on imprinting. New technical steps have been recently added to the IVF/ICSI procedures, like testicular/ovarian tissue cryopreservation and oocyte *in vitro* maturation (21) as well as preimplantation genetic diagnosis. It is presently not known whether these may expose the gametes or early embryos to risks of imprinting defects.

Table 1. *Selected human disorders linked to an imprinting defect, that have been reported after ART*

Disorders	Candidate chromosomal location	Reported cases linked to ART (Ref)
BWS	11p15	(22–26,36)
AS	15q11-13	(32–34)
PWS	15q11-13	(35,36)
SRS	7	(35)
Isolated hemihyperplasia	11p15	(38)
Autism	15q11-13	NR
Bipolar disorder	18p11.2	NR
Schizophrenia	18p11.2	NR
Late-onset Alzheimer disease	10 and 12	NR
Transient neonatal diabetes mellitus	6q24	NR
Albright hereditary osteodystrophy	20q13.2	NR
Retinoblastoma	13q	(39)
Preeclampsia	10q22	NR
Biparental complete HM	19q13.4	NR

NR, not reported.

Recent studies have suggested that a number of specific imprinting disorders might be more frequent in children conceived by ART than naturally.

BWS

In a prospective study on BWS, DeBaun *et al.* (22) identified seven sporadic cases who were conceived by ART. In six of them, they identified the specific epigenetic alterations generally associated with BWS, *i.e.* LOI at KCNQ1OT1 or H19. Their results showed, in children with BWS, a 6-fold higher prevalence of ART- *versus* natural conception (4.6% *versus* 0.8%, respectively). Gicquel *et al.* (23) found in their BWS patient series a three-time over-representation of ART compared with the general population (4% *versus* 1.3). All their patients presented a KCNQ1OT1 LOI. Maher *et al.* (24) studied 149 sporadic BWS cases and looked for a possible association with ART (24). A conception by ART was recorded for 4% of BWS cases to be compared with 1.2% in their control population. Among the reported cases, 2 had a KCNQ1OT1 LOI.

Halliday *et al.* (25), in a large case-control study analyzed the frequency of BWS in 14′894 babies born after ART compared with 1′316′500 live births. They detected 37 cases of BWS, corresponding to an overall risk of BWS 9 times higher in the ART group, than in their general population.

In a retrospective study, Chang *et al.* (26) identified 19 BWS children (out of a 341 BWS registry) who were conceived by ART. The latter had similar clinical features as naturally conceived children. Interestingly, no specific aspect of the ART procedure, like the use of specific culture media, or the timing for transfer of embryo could be associated with BWS.

Rossignol *et al.* (27) examined the methylation status of various imprinted genes in 40 BWS displaying a KCNQ1OT1 LOI, either conceived by ART or naturally. They showed in both groups that some BWS patients presented abnormal methylation patterns at loci other than KCNQ1OT1. Their results suggest that ART was not associated to a locus-specific distribution of imprinting defects.

Interestingly, a number of monozygotic female twin pairs discordant for BWS have been reported. Weksberg *et al.* (28,29) showed that the incidence of female monozygotic twins among patients with BWS was indeed dramatically increased over that of the general population. In their series, each affected twin had an imprinting defect at KCNQ1OT1. It was proposed that a lack of maintenance of DNA methylation at a critical stage of preimplantation development causes a LOI in KCNQ1OT1 and that this LOI may increase the probability of monozygotic twinning or conversely the monozygotic twinning phenomenon may increase the probability of epigenetic alterations at KCNQ1OT1 (28). Smith *et al.* (30) reported two male monozygotic twin pairs with BWS, one discordant and the other concordant for the condition. These carried molecular defects associated with BWS other than KCNQ1OT1 LOI. These authors concluded that male monozygotic twins with BWS, rarer than female monozygotic twins with BWS, might carry heterogeneous molecular defects.

AS AND PWS

Concerning the occurrence of AS and PWS, Manning *et al.* (31) examined the DNA-methylation status of the 15q11-q13 chromosomal region (involved in the pathogenesis of these two syndromes) in 92 children born after an ICSI procedure. They did not observe any abnormal methylation patterns. Two years later, Cox *et al.* (32) reported the case of two children conceived by ICSI who had developed AS. Both patients had an imprinting defect in the 15q11-q13 chromosomal region. Orstavik *et al.* (33) also reported a case of AS children conceived by ICSI. More recently, Ludwig *et al.* (34) reported an increased prevalence of imprinting defects in AS patients conceived by subfertile couples (naturally, after hormonal stimulation alone or by ICSI). Interestingly, the increased risk of imprinting defects was independent of the type of conception. These data suggest that, rather than the ART itself, it is the subfertility that might be the cause of imprinting defects.

Kallen *et al.* (35) compared the Swedish registry medical data from 16,280 children born after ART (IVF/ICSI) to the data from more than 2 million naturally conceived. They found one case of PWS and one of SRS in the ICSI-conceived group. The occurrence of these two such cases among their ICSI-conceived group was considered by the authors as suggestive of a link between ART and imprinting defects.

Sutcliffe *et al.* (36), by examining, in a British survey, the use of ART in families of children with syndromes linked to imprinting defects, confirmed an association between ART and BWS but did not support a significant association between ART and PWS or transient neonatal diabetes mellitus.

OTHER DISEASES

Lidegaard *et al.* (37) analyzed the frequency of imprinting disorders in 6052 children conceived by IVF compared with 442,349 singleton conceived naturally. They found no indication after IVF of an increased risk of diseases potentially linked to imprinting defects, such as congenital syndromes, childhood cancers, mental diseases and developmental disturbances.

Shuman *et al.* (38) analyzed 51 patients with isolated hemihyperplasia, a disease reported to result from various molecular defects among which changes at the 11p15 imprinted locus. Eight of their 51 patients displayed an uniparental disomy in the 11p15 region. Interestingly, two of them had been conceived by ART.

Relative risks for retinoblastoma were reported as significantly raised for IVF-born babies to develop retinoblastoma, in a study performed in the Netherlands (39). However, the mechanism by which an imprinting abnormally may underlie retinoblastoma is still unraveled.

The interpretation of the studies available to date is difficult and confounded by their different methodological approaches, as, for example, the registry-based *versus* case reports of ART-conceived children showing by imprinting defect syndromes. Furthermore, there are very few follow-up studies providing information on the growth and developmental parameters of children conceived by ART, as most of them are still under the age of 20. The longest follow-ups performed to date concern 8-y-old children conceived by ICSI (40,41). It has to be emphasized that if a risk of an imprinting disorders, such as BWS is really linked to ART, it is still low ($<1\%$), compared with the probability of a healthy birth.

IMPRINTING DEFECTS AND MALE INFERTILITY

Effects of DNA methylation on the expression of genes involved in male reproductive organ development, spermatogenesis, and male sexual behavior have been reported (42).

Some of the imprinting disturbances suggested to be associated with ART may indeed be already present in the gametes of infertile men.

It has been hypothesized that germ cells from infertile men, such as those being used for ICSI, may contain, among other genetic defects, imprinting abnormalities. Marques *et al.* (43) have compared the imprinting of the paternally expressed MEST/PEG1 and the maternally expressed H19, in the spermatozoan DNA, of a cohort of 123 oligozoospermic investigated for infertility and normozoospermic patients. They found normal unmethylated patterns for MEST/PEG1 but sporadic hypomethylated H19 CpG sites in oligozoospermic patients. Their data suggest an association between hypospermatogenesis and defective genomic imprinting. Hartmann *et al.* (44) also analyzed imprinting in disruptive spermatogenesis. They explored the methylation pattern of SNRPN (paternally expressed) and H19 gene in different germ cell types obtained by testicular biopsies of a few infertile patients. They demonstrated correct genetic imprints for SNRPN and H19, in spermatogonia, primary spermatocytes and sper-

matids selected from seminiferous tubules exhibiting spermatogenic arrest.

The discordant results of these two reports emphasize the need for case-control studies involving a large number of individuals. Also, the analysis of the full DMD of various imprinted genes would provide a clearer picture of the implication of methylation changes than the analysis of a small number of CpG di-nucleotides in a DMD portion. Although to date no technique exists for the serial-analysis of methylation, the developments of molecular genetics will certainly permit this approach in the future.

IMPRINTING AND PLACENTA

One of the most important organ for the imprinted gene action is the placenta and several genes show a tissue-specific placental imprinting (45).

Most maternally imprinted genes enhance, whereas most paternally imprinted genes diminish or suppress, fetal growth. Most paternally expressed genes enhance placental growth, while most maternally expressed genes reduce placental size (46). Figure 1 gives a schematic view of this concept. Among the imprinted genes acting on fetoplacental growth are the paternally expressed IGF2, MEST/PEG1, PEG3, INS1, INS2, and MEST and the maternally expressed IGF2R, H19, and GRB10 (47). Imprinted genes products may act on fetal growth by modulating nutrient supply, by controlling either the optimal growth and development of all/part of the placenta, or the exchange of nutrients across the placenta. The imprinted IGF2-H19 gene complex plays a key role in the nutrient-transfer capacity of the placenta (47). This was shown in a study, in which Constancia *et al.* (48), using genetic mouse models of impaired fetal growth, showed that the imprinted IGF2 gene was playing a key role in the nutrient supply by modulation of activity and expression of placenta-specific nutrient transporters.

Placenta-specific imprinted genes seem therefore to play a key role in the control of fetal growth.

IUGR

IUGR, defined as an impaired growth and development of the embryo/fetus or its organs during pregnancy, is a medical condition that is frequently observed and predisposes to perinatal mortality. It can be caused by several genetic defects, among which chromosomal abnormalities.

Several imprinting disorders and uniparental disomies, involving imprinted chromosomal regions, are also associated with IUGR (49–51).

Studies in mouse have suggested that imprinting defects could affect the maternal supply of nutrients to the fetus, and consequently the intrauterine growth. In human, consistent with the role of imprinted genes in placental function, several uniparental disomies such as maternal uniparental disomy 7, maternal uniparental disomy 14, paternal uniparental disomy 6q24, and maternal uniparental disomy 20 have been shown to be associated with IUGR (52). McMinn *et al.* (53) analyzed the expression of six imprinted genes in late-gestation placental samples from nonsyndromic human IUGR. They reported a significantly increased expression of paternally-imprinted PHLDA2 and decreased expression of the maternally imprinted MEST/PEG1 and PLAGL1/ZAC1, and of the paternally imprinted MEG3, GATM, and GNAS in IUGR placen-

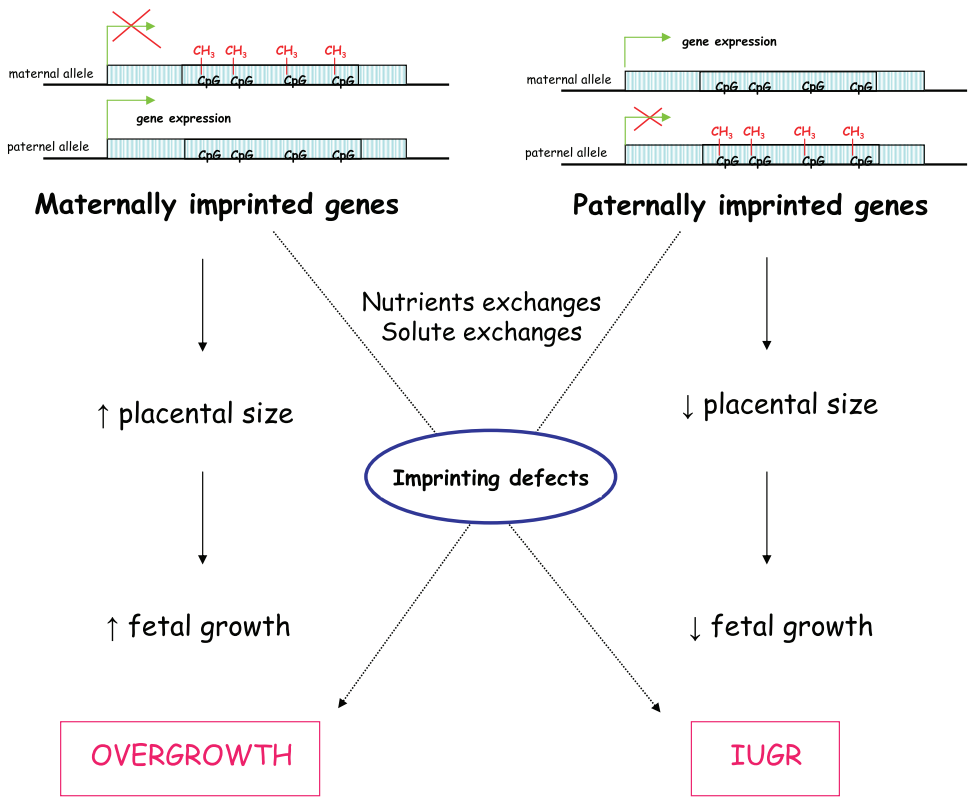

Figure 1. Effects of maternally/paternally imprinted genes on placental growth and possible pathologic consequences of imprinting defects in these genes.

tas. These results emphasize the hypothesis that, in IUGR, placenta-specific imprinted genes may be dysregulated.

Another placental disease, preeclampsia, has also recently been proposed to be linked to imprinted genes disturbances at the 10q22 chromosomal locus (54,55).

OVERGROWTH

As might be expected, if imprinted genes play a role in the control of fetal growth, imprinting disturbances may also lead to fetal overgrowth. In human and mice, fetal overgrowth has been described in association with the abnormal expression of various imprinted genes, as H19, IGF2 and IGF2R (56). Furthermore, BWS is also associated with a phenotype of overgrowth. Gene disruption experiments have shown that inactivation of the mouse H19 gene led to biallelic IGF2 expression and extensive somatic fetal growth in animals inheriting the H19 mutation from their mothers. Paternal inheritance of the disruption had no effect, reflecting the normal inactivity state of H19 when paternally inherited (57). Disruption of the maternal allele of GRB10 in mice also resulted in overgrowth of both the embryo and placenta, with mutant larger than normal at birth (58). Overexpression of IGF2 genes also resulted in fetal overgrowth (46,59). Morison *et al.* (60) detected constitutional LOI of IGF2 in four children with somatic overgrowth but none of BWS features. Among them, three children showed H19 methylation abnormalities. In animals such as bovines, a particular overgrowth syndrome known as "large offspring syndrome" with a significant increase in birth weight, polyhydramnios, hydrops fetalis, altered organ growth, and various placental and skeletal defects was described after *in vitro* culture of preimplantation embryos (56,61). Although the underlying mechanisms are only partially understood, methylation defects of imprinted genes may be the cause of both overgrowth and growth restriction abnormalities observed in humans.

HM

HM is an abnormal pregnancy characterized by excessive trophoblastic proliferation and a reduced/lack of embryonic development. Most HM are sporadic, and their occurrence is approximately 1/500 to 1/1000 pregnancies (62). HM can be divided into two subtypes: complete HM or partial HM. Most complete HM are sporadic and exhibit a diploid genome that is entirely paternally derived (*i.e.* androgenetic). Two mechanisms underlie the androgenetic constitution: an anuclear oocyte fertilized by two sperms or, most frequently, an anuclear oocyte fertilized by one sperm with subsequent duplication of the paternal genome. Most partial HM have a triploid genome, with three copies of each chromosome, two of them being paternally and one maternally inherited (63). The mechanisms leading to the different types of HM are summarized in Figure 2. In complete HM, morphologically and histologically, embryonic development is usually absent and all villi are cystic. Embryonic development is observed and a wide range of normal to abnormal cystic villi is observed in partial HM.

In a rare type of complete HM, a biparental origin of the chromosomes has been found: these are referred to as biparental complete HM (64). It has been shown that complete HM and biparental complete HM are pathologically indistinguishable (65). A small number of women presented a disorder characterized by highly recurrent biparental complete HM, with a diploid biparental inheritance (66,67). The pedigrees were consistent with an autosomal recessive transmission (68), possibly disturbing the expression of imprinted genes in the pregnancies.

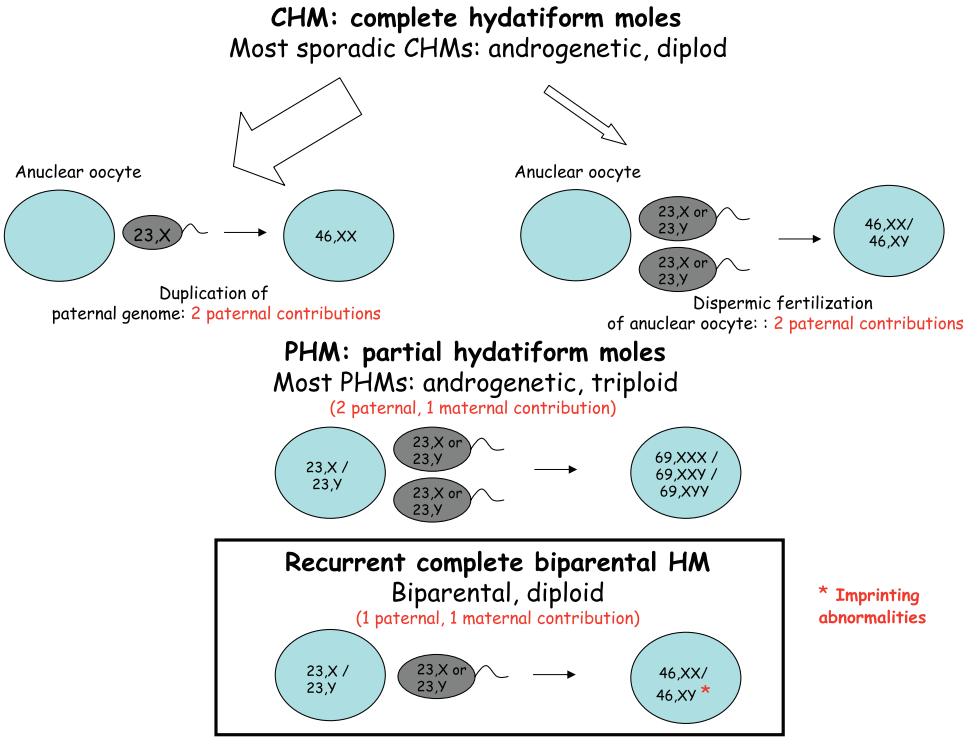

Figure 2. Karyotypes of HM. Complete HM are mostly sporadic and androgenetic. They result from the fertilization of an anuclear oocyte by a single sperm with duplication of paternal genome (most frequent mechanism, *thick arrow*) or by dispermic fertilization of a single oocyte (less frequent mechanism, *thin arrow*). Partial HM are mostly androgenetic triploid. They result from the fertilization of an oocyte by two sperms. Recurrent biparental HM are biparentally inherited diploid. They result from the fertilization of an oocyte by a single sperm.

Moglabey *et al.* (69) and El Maarri *et al.* (67) mapped a maternal locus responsible for biparental complete HM to 19q13.4. The imprinted gene PEG3, mapping to the region of interest, was suggested initially as a candidate for the biparental complete HM but later excluded when the candidate region was refined to a 1.1 Mb region at in 19q13.42 (70,71). In some pedigrees, linkage to chromosome 19q13.42 could not be established, suggesting a genetic heterogeneity in biparental complete HM (72). It is also possible that a defective gene in biparental complete HM regulates the expression of genes in this specific 19q13.42 chromosomal region. In a case of biparental complete HM that did not map to this region, Judson *et al.* (66) observed abnormalities in the methylation status of the maternally imprinted KCNQ1OT1, SNRPN, MEST/PEG1, and PEG3. In contrast, they found that the paternally imprinted H19 remained unaffected. Their results suggested that biparental complete HM can be caused by a recessive maternal mutation, which leads to the failure of establishment of maternal imprints and therefore to a paternal pattern of imprint in the maternal alleles. Very recently, Murdoch *et al.* (73) screened various genes of the 19q13.4 biparental complete HM candidate region and identified different mutations in the NALP7 gene in two families, establishing NALP7 as the causative gene for the biparental complete HM in his cases. NALP7 shares no structural homology with proteins involved in DNA methylation, and the authors suggested that the abnormal imprinting patterns observed in molar tissues could then be a consequence of a defective oocyte growth and/or maturation, during which maternal methylation marks were added.

CONCLUSION

The understanding of the role of defective imprinting in the development of human diseases has just begun. The reprogramming of the genomic imprints certainly represents a key period for the adequate resetting of the imprints and therefore also a target for a disturbance of this subtle phenomenon. We may indeed observe in the next decade that various environmental factors, such as gamete *in vitro* manipulation, or exposure to specific compounds during pregnancy may lead to changes in the imprinting patterns of genes and affect gametogenesis and embryonic development. The actual state of the research does not allow to draw any conclusion yet, but certainly to express a warning. As we cannot yet evaluate precisely the consequences of ART on imprinting, long-term, large follow-up studies of the ART-conceived children must be performed. As well, worldwide standardization of the technologies used in ART must be performed. Furthermore, as imprinting defects may also be involved in the pathogenesis of reproductive diseases such as male infertility or placental defects, the search for abnormalities in the methylation pathways has to be emphasized. The development of serial analysis methods for exploring the methylation pattern of imprinted genes will also be needed to assess these possible changes.

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