Chapter 5

Genomic Imprinting and Human Reproduction

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Additional information is available at the end of the chapter

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1. Introduction

The high frequency of reproductive losses is specific to the human race. The chances of conceiving and giving birth to a healthy child for women between 20 and 30 years old are estimated to be as low as 21-28% per cycle [1]. About 60% of zygotes are eliminated during the pre-implantation or early post-implantation developmental stages and 15-20% of clinically recognized pregnancies are lost during the first trimester [2]. Approximately 50-60% of spontaneously aborted embryos have chromosomal abnormalities which are not compatible with prenatal development [3]. At the same time, the death of another considerable number of embryos with normal karyotypes cannot be explained by existing cytogenetic theories. Considering ontogenesis as a result of the unrolling of the strict developmental program, the epigenetic basis of this process may be of outstanding significance.

According to the classical definition, coined by Conrad Waddington in 1942, epigenetics is a branch of biology which studies "causal mechanisms" by which "the genes of the genotype bring about phenotypic effects" [4]. In its beginning, epigenetics was a synonym for developmental genetics. However, in contrast to classical genetic theories, the subject of epigenetics has a wider diversity of phenomena, which may be unrelated to changes in gene nucleotide sequences [5]. A strong surge of interest in studying the epigenetic basis of human hereditary pathology has been noted over the last several years [6, 7]. New classes of epigenetic diseases, namely chromatin diseases [8] and imprinting disorders [9] have been identified. However, little is known about the features and nature of epigenetic abnormalities, i.e., epimutations, [10] during human prenatal development. In this chapter information on the impact of genomic imprinting abnormalities on embryo development is summarized and discussed.

2. Genomic imprinting and its role in embryogenesis

Genomic imprinting is an epigenetic phenomenon, which is related to differential parent-of-origin gene expression. The term "imprinting" was taken from physiology. It was Konrad Lorenz, an Austrian zoologist, ethologist and ornithologist, who, when working with geese, rediscovered the principle of imprinting (originally described by Douglas Spalding in the 19th century) in the behaviour of nidifugous birds when a young bird acquired several of its behavioural characteristics from one parent.

The term "chromosomal imprinting" was coined in 1960 by Helen Crouse, one of only three PhD students trained by Nobel Laureate Barbara McClintock. Crouse described the selective elimination of paternal chromosomes in the male meiosis in *Sciara* fly [11]. In the first meiotic division of spermatogenesis, a monopolar spindle forms upon which all the maternally derived homologues move to a single pole and all the paternally derived homologues move away from the single pole and are completely eliminated from the cell as a nucleoplasmic bud. Crouse wrote, "the 'imprint' a chromosome bears is unrelated to the genetic construction of the chromosome and is determined only by the sex of the germ line through which the chromosome has been inherited" (cited in [12]). At the time that Crouse first used the term, chromosome imprinting was known to occur in *Sciara* spermatogenesis. This mechanism of selected chromosome segregation remains enigmatic, although much headway has been made in mammalian systems [12].

The first evidence of the parental genome's memory in mammals came from experiments conducted by the Surani, McGrath and Solter groups with pronuclei transplantation in mouse zygotes in 1984 [13, 14]. These studies were aimed at answering the question about the absence of parthenogenesis in mammalian reproduction. It was discovered that diploid androgenic mouse embryos derived from zygotes, which contained two paternal pronuclei and none of the maternal pronuclei, demonstrated an extensive proliferation of extraembryonic tissues but poor development of the embryo *per se*, which usually did not reach a 4-6 somite stage. In contrast, gynogenic zygotes with two maternal pronuclei and an absence of the paternal genome resulted in embryos which developed until the stage of early somites, but then died due to the poor development of supportive extraembryonic tissues. These pioneers' studies established that diploidy alone is not sufficient for embryonic development, but that a balance of maternal and paternal genomes is strongly required for normal embryogenesis. Moreover, the impact of parental genomes on embryo development is different. It seems that the maternal genome is responsible for the development of the embryo body to a greater extent, whereas the paternal one is involved in the support of extraembryonic tissue differentiation.

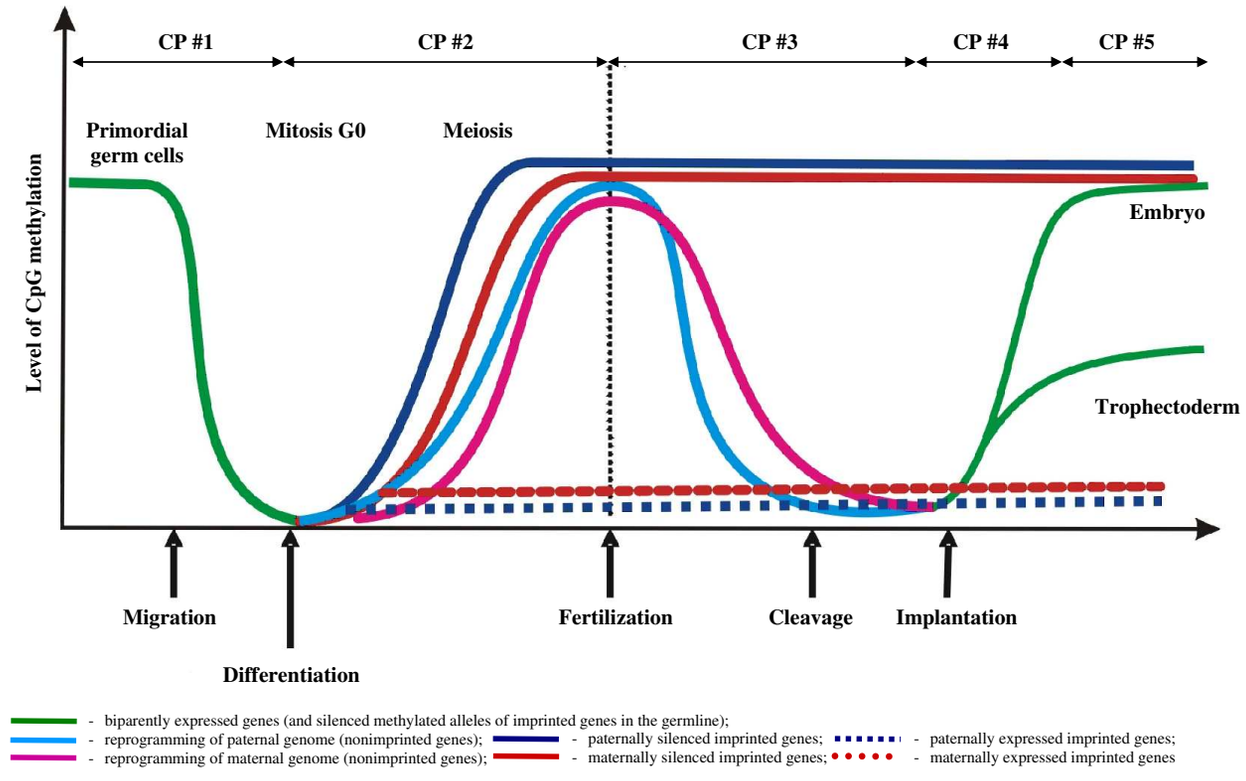
It is interesting to note that a similar effect of the increasing number of paternal genomes in the zygote is also observed in humans due to an abnormality of fertilization [15]. Fertilization of a diploid oocyte by normal haploid sperm or double fertilization of a normal oocyte by two haploid sperms leads to triploidy in the zygote. In this case, the partial hydatidiform mole (PHM) arises. PHM is characterized by the cystic degeneration of chorionic villi and the presence of a visible embryo in the foetal sac. In the case of the extrusion of the maternal pronucleus from such a triploid zygote, a diploid karyotype is restored through the postzygotic

triploid diploidization mechanism [16], but both haploid genomes are paternal in their nature. This bipaternal karyotype is not compatible with the development of an embryo body, but leads to a hyper proliferation of trophoblasts cells and a complete hydatidiform mole (CHM) with an increased risk of chorioepithelioma. The observed effect may be explained by the double increase in the dose of imprinted genes expressed from paternal chromosomes, which promotes proliferative and invasive activity of the trophoblasts cells as well as an absence of activity of the maternal imprinted genes, which, in turn, must suppress trophoblast proliferation.

The parental differences in imprinted genes expression are epigenetic in their nature. They are established during gamete differentiation by sex-dependent epigenetic chromatin modifications, mainly by the differential DNA methylation of promoter regions of imprinted genes or regulatory imprinted centres, which are further stably inherited in the somatic cells of the progeny. These regular and consecutive alterations of chromatin organization are referred to as epigenetic genome reprogramming [17, 18] (Figure 1). This starts in the primordial germ cells when they enter the gonads. Both imprinted and non-imprinted loci become demethylated. This total erasure of epigenetic information is required for the totipontency of future germ cells, imprinting switching and for the prevention of the inheritance of epigenetic defects. The demethylated chromatin's state remains until the duration of the mitotic arrest in male germ cells and the meiotic arrest in female ones. When mitotic divisions of spermogonia are resumed, *de novo* DNA methylation begins, which terminates completely by the time of pachytene of meiosis I. In oocytes, *de novo* DNA methylation starts only at their maturation and ends by metaphase II. In this period, sex-specific methylation of imprinted genes is established. In one imprinting genes, DNA methylation occurs exclusively in oogenesis, whereas in others it occurs during spermatogenesis [19].

The second wave of epigenetic genome reprogramming, which involves somatic cells, begins immediately after fertilization. The paternal chromosomes became decondensed, protamines in the chromatin are replaced by histones and fast demethylation of paternal genome is triggered. The maternal genome undergoes slow passive demethylation. It is believed that demethylation of parental genomes is required to induce pluripotency in embryonic stem cells. Later, during implantation, *de novo* DNA methylation is launched again, which results in specific methylation patterns of particular genomic regions in different cells and tissues. This process, in fact, provides one of the most important mechanisms for committing and regulating tissue specific gene expression during ontogeny. It is significant that imprinted genes avoid this reprogramming wave in somatic cells, preserving their differential methylation pattern inherited from the parents. Genomic imprinting is also regulated through other epigenetic mechanisms, such as histone modifications, antisense transcripts and small non-coding RNA, which have been discussed in detail in some comprehensive reviews [19, 20].

At present, (August 2013), it is reported that there are about 90 imprinted genes in a human genome [21]. Most of them are involved in the regulation of intrauterine foetal development through the control of cell proliferation and the differentiation of placental tissues, regulation of metabolism of some hormones and growth factors [22]. The evolutionary reverse to the haploid expression of a subset of genes in mammalian and flowering plants genomes was a



CP – critical periods of reprogramming

Figure 1. Dynamics of epigenetic genome reprogramming

great surprise. Several hypotheses were introduced to explain this intriguing fact, but the "sex conflict" was one the most popular among them [23]. According to this hypothesis, maternal imprinted genes in mammals are responsible for the suppression of foetal growth in order to save maternal resources for subsequent pregnancies. In contrast, paternal imprinted genes are involved in the promotion of foetal growth that provides higher chances of survival for many offspring.

Testing this hypothesis in mouse models, the direct evidence for the significant role of genomic imprinting in mammalian embryo development was obtained. The generation of uniparental disomies (UPD) in progeny of translocation carrier's mice gives nonviable embryos [24]. This fact leads to the idea of searching for UPD in human spontaneous abortions in order to estimate the impact of genomic imprinting abnormalities on prenatal death.

3. UPD in spontaneous abortions

To date, eight studies have been performed to find UPD in spontaneous abortions [25-32] (Table 1). However, the obtained results were modest. Only seven cases of UPD (2.3%) among a total of 305 spontaneous abortions were found and most of them involved chromosomes which did not contain known imprinted genes. Only in three cases segmental UPD (16p/16q (mat), 14q (pat) and 7q (mat)) embryo death can be connected with a disturbance of the dose

of imprinted genes localized on these chromosomal regions. Moreover, spontaneous abortions without previous cytogenetic analysis were included in some studies that could have led to overestimation the obtained rate. As a result, the frequency of UPD for chromosomes, which contain known imprinted genes in spontaneous abortions were estimated to be 1% (3/305) or 1.14 per 1,000 occasions of chromosome inheritance from parents to progeny. The latter figure was obtained from the investigation of 6,156 cases of chromosome inheritance by DNA microsatellite analyses and seven cases of UPD were found in the eight cited studies. This figure does not significantly differ from the expected frequency (1.65:1,000), predicted from data about frequencies of chromosome segregation errors in gametogenesis and early embryogenesis, which can lead to uniparental inheritance in humans [33].

Thus, it seems that UPD is a selectively neutral phenomenon in human reproduction. Moreover, UPD for some chromosomes (6, 7, 11, 14 and 15) are compatible with postnatal life leading

Samples	Number of spontaneous abortions	UPD cases	References
Anembryonic pregnancies without cytogenetic analysis	23	UPD(21)mat, UPD(21)mat in combination with trisomies 7 and 9	[25]
Spontaneous abortions: - first trimester, without cytogenetic analysis	18	0	[26]
- with normal karyotype	35	0	[27]
- 6-22 weeks, with normal karyotype	71	UPD(9)mat UPD(21)mat	[28]
- with normal karyotype	24	Maternal segmental heterosomy 16pter-D16S3107 and isodisomy D16S3018-qter	[29]
- with normal karyotype	81	Segmental UPD(14q)pat, UPD(7q)mat	[30]
Missed abortions and anembryonic pregnancies: - with 46,XX karyotype (analysis of X-chromosome inheritance only)	52*	0	[31]*
- with normal karyotype	87*	0	[32]*
Total:	305	7 (2,3%)	

Note: * - samples partially overlap.

Table 1. Results of UPD studies in spontaneous abortions

to a formation of specific genomic imprinting disorders: transient neonatal diabetes mellitus (TNDM), Silver-Russell syndrome (SRS), Beckwith-Wiedemann syndrome (BWS), Wang and Temple syndromes, Prader-Willi syndrome (PWS) and Angelman syndrome (AS), respectively [34]. It became clear that UPD is a rare cytogenetic phenomenon, which cannot explain the mechanisms of imprinted genes disturbances in human pregnancy loss. The only evidence for the pathogenetic role of genomic imprinting abnormalities in human reproduction remains from studies on a hydatidiform mole which originated from the doubling of paternal genome in conception [15]. However, this conclusion does not offer an answer on the possible mechanisms of imprinting disturbances associated with early pregnancy loss.

4. Epimutations of imprinted genes in spontaneous abortions

Taking into account the epigenetic nature of genomic imprinting, we proposed a hypothesis that expected the deleterious effect of abnormal imprinted genes expression to be visible at the epigenetic rather than cytogenetic level [35]. Indeed, UPD formation requires a combination of several subsequent errors in chromosomal segregation during parental meiosis, fertilization and embryo development. For example, the most frequent mechanism of UPD formation is trisomy rescue. It arises from chromosomal nondisjunction in meiosis, trisomy formation in the zygote after fertilization and the loss of additional chromosome in some somatic cells during subsequent mitotic divisions. In a third of the cases of such correction, the situation of inheritance of both homologues from one parent may be observed. If involved chromosome contains an imprinted gene, then the double increase or complete loss of expression of imprinted genes may be detected and it is dependent on the parental origin of expressed allele.

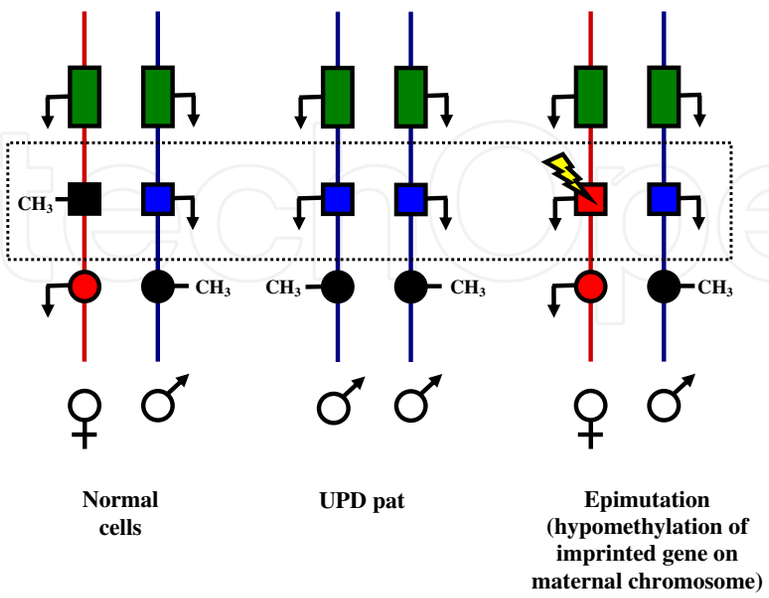


Figure 2. Effects of UPD and epimutations on expression of imprinted genes.

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On the other hand, the change of the imprinted gene dose may be achieved by epimutations, i.e., abnormal methylation of the expressed allele or demethylation of the silenced allele. From a functional point of view, epimutations and UPD influences on imprinted genes expression should be similar (Figure 2). It is important that epimutation on a single allele is enough to achieve the imprinted gene dysfunction in a dominant manner.

Before the testing of the hypothesis about the influence of methylation defects in imprinted genes on the aetiology of early pregnancy loss, a classification of epimutations was introduced [36]. They were divided into the following several groups depending on their germinal or somatic origin, hyper- or hypomethylation of active or silenced alleles, and affected parental chromosomes (Table 2):

1. Types of epimutations of imprinted genes by the loci involved:
    - 1.1. Epimutations causing a global disturbance of genomic imprinting at the genome level.
    - 1.2. Epimutations at the imprinting centres causing a disturbance of imprinting of neighbouring genes.
    - 1.3. Epimutations at the imprinted genes.
2. Types of epimutations of imprinted genes by their origin:
    - 2.1. Germinal epimutations
        - 2.1.1. Errors of genomic imprinting erasure in primordial germ cells with retention of methyl groups (Critical Period 1, CP #1, on Figure 1). These errors may lead to transgenerational inheritance of epigenetic defects.
        - 2.1.2. Errors of imprinting establishment during gametogenesis (CP #2 on Figure 1)
            - 2.1.2.1. Absence of methylation of imprinted genes alleles that normally should be methylated in sperm or oocytes.
            - 2.1.2.2. Aberrant methylation of imprinted genes alleles that normally should be unmethylated in sperm or oocytes.
    - 2.2. Somatic epimutations
        - 2.2.1. Abnormal hypomethylation of inactive parental alleles of imprinted genes during epigenetic genome reprogramming (CP #3 on Figure 1).
        - 2.2.2. Abnormal methylation of expressed parental alleles of imprinted genes during de novo DNA methylation upon epigenetic genome reprogramming (CP #4 on Figure 1).
        - 2.2.3. Spontaneous hypomethylation of inactive parental alleles of imprinted genes in somatic cells after epigenetic genome reprogramming (CP #5 on Figure 1).
        - 2.2.4. Spontaneous hypermethylation of expressed parental alleles of imprinted genes in somatic cells after epigenetic genome reprogramming (CP #5 on Figure 1).
3. Types of epimutations of imprinted genes by their functional consequences and affected parental alleles:
    - 3.1. Hypomethylation of the inactive maternal allele of the imprinted gene.
    - 3.2. Hypomethylation of the inactive paternal allele of the imprinted gene.
    - 3.3. Hypermethylation of the expressed maternal allele of the imprinted gene.
    - 3.4. Hypermethylation of the expressed paternal allele of the imprinted gene.

	Type of epimutations	Abnormal hypomethylation of		Abnormal hypermethylation of	
		maternal allele	paternal allele	maternal allele	paternal allele
Germinal epimutations	Errors of genomic imprinting erasure with retention of methyl groups (CP #1)	Not applicable	Not applicable	*IGF2/H19* (BWS)	*SNURF-SNRPN* (PWS); *PEG1/MEST* (SRS)
	Absence of imprinted genes alleles methylation (CP #2)	BiCHM; *SNURF-SNRPN* (AS); *KCNQ1OT1* (BWS, TNDM); *PLAGL1* (TNDM, BWS)	*IGF2/H19* (SRS)	Not applicable	Not applicable
	Aberrant hypermethylation of imprinted genes alleles (CP #2)	Not applicable	Not applicable	*IGF2/H19* (BWS)	*SNURF-SNRPN* (PWS); *PEG1/MEST* (SRS)
Somatic epimutations	Abnormal hypomethylation of inactive parental alleles of imprinted genes during epigenetic genome reprogramming (CP #3)	*SNURF-SNRPN* (mosaic forms of AS). MHS; mosaic forms of TNDM	Partial hypomethylation of *IGF2/H19* (SRS)	Not applicable	Not applicable
	Abnormal hypermethylation of expressed parental alleles of imprinted genes during epigenetic genome reprogramming (CP #4)	Not applicable	Not applicable	*IGF2/H19* (BWS)	*SNURF-SNRPN* (PWS)
	Stochastic epimutations (hypo- and hypermethylation) in somatic cells after epigenetic genome reprogramming (CP #5)	*KCNQ1OT1* (oesophagus carcinoma, liver cancer); *PEG3* (choriocarcinoma)	*P73* (renal carcinoma; lung cancer)	*IGF2/H19* (Wilm's tumour); *P73* (acute leukaemia, Burkitt's lymphoma); *DLK1/GTL2* (renal carcinoma)	*SNURF-SNRPN* (mosaic forms of PWS); *PEG3* (cervical and endometrial carcinomas); *PLAGL1* (ovarian cancer)

Table 2. Types of epimutations of imprinted genes in human (according to [36] with modifications)

The first evidence of epimutations in imprinted genes in reproductive losses came from studies of biparental complete hydatidiform mole (BiCHM, MIM #231090). This pathology, as opposed to classical androgenic complete mole, arose in the case of normal biparental karyotype [37] (Figure 3). It was shown that imprinted genes, which are methylated on maternal chromosomes in normal embryos, became hypomethylated in the case of BiCHM both on the paternal and maternal homologues [15]. Functionally, this epigenetic status is the same as an androgenic

complete mole. Subsequent studies revealed that BiCHM arose due to germinal epimutations, namely the absence of *de novo* methylation of imprinted genes in oogenesis (type # 2.1.2.1 according to classification) [38]. However, these findings do not offer an answer about the prevalence of imprinting defects in human reproductive losses. The convenient model system for studies became spontaneous abortions.

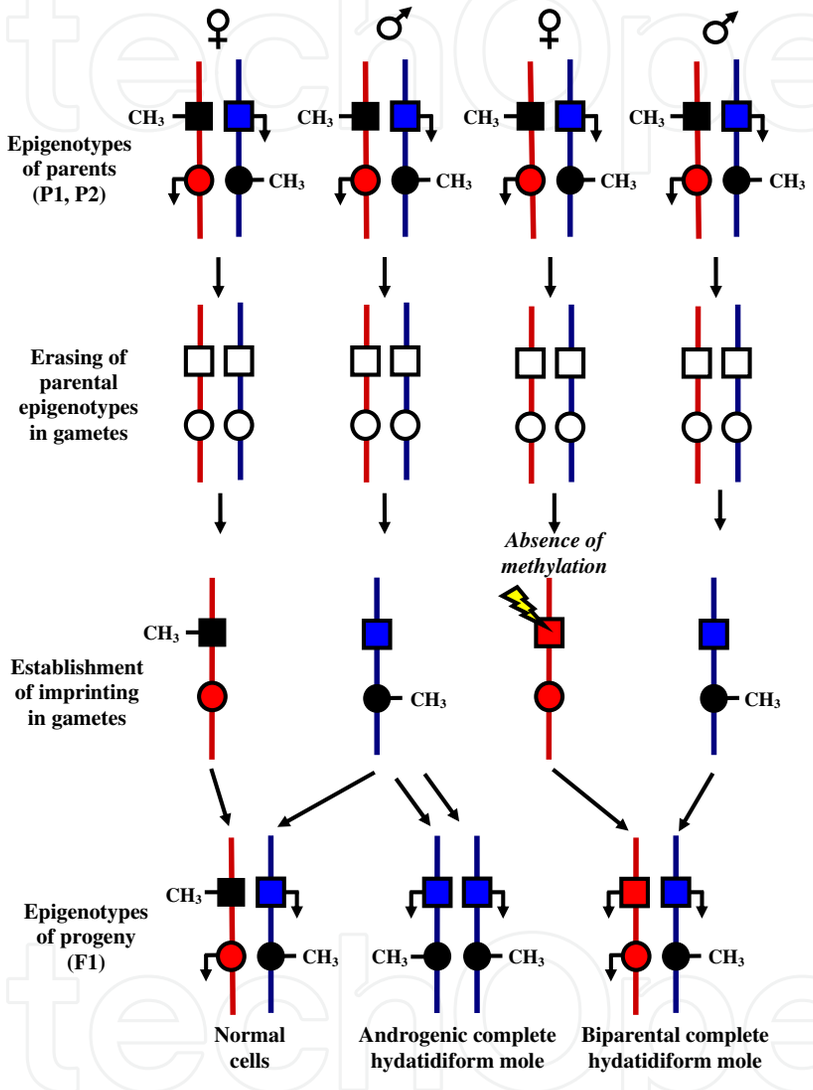

Figure 3. Cytogenetic and epigenetic mechanisms of hydatidiform mole formation.

The first study devoted to the analysis of methylation status of *SNRPN* imprinted gene was published by our group in 2006 [39]. However, it was shown that all investigated spontaneous abortions, as well as the control sample of induced (therapeutic) abortions, had normal differential methylation of parental alleles of this imprinted gene.

Our further studies on imprinted genes *PLAGL1 (ZAC), CDKN1C* and regulatory imprinting centres *IGF2/H19* and *KCNQ1OT1* (*LIT1*) in spontaneous abortions revealed that 9.5% and 10.3% of embryos with normal karyotype had hypomethylation of *KCNQ1OT1* and *PLAGL1*,

respectively, on maternal chromosomes [35, 40]. It is interesting that in two embryos from the investigated group (2.3%) epimutations were detected in both genes. In both families, the women had not had a successfully delivered pregnancy. In one family, the woman had had four spontaneous abortions, in the other, the woman had had two spontaneous abortions and a stillbirth. It was shown that the frequency of recurrent pregnancy loss (i.e., the loss of three or more consecutive pregnancies) was significantly higher in woman who had had a loss of methylation in *PLAGL1* in spontaneous abortions tissues, in comparison with woman without epimutations in the aborted embryo (33% and 8%, respectively, $p < 0.05$). A similar tendency was observed for *KCNQ1OT1* epimutations, but it does not reach a statistically significant difference.

It was remarkable also that all detected epimutations were confined to one placental tissue (cytotrophoblast or extraembryonic mesoderm) only indicating their somatic origin in post-implantation stages of development after the divergence of embryonic and extraembryonic cell lineages. This observation has an important value for the discussion on the increase of genomic imprinting disorders in children born after the application of assisted reproductive technologies (ART) [20, 41-44]. Indeed, several cases of children with BWS, SRS and AS born after *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) have been reported. However, it is more significant that all patients with confirmed molecular diagnosis had hypomethylation of imprinted genes on maternal chromosomes as a cause of disorder among all the other known possible mechanisms which can alter imprinted genes expression – UPD, chromosomal microdeletions or microduplications, point mutations in imprinted genes or regulatory imprinted centres. This fact is supported by results of *in vitro* studies in model organisms [45]. This often leads to a hasty one-sided conclusion that manipulations with gametes and embryos in an artificial medium cannot support correct recording and maintenance of genomic imprinting and they are responsible for an increase of imprinting defects in children after ART. Our, and other, recently published data from spontaneous abortions studies [46] indicates that the same epimutations with relatively high frequency can be observed in embryos from natural cycles also and not only from IVF or ICSI pregnancies. This means that epimutations of imprinted genes arising during human embryo development, and infertile pairs or couples with the history of recurrent pregnancy losses (typical patients of ART clinics), may have increasing chances to produce progeny with epimutations after overcoming the natural reproductive barriers by IVF or ICSI. This conclusion implies that the nature of epimutations involves the interaction of hereditary and environmental factors.

Evidence for epimutations of imprinted genes in spontaneous abortions was also noted in other subsequent studies (See tables 3 and 4). For example, multiple hypermethylation of imprinted genes was detected in 4% (2 out of 55) of spontaneous abortions and 18% (10 out of 57) of stillbirths [47]. In this study, *H19, KCNQ1OT1, PEG3* and *SNRPN* genes with paternal expression and *MEG3* and *NESP55* with maternal expression were investigated. In other studies, the expression level of several imprinted genes was compared between placental tissues from normal pregnancies and pregnancies complicated by intrauterine growth retardation (IUGR) [48]. It was found that *PHLDA2, ILK2, NNAT, CCDC86* and *PEG10* genes had increased expression in IUGR pregnancies, whereas the expression level of *PLAGL1, DHCR24,*

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ZNF331, and *CDKAL1* was decreased. In this study, hypomethylation of *DLK1, H19,* and *SNRPN* was demonstrated also in the placental tissues of foetuses with IUGR. In another study, expression levels of four imprinted genes (*IGF2, PEG10, PHLDA2,* and *CDKN1C*) were investigated in embryonic and placental tissues of 38 spontaneous abortions [49]. The increased expression of *PHLDA2* was found both in the embryonic and placental tissues in the first trimester. During the second trimester, elevated expression levels of all four genes were observed in both tissues, however, for the *PHLDA2* gene it was specific to the embryonic tissues only. A decreased expression of *PEG3* was detected in embryonic tissues during the third trimester.

Pathology	Sample size	Tissue	MLMD frequency	*PLAGL1* (6q24)	*PEG10* (7q21) *DLK1* (14q32)	*PEG3* (19q13.4)	*LIT1* (11p15)	*INS* (11p15)	*SNRPN* (15q11)	*WT1* (11p15)	*KCNQ1* (11p15)	*GABRB3* (15q11)	*HTR2A* (13q14)	*TRPM5* (11p15)	*MEST* (7q32) *GABRA5* (15q11)	Ref.
SA	55	MT	3.6% (2)	-	-	-	2↑	1↑	-	1↑	-	-	-	-	-	-	[47]
SB	57	MT	17.5% (10)	-	-	-	6↑	2↑	-	9↑	-	-	-	-	-	-	[47]
SA	87	CC	2.3% (2)	2↓	-	-	-	2↓	-	0	-	-	-	-	-	-	[40]
SA	13	CC	100% (13)	1↓	3↓	0	0	0	2↑	2↑	4↓	5↓	3↓	2↓	1↑	0	3↑	[50]
SA	13	EM	100% (13)	2↓	9↓	9↓	0	0	7↑	10↑	10↓	3↓	5↓	6↓	5↑	0	7↑	[50]
SA	165	CC	3.6% (6)	-	-	-	-	6↑	-	7↑	-	-	-	-	-	-	-	[46]
SA	29	CC	10.3% (3)	-	-	-	1↓, 2↑	2↑	-	1↑	-	-	-	-	-	1↑	-	[51]
Total	406		12.1% (49)	5.0% (5/100)	46.2% (12/26)	34.6% (9/26)	7.1% (11/154)	3.2% (13/406)	34.6% (9/26)	7.4% (30/406)	53.8% (14/26)	30.8% (8/26)	30.8% (8/26)	30.8% (8/26)	23.1% (6/26)	1.8% (1/55)	38.5% (10/26)	
				↓ - 16.0% (65/406); ↑ - 18.7% (76/406)														

Notes for tables 3 and 4: SA – spontaneous abortion; SB – stillbirth; MT – muscular tissues of embryo; CC – chorion cytotrophoblast; EM – extraembryonic mesoderm; MLMD – multilocus methylation defects (number of embryos with MLMD is indicated in parentheses); ↓ – hypomethylation; ↑ – hypermethylation.

Table 3. Frequency of epimutations in paternally expressed imprinted genes in spontaneous abortions

Pathology	Sample size	Tissue	MLMD frequency	*GRB10 (7q21)*	*CPA4 (7q32)*	*PHLDA2 (11p15)*	*ZNF215 (11p15)*	*H19 (11p15)*	*MEG3 (14q32)*	*NESP55 (20q13.3)*	Ref.
SA	55	MT	3.6% (2)	-	-	-	-	0	1↑	0	[47]
SB	57	MT	17.5% (10)	-	-	-	-	4↑	8↑	6↑	[47]
SA	87	CC	2.3% (2)	-	-	-	-	0	0	-	[40]
SA	13	EM	100% (13)	1↓	8↓	2↓	8↓	1↑	0	0	[50]
SA	13	CC	100% (13)	5↓	5↓	9↓	4↓	6↑	0	0	[50]
SA	165	CC	3.6% (6)	-	-	-	-	4↑	-	-	[46]
SA	29	CC	10.3% (3)	-	-	-	-	0	0	0	[51]
Total	406		12.1% (49)	23.1% (6/26)	50.0% (13/26)	42.3% (11/26)	46.2% (12/26)	3.7% (15/406)	3.7% (9/241)	3.9% (6/154)	
				↓ - 10.3% (42/406); ↑ - 7.4% (30/406)							

Table 4. Frequency of epimutations in maternally expressed imprinted genes in spontaneous abortions

It is important to note that all the above mentioned studies were performed by the "candidate gene" approach, i.e., only several interesting genes were tested based on their supposed functions in embryogenesis. A new era in this area of research began with the application of array technologies. They provide comprehensive and unbiased analysis of the human imprintome. Results of the first studies revealed a new, intriguing phenomenon of multilocus methylation defects (MLMD) on imprinted genes. This effect is presented by multiple epimutations (hypo- and/or hypermethylation) affecting several imprinted genes simultaneously in the genome in different combinations. In fact, the first example of MLMD is a BiCHM, which results from errors of imprinting establishment on maternal chromosomes during epigenetic genome reprogramming in oocytes leading to the hypomethylation of maternal alleles of different imprinted genes [38]. MLMD were detected in human reproductive losses [50] as well as in patients with genomic imprinting disorders [52]. Importantly, in one recent study 15 (8%) probands among 194 patients with clinical features of an imprinting disorder but no molecular diagnosis had methylation anomalies, including missed and unexpected molecular diagnosis [53].

Evidence of MLMD in 13 first trimester spontaneous abortions were obtained in our study by DNA methylation array the GoldenGate Methylation Cancer Panel I (Illumina) analysis of 51 imprinted genes [50]. Multiple methylation defects affecting from four to 12 genes in each embryo were found. Epimutations were presented by the hypomethylation of paternal alleles of *GRB10*, *CPA4*, *PHLDA2*, *ZNF215* genes, and maternal alleles of *PEG10*, *PLAGL1*, *WT1*, *HTR2A*, *DLK1*, *GABRB3*, *KCNQ1* genes. Hypermethylation was observed on paternal allele of

H19 and on maternal alleles of *INS*, *TRPM5*, *PWCR1*, *GABRA5* genes. The majority of epimutations (78%) were confined to single placental tissue (extraembryonic mesoderm or cytotrophoblast), indicating postzygotic errors in imprinting maintenance in somatic cells.

Summarizing the published data, it is possible to note that MLMD were observed in 49 of 406 investigated spontaneous abortions (12%) (See tables 3 and 4). It is evident that this value, of a significant magnitude greater than UPD frequency in miscarriages, indicates an appreciable effect of epigenetic defects on imprinted genes in pregnancy losses. The incidence of epimutations for different genes varied from 1.8% (*MEST*) to 53.8% (*WT1*). For genes expressed from maternal chromosomes, the frequency of epimutations was 17.7%, with 10.3 % of these presented by the hypomethylation of respective inactive paternal alleles and 7.4% by the hypermethylation of active maternal alleles. For genes expressed from the paternal allele, the total frequency of epimutations was twice as frequent (34.7%). These epimutations were presented by the hypomethylation of respective inactive maternal alleles (16%) and the hypermethylation of expressed paternal alleles (18.7%).

The results of the studies suggest possible mechanisms of the selective influence of epimutations of imprinted genes on early embryo development. As it was mentioned early, the "sex conflict" hypothesis is the most popular in explaining the imprinted mode of gene expression. From its point of view, the expected suppressive effect of epimutations should emerge from the hypomethylation of paternal alleles, which leads to a loss of imprinting and the biallelic expression of maternal genes responsible for foetal growth suppression, as well as from hypermethylation of paternal alleles, which leads to the absence of products responsible for foetal growth stimulation. The total incidence of such types of epimutations in spontaneous abortions is 29% (10.3 + 18.7). At the same time, the total incidence of epimutations, which can lead to the promotion of foetal growth (hypo- and hypermethylation of maternal alleles), was 23.4% (16 + 17.4). The differences between frequencies of "suppressive" and "promotion" epimutations were not statistically significant ($p = 0.07$). However, this is a relative estimation for several reasons, including complete acceptance of the "sex conflict" hypothesis for each imprinted gene (maternal genes are suppressors, paternal genes are activators) and the idea that there is a strong reverse correlation between gene methylation and its expression. To obtain more precise and weighted estimations, further studies of imprinted gene functions and molecular mechanisms of their expression regulation are encouraged.

Does MLMD arise spontaneously in different loci or is it driven by mutations in a candidate's genes responsible for genomic imprinting establishment and maintenance? To answer this question the evidence of MLMD in patients with imprinting disorders should be discussed.

5. Multiple methylation defects in patients with imprinting disorders

The first evidence of multiple epimutations of imprinted genes was obtained in 2005 in the comparative analysis of methylation status of two genes – *ZAC (PLAGL1)* and *LIT1 (KCNQ1OT1)* in eight patients with BWS and 17 patients with TNDM [54]. The idea for this study was based on the fact that there is partial overlapping of some clinical features in these

syndromes. This observation may reflect possible interactions between *PLAGL1* and $P57^{KIP2}$ genes. According to the author's suggestion, an overexpression of *PLAGL1* due to the loss of methylation of inactive maternal allele may lead to the suppression or inhibition of cyclin-dependent kinases $P57^{KIP2}$ through the hyperactivation of *LIT1 (KCNQ1OT1)*. It was shown that all eight BWS patients had normal *PLAGL1* methylation, whereas in two patients with TNDM hypomethylation at *LIT1* was detected. The first patient had UPD(6)pat. The second one had a normal karyotype, but, typically for TNDM, a loss of methylation at *PLAGL1* on maternal chromosome 6. It is interesting that in the latter patient's situation, clinical signs of TNDM were supplemented with umbilical hernia and macroglassia that are typical for BWS.

In 2006, Mackay and colleagues reported on a study of two patients with TNDM and IUGR who had hypomethylation of *ZAC* and *KCNQ1OT1* on maternal chromosomes 6 and 11 respectively [55]. Significantly, both patients did not have the overgrowth which is typical for BWS in the case of *KCNQ1OT1* hypomethylation. However, they had moderate macroglassia, which is not typical for TNDM, and abdomen wall defects and exomphalos that are frequent in both syndromes.

Later in 2006, Mackay and colleagues described another 12 patients with TNDM and hypomethylation of *ZAC* on maternal chromosome 6q24 [56]. In six patients (50%) additional methylation defects were found in different imprinted genes. It was hypomethylation of *GRB10, PEG1/MEST, KCNQ1OT1,* and *PEG3* on maternal chromosomes in different combinations. All patients with multiple methylation defects had higher birth weight and were more phenotypically diverse than other TNDM patients with different genetic aetiologies, except for *ZAC* hypomethylation, presumably reflecting the influence of dysregulation of multiple imprinted genes. It was proposed the existence of a "maternal hypomethylation syndrome" (MHS), when a patient with the loss of methylation at one maternally-methylated locus may also manifest DNA methylation loss at other loci, potentially complicated or even confounded the clinical presentation [56].

Another specific finding of this study which is also very intriguing, is that the level of mosaicism for the DNA methylation index varied in different investigated tissues (blood, mouthbrushes, fibroblasts) and affected different genes. The presence of mosaicism indicates postzygotyic errors of imprinting maintenance in somatic cells. However, it is more significant that such errors affected only maternally, but not paternally inherited alleles. This type of epigenetic mosaicism may explain another interesting fact that all patients with MHS had a major clinical manifestation of TNDM but no other genomic imprinting disorders that can be expected from the involvement of different imprinted genes. It is reasonable to assume that germinal epimutations at *ZAC*, which in theory must be presented (or at least maintained) in all somatic cells, should lead to TNDM symptoms, whereas somatic epimutations at other different genes can partially modify only a clinical manifestation of the "main" hereditary imprinting syndrome.

Later, MLMD were also reported in other imprinting disorders. However, both maternal and paternal alleles were affected in comparison with MHS. Hypermethylation and hypomethylation of imprinted genes were reported also. For example, in some cases of BWS, loss of methylation at *KCNQ1OT1* was accompanied by hypomethylation at *PLAGL1, PEG/MEST* and

SNURF-SNRPN [57]. In some patients with SRS, hypomethylation at *GRB10* and *IGF2*/*H19* was observed [58, 59]. Multiple epimutations were observed at *H19*, *PEG3*, *NESPAS* and *GNAS* genes in one patient with overlapping clinical symptoms of PWS and BWS [60].

It was mentioned earlier that microarray technologies allow us to obtain comprehensive data sets about the methylation status of imprinted genes over the whole genome. In a recent study, 65 patients with different genomic imprinting disorders (BWS, SRS, PWS, AS, TNDM and pseudohypoparathyroidism (PHP-1B)) were investigated by using "GoldenGate Cancer Panel I" (Illumina) DNA methylation microarray [61]. MLMD were detected in all the diseases except PWS and AS, which demonstrated methylation defects at *SNRPN* only. Multiple epimutations were observed in 33% of BWS patients with *KCNQ1OT1* hypomethylation, 75% of TNDM patients with *PLAGL1* hypomethylation, 50% of PHP-1B patients with *GNASXL*/*EX1A* hypomethylation, and in 17% of SRS patients with *H19* hypomethylation. MLMD involved an additional one to 16 imprinted genes in each patient.

Thus, examinations of patients with imprinting disorders indicate that the epigenetic basis of these diseases may, in some cases, be supplemented by multiple methylation defects in several other imprinting genes in addition to epimutation at the disease-specific gene, which is responsible for the pathogenesis of major clinical features of a given syndrome. The incidence of MLMD in different imprinting disorders varies from 8.9% (SRS) to 56.3% (TNDM) (Table 5). The incidence of methylation defects at different genes varies also. The *KCNQ1OT1*, *H19*, and *GNAS*/*NESPAS* genes are the most frequently involved in MLMD. On the other hand, *SNURF-SNRPN* imprinting centre is a very rare subject in the study of methylation defects in BWS and SRS patients, and scarcely noted in the list of MLMD affected genes in patients with TNDM and MHS.

Is the combination of imprinted genes affected by MLMD in different syndromes and pregnancy losses non-random? Are there any specific features of nucleotide sequences and mechanisms of expression regulation of imprinted genes with different incidence of methylation defects? Does an interaction between imprinted genes that may lead to formation of specific epigenotype and phenotype exist? Further studies are necessary to obtain answers to these questions. However, there is some data which indicates the existence of coordination mechanisms in the regulation of epigenetic status of human imprintome. For example, it was shown that epigenetic changes in the cases of BiCHM and TNDM affected imprinted genes on maternal chromosomes only, whereas other non-imprinted genes were not a subject for epimutations [68, 69]. On the other hand, multiple methylation defects were observed by DNA methylation array analysis both in imprinted and non-imprinted genes in spontaneous abortions [50]. It is also possible that MLMD may have a different molecular nature in comparison with a methylation defect at a single locus. For example, multiple methylation defects in patients with BWS were observed only in the cases of *KCNQ1OT1* hypomethylation, but not in the ones with *H19*/*IGF2* hypermethylation (Table 5). The possible regulation genetic mechanisms for epigenetic status of imprinted genes are discussed in the last part of the chapter.

Syndrome	Sample size	Disease-specific epimutation	MLMD frequency	*PLAGL1* (6q24)	*MEST/PEG1* (7q32)	*LIT1* (11p15)	*DLK1/GTL2* (14q32)	*SNRPN* (15q11)	*IGF2R* (6q25)	*GRB10/MEG1* (7p13)	*H19* (11p15)	*GNAS/NESPAS* (20q13)	Ref.
				Genes with paternal expression					Genes with maternal expression				
TNDM	12	*PLAGL1* hypometh	50% (6)	12↓	5↓	3↓	0	0	n.a.	3↓	0	n.a.	[56]
TNDM	4	*PLAGL1* hypometh	75% (3)	4↓	0	0	0	0	1↓,1↑	1↓	0	0	[61]
Total	***16***	***PLAGL1 hypometh***	***56.3% (9)***	***100%***	***31.4% (5/16)***	***18.8% (3/16)***	***0***	***0***	***12.5% (2/16)***	***25.0% (4/16)***	***0***	***0***	
BWS	40	*LIT1* hypometh	25% (10)	n.a.	3↓	40↓	n.a.	1↓	6↓	n.a.	n.a.	n.a.	[62]
BWS	81	*LIT1* hypometh	21% (17)	7↓	6↓	81↓	0	0	6↓	4↓	0	10↓	[63]
BWS	68	*LIT1* hypometh	23.5% (16)	6↓	6↓	68↓	0	1↓	10↓	n.a.	1↓	n.a.	[64]
BWS	11	*LIT1* hypometh	45.5% (5)	0	1↓	11↓	2↓	n.a.	2↓	2↓	2↓	2↓	[65]
BWS	24	*LIT1* hypometh	25% (6)	2↓	4↓	24↓	0	1↓	n.a.	n.a.	n.a.	n.a.	[57]
BWS	43	*LIT1* hypometh	33% (14)	3↓	3↓	43↓	0	0	3↓	3↓	2↓	3↑	[61]
Total	***267***	***LIT1 hypometh***	***25.5% (68)***	7.9% (18/227)	8.6% (23/267)	***100%***	0.9% (2/227)	1.2% (3/256)	***10.9% (27/243)***	6.7% (9/135)	2.5% (5/203)	11.9% (16/135)	
BWS	20	***H19*** hypermeth	0	0	0	0	0	0	0	0	20↑	0	[63]
SRS	23	***H19*** hypometh	8.2% (2)	0	0	1↓	0	n.a.	1↓, 2↑	1↓	23↓	2↓	[66]
SRS	74	***H19*** hypometh	9.5% (7)	2↓	3↓	3↓	5↓	1↓	2↓	n.a.	74↓	n.a.	[64]
SRS	65	***H19*** hypometh	7.7% (5)	n.a.	1↓	3↓	1↓	n.a.	3↓	0	65↓	n.a.	[67]
SRS	6	***H19*** hypometh	16.6%(1)	0	1↓	0	0	0	0	0	6↓	0	[61]
Total	***168***	***H19 hypometh***	***8.9% (15)***	2.1% (2/103)	2.5% (5/168)	4.3% (7/168)	3.7% (6/168)	1.4% (1/80)	4.9% (8/168)	1.1% (1/94)	***100%***	8.7% (2/29)	
PWS/BWS	1	***SNRPN*** hypometh	–	n.a.	n.a.	1↓	n.a.	1↓	n.a.	n.a.	1↓	1↓	[60]
PHP-1B	10	***GNAS*** hypometh	50% (5)	0	2↓	0	0	0	1↓	0	0	10↓	[61]
Total	***476***		***20.4% (97/476)***	***10.3% (38/370)***	***7.4% (35/475)***	***58.4% (278/476)***	***1.8% (8/435)***	***1.1% (4/377)***	***8.7% (38/439)***	***5.2% (14/269)***	***47.1% (194/412)***	***15.0% (29/193)***	
				↓ – 75.6% (360/476); ↑ – 0.6% (3/476)					↓ – 52.9% (252/476); ↑ – 4.8% (23/476)				

Note: MLMD – multilocus methylation defects (number of patients with MLMD is indicated in parentheses); n.a. – not analysed; ↓ – hypomethylation; ↑ – hypermethylation.

Table 5. Incidence of methylation defects in patients with imprinting disorders

6. Genetic control of epigenetic status of imprinted genes

The story begins again with BiCHM. Recurrent cases of this pathology or classical CHM and PHM in anamnesis, the occurrence of several cases within one pedigree, and the appearance in consanguineous couples provide evidence for an autosomal recessive mode of inheritance of BiCHM by maternal lineage. The first candidate's genes were DNA methyltransferases, however, the sequencing of it in women with a history of BiCHM did not reveal the presence of mutations. Subsequent genome-wide association studies and homozigosity mapping indicated the linkage of BiCHM with chromosomal segment 19q13.42, in which the *NLRP7 (NALP7)* gene was mapped [70]. In this first study, five mutations of *NLRP7* in familial and recurrent cases of hydatidiform mole were reported. Two mutations, IVS3+1G-A and IVS7+1G-, were found in introns 3 and 7, respectively. Three single nucleotide changes were detected in another three families: p.R693W, p.R693P, and p.N913S. All these mutations were absent in the 348 individuals from the control group.

Homozygous c.295G>T (p.Glu99X) and heterozygous c.1970A>T (p.Asp657Val) mutations were observed in a woman with four hydatidiform moles [71]. Her sister with two moles and brother were compound heterozygotes for these mutations. Her father had a homozygous p.Asp657Val mutation. Her mother had a homozygous p.Glu99X mutation, three successful pregnancies and a stillbirth in anamnesis. In another studied family, a brother and his three sisters with recurrent hydatidiform moles (two, three, and five cases in anamnesis, respectively) were homozygous for p.Arg693Pro mutations, but the brother did not have any reproductive problems. The authors of this study made a very important conclusion that mutations of *NLRP7* do not affect the foetal development in the case of paternal inheritance, but lead to recurrent hydatidiform moles when transmitted from the mother.

Several studies combined methylation analysis of imprinted genes and the search for *NLRP7* mutations. For example, homozygous c.2248C>G (p.L750V), c.2471+1G>A missense mutations and heterozygous c.2248C>G (p.Leu750Val), c.2810+2T>G *NLRP7* mutations in women with BiCHM and multiple hypomethylation at the *PEG3, SNRPN, KCNQ1OT1, GNAS* imprinted genes were reported [72]. Studying 11 families with BiCHM, Hayward and colleagues performed methylation analysis of the *ZAC*, *GNAS-NESP55*, *LIT1*/*KCNQ1OT1*, and *SGCE*/*PEG10* genes in four families. Hypomethylation of maternal alleles at the *ZAC* and *LIT1* as well as hypermethylation of maternal alleles at the *NESP55*, which is unusual for BiCHM, were observed in all four families. The sequencing of *NLRP7* revealed eight not previously described mutations: p.K116X, p.L398R, p.S673X, p.W778X, c.939_952dup14, c.1456dupG, c.2030delT, and c.277+1G>C [73]. Moreover, p.L398R was associated with multiple epimutations of imprinted genes indicating that some mutations in *NLRP7* may be connected with different types of methylation defects, but not restricted by the hypomethylation of maternal alleles only.

A search for *NLRP7* mutations was performed in 40 Tunisian families with sporadic hydatidiform moles [74, 75]. Two sisters in one family had a homozygous mutation p.E570X. Heterozygous mutations were found in 11 patients. There were several new mutations among them: c.544G>A (p.Val182Met), c.1480G>A (p.Ala494Thr), c.1532A>G (p.Lys511Arg) and c.

2156C>T (p.Ala719Val). The authors concluded that the presence of some heterozygous mutations of *NLRP7* in woman may be a risk factor not only for BiCHM, but also for a sporadic mole.

There are current reports on mutations at almost all exons and introns of *NLRP7* gene, which were associated with BiCHM. The product of the gene belongs to CATERPILLER proteins family. These NLRP proteins are implicated in the activation of proinflammatory caspases through multiprotein complexes called inflammasomes. This gene may act as a feedback regulator of caspase-1-dependent interleukin 1-beta secretion, which is pleiotropic cytokine involved in trophoblast invasion in the uterus during implantation [76].

As mentioned previously, *NLRP7* may be a gene with a maternal effect. The products of such genes are necessary for oocytes to support early embryo development before the activation of embryo genome. These genes do not have an influence on ovulation and fertilization, but the absence of their products leads to the termination of early embryo development. This feature may explain the lack of reproductive problems in *NLRP7* mutation's male carriers. It seems that another gene from the NRLP family in mice – *Nalp5*, is a gene with maternal effect also. *Nalp5-/-* females had normal ovaries. Their oocytes fertilized normally, but embryos arrested their development at the 2-cell stage [77].

NLRP7 protein has no DNA-binding motifs in its sequence, which is why it is unclear how it may be involved in imprinting recording during oogenesis. In this situation, an alternative hypothesis is attractive. According to this hypothesis, the involvement of NLRP7 in BiCHM pathogenesis may be related to its participation in inflammation and autoimmune response. It was found that patients with *NLRP7* mutations cannot provide specific responses to different antigens [78]. As a result, in such women androgenic blastocysts, which are in fact are complete allografts, can implant and develop without rejection from the maternal side. It is also possible that androgenic blastocysts arise spontaneously *de novo* with definite frequency due to errors of fertilization or through epigenetic mechanisms independently from *NRLP7* mutations. In women without *NRLP7* mutations and normal immune systems, such androgenic blastocysts die or stop in development. It is interesting that mutations in other members of the CATERPILLER family – *NLRP1* and *NOD2* were also associated with some clinical forms of vitiligo (MIM #606579) and inflammatory bowel disease (MIM #266600) [78]. There were no reports on the association of molar pregnancies with these autoimmune diseases, except for one study [79]. However, the association of Crohn's disease with recurrent pregnancy loss was noted repeatedly [80, 81]. It is possible that this association is based on common pathogenic mechanisms involved in the disturbance of autoimmune response regulation.

Mutations at the *NLRP7* gene have not been found in every case of BiCHM and only 48-60% of patients with recurrent hydatidiform moles indicate a heterogeneous nature of this reproductive pathology. Indeed, in 2011 a type II of BiCHM was described (MIM #614293), which is clinically indistinguishable from the classical variant of the biparental complete mole. It was related to mutations at the *KHDC3L* (*C6orf221*) gene in 6q13. The search for mutations in this gene was performed in 14 pedigrees with BiCHM without *NLRP7* mutations. As a result, homozygous change c.3G>T, deletion c.322_325delGACT and compound heterozygote c. 322_325delGACT were found in three families [82]. This study revealed that *KHDC3L* is

mutated in 14% of patients with recurrent a hydatidiform mole who are negative for *NLRP7* mutations.

Sequencing of *KHDC3L* in 97 patients with recurrent moles, reproductive loss and absence of *NLRP7* mutations allows to identify three unrelated patients, each homozygous for one of the two protein-truncating mutations, a novel 4-bp deletion resulting in a frame shift c. 299_302delTCAA (p.Ile100Argfs*2), and a previously described 4-bp deletion c.322_325delGACT (p.Asp108Ilefs*30), transmitted on a shared haplotype to three patients from different populations [83]. It was also shown that molar tissues from one of the spontaneous abortions were diploid and biparental. In this study, immunofluorescence analysis revealed colocalization of KHDC3L and NLRP7 proteins in lymphoblastoid cell lines from normal subjects. Using cell lines from patients, it was demonstrated that the *KHDC3L* mutations do not change the subcellular localization of protein in haematopoietic cells. This finding highlights the similarities between the two causative genes for recurrent moles, *KHDC3L* and *NLRP7*, in their subcellular localization, the parental contribution to the mole tissues caused by them, and the presence of several founder mutations and variants in different populations in both of them indicating positive selection and adaptation.

It is probable that some patients with imprinting disorders and MLMD also have mutations in two other genes – *NLRP2* (*NALP2*) (19q13.42) and *ZFP57* (6p22.1), which may be involved in imprinting maintenance. The NALP2 is a cytosolic protein of the CATERPILLER's subfamily. It is suggested that NALP2 is a component of inflammosomes, like NLRP7. Mutation c. 1479delAG (p.Arg493SerfsX32) at the *NLRP2* was found in a family with one healthy child and two children with BWS [84]. *KCNQ1OT1* imprinted centres were hypomethylated in both affected children, whereas *PEG1* hypomethylation was observed in only one of them. Homozygous mutation at the *NLRP2* was detected in the mother and the affected child, whereas the father, the healthy child and the other diseased child were heterozygotes. The mutation was absent in the control group. This data is in agreement with the hypothesis that *NLRP2* is a maternal-effect gene, like *NLRP7*. It is interesting that *NLRP2 per se* is imprinted. Its monoallelic expression from the maternal chromosome was detected in decidual tissue, foetal heart and liver [85]. However, there is a single report, which does not support the maternal effect of *NLRP2*. In 2013, a paternally inherited mutation c.2077C>T (p.R693W) was observed in BWS patient with *KCNQ1OT1* hypomethylation [61]. This mutation was absent in individuals from the control group and may be pathogenic according to the "PolyPhen-2" database. However, it seems that this example does not cover multiple methylation defects.

In 2008, mutations at the *ZNF57* were detected for the first time in seven out of twelve families with TNDM and multiple hypomethylation of imprinted genes *PLAGL1, CRB10, PEG3, KCNQ1OT1, PEG1/MEST*, and *NESPAS* [86]. Homozygous mutation p.C241X was found in a brother with hypomethylation at the *KCNQ1OT1, NASPAS* and *PEG1/MEST* and his sister with *KCNQ1OT1* and *PEG1/MEST* hypomethylation. Proband in another family had deletion c.257_258delAG (p.E86VfsX28). His father was a heterozygote for this deletion. Compound heterozygous mutation c.683G>A (p.R228H) was detected in two families, including one patient with hypomethylation of *PEG1/MEST* and *NESPAS*. None of the mutations were found in 200 individuals from the control group.

Two years later, Mackay and Temple reported about *ZFP57* mutations in 10 out of 16 patients with TNDM and hypomethylation at the *PLAGL1, GRB10* and *PEG3* genes [87]. It is interesting that all patients with maternal hypomethylation syndrome, *ZFP57* mutations and *PLAGL1* hypomethylation also had *PEG3* and *GRB10* hypomethylation. However, in the absence of *ZFP57* mutations, *PEG3* and *GRB10* genes were infrequently affected by methylation defects. This observation indicates that *ZFP57* may be involved in imprinting maintenance in somatic cells in contrast to germinal methylation defects, which are associated with mutations at the *NLRP7*.

ZFP57 mutations were also observed in patients with other imprinting disorders, like SRS and BWS. For example, seven mutations at the *ZFP57* were found in 30 patients with SRS and hypomethylation of imprinting centre *H19/IGF2* on chromosome 11 [88]. Other imprinted genes were not tested in this study. Six nucleotide changes were recognized as known polymorphisms, whereas one patient had not previously detected homozygous mutation p.R125Q in the exon 6. This mutation was also found in heterozygous form in two of the 80 (2.5%) healthy individuals.

Twenty-seven BWS patients with *KCNQ1OT1* hypomethylation were tested for the presence of *ZFP57* mutations [89]. As a result, three new nucleotide changes were found. Two twin girls and their father were heterozygous for the c.503C>T (p.Ser168Phe). Two other changes c. 723C>T and c.1026T>C were detected in a diseased child and his father.

ZFP57 mutations were found in a recent study of patients with TNDM, SRS, BWS and methylation defects [61]. New homozygous deletion c.371delC was identified in a patient with TNDM and maternal hypomethylation of five imprinted genes, including *PLAGL1*. Both parents in this family were heterozygous carriers of deletion, whereas it was absent in the 180 individuals from the control group. Two cases of maternal inheritance of the mutation c. 374G>A (p.R125Q) were observed in a SRS patient with *H19/IGF2* hypomethylation and in a BWS patient with a loss of methylation at the *KCNQ1OT1*. This mutation was not found in the control sample. The mother of the SRS patient was a compound heterozygote for p.R125Q and c.559G>A (p.R187C) mutation.

The protein encoded by *ZFP57* is a zinc finger protein containing the Kruppel-associated box repressor (KRAB) domain, which acts as transcription repressor. It is interesting that this domain may be involved in the *de novo* DNA methylation during mouse embryogenesis [90]. It was also shown that *Zfp57* mutations in mice may induce multiple methylation defects of imprinted genes. Partial hypomethylation of maternal and paternal alleles was noted in progeny with *Zfp57-/-* genotype in zygote [Li et al., 2008]. The authors of this study suggested that *Zfp57* is a maternal-zygotic effect gene and its product is required for imprinting maintenance in different genomic loci.

Taking the discussed results together, it is possible to make an unexpected conclusion that some part of imprinting diseases and reproductive disorders associated with abnormal imprinting are related to defects in gene (or genes) involved in the establishment and maintenance of epigenetic organization of imprinted loci. In other words, imprinting diseases, or at least some part of them, that were usually considered epigenetic in nature, have, in fact, a

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single gene basis sometimes modified by parental-of-origin effects. A similar situation is specific for chromatin diseases (ICF, Rett, Rubinshtein-Taybi, Coffin-Lowry, ATR-X syndromes), which arise due to mutations in genes involved in the control of chromatin organization [8]. From this point of view, the presence of one form of TNDM, a classical imprinting disorder, in the OMIM catalogue (MIM # 601410) as a result of *ZFP57* mutations is not unexpected.

Considering the high incidence of reproductive losses in humans and the elevated level of methylation defects at different imprinted loci in spontaneous abortions, the search for mutations in genes involved in the control of genomic imprinting is a challenge for modern reproductive epigenetics and medicine. In our preliminary study, 11 first trimester missed abortions with MLMD of imprinted genes were tested for the presence of *NLRP7* mutations [92]. Nine genetic variants of *NLRP7* were found. Seven of them were presented in specific databases for women with BiCHM and normal reproductive outcomes. Whereas two new changes, c1405delC and c1444delC, in homozygous form were found in spontaneous abortion with hypomethylation at the *PEG10, KCNQ1, WT1, ZNF215* and hypermethylation at the *INS, PWCR1* and *GABRA5* genes.

7. Conclusion

The reviewed data clearly indicates that epigenetic abnormalities are the leading cause of imprinted gene dysfunction in pregnancy complications and losses. This is not surprising because of the fact that the rate of epimutations is estimated to be one or two orders higher than the incidence of classical gene mutations. It is import to also note that epimutation in one allele is enough to cause the loss of imprinting or the silencing of an imprinted gene due to one of its main inherent features, namely monoallelic expression.

The application of genome wide technologies of DNA methylation analysis revealed the phenomenon of multiple methylation defects at imprinting genes both in spontaneous abortions and in some patients with imprinting disorders. Today we are witnesses of data accumulation about spectrum and the incidence of this type of methylation abnormalities in different diseases. However, cautious estimations should be provided because of a lack of current data about possible benign epipolymorphisms of imprinted genes. However, obtained results change and supplement existent concepts about pathogenesis of imprinting disorders. One of the most intriguing findings is that some part of epigenetic imprinting defects has, in fact, a genetic nature due to mutations in genes, which are responsible for imprinting regulation. This remark may have obvious significance for the likes of molecular genetic diagnosis in the light of the application of high-throughput genomic and post-genomic technologies and for medical genetic counselling. Carriers of mutations in imprinting control genes may have incorrect or instable epigenomes in their gametes or progeny which will be not compatible with fertilization, implantation, normal prenatal development or the delivery of a healthy child. Preimplantation genetic diagnosis for the excluding of embryo transfer with mutations in such genes may be a successful reproductive choice for such couples.

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