

# **Genomic imprinting and assisted reproduction**

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## **Abstract**

Imprinted genes exhibit a parent-of-origin specific pattern of expression. Such genes have been shown to be targets of molecular defects in particular genetic syndromes such as Beckwith-Wiedemann and Angelman syndromes. Recent reports have raised concern about the possibility that assisted reproduction techniques, such as in vitro fertilization or intracytoplasmic sperm injection, might cause genomic imprinting disorders. Although the number of reported cases is still too small to permit any firm conclusion to be drawn, the safety of these widely used techniques must be further evaluated.

## Introduction

The first in vitro fertilization (IVF) baby was born in 1978 and intracytoplasmic sperm injection (ICSI) was introduced in 1992 for the treatment of male infertility. Both these techniques have been continually amended and access to them improved for infertile couples. Indeed, assisted reproduction now accounts for 1% to 3% of births in many developed countries [1]. Until recently, these techniques were considered accurate substitutes for natural oocyte fertilization, and were therefore regarded as safe. However, reports of children conceived using assisted reproduction technologies (ART), and presenting with congenital anomalies have been published during the last 3 years. Beckwith-Wiedemann and Angelman syndromes were among the abnormalities seen, suggesting a possible link between these techniques and an increased incidence of genetic or epigenetic defects. Even though the number of reported patients is still small, and the significance of the relationship between ART and congenital anomalies difficult to determine, the safety of these techniques must be questioned. In particular, the relationship between the techniques and the occurrence of imprinting defects must be clarified.

## Epigenetics and DNA methylation

Heritable information, or instructions that govern gene expression can be passed on from cell to cell or from parent to offspring, mainly in the form of DNA sequence changes. Epigenetic modifications are changes that do not modify the DNA sequence *per se* but that can affect gene expression and function as heritable bits of information. Epigenetic modifications themselves might therefore explain how environmental factors modulate gene expression without altering the DNA sequence. The best, although not the only, characterized epigenetic phenomenon is DNA methylation [2].

DNA methylation is the addition of a methyl groups to cytosine bases located 5' of guanines (within cytosine-phospho-guanine (CpG) dinucleotides sequences). Methylation is catalyzed by the DNA cytosine-5-methyltransferase (DNA-MTase) enzyme family. Methylation induces changes in chromatin structure and is generally associated with silencing of gene expression, thus providing a way to control gene expression [3]. Indeed, methylation patterns are the result of complex interactions between *de novo* methylation, the maintenance of existing methylation, and demethylation [4].

## Imprinting

The majority of mammalian genes are expressed from both maternal and paternal alleles (biallelic expression). Genomic imprinting is a mechanism of gene regulation in which only one of the parental alleles of a gene is expressed, often in a tissue-specific or developmental stage-specific manner [5]. The expressed allele is therefore determined by the sex of the transmitting parent. Imprinting is controlled by DNA methylation. It is estimated that the total number of imprinted genes in the human and mouse genomes ranges between 100 and 200 [6]. Imprinted genes are more often grouped into clusters than scattered throughout the genome and this organization most likely reflects a coordinated way of gene regulation in a chromosomal region [7]. Two features are characteristic, although not specific, to imprinted genes. The first one is the unusual richness in CpG islands onto which imprinted patterns of methylation are placed, and the second one is the presence of clustered direct repeats near or within the CpG islands [8].

## Imprinting in development

During the development of primordial germ cells (PGC), imprinted methylation patterns are removed by a mechanism of erasure [9]. Both passive and active demethylation probably occur, although no enzymes have yet been identified for this second process. This is followed thereafter by the establishment of sex-specific patterns of methylation during gametogenesis. The timing of erasure in PGCs is thought to be crucial. From mouse studies, it seems that erasure occurs at the time that primordial germ cells enter into the gonads [9] [10]. Imprint establishment during gametogenesis occurs with a different timing between the male and female germ lines. In males it is completed by the haploid (meiotic) phase of spermatogenesis and in females imprint acquisition occurs in fully grown oocytes around the time of completion of the first meiotic division [6]. Furthermore, it seems that, at least in oocytes, methylation might be acquired at different times (asynchronously) for different genes [6]. Epigenetic reprogramming of germ cells is important for accurate development, as it controls expression of early embryonic genes, cell cleavage and cell determination [11]. The different stages of imprint establishment/maintenance as well as the possible manipulations which might be disturbed by those are illustrated in Figure 1.

The main consequence of the sex-specific establishment and maintenance of imprinted methylation patterns is the creation of maternal- and paternal-allele methylation differences (differentially methylated domains or DMDs) in or around imprinted genes. A primary DMD is established during gametogenesis and secondary DMDs develop during embryogenesis, most likely due to a direct influence of a nearby primary DMD [12].

Imprinted genes are implicated in the regulation of embryonic and fetal growth, as well as many aspects of placental function, including placental growth and the activity of transplacental transport systems [13]. Indeed, in ruminants, such as sheep and cattle, a particular overgrowth syndrome known as "large offspring syndrome" (LOS) was reported after in vitro culture of embryos, LOS is caused by abnormal methylation of the IGF2R gene [14]. Imprinted genes also are major participants in postnatal development and behaviors. Based on the functions of imprinted genes, disruptions of normal imprinting have predictable consequences such as embryonic death, excessive or defective fetal growth and a spectrum of intrauterine growth retardation.

### **Imprinting defect syndromes in human**

Several human syndromes are known to be associated with defects in gene imprinting, including Prader-Willi, Angelman, Beckwith-Wiedemann, Silver-Russell and Albright hereditary osteodystrophy syndromes [1]. Aberrant imprinting might also play a role in cancers and neurobehavioural disorders such as autism.

The Beckwith-Wiedemann syndrome (BWS), whose frequency in the general population is about 1/14'000, is characterized by somatic overgrowth, congenital malformations and a predisposition to embryonic neoplasia. It is associated with abnormalities in a cluster of imprinted genes within a chromosomal region of approximately 1 Mb in the 11p15 region. The imprinted cluster of genes on 11p15 contains at least 12 imprinted genes and includes the paternally expressed genes IGF2 and KCNQ1OT1, and the maternally expressed genes H19, CDKN1C and KCNQ1 [15]. Approximately 25 to 50% of BWS patients have a biallelic expression of the IGF2 gene, and some of these cases exhibit a loss of imprinting (LOI) of IGF2 which is dependent on hypermethylation changes of H19 [15]. Approximately 50% of the sporadic BWS have a loss of methylation associated to a LOI at KCNQ1OT1, an untranslated RNA within the KCNQ1 gene [16]. Some BWS cases exhibit LOI for KCNQ1OT1 as well as LOI for IGF2 [16]. It has been shown in BWS patients that aberrant methylation of KCNQ1OT1 is specifically associated with overgrowth and birth defects, whereas aberrant methylation of H19 is specifically associated with an increased risk of developing tumors [17].

The Prader-Willi and Angelman syndromes (PWS/AS), whose frequencies in the general population are 1/10'000 and 1/15'000, respectively, are typical examples of imprinting dysregulations leading to severe neurobehavioral disturbances. The domain involved in these 2 pathologies is a 2Mb domain on the 15q11-13 chromosomal region. PWS is due to a defect in the expression or silencing of paternally expressed genes within this region, whereas AS is due to defect in the expression or silencing of maternally expressed genes within this same region. Imprinting within this 2 Mb domain is thought to be under the control of an imprinting center comprising 2 regulatory regions: the PWS-shortest region of overlap (SRO) and the AS-SRO [18]. It is now thought that PWS-SRO and AS-SRO operate in a stepwise way to establish imprinting during the early developmental stages [19]. Indeed, imprinting at the AS-SRO might cause maternal allele-specific repression of the PWS-SRO, preventing activation of the corresponding genes [18].

In addition to the nervous system phenotypes associated with PWS and AS, imprinting may have a more far-reaching impact on neurological development

and behaviour. There is a rapidly accumulating body of evidence suggesting a parent-specific imprinting defect in many common neurobehavioral disorders. Autism, bipolar affective disorder, schizophrenia [20] and other complex neurobehavioral phenotypes such as alcohol abuse and audiogenic seizures [21] may be linked to imprinting disturbances. That is, the transmission of abnormalities has been shown to be dependent upon which parent transmits the disease susceptibility. Such parent-of-origin effects on disease manifestation may be explained by a number of genetic mechanisms, one of them being genomic imprinting [22]. For instance, a lower age of onset of symptoms following paternal inheritance of one subtype of schizophrenia and following maternal inheritance of Tourette's syndrome suggests that imprinted genes are involved in the pathophysiology of these syndromes. As well, there are parent-specific components to acquiring late-onset Alzheimer's disease (paternal-specific component) or familial neural tube defects (maternal-specific component) [21].

### **Cases of defective imprinting in ART conceptions**

Prior to the establishment of sex-specific imprints in male and female germ cell lineages, imprints are erased. After erasure of the pre-existing imprints, the timing of acquisition of imprints is significantly different between the 2 germ lines [7]. In the female germ line, methylation occurs in the postnatal growth phase while oocytes are arrested at the diplotene stage of prophase I [23], whereas during spermatogenesis, methylation takes place before meiosis [24]. Maternal imprints are continually established as oocyte mature in the fertile female, and paternal imprints are established as long as spermatogonia proliferate in the fertile male. Thus, paternal imprints seem to be established earlier than maternal ones. It has been shown that this sex-specific methylation is intrinsic and cell-autonomous, and is not due to any influence of the genital ridge somatic cells, or gonadal environment on the primordial germ cells [25]. Imprinting defects in the course of assisted reproduction could theoretically occur at several steps of the methylation erasure/re-methylation processes, both on male and female germ cell lineages as well as during the early stages of in vitro embryonic development.

The first baby conceived by IVF was born 26 years ago. Intracytoplasmic sperm injection (ICSI) was developed approximately 10 years ago, as the reproductive solution for severe male infertility. Several studies have established the general safety of both IVF and ICSI [26]. Nevertheless, it was recently reported that both IVF and ICSI may be associated with an

increased risk of major birth defects. Hansen et al (2002) in a study on 837 infants conceived by IVF and 301 infants conceived by ICSI reported rates of major birth defects (musculoskeletal, cardiovascular, urogenital, gastrointestinal, central nervous system, metabolic and poorly defined ones), as high as 9.0% of IVF and 8.6% of ICSI conceptions, respectively, compared to the 4.2% rate in physiological conceptions. The possible link with imprinting disturbances was not considered in this study. Schieve et al. [27] studied 42,463 infants conceived with assisted reproductive technology and reported a higher occurrence of low (less-than-or-equal 2500 g) and very low (less-than 1500 g) birth weight in these compared to the control population of children physiologically conceived. These results were in part due to the increase in multiple pregnancies, known to be associated to ART, but also due to a higher rate of low birth weight among singleton pregnancies. In addition to these associated defects, a higher incidence of sex-chromosome aneuploidy has also been reported in ART conceptions [28].

To date, a total of 24 cases of BWS patients conceived by ART have been reported [6]. DeBaun et al. [29] recently reported 7 cases of BWS conceived by ART, 6 of these showing an imprinting defect at KCNQ1OT1 or H19. By comparing this rate of ART-conceived BWS to the rate of ART in the general population at the same period, they determined that sporadic cases of BWS were approximately six times more likely to have been conceived by ART than not. They suggested that causative factors may include the in-vitro culture conditions before and/or after in vitro fertilization, or the exposure of the gametes or embryos to specific media or growth factors.

Maher et al. reviewed a different set of sporadic BWS cases and looked for an association with ART [30]. They found that 6 out of the 149 BWS cases examined were conceived by ART, and many of these had a KCNQ1OT1 loss of imprinting as the causative molecular defect. Indeed, when compared to the incidence in the general population, ART had a four-fold greater likelihood of being associated with BWS. The cases reported both by DeBaun et al [29] and Maher et al. [30] were recruited through registries of BWS patients. However, it can be debated if parents with BWS born after ART are more prone to join BWS registries, therefore introducing a bias in recruitment through registries.

Recently, a case-control study analyzed the frequency of BWS in 1'316'500 live births and 14'894 babies born after an IVF procedure [31]. They reported

the risk of BWS as 9 times higher in the IVF population, than in their general population.

An initial study found no evidence in 92 children conceived by ICSI of abnormal methylation patterns at 15q11-13, the locus linked to the pathogenesis of AS and PWS [32]. However, Cox et al. [33] and Orstavik et al. [34] reported a total of 3 Angelman syndrome children conceived by ICSI, They demonstrated that it was due in all 3 cases to loss of imprinting within the 15q11-13 imprinted gene cluster.

### **Why might ART be harmful for the imprints**

For assisted reproduction by intracytoplasmic sperm injection (ICSI), the injection of a spermatozoon into the ovum by micro-manipulation bypasses several of the steps involved in fertilisation. However, in male germ cell imprinting establishment, it seems that the paternal imprints are well established in the mature, meiotic stages of spermiogenesis. Furthermore, round spermatid microinjections have confirmed that paternal imprints are completely established in primary spermatocytes [35]. This point is relevant to the recent use of ICSI using round spermatids. Manning et al. [36] have analysed the methylation pattern in immature testicular sperm cells at different developmental stages and found that the ejaculated spermatozoa and elongated spermatids had completed the establishment of paternal methylation imprints. However, spermatozoa used for ICSI generally originate from men with perturbed semen parameters that may have adversely affected the establishment of imprints. Moreover, immature spermatozoa for ICSI can also be directly collected from the testes of infertile males. It has been hypothesized that spermatozoa from men with fertility problems, such as those being used for assisted reproduction by ICSI, contain a higher number of gametes with chromosomal abnormalities [37]. Among the possible sperm abnormalities, a defect in gene imprinting can also be considered. Indeed, a recent report has analysed the imprinting of two oppositely imprinted genes (MEST and H19) in spermatozoan DNA from normozoospermic and oligozoospermic patients. Their data suggest an association between abnormal genomic imprinting and hypospermatogenesis [38]. It is also theoretically possible that freezing of mature sperm or the cryoprotectants used might disturb the established male imprints in mature spermatozoa or round spermatids.

It may also be true that women with a variety of fertility problems, such as ovarian failure and/or hormonal disturbances, may be more prone to produce gametes with inherent imprinting defects. This is purely speculative at this point but should be considered because of the establishment of maternal imprints during the final phase of oocyte growth and meiotic maturation.

In addition to the theoretical possibility that there may be innate defects in oocytes used in ART, the in vitro treatment of oocytes and embryos during ART procedures might affect the establishment of imprints in female germ cells. For example, superovulation or in vitro maturation of oocytes might affect the establishment of the complete array of normal maternal imprints. Oocytes used for assisted reproduction usually originate from women who undergo a hormonal hyperstimulation protocol followed by fertilization in vitro. It is not clear from experiments published to date in human if the clinical use of high doses of gonadotrophins might alter imprint acquisition. Gonadotrophins might theoretically cause the premature release of immature oocytes having not completed the establishment of their imprints and establishment may not be completed during in vitro maturation. The possible effect of ovarian endocrine hyperstimulation on methylation of imprinted genes has been analysed in mice by Shi and Haaf [39]. Using an immunostaining method to assess the extent of genomic cytosine methylation, these authors reported abnormal methylation patterns in 2-cell embryos from superovulated females as compared to non-superovulated ones. However, no data has been published in human concerning the possible effects of ovarian hyperstimulation on imprinting. Concerning the in vitro maturation of oocytes, preliminary studies in mouse suggest that an extended culture might lead to loss of methylation at certain imprinted loci, such as *Igf2r* and gain of methylation at other loci, such as *H19* [40].

Potential disruption of the normal imprinting could result from the in vitro manipulation of early stage embryos. Indeed, in vitro culture with the use of slightly different culture media led to decreased fetal viability and imprinting disturbances in mice. Doherty et al. [41] first reported the differential effects of culture media in preimplantation mouse embryos on the *H19* imprinted gene. They demonstrated that a loss of methylation at *H19* gene was associated with culture in Whitten's media, resulting in LOI in the imprinting control domain upstream the start of *H19* transcription. As well, Khosla et al. [42] examined mouse preimplantation embryos cultured in different culture media and transferred into recipient mothers. They were able to show that fetal development as well as the expression pattern of

imprinted genes, including the *Igf2* and *H19* genes, was influenced by the addition of fetal calf serum (FCS) in the culture media. The mechanism by which culture media and other gamete or embryo handling might induce defects and lack of maintenance of methylation at imprinted loci is not clear. They might facilitate the removal of methyl groups on cytosine bases or perhaps disturb the program of gametic development in such a way that the completeness of imprint erasure and/or establishment is compromised [11]. Furthermore, cryopreservation of embryos could potentially affect the cytoskeleton, chromatin structure and the availability of methylation and/or demethylation enzymes during preimplantation development. Indeed, the processes of imprint establishment and of imprint maintenance might be disturbed during the *in vitro* stages of embryonic development. However, it is presently not known if culture of human preimplantation embryos in different media or for longer periods, in order to perform late-stage embryo transfer might lead to such disturbances in genomic imprinting.

It also might be imagined that the disturbances in imprinting affect the germline cells of the embryo conceived by assisted reproduction and that the problems of imprinting might occur in the children of the subsequent generation [11]. Therefore, follow-up of these individuals is necessary to further define the possible risks associated with ART.

### **Imprinting and placenta**

A critical way of regulating intrauterine development is through placental function and growth. Interestingly, most imprinted genes are expressed both in fetal and placental tissues, and involved in fetal growth [13]. In general, paternally expressed imprinted genes enhance fetal growth whereas maternally expressed imprinted ones suppress it (Reik and Walter, 2001). Among the genes expressed in placenta, the *Mash2* gene was shown to regulate the development of spongiotrophoblast [43]. *Igf2* transcripts are found specifically in the labyrinthine trophoblast [44], and *Ascl2* is a transcription factor expressed in the spongiotrophoblast and labyrinthine layers [6]. Indeed, mice with deletions of *Igf2* and *Ascl2* genes exhibited fetal growth retardation and death during embryonic development, respectively [44] [43].

In humans, several imprinting disorders are associated with intrauterine growth retardation (IUGR) [45]. Studies on human placental imprinted genes and on the different roles of the maternally and paternally expressed ones

are certainly needed for the understanding the placenta's role in normal embryonic and fetal development. Furthermore, analyses of placental samples obtained after ART conceptions might provide us answers to some important questions about the possible links between ART and genomic imprinting.

## **Conclusion**

Concern has been recently raised about the possible increased incidence of genetic syndromes due to imprinting defects in children conceived by assisted reproduction. In particular, experimental reports in mice have raised the question that some of the steps involved in these techniques, such as ovarian hyperstimulation or the culture media for in vitro culture of embryos might be detrimental to the formation of genomic imprints. In order to be able to give adequate genetic counseling to infertile couples reproducing through ART, there must be large case-control studies as well as cautious long-term evaluation of the safety of these techniques. Although the unraveling of the mechanisms underlying genomic imprinting is now only beginning, there is a clear need to investigate and better understand the regulation of this process during fecundation and embryogenesis.

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## **Competing interests**

None declared

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## Figure 1

Possible interactions between different steps of assisted reproduction procedures and imprint establishment or maintenance through different stages of development. PGC: primordial germ cell. PGD: preimplantation genetic diagnosis.

Figure 1

