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Imprinting and assisted reproductive technology

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In the past 25 years, the frequency of assisted reproductive technology (ART) births has increased rapidly to account for 1–2% of all births in many developed countries. ART procedures such as *in vitro* fertilization and intracytoplasmic sperm injection are generally considered to be safe, but recent studies suggest a small excess of birth defects and low-birth weight in ART children. In addition, several clinical studies have reported an increased frequency of ART conceptions among children with Beckwith–Wiedemann syndrome or Angelman syndrome caused by an imprinting defect. Although these studies require further confirmation, they are consistent with animal studies reporting disordered expression and epigenetic changes in imprinted genes following *in vitro* embryo culture. The absolute risk of an imprinting disorder after ART appears to be very small, but further data are required to determine whether the association between ART and human imprinting disorders reflects the effect of embryo culture (or some other aspect of ART) and/or a common mechanism for infertility and imprinting disorders. Retinoblastoma and neurodevelopmental defects have been only tentatively linked to ART, but in view of the role of epigenetic processes in the regulation of gene expression in development and cancer, further research is required into long-term health outcomes for ART children and the epigenetic consequences of ART protocols.

INTRODUCTION

The first *in vitro* fertilization (IVF) baby was born in 1978, and intracytoplasmic sperm injection (ICSI) was introduced as a treatment for male infertility in the early 1990s. Assisted reproductive technology (ART) births now account for 1–3% of all births in developed countries, and recent trends in ART include prolongation of *in vitro* embryo culture times and increasing use of ICSI such that in some centres ICSI accounts for up to 80% of ART procedures. Initially, there were concerns that ICSI would increase the risk of birth defects and genetic disorders as (a) it bypasses almost all the natural selection mechanisms that operate in natural conception and (b) aspects of the ICSI procedure (e.g. possible mechanical damage to the sperm, introduction of acrosome and media components into the egg, etc.) could have a deleterious effect. Although there is evidence for an increase in chromosome abnormalities in ICSI conceived pregnancies, until recently there was little concern otherwise that ART conceived children might be less healthy than their naturally conceived counterparts (1). Within the past few years, however, several reports have suggested that there may be links between ART and an increased risk of low-birth weight and birth defects, specific imprinting disorders and, possibly, childhood cancer (2–9). Although the precise significance and origin of these associations require confirmation and clarification, available evidence supports the case for systematic studies to establish the long-term safety of ART procedures.

ART AND IMPRINTING DISORDERS

A link between ICSI and Angelman syndrome was suggested by Cox *et al.* (4) who reported two children conceived by ICSI, who developed Angelman syndrome (10). Most children with Angelman syndrome have a germline deletion or a uniparental disomy of chromosome 15 (Fig. 1). However, molecular analysis of both cases associated with ICSI revealed an infrequent sporadic imprinting defect [loss of normal maternal allele methylation at the *SNRPN* differentially methylated region (DMR) without an imprinting centre

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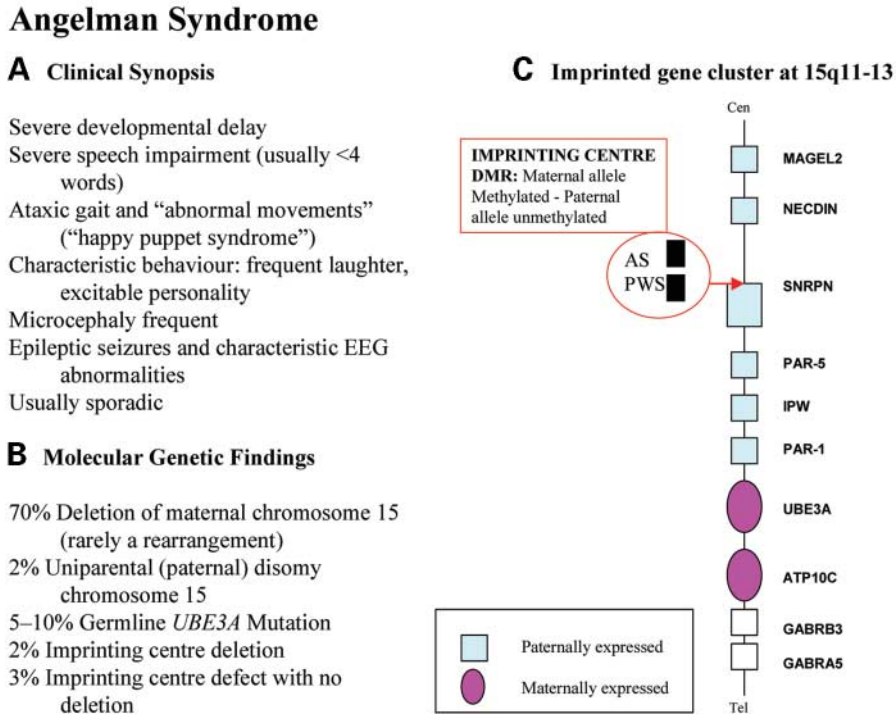

Figure 1. Clinical and molecular background information on Angelman syndrome. (**A**) Summary of clinical phenotype. (**B**) Frequency of molecular genetic abnormalities in Angelman syndrome. Mutation, deletion or loss of expression of *UBE3A* results in Angelman syndrome. Loss of expression of the maternally expressed *UBE3A* may result from gene deletion, paternal uniparental disomy or an imprinting centre defect. An imprinting centre defect may be caused by a deletion or an epimutation, which is characterized by loss of maternal allele methylation at the DMR within the imprinting centre. (**C**) Schematic map (not to scale) of the Angelman syndrome domain showing location of the imprinting control centre/*SNRPN* DMR. The imprinting centre has a bipartite structure and regulates expression of several paternally expressed genes (e.g. *SNRPN, MAGEL2*) and two maternally expressed genes (*UBE3A* and *ATP10C*). The bipartite structure of the imprinting control centre was revealed by the smallest region of overlap in patients with Angelman syndrome and Prader–Willi syndrome and an imprinting centre deletion.

deletion] (Fig. 1). Such imprinting defects are usually found in <5% of all Angelman syndrome cases and have an expected incidence of 1 in 300 000 (11). The suggestion that ICSI might be an aetiological factor in these cases is consistent with the observation that the maternal allele *SNRPN* methylation imprint is established at fertilization or later (12). Further evidence implicating ICSI in the pathogenesis of Angelman syndrome patients with rare sporadic imprinting defects was provided in a follow-up report by Orstavik *et al.* (5) who described an additional case associated with ICSI. These reports provoked considerable interest as three children with Angelman syndrome caused by epimutations would be predicted to occur in ~1 in 900 000 births, but the worldwide estimated total of ART births was ~1 000 000 (13). Thus, unless these three cases represented complete ascertainment of all sporadic Angelman syndrome cases following ART, there appeared to be an increased frequency of a specific subgroup of Angelman syndrome. Subsequently, reports of an association between ART and a second classical imprinting disorder, Beckwith–Wiedemann syndrome (BWS), reinforced concerns about ART, epigenetic abnormalities and imprinting disorders (6–8). Thus, in retrospective studies in the UK and France, an increased frequency of ART children was observed in cohorts of children with BWS [relative risk ~4 ($P = 0.009$) and 3.2 ($P = 0.01$), respectively] (6,8). These studies may have underestimated the risks as a detailed reproductive history was not available for all patients. However, in a study in the USA in which a detailed reproductive history was obtained, the prevalence of ART was six times higher in BWS children (Table 1) (7). In addition, a retrospective case–control study of BWS and IVF undertaken in Australia reported a 10.8% frequency of IVF in BWS children (4/37) compared with 0.7% (1/148) in matched controls ($P = 0.006$, odds ratio = 17.8 with 95% CI 1.8–432.9) (9). It was also estimated that the risk of BWS after IVF is ~1/4000, which was 9-fold higher than the population risk (9). Despite the occasional reports of other imprinting disorders (e.g. Prader–Willi or Silver–Russell syndrome) in ART children, to date an increased frequency of ART has not been reported in other cohorts with imprinting disorders.

EPIGENETIC ALTERATIONS AND ART

A common feature of ART-associated BWS and Angelman syndrome cases is a strong association with epimutations involving loss of maternal allele methylation at critical imprinting control region/differentially methylated region (SNRPN DMR and KvDMR1) (Figs 1 and 2). Thus, 23 of 24 ART-associated BWS cases for whom molecular genetic data is available have demonstrated loss of methylation (LOM) at the 11p15.5 DMR within the *KCNQ1* gene

Table 1. Details of studies reporting an increased frequency of ART births in BWS

Location	Study design	ART in BWS cohort	Number of BWS ART cases treated with ICSI	Number of BWS ART cases with KvDMR1 (LOM/number tested)	Reference
UK	Retrospective cohort	6/149, expected 1.5 ($P = 0.009$)	3/6	2/2	Maher *et al.* (6)
USA	Retrospective and prospective cohorts	7	5/7	5/6	DeBaun *et al.* (7)
	Prospective cohort	3/65, expected 0.49			
France	Retrospective cohort	6/149, expected 1.94 ($P = 0.01$)	2/6	6/6	Gicquel *et al.* (8)
Australia	Retrospective case–control	4/37 versus 1/148 controls ($P = 0.006$)	1/4	3/3	Halliday *et al.* (9)

(KvDMR) (IC2 see Fig. 2) (unpublished data; 6–9). LOM at the maternally methylated/paternally unmethylated KvDMR is detected in 40–50% of all sporadic BWS cases; therefore, KvDMR1 LOM is over-represented in ART-associated BWS ($P < 0.001$) (14–17). Loss of KvDMR1 methylation is associated with downregulation of the maternally expressed growth suppressor *CDKN1C* and, in some cases, loss of imprinting (biallelic expression) of *IGF2* (a paternally expressed growth promoter) (15,16,18). Although KvDMR1 LOM may result from a germline deletion (19), most sporadic cases result from an epimutation suggesting that the association of BWS with ART appears to result predominantly from an increased susceptibility to KvDMR1 demethylation following ART. This interpretation would be consistent with the results of molecular analysis of post-ART Angelman syndrome cases (discussed earlier) and animal studies (discussed subsequently).

EPIGENETIC ALTERATIONS AND ART: ANIMAL STUDIES

Animal data have demonstrated that *in vitro* embryo culture, and related procedures, may be associated with epigenetic changes, disordered genomic imprinting and alterations in intrauterine growth. Thus, in sheep and cattle the large offspring syndrome (LOS) is characterized by increased birth weight and perinatal morbidity after embryo culture and LOM at an imprinting control element in the maternally expressed *IGF2* receptor (*IGF2*R) is found in some cases (20). Despite the phenotypic similarities between BWS and LOS, epigenetic alterations at *IGF2R* do not appear to be directly relevant to growth abnormalities following ART as (a) *IGF2R* is frequently not imprinted in humans, (b) epigenetic alterations at *IGF2R* are rare in human growth disorders and (c) there is an increased frequency of intrauterine growth retardation, rather than overgrowth, in children conceived by ART (2,21,22). Nevertheless, studies of preimplantation mouse embryos have demonstrated that embryo culture conditions (e.g. presence of fetal calf serum) can influence imprinted gene (*IGF2* and *H19*) expression and methylation status (23).

ORIGIN OF IMPRINTING DISORDERS AFTER ART

The initial reports linking ART with Angelman syndrome appeared to suggest a specific association with ICSI (4,5). However, of 23 ART-related BWS cases reported in the four recent studies (6–9), only 10 have involved ICSI. Thus, it appears that ICSI *per se* is not the major determinant of the observed association between ART and imprinting disorders. In view of the association between embryo culture, epigenetic alterations and disordered imprinting in animal studies (20,23), a plausible hypothesis is that *in vitro* embryo culture might predispose to LOM at the KvDMR1 or *SNRPN* DMRs causing ART-associated *in vitro* embryo culture imprinting disorders. If this hypothesis is correct then changes in human ART embryo culture protocols might reduce (or increase) the risk of an imprinting disorder. Thus, in studies of cultured mouse embryos, Mann *et al.* (24) found that loss of imprinting of *H19* (and loss of DMR methylation) was enhanced by culture in Whitten's medium. Loss of *H19* imprinting occurred between the two-cell and blastocyst stages suggesting that the precise conditions of *in vitro* embryo culture might influence the risk of epigenetic alterations following human ART.

An alternative hypothesis is that the apparently increased risk of an imprinting disorder following ART might be because of an association with infertility rather than with *in vitro* embryo culture. Thus, treatment for infertility (e.g. medically induced ovarian hyperstimulation leading to harvesting of immature oocytes) might be implicated and/or susceptibility to epigenetic defects might be responsible for both infertility and an increased risk of imprinting defects. Recently, Ludwig *et al.* (25) identified 16 Angelman syndrome patients born to subfertile couples and found an increased frequency of imprinting defects (25 versus expected 4%). One of four children with an imprinting defect was conceived by ICSI, but the highest risk of a child with an imprinting defect (RR 12.5) was in couples with prolonged infertility (time to pregnancy >2 years) and a history of infertility treatment. They hypothesized that imprinting defects and subfertility might have a common cause, and superovulation rather than ICSI may further increase the risk of conceiving a child with an imprinting defect (although the absolute risk is very small).

IMPLICATIONS OF EPIGENETIC ALTERATIONS AFTER ART

Follow-up studies of ART children have concentrated on neonatal and early childhood outcomes. There is relatively little longer term follow-up information and, of course, no data are available for adult-onset disorders. The best-documented complication of ART is multiple births. Although most cases

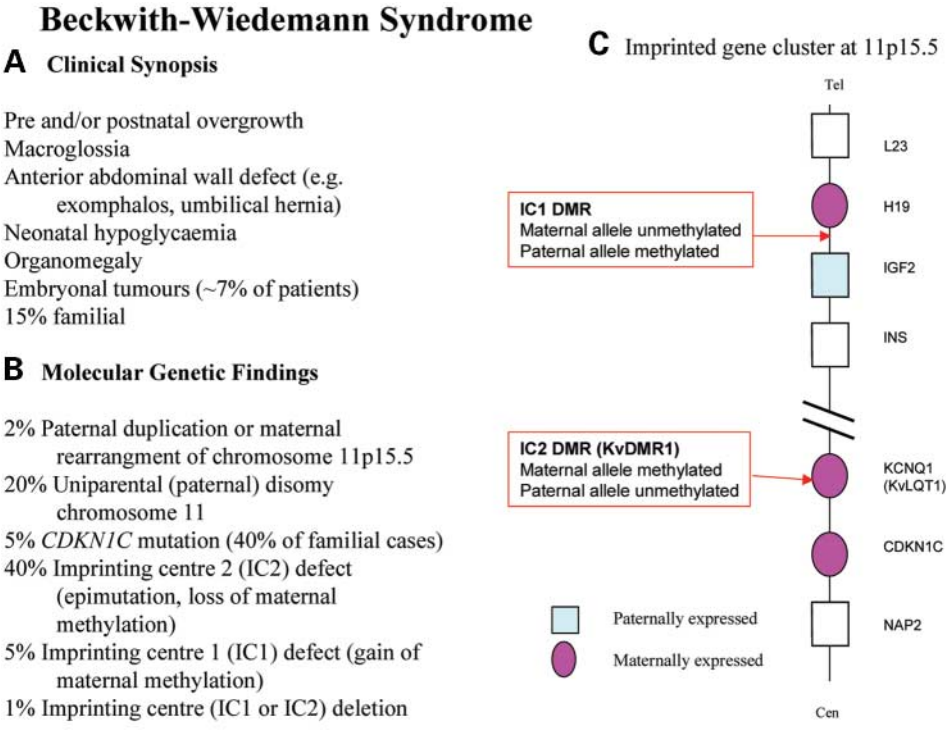

Figure 2. Background information on BWS. (**A**) Summary of clinical phenotype. (**B**) Frequency of molecular genetic abnormalities in BWS. The key events in the pathogenesis of BWS appear to be increased expression of the paternally expressed growth promoter *IGF2* and/or reduced expression of the maternally expressed growth suppressor *CDKN1C*. Germline mutations in CDKN1C may cause familial BWS, but most cases are sporadic and result from uniparental disomy (paternal isodisomy) of 11p15.5 or imprinting error involving the two imprinting centres (IC1 and IC2). (**C**) Schematic map (partial and not to scale) of the BWS imprinted gene cluster region showing the location of two imprinting control regions (IC1 and IC2). Both are associated with a DMR. IC1 is upstream of H19 and the associated DMR shows paternal allele methylation and regulates imprinting of IGF2 and H19. In the subgroup of children with BWS and an IC1 defect, both alleles are methylated and there is silencing of H19 expression and biallelic (loss of imprinting) IGF2 expression. IC2 is contained within an intron of KCNQ1 and regulates imprinting of CDKN1C but not H19. The associated DMR (known as KvDMR1) shows maternal allele methylation and in the subgroup of children with BWS and an IC2 defect, both alleles are unmethylated and there is loss of maternal allele expression of CDKN1C (and in some cases loss of imprinting of IGF2). Rarely, IC1 and IC2 defects may be caused by a germline deletion but most cases appear to have an epimutation.

result from multiple embryo transfer, an increased risk of monozygotic twinning has also been reported (26,27). ART is associated with an increased frequency of low-birth weight in babies (2,28,29). Thus, Schieve *et al.* (2) reported a 2-fold increase in low- and very low-birth weight after ART. However, whether these risks relate to ART *per se* or are associated with infertility has not been defined clearly. In view of the epidemiological studies that suggest links between low-birth weight and adult insulin insensitivity and cardiovascular disease, factors which predispose to reduced intrauterine growth may have lifelong implications for health (30,31). In a population-based study, Hansen *et al.* (3) reported a 2-fold excess of major birth defects in IVF and ICSI children. In another study, an increased risk of birth defects was also detected, but it was suggested that infertility rather than ART might be the major risk factor (32).

The reported associations between ART and imprinting disorders, such as BWS and Angelman syndrome, require further confirmation. However, as imprinting disorders are rare, an increased relative risk associated with ART (e.g. a 9-fold risk of BWS) (9) translates into a low absolute risk and is unlikely to be a major concern for prospective parents. Furthermore, the risks of BWS or Angelman syndrome are too low to justify routine screening following ART conceptions. Of potentially greater significance is the possibility that ART-associated susceptibility to epigenetic alterations might cause or predispose to disorders that are not currently recognized as 'epigenetic or imprinting disorders'. Approximately 75 imprinted genes identified to date appear to be preferentially involved in prenatal growth and neurodevelopment and epigenetic alterations have a major role in the pathogenesis of many human cancers (33,34). However, there is no direct evidence to implicate disordered imprinting in the pathogenesis of the increased risk of low-birth weight after ART. Similarly, most studies do not suggest that ART children have an increased frequency of neurodevelopmental abnormalities (35). However, recent studies of preimplantation mouse embryos have suggested that *in vitro* culture conditions can produce long-term neurodevelopmental and behavioural effects (36,37). These findings and those of a population-based report suggesting an increased risk of cerebral palsy and developmental delay (possibly independent of low-birth weight) in ART children (38), support the case for further neurodevelopmental and behavioural studies in ART children. Likewise, although initial reports demonstrated no increased risk of cancer in ART children up to 6

years of age (39,40), a recent, as yet unconfirmed, study reported an increased frequency of ART in children with retinoblastoma (41). Somatic epigenetic changes have a major role in the pathogenesis of many adult and paediatric cancers (see Laird, this issue), and it is conceivable that epigenetic events occurring in early life might influence susceptibility to cancer and other common diseases. In particular, loss of imprinting of IGF2 in normal colonic mucosa has been linked to an increased risk of colorectal cancer (42,43). The possibility that disordered imprinting in a subset of ART children (whether related to ART or associated with infertility) might predispose to late-onset disease must be considered speculative at present. However, human and animal studies indicate a need for both (a) large-scale detailed studies of cohorts of ART children to define precise risks (and causes) of birth defects, neurodevelopmental abnormalities and cancer and (b) investigations to establish whether subclinical imprinting and epigenetic abnormalities are more common in ART children. Such findings might provide insights into whether a subset of ART children are likely to be at increased risk for late-onset disease (and so may benefit from targeted screening) and biological markers for monitoring the effects of changes in ART protocols.

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