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...
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). 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...
(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 IGFB2 receptor (IGFB2R) is found in some cases (20). Despite the phenotypic similarities between BWS and LOS, epigenetic alterations at IGFB2R do not appear to be directly relevant to growth abnormalities following ART as (a) IGFB2R is frequently not imprinted in humans, (b) epigenetic alterations at IGFB2R 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 imprintened gene (IGFB2 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 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

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

<table>
<thead>
<tr>
<th>Location</th>
<th>Study design</th>
<th>ART in BWS cohort</th>
<th>Number of BWS ART cases treated with ICSI</th>
<th>Number of BWS ART cases with KvDMR1 (LOM/number tested)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK</td>
<td>Retrospective cohort</td>
<td>6/149, expected 1.5 (P = 0.009)</td>
<td>3/6</td>
<td>2/2</td>
<td>Maher et al. (6)</td>
</tr>
<tr>
<td>USA</td>
<td>Retrospective cohort</td>
<td>7</td>
<td>5/7</td>
<td>5/6</td>
<td>DeBaum et al. (7)</td>
</tr>
<tr>
<td>France</td>
<td>Retrospective cohort</td>
<td>3/65, expected 0.49</td>
<td>2/6</td>
<td>6/6</td>
<td>Gicquel et al. (8)</td>
</tr>
<tr>
<td>Australia</td>
<td>Retrospective case–control</td>
<td>4/37 versus 1/148 controls (P = 0.006)</td>
<td>1/4</td>
<td>3/3</td>
<td>Halliday et al. (9)</td>
</tr>
</tbody>
</table>
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 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.

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 IGFI2 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.

REFERENCES


