Human Reproduction Vol.18, No.12 pp. 2508–2511, 2003 DOI: 10.1093/humrep/deg486

DEBATE—continued

Epigenetic risks related to assisted reproductive technologies: Epigenetics, imprinting, ART and icebergs?

Eamonn R.Maher[1,3], Masoud Afnan[2] and Christopher L.Barratt[2]

[1]Section of Medical and Molecular Genetics, Division of Reproductive and Child Health, University of Birmingham Medical School, Birmingham and West Midlands Regional Genetics Service, Birmingham Women's Hospital, Edgbaston, Birmingham and [2]Assisted Conception Unit, Birmingham Women's Hospital, Edgbaston, Birmingham and Division of Reproductive and Child Health, University of Birmingham Medical School, Birmingham, UK

[3]To whom correspondence should be addressed at: Section of Medical and Molecular Genetics, Department of Paediatrics and Child Health, University of Birmingham, The Medical School, Edgbaston, Birmingham B15 2TT, UK. E-mail: E.R.Maher@bham.ac.uk

Recently, a series of case reports and small studies has suggested that births involving assisted reproductive technology (ART) may have an increased risk of imprinting disorders such as Beckwith–Wiedemann syndrome and Angelman syndrome. Herein, the significance and implications of these findings are discussed. It is speculated that, although such imprinting disorders may be shown to be only rare complications of ART, epigenetic errors might account for a much wider spectrum of ART-related complications than is recognized currently. Addressing these questions should be a priority for research on cohorts of ART children.

Key words: ART/epigenetics/imprinting/safety

Introduction

Births associated with assisted reproductive technologies (ART) now account for ~1% of all UK births, and the popularity of some technologies such as ICSI is increasing. Whilst some follow-up studies of ART children have suggested that ART poses little risk to normal development and child health, other investigators have reported a two-fold excess of major birth defects in ART children (Hansen *et al*., 2002). In addition, compared with babies conceived naturally, there is an increased frequency of intrauterine growth retardation (IUGR) in babies after ART conceptions (Doyle *et al*., 1992; Buitendijk, 1999; Schieve *et al*., 2002). Thus, in a recent study (Schieve *et al*., 2002) it was reported that ART accounts for a disproportionate number of low-birth-weight and very-low-birth-weight infants in the United States. Whilst determining the significance of potential contributing factors such as multiple births, maternal age and the nature of the ART procedure to the excess of IUGR in ART children may be difficult, it was concluded (Schieve *et al*., 2002) that ART *per se* is a significant factor in the excess of low-birth-weight babies born after ART. As epidemiological studies have suggested there to be links between birth weight and adult insulin insensitivity and cardiovascular disease (Barker *et al*., 1993; Forsen *et al*., 2000), reduced intrauterine growth may have important lifelong implications for health.

Genomic imprinting disorders and ART

Animal data have demonstrated that in-vitro embryo culture and related procedures may be associated with disordered genomic imprinting and produce alterations in intrauterine growth (Khosla *et al*., 2001a; b). Imprinted genes show parent-of-origin specific differences in allelic expression (e.g. *IGF2* is expressed solely from the paternally inherited allele and *H19* from the maternally inherited allele), and although only a minority of human genes (~50) are known to be imprinted, most of those identified to date have a role in regulating pre- and/or post-natal growth (Reik and Walter, 2001). Genomic imprinting is an epigenetic process, and allele-specific gene silencing is maintained by a variety of imprinting control mechanisms including differential regulation of methylation and/or chromatin structure according to parental origin (Reik and Dean, 2001).

A direct link between ART, disordered imprinting and fetal growth in sheep was provided by the demonstration that the 'large offspring syndrome' (LOS) is associated with loss of methylation at an imprinting control element in *IGF2R* (Young *et al*., 2001). However, whether IGF2R is imprinted in humans is controversial, and so the relevance of these findings for clinical medicine was unclear. Furthermore, analysis of the methylation status in the Prader–Willi/Angelman syndrome-imprinted gene cluster at 15q11-q13 provided no evidence for

epigenetic changes in children born after ICSI (Manning *et al*., 2000).

A central tenet of medical genetics practice is that the study of rare genetic disorders may provide insights into the pathogenesis of more common, but often less clearly defined, conditions. Hence, recent reports of an excess of ART children among patients with Beckwith–Wiedemann (BWS) and Angelman syndromes have been the subject of intense interest as they potentially provide 'proof of principle' of a link between ART and disordered genomic imprinting in humans. Conversely, some sceptics have doubted the significance of reports of small numbers of children with rare disorders and suggested that, if real, an increased risk of such rare disorders will not have a major impact on ART children.

A link between ICSI and Angelman syndrome has been suggested in a report of two children who were conceived in such a manner and subsequently developed the syndrome (Cox *et al*., 2002). Molecular analysis revealed an abnormal methylation pattern at the *SNRPN* differentially methylated region in both cases, with one child showing complete loss of normal maternal allele *SNRPN* methylation, and the other partial loss. Neither patient had evidence of an imprinting centre deletion, so both had a sporadic imprinting defect (epimutation) that accounts for <5% of all Angelman syndrome cases and is estimated to have an incidence of ~1 in 300 000 newborns. 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 (El-Maarri *et al*., 2001). Further evidence implicating ICSI in the pathogenesis of rare sporadic imprinting defect patients was provided in a follow-up report in which an additional case of Angelman syndrome with an imprinting defect conceived by ICSI was described (Orstavik *et al*., 2003). Since: (i) three children with Angelman syndrome caused by epimutations would be predicted to occur in 900 000 births; (ii) the worldwide total of ART births since 1978 is ~1 000 000 (Schultz and Williams, 2002); and (iii) complete ascertainment of all sporadic Angelman syndrome cases following ART would seem unlikely, these two reports (Cox *et al*., 2002; Orstavik *et al*., 2003) raised the possibility of an association between ART and a specific imprinting disorder.

Reports of significant associations between ART and a second classical imprinting disorder, BWS, have substantiated concerns about ART and imprinting disorders and shown that such concerns are not restricted to ICSI procedures (DeBaun *et al*., 2003; Gicquel *et al*., 2003; Maher *et al*., 2003). Thus, initially, two independent studies from the UK and USA reported an increased frequency of children conceived by ART among children diagnosed with BWS. In a retrospective UK study, it was observed that six of 149 children with BWS had been born after ART procedures compared with an expected 1.5 children ($P = 0.009$) (Maher *et al*., 2003). In the USA, others (DeBaun *et al*., 2003) identified seven BWS children born after ART and, in a prospective study, the prevalence of ART was 4.6% in BWS children compared with a control rate of 0.8%. Thus, two independent studies undertaken in two continents, both identified an association between ART and BWS. Recently, a third report from France (Gicquel *et al*., 2003) has confirmed these findings whereby, in a retrospective survey of 149 BWS patients, six children were identified as being born after ART (expected 1.94, $P = 0.01$). The relative risks in the retrospective UK and French studies were ~4 and 3.2 respectively, while the prospective component of the US study estimated a ~6-fold increase. It has been suggested by these two groups (Gicquel *et al*., 2003; Maher *et al*., 2003) that the frequency of ART cases may have been underestimated, as a detailed reproductive history was not available for all BWS patients in these retrospective studies.

Further evidence that the observed findings represent a real association between ART and disordered imprinting causing BWS is that 13 of 14 ART-associated BWS cases analysed to date have demonstrated loss of methylation at a differentially methylated region (KvDMR) within the *KCNQ1* gene (DeBaun *et al*., 2003; Gicquel *et al*., 2003; Maher *et al*., 2003). Normally, the paternally inherited KvDMR is unmethylated and the maternally inherited allele is methylated. In 40–50% of sporadic (non-ART-associated BWS cases), there is loss of KvDMR1 maternal allelic methylation (Lee *et al*., 1999; Smilinich *et al*., 1999; Engel *et al*., 2000). Loss of KvDMR1 methylation may be associated with alterations in expression of two candidate BWS genes, *CDKN1C* and *IGF2*, such that *CDKN1C* expression is down-regulated (Diaz-Meyer *et al*., 2003) and there may be loss of imprinting *IGF2* (i.e. biallelic expression) (Smilinich *et al*., 1999). Most BWS cases with KvDMR1 loss of methylation are sporadic and likely result from an epimutation (imprinting error). The frequency of KvDMR1 loss of methylation among ART-associated BWS cases is significantly greater than that in sporadic non-ART BWS children (Engel *et al*., 2000), so that the association of BWS with ART appears to result predominantly from an increased frequency of epimutations at KvDMR1.

Risks of specific ART procedures

The initial reports linking ART with Angelman syndrome seemed to suggest that ICSI might be the predominant risk factor (Cox *et al*., 2002; Orstavik *et al*., 2003). However, of 19 ART- related BWS cases reported in the three recent studies (DeBaun *et al*., 2003; Gicquel *et al*., 2003; Maher *et al*., 2003), only nine have involved ICSI. In some centres, ICSI now accounts for up to 80% of ART procedures, and concern has been expressed that: (i) ICSI bypasses almost all of the natural selection mechanisms that operate in natural conception; and (ii) aspects of the ICSI procedure (e.g. possible mechanical damage to the sperm, introduction of acrosome and media components into the egg, etc.) would have a deleterious effect. However, it is clear that ICSI *per se* is not the major determinant of the observed association between ART and imprinting disorders.

BWS is characterized by a triad of pre- and/or post-natal overgrowth, macroglossia and anterior abdominal wall defects. In addition, ~7% of BWS children develop a tumour, most commonly Wilms' tumour (Elliott and Maher, 1994; Maher and Reik, 2000). Pre-natal overgrowth is a feature of BWS in humans and of LOS in ruminant mammals. LOS is associated with IVF techniques and with loss of maternal allele

methylation at a DMR within the imprinted IGF2 receptor gene (*IGF2R*) locus (Young *et al*., 2001). Intriguingly, the frequency of epigenetic changes associated with in-vitro embryonal culture can be influenced by the constituents of the culture media used (Reik *et al*., 1993; Dean *et al*., 1998; Khosla *et al*., 2001a). However, the imprinting status of *IGF2R* in humans is controversial and so it was unclear if these findings are germane to clinical medicine, particularly as IUGR rather than overgrowth is the major ART-associated growth disorder in humans. However, epigenetic alterations at *IGF2R* are not thought to account for all aspects or cases of the LOS phenotype. Thus, epigenetic changes at additional imprinted genes are also likely to be implicated in the pathogenesis of LOS (Young, 2003). Nevertheless, studies into the aetiology of LOS do suggest that in-vitro culture conditions might be implicated in the pathogenesis of the epimutations in ART-associated BWS and Angelman syndrome children. It is striking that ART-associated epimutations in (most) BWS and Angelman syndrome cases and in LOS involve loss of maternal allele methylation. As ICSI is often performed for poor sperm function, it might have been expected, a priori, that ART-related imprinting disorders would have been related to paternal genome mutations or epimutations if the ICSI procedure *per se* was the major determinant of human cases.

Implications for clinical practice

The biggest risk to children born following IVF or ICSI remains multiple pregnancy—particularly higher-order multiple pregnancy. Angelman syndrome is a serious neurodevelopmental disorder however (Clayton-Smith and Laan, 2003), and although there are no estimates of its absolute risk after ART—even if the relative risk of epimutations causing the syndrome are increased 100-fold—the absolute risk would be small (~1 in 3000). It seems unlikely therefore that this would cause many couples requesting ART to decline treatment. Epimutations causing BWS are more frequent than those causing Angelman syndrome but, notwithstanding the risk of serious complications such as exomphalos and embryonal tumours, BWS is usually compatible with a normal life. In order to provide prospective parents with accurate risk information, there is a pressing need to define the absolute risk of imprinting disorders after ART by prospectively following a cohort of ART children. It is acknowledged that many couples will still choose to try for a pregnancy despite known and unknown risks to the child (e.g. Siegel and Scrimshaw, 2001). Perhaps the most significant implication of the recent reports linking ART with imprinting disorder is the possibility that these reports might indicate a 'tip of the iceberg' situation. Two possible 'iceberg scenarios' might be proposed. First, if in-vitro culture is a major factor in the predisposing to imprinting errors, the recent trend to prolong in-vitro blastocyst culture times might be followed by an increased frequency of imprinting disorders. The converse of this is that pinpointing the precise risk factors for epimutation after ART may allow protocols to be altered so that the risks are minimized.

The second 'iceberg scenario' is that while Angelman syndrome and BWS may be rare complications of ART, epigenetic changes that are not yet recognized to be associated with specific phenotypes might also be more frequent and have a significant influence on the long-term health of ART children. Thus, according to this hypothesis, loss of methylation at KvDMR1 causing BWS is not a rare event that occurs because the KvDMR1 locus is particularly sensitive to epimutations, but rather that epimutations are equally likely to occur at other loci and result in phenotypes such as IUGR (or fetal loss). The major difference between BWS and IUGR after ART is that the epigenetic changes that lead to BWS are well defined and can be tested for, but the epigenetic alterations that might lead to IUGR or fetal loss in humans have not been defined. Nevertheless, this hypothesis does provide a testable theory, such that the epigenotype of candidate genes and imprinting control regions in ART children with and without IUGR and normal controls can be defined. The recent report of an association between ART and retinoblastoma in a Dutch cohort (Moll *et al*., 2003) has provided further reasons to carefully document the possible implication of ART procedures for long-term health. At present, the Dutch retinoblastoma study is an isolated (and somewhat unexpected) finding that requires confirmation. However, somatic epigenetic changes have a major role in the pathogenesis of many adult and paediatric cancers, and it is conceivable that epigenetic events occurring in early life might influence susceptibility to cancer and other common diseases. Ultimately, time will tell whether the recent reports of associations between ART and rare imprinting disorders are viewed as rare events without wider implications, or as seminal findings that indicated a significant role for epigenetics in human disease. Nevertheless, it is suggested that the possibility of epigenetic 'icebergs' after ART now requires urgent and careful investigation.

Acknowledgements

The authors thank SPARKS and the Birmingham Children Hospital's Endowment Fund for financial support.

References

Barker, D.J., Gluckman, P.D., Godfrey, K.M., Harding, J.E., Owens, J.A. and Robinson, J.S. (1993) Fetal nutrition and cardiovascular disease in adult life. *Lancet*, **341**, 938–941.

Buitendijk, S.E. (1999) Children after *in vitro* fertilization. An overview of the literature. *Int. J. Technol. Assess. Health Care*, **15**, 52–65.

Clayton-Smith, J. and Laan, L.A.E.M. (2003) Angelman syndrome: a review of the clinical and genetic aspects. *J. Med. Genet.*, **40**, 87–95.

Cox, G.F., Burger, J., Lip, V., Mau, U.A., Sperling, K., Wu, B.L. and Horsthemke, B. (2002) Intracytoplasmic sperm injection may increase the risk of imprinting defects *Am. J. Hum. Genet.*, **71**, 162–164.

Dean, W., Bowden, L., Aitchison, A., Klose, J., Moore, T., Meneses, J.J., Reik, W. and Feil, R. (1998) Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. *Development*, **125**, 2273–2282.

DeBaun, M.R., Niemitz, E.L. and Feinberg, A.P. (2003) Association of *in vitro* fertilization with Beckwith-Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *Am. J. Hum. Genet.*, **72**, 156–160.

Diaz-Meyer, N., Day, C., Khatod, K., Maher, E.R., Cooper, W., Reik, W., Junien, W., Graham, G., Algar, E., Der Kaloustian, V.M. and Higgins, M.J. (2003) Silencing of CDKN1C (p57KIP2) is associated with

hypomethylation at KVDMR1 in Beckwith-Wiedemann syndrome. *J. Med. Genet.* (in press)

Doyle, P., Beral, V. and Maconochie, N. (1992) Preterm delivery, low birthweight and small-for-gestational-age in liveborn singleton babies resulting from in-vitro fertilization. *Hum. Reprod.*, **7**, 425–428.

Elliott, M. and Maher, E.R. (1994) Beckwith-Wiedemann syndrome. *J. Med. Genet.*, **31**, 560–564.

El-Maarri, O., Buiting, K., Peery, E.G., Kroisel, P.M., Balaban, B., Wagner, K., Urman, B., Heyd, J., Lich, C., Brannan, C.I., Walter, J. and Horsthemke, B. (2001) Maternal methylation imprints on human chromosome 15 are established during or after fertilization. *Nature Genet.*, **27**, 341–344.

Engel, J.R., Smallwood, A., Harper, A., Higgins, M.J., Oshimura, M., Reik, W., Schofield, P.N. and Maher, E.R. (2000) Epigenotype-phenotype correlations in Beckwith-Wiedemann syndrome. *J. Med. Genet.*, **37**, 921–926.

Forsen, T., Eriksson, J., Tuomilehto, J., Reunanen, A., Osmond, C. and Barker, D. (2000) The fetal and childhood growth of persons who develop type 2 diabetes. *Ann. Intern. Med.*, **133**, 176–182.

Gicquel, C., Gaston, V., Mandelbaum, J., Siffro, J.-P., Flahault, A. and Le Bouc, Y. (2003) *In vitro* fertilization may increase the risk of Beckwith-Wiedemann syndrome related to abnormal imprinting of the KCNQ1OT gene. *Am. J. Hum. Genet.*, **72**, 1338–1341.

Hansen, M., Kurinczuk, J.J., Bower, C. and Webb, S. (2002) The risk of major birth defects after intracytoplasmic sperm injection and *in vitro* fertilization. *N. Engl. J. Med.*, **346**, 725–730.

Khosla, S., Dean, W., Brown, D., Reik, W. and Feil, R. (2001a) Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. *Biol. Reprod.*, **64**, 918–926.

Khosla, S., Dean, W., Reik, W. *et al*. (2001b) Epigenetic and experimental modifications in early mammalian development: Part II. *Hum. Reprod. Update*, **7**, 419–427.

Lee, M.P., DeBaun, M.R., Mitsuya, K., Galonek, H.L., Brandenburg, S., Oshimura, M. and Feinberg, A.P. (1999) Loss of imprinting of a paternally expressed transcript, with antisense orientation to KCNQ1, occurs frequently in Beckwith-Wiedemann syndrome and is independent of insulin-like growth factor II imprinting. *Proc. Natl Acad. Sci. USA*, **96**, 5203–5208.

Maher, E.R. and Reik, W. (2000) Beckwith-Wiedemann syndrome imprinting in clusters revisited. *J. Clin. Invest.*, **105**, 247–252.

Maher, E.R., Brueton, L.A., Bowdin, S.C., Luharia, A., Cooper, W., Cole, T.R., Macdonald, F., Sampson, J.R., Barratt, C.L., Reik, W. and Hawkins, M.M. (2003) Beckwith-Wiedemann syndrome and assisted reproduction technology (ART). *J. Med. Genet.*, **40**, 62–64.

Manning, M., Lissens, W., Bonduelle, M., Camus, M., De Rijcke, M., Liebaers, I. and Van Steirteghem, A. (2000) Study of DNA-methylation patterns at chromosome 15q11-q13 in children born after ICSI reveals no imprinting defects. *Mol. Hum. Reprod.*, **6**, 1049–1053.

Moll, A.C., Imhof, S.M., Cruysberg, J.R., Schouten-van Meeteren, A.Y., Boers, M. and van Leeuwen, F.E. (2003) Incidence of retinoblastoma in children born after in-vitro fertilisation. *Lancet*, **361**, 309–310.

Orstavik, K.H., Eiklid, K., van der Hagen, C.B., Spetalen, S., Kierulf, K., Skjeldal, O. and Buiting, K. (2003) Another case of imprinting defect in a girl with Angelman syndrome who was conceived by intracytoplasmic semen injection. *Am. J. Hum. Genet.*, **72**, 218–219.

Reik, W. and Dean, W. (2001) DNA methylation and mammalian epigenetics. *Electrophoresis*, **22**, 2838–2843.

Reik, W. and Walter, J. (2001) Genomic imprinting: parental influence on the genome. *Nature Rev. Genet.*, **2**, 21–32.

Reik, W., Romer, I., Barton, S.C., Surani, M.A., Howlett, S.K. and Klose, J. (1993) Adult phenotype in the mouse can be affected by epigenetic events in the early embryo. *Development*, **119**, 933–942.

Schieve, L.A., Meikle, S.F., Ferre, C., Peterson, H.B., Jeng, G. and Wilcox, L.S. (2002) Low and very low birth weight in infants conceived with use of assisted reproductive technology. *N. Engl. J. Med.*, **346**, 731–737.

Schultz, R.M. and Williams, C.J. (2002) The science of ART. *Science*, **296**, 2188–2190.

Siegel, K. and Scrimshaw, E. (2001) Reasons and justifications for considering pregnancy among women living with HIV/AIDS. *Psychol. Women Q.*, **25**, 112–123.

Smilinich, N.J., Day, C.D., Fitzpatrick, G.V., Caldwell, G.M., Lossie, A.C., Cooper, P.R., Smallwood, A.C., Joyce, J.A., Schofield, P.N., Reik, W. *et al*. (1999) A maternally methylated CpG island in KCNQ1 is associated with an antisense paternal transcript and loss of imprinting in Beckwith-Wiedemann syndrome. *Proc. Natl Acad. Sci. USA*, **96**, 8064–8069.

Young, L.E. (2003) Scientific hazards of human reproductive ''cloning''. *Hum. Fertil. (Camb.)*, **6**, 59–63.

Young, L.E., Fernandes, K., McEvoy, T.G., Butterwith, S.C., Gutierrez, C.G., Carolan, C., Broadbent, P.J., Robinson, J.J., Wilmut, I. and Sinclair, K.D. (2001) Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nature Genet.*, **27**, 153–154.

Submitted on June 17, 2003; accepted on August 27, 2003