Human Reproduction Vol.17, No.10 pp. 2487–2494, 2002

DEBATE

Epigenetic risks related to assisted reproductive technologies

Risk analysis and epigenetic inheritance

M.De Rycke[1,3], I.Liebaers[1] and A.Van Steirteghem[2]

[1]Centre for Medical Genetics, and [2]Centre for Reproductive Medicine, University Hospital of the Dutch-speaking Brussels Free University, Laarbeeklaan 101, 1090 Brussels, Belgium

[3]To whom correspondence should be addressed. E-mail: lgendrem@az.vub.ac.be

A broad spectrum of assisted reproductive technologies has become available for couples with fertility problems. Follow-up studies of children born as a result of assisted reproduction have shown that neonatal outcome and malformation rates are not different from those of the general population, except for a low birthweight and a slight increase in chromosomal abnormalities. The safety aspect of assisted reproduction at the epigenetic level has not been well studied. Epigenetics refers to phenomena where modifications of DNA methylation and/or chromatin structure underlie changes in gene expression and phenotype characteristics. This article intends to analyse epigenetic risks related to assisted reproduction on the basis of an overview of epigenetic reprogramming events in the gamete and early embryo. Two epigenetic modifications, methylation and imprinting, are considered in more detail. The interference of in-vitro embryo culture, immature sperm cells and nuclear transfer with epigenetic reprogramming is discussed, as well as the possibility of epigenetic inheritance.

Key words: assisted reproductive techniques/epigenetic risks/imprinting/in-vitro culture/methylation

Introduction

Since the introduction of IVF more than 20 years ago, assisted reproductive technologies have evolved constantly. Various techniques have been developed, some of which are quite invasive: ICSI with mature and immature sperm cells, embryo biopsy for preimplantation genetic diagnosis (PGD) and transfer of ooplasm to a recipient oocyte. Although follow-up studies of children born as a result of assisted reproduction indicate normal development, the safety aspect of assisted reproduction at the epigenetic level has not been well studied yet. In order to be able to estimate the potential long-term risks related to the possible disturbance of epigenetic phenomena after assisted reproduction, we first describe the spectrum of assisted reproductive technologies, then outline the different epigenetic reprogramming events in the gametes and early embryos. The interference of in-vitro culture, immature sperm cells and nuclear transfer with epigenetic reprogramming is discussed, as well as the possibility of epigenetic inheritance.

Spectrum of assisted reproductive technologies

After the birth of the first IVF baby in 1978, assisted reproductive technologies increased in number and their spectrum has widened. ICSI was introduced about a decade ago to treat male-factor infertility (Palermo *et al*., 1992; Van Steirteghem *et al*., 1993). Initially the technique involved the injection of a single sperm, derived from an ejaculate, into an oocyte. Later, surgically-obtained immature sperm and elongated spermatids from the epididymis or testis were also used (Devroey *et al*., 1996). A few case series of ICSI with round spermatids were reported but the practice of round spermatid injection has not been introduced in clinical practice because of ill-defined indications and poor results (Tesarik *et al*., 1995; Verheyen *et al*., 1998; Sousa *et al*., 1999; Silber *et al*., 2000).

Assisted reproduction together with embryo biopsy procedures and molecular genetic techniques at the single-cell level have led to the development of PGD for couples at risk of transmitting a genetic disease (Handyside *et al*., 1990). PGD is an early form of prenatal diagnosis, aimed at selecting embryos not affected by the genetic disease under investigation, before their transfer to the uterus. There are two major techniques used in PGD: (i) the presence of chromosomal aberrations is analysed using fluorescence in-situ hybridization (FISH); (ii) single-gene defects are detected using polymerase chain reaction (PCR). Both techniques are becoming increasingly established as reported by the International Working Group on PGD. Up to 2001, ~500 healthy babies have been born (International Working Group on Preimplantation Genetics, 2001).

The procedure of ooplasmic transfer was developed for patients with advanced maternal age and/or with poor embryo

development and recurrent implantation failure. About 30 babies have been born using a modified ICSI protocol for the injection of a sperm together with 5–15% good-quality donor ooplasm (Barritt *et al*., 2001). Although the presence of donor mitochondrial (mt) DNA was observed in some of the babies, it is not clear whether this had a therapeutic effect (Brenner *et al*., 2000). Ooplasmic transfer remains an experimental technique since the underlying factors and mechanisms leading to the observed increased embryo implantation rate are not yet known.

Other techniques, such as metaphase II (MII) spindle transfer, are currently under study in animal models (Wang *et al*., 2001). Abnormal spindle formation as seen in oocytes from women of advanced age might be due to aberrant control mechanisms in the ooplasm (Battaglia *et al*., 1996). Transferring the spindle and bringing it under control of a recipient MII ooplasm might be beneficial in patients with poor oocyte and embryo quality.

Finally, reproductive cloning by nuclear transfer has been achieved in different animal species, but the success rate is low: only one embryo out of 100 will develop into an adult cloned animal (Solter, 2000). The possibility of reproductive human cloning raises major legal, ethical and social issues, and so far it has been banned in many countries.

The widespread, almost immediate clinical application of IVF and ICSI in assisted reproduction, without any adequate experimental phase, as well as the invasive nature of some of the techniques, have led to a debate concerning the safety of these techniques. Retrospective and prospective follow-up studies have shown that the major and minor malformation rates for IVF and ICSI babies were similar to those in the general population. Some studies showed an increase in de-novo chromosomal aberrations in ICSI babies (Tarlatzis and Bili, 1998; Bonduelle *et al*., 1999). Neonatal data further indicate that the rates of low and very low birthweight observed in the IVF and ICSI singletons are higher than in the general population (Doyle *et al*., 1992; Buitendijk, 1999; Sutcliffe *et al*., 2001; Bonduelle *et al*., 2002).

With time, IVF and ICSI technology were complemented with cryopreservation and blastocyst culture/transfer/freezing techniques as a means to increase pregnancy rates and to avoid multiple pregnancies. The cryopreservation process appeared to have no negative influence on neonatal outcome and child development as compared with fresh embryo transfer (Wennerholm, 2000). Blastocyst culture is used in some centres for selected patients as well as in PGD cycles. No differences have been observed in birthweight when comparing blastocyst transfers with early cleavage-stage embryo transfers, but birthweights were lower than in control populations (Ménézo *et al*., 1999; Kausche *et al*., 2001).

Epigenetic reprogramming

The intention of this article is not to overview all the effects of assisted reproduction on children so conceived, but to focus on potential consequences at the epigenetic level.

The genetic information of a DNA sequence is complemented by epigenetic modifications. Epigenetics covers a broad range of effects: DNA methylation, imprinting, RNA silencing, co-valent modifications of histones and remodelling by other chromatin-associated complexes. All these effects involve mechanisms in the regulation of gene expression. Epigenetic patterns are imposed on the genome during differentiation through predetermined programmes (genetic factors). Additional epigenetic changes enable cells to respond to environmental factors by altering the expression level of the gene without having to change the DNA code itself. There are at least two critical periods in which epigenetic reprogramming occurs, one during gametogenesis and another during the preimplantation embryonic stage (Reik *et al*., 2001). Reprogramming during gametogenesis is essential for the imprinting mechanism. Imprinting regulates the differential expression of paternally and maternally derived genes. Imprints are established differentially in sperm and oocyte and are maintained in the zygote and further through all somatic cell divisions. They are reset in the germline in a sex-specific way. Epigenetic germline reprogramming starts when primordial germ cells (PGC) enter the gonads. Demethylation processes occur for non-imprinted genes as well as for imprinted genes, after which cell division is arrested at mitosis for male gametes and at meiosis for female gametes. Erasure of epigenetic modifications in the PGC ensures genetic totipotency which is necessary for the development of a new organism. At cell cycle resumption, methylation and chromatin remodelling take place, providing the genome in the gametes with molecular programmes for oocyte activation and embryonic development. Mature oocytes and sperm have a quite different epigenetic organization. The sperm genome is more methylated than the oocyte genome, chromatin has been compacted with protamines in sperm cells and with histones in oocytes, the oocyte chromatin structure is more repressive than that of sperm cells and, finally, differentially methylated regions have been established at imprinted loci. Both genomes are in a silent state when they come together at fertilization. After fertilization, paternal chromosomes decondense and are remodelled, protamines are exchanged for maternal histones and active, rapid demethylation takes place (Mayer *et al*., 2000). The maternal genome is demethylated more gradually by a passive mechanism. At the time of implantation, a genome-wide methylation takes place. Epigenetic reprogramming during the preimplantation period is important for accurate development, as it controls expression of early embryonic genes, cell cleavage and cell determination. In adult tissues, the early embryonic genes are repressed and tissue-specific genes are activated. Imprinted genes maintain their methylation marks from the gamete stage and they escape these general (de)methylation processes of the preimplantation stage.

Disturbance of epigenetic reprogramming may influence gene expression and phenotype characteristics. Moreover, epigenetic changes that occur shortly after fertilization, before specification of the germ line, will involve both somatic cells and germ-line cells and may lead to the inheritance of an epigenetic trait resulting in transgenerational phenotypes.

Possible epigenetic risks linked to assisted reproduction may result from the use of immature sperm cells with incomplete reprogramming or from the use of in-vitro embryo procedures during a time window when epigenetic reprogramming occurs.

Methylation

Very few of the factors involved in epigenetic reprogramming have been identified. The best studied epigenetic modification until now has been DNA methylation of CpG motifs (Jones and Takai, 2001). The establishment and maintenance of methylation patterns depend on at least three different DNA methyltransferases, one 'maintenance' methyltransferase, DNMT1, which prefers hemi-methylated DNA as a substrate, and two 'de-novo' methyltransferases, DNMT3A and DNMT3B, which act preferentially on non-methylated DNA. Loss of *Dnmt3b* and *Dnmt1* genes causes embryonic lethality in mice at mid-gestation. *Dnmt3a* knockout mice are live-born but they fail to thrive and die shortly after birth (Okano *et al*., 1999).

DNA (hypo)methylation is linked to chromatin structure and transcriptional regulation. Transcriptional repression is established through the interaction of methyl-CpG binding proteins with chromatin remodelling complexes. Methyl-CpG binding proteins, such as MECP2, bind selectively to methylated regions, recruit histone deacetylase and direct transcriptional repression by deacetylating histones and assisting the formation of a stable, repressive chromatin structure in promoter regions. Transcriptional activation of a gene has been linked with unmethylated CpG motifs in the promoter region. A transcriptional activator that binds specifically to unmethylated CpG dinucleotides has been identified: CGBP (Voo *et al*., 2000). Unsurprisingly, mouse embryos lacking the *Cgbp* gene are viable only up to the blastocyst stage, showing that loss of methylation control in the early embryo will lead to aberrant development and even cell death (Carlone and Skalnik, 2001). In humans, several genetic diseases have been associated with methylation defects (Robertson and Wolffe, 2000). *ICF*, for *Immunodeficiency, Centromeric instability and Facial anomalies*, involves pericentromeric hypomethylation due to mutations in the methyltransferase *DNMT3B* gene. Rett syndrome (RTT) is characterized by initial normal development, followed by progressive degeneration of acquired motor skills, loss of speech and mental retardation (Amir and Zoghbi, 2000). RTT is an X-linked dominant disorder limited to girls, as the few males born alive die before the age of 2 years. Mutations in the methylCpG binding gene *MECP2* underlie the disease. Mutations in the same gene, but distinct from those involved in RTT, have been identified as the cause of non-specific X-linked mental retardation (Couvert *et al*., 2001). Fragile X mental retardation is associated with aberrant de-novo methylation and histone acetylation of an expanded polymorphic CGG repeat (>200 repeats) in the 5′ untranslated region of the *FMRI* gene (Oberlé *et al*., 1991; Verkerk *et al*., 1991; Yu *et al*., 1991). This methylation spreads to the CpG island in the promoter region and silences the gene. *FMR1* plays a role in protein synthesis in neurons. ATR-X (X-linked α-thalassaemia/mental retardation) is characterized by mental retardation, facial dysmorphism, α-thalassaemia and urogenital abnormalities. The ATRX protein is a transcriptional regulator acting through chromatin remodelling. Mutations in *ATRX* induce changes in the methylation patterns of highly repeated sequences (Gibbons and Higgs, 2000; Gibbons *et al*., 2000). A common aspect to the above diseases is mental retardation. It seems that DNA promoter methylation is an important mechanism of transcriptional regulation in neural cells. Identification of the target genes will help to understand how mutations in *MECP2* and *DNMT3B* relate to defective brain development.

Methylation defects may not only arise by genetic mutations in the methylation machinery, but also by epigenetic alterations at their target genes. Moreover the methyltransferases themselves may be deregulated epigenetically by time variations. DNMT1, 3A and B and chromatin remodelling complexes are cell-cycle regulated. Changes in embryo developmental timing, such as can be caused by embryo culture, may interfere with their activity and finally with methylation patterns and expression levels. Few studies have been performed on the possible effects of assisted reproduction on the expression of non-imprinted genes. Using RT–PCR for mRNA phenotyping in bovine preimplantation embryos, it has been shown that the expression patterns of several genes important for development differ between embryos cultured in-vitro and embryos obtained in-vivo. Furthermore, the mRNA levels of in-vitro-produced embryos are altered by the presence or absence of serum in the medium (Niemann and Wrenzycki, 2000). In the follow-up studies of IVF and ICSI children, little attention has been given to methylation defective disorders in the form of, for example, neurodevelopmental problems. Often, the study groups are relatively small and most surveys stop when the children reach the age of 2 years, which means that problems which become manifest only later in development will not be detected. An Australian study showed that there was an increased proportion of 1 year old ICSI children with a mildly delayed mental development, compared with IVF or naturally conceived children (Bowen *et al*., 1998). Re-examination of the group of ICSI children at the age of 5 years did not show a difference in full-scale IQ (Leslie *et al*., 2001). The overall results of a similar study looking at ICSI and IVF children of 2 years old showed no mental developmental difference between these children and the general population (Bonduelle *et al*., 1998). A more recent UK case–control study again found no difference in mean neurodevelopmental score between ICSI children and a control group (Sutcliffe *et al*., 2001).

Imprinting

Another critical step, apart from the initiation of general (de)methylation patterns at the preimplantation stage, is the resetting and the maintenance of differential methylation marks at imprinted loci in the germline and zygote, followed by their differential expression (Reik and Walter, 2001). Imprinted genes play key roles in embryonic growth and behavioural development and they are also involved in carcinogenesis. Several human syndromes are known to be associated with imprinted genes including Silver–Russell, Albright, Prader–Willi, Angelman, Beckwith–Wiedemann and Wilms' tumour

(Falls *et al*., 1999). Aberrant imprinting is also suspected to play a role in other neurobehavioural disorders such as autism, bipolar affective disorder and schizophrenia (Isles and Wilkinson 2000; Skuse, 2000). Imprinted genes occasionally function as oncogenes or tumour suppressor genes (*IGF2*, *WT1*, *M6P/IGF2R*, *p73*). The functional monoallelic loci are particularly vulnerable to proto-oncogene activation and tumour suppressor inactivation (Jirtle *et al*., 1999).

The effect of aberrant imprinting during the preimplantation developmental window may have several consequences. There may be consequences in the long term such as cancer susceptibility. Otherwise, early deregulation of imprinted genes may lead to biallelic expression or absence of expression. Taking into account the essential function of imprinted genes in embryonic growth, this may lead to embryo developmental arrest and death, fetal growth retardation or overgrowth, or it may cause one of the clinical syndromes already mentioned. Again, as with the other epigenetic phenomena, the relative timing of imprint processes with respect to the developmental stage is critical. Small differences between the embryonic clock and the timing of the imprinting mechanism may cause major disturbances. This is illustrated by the explicit activity of the oocyte-specific isoform of *Dnmt1* during the 8-cell stage of the mouse embryo. This maintenance methyltransferase protein is localized in the cytoplasm of oocytes and blastomeres until postimplantation stage, but at the 8-cell stage it enters the nucleus to ensure maintenance of imprinted methylation marks. Mice deficient in the *Dnmt1* isoform die at different times pre- and postnatally (Dean and Ferguson-Smith, 2001; Howell *et al*., 2001).

Aberrant imprinting may arise not only at the time of preimplantation, but defects may be generated at an earlier step, at the time of imprint resetting in the gametes. The issue of incomplete imprint resetting during gametogenesis is discussed further in the section about ICSI with immature sperm cells.

There are few reports on imprinting analysis in the context of possible effects of assisted reproduction. In one molecular study focusing on the imprint control region involved in Prader–Willi and Angelman syndrome, no imprinting defects were detected in a group of nearly 100 children of which the majority were conceived after ICSI with ejaculated sperm (Manning *et al*., 2000). In another study, the expression of several imprinted genes was analysed in human oocytes and early embryos (Salpekar *et al*., 2001). The expression was variable from embryo to embryo. A follow-up study on 73 infants reported a boy with Beckwith–Wiedemann syndrome (Olivennes *et al*., 2001). Unfortunately this patient was lost to further follow-up and it could not be determined whether the syndrome was due to loss of imprinting.

Interference of assisted reproduction with epigenetic reprogramming

In-vitro culture

All assisted reproductive technologies have in common that they involve gamete, zygote and embryo incubation in synthetic culture medium. In-vitro culture and embryo manipulation have been associated with aberrant fetal growth. In mice and humans, there seems to be a reduction in birthweight, whereas in cattle and sheep several reports have described an enhancement in fetal growth, heavier newborn animals and an increased mortality during pregnancy and at birth, all major characteristics of what is referred to as the large offspring syndrome (LOS). The LOS phenotype has also been described in reports on cloned animals. So far, it has not been observed in humans born as a result of assisted reproduction. It has been suggested that at least some of the problems may result from an accumulation of epigenetic alterations during embryo culture (Young and Fairburn, 2000). The part of the genome that is particularly prone to epigenetic alterations during the preimplantation stage is the imprinted genes. Many of the imprinted genes have an essential function in embryonic growth, and aberrant imprinting may cause growth disturbances. The hypothesis of the occurrence of epigenetic misprogramming during in-vitro culture predicts that: (i) diverse outcomes will be found depending on the types of epigenetic disturbances and on the developmental stage at which they occur; (ii) similar disturbances will occur in different species and result in similar phenotypes, but some might lead to different phenotypes as a result of species-specific differences in embryonic development and/or differences in epigenetic programming. For instance, it has been shown that imprinting may vary between species, tissues, cells and stage of embryonic development. It is therefore possible that the contradictory growth disturbances—a reduction in mice and humans versus an increase in fetal growth in cattle—result from similar epigenetic disturbances. It was demonstrated that fetal overgrowth in sheep after embryo culture was associated with reduced expression of *M6P/IGF2R* through loss of methylation (Young *et al*., 2001). *M6P/IGF2R* plays a role in fetal organogenesis, tumour suppression and T-cell-mediated immunity. The locus is imprinted in mice, sheep, cows and pigs, but not in humans (Killian *et al*., 2001). The absence of imprinting at this particular locus may render human cells less susceptible to epigenetic disturbances. Down-regulation of the *M6P/IGF2R* tumour suppressor gene in humans will be mediated by two epigenetic 'hits', while in cattle and mice only one 'hit' will be required, since the second allele is already inactivated by the imprinting mechanism. The hypothesis of aberrant phenotypes as a result of epigenetic deregulation during in-vitro culture is supported by a number of studies. Alterations in methylation and expression levels were observed for the imprinted *Igf2/H19* locus in preimplantation mouse embryos (Khosla *et al*., 2001). One group (Doherty *et al*., 2000) reported that culture of preimplantation mouse embryos in media without serum resulted in loss of methylation at the *H19* imprinted locus. However, the imprinted expression of *Snrpn* was maintained, indicating that culture conditions can selectively affect the expression of imprinted genes.

The exact mechanism(s) by which culture media induce abnormal epigenetic modifications are not known. Media components could remove or interact with methyl groups on DNA or on histone tails. Another explanation may be that embryonic developmental timing is disturbed by the synthetic

media and that this interferes with epigenetic reprogramming and gene expression.

The use of prolonged culture systems may deregulate epigenetic mechanisms to a further extent. In-vitro culture up to the blastocyst stage is used in some centres for selected patients and in PGD cycles. Follow-up studies comparing blastocyst transfers versus early cleavage-stage embryo transfers showed no differences in birthweight (Ménézo *et al*., 1999; Kausche *et al*., 2001).

Immature sperm cells

Immature sperm cells are epigenetically quite different from mature sperm cells. A first issue of concern is the imprinting status. It is unclear whether imprint establishment has been completed in immature gametes. Most studies on imprint resetting have been performed in mice and almost no studies have addressed the problem in humans. In mice it was demonstrated that imprint resetting for the paternally expressed *Snrpn*, *Igf-2*, *Peg1* and the maternally expressed *Mash2*, *Igf-2r* and *H19* has been largely or entirely completed by the time the spermatid stage is reached (Shamanski *et al*., 1999). In humans, it has been shown that the methylation patterns of *H19* and *MEST*/*PEG1* are established during spermatogonial differentiation (Kerjean *et al*., 2000). From the limited data available and assuming that the resetting mechanism is similar in mice and humans, it appears that imprint establishment is completed by the time the spermatid stage is reached.

Another difference in epigenetic organization between immature and mature sperm cells is that spermatid chromatin has not yet been so densely packed. The genome may therefore be less protected against events of delayed oocyte activation, such as the delayed inactivation of the metaphase-promoting factor that can cause aneuploidy in the embryo (Tesarik, 1998). Another point of concern relates to the finding that spermatogenesis-specific genes undergo late epigenetic reprogramming at the level of the epididymis (Ariel *et al*., 1994). Genome-wide methylation patterns have been initiated at the prospermatogonia stage in the testes. However, genes involved in spermatogenesis are specifically demethylated for transcription and they become remethylated again in the epididymis. This implies that methylation patterns of spermatids and sperm derived from testes and epididymis will differ from the hypermethylated patterns found in ejaculated sperm. Follow-up studies of children born after ICSI with epididymal and testicular sperm have shown no additional risks as compared with children born after ICSI with ejaculated sperm (Bonduelle *et al*., 2002). However, there is a case report of two major malformations out of four pregnancies obtained after ICSI with elongated spermatids (Zech *et al*., 2000). Another study on a larger series did not detect an increased incidence of malformations (Sousa *et al*., 2000).

Furthermore, the mature sperm genome is in a silent state, but the spermatid genome is transcriptionally active and the introduction of spermatid transcripts into the oocyte may interfere with epigenetic reprogramming during the preimplantation stage. A comparative expression study performed on preimplantation mouse embryos derived from oocytes injected with round spermatids or sperm showed that activation of the embryonic genome is much less efficient with spermatids. A higher rate of developmental arrest has indeed been found in embryos derived from round spermatid injection compared with embryos obtained after standard ICSI (Vicdan *et al*., 2001). The expression patterns of several genes were disturbed in early embryos (4-cell stage) derived from spermatids. The results also suggested that regulatory mechanisms were activated in the oocyte to repress inappropriate spermatid transcription (Ziyyat and Lefèvre, 2001).

Ooplasmic transfer and cloning

Some small series of clinical cases of ooplasmic transfer have been reported (Barritt *et al*., 2001). Very little is known about the factors and organelles that are possibly transferred. The presence of donor mtDNA was observed in some of the babies, but it is not clear whether this had a therapeutic effect (Brenner *et al*., 2000). The function of mitochondria is controlled by interactions between both nuclear and mitochondrial genes. The dual nature of this control sets up potential conflicts between the different genome parts. Ooplasmic transfer into human oocytes may induce conflicts between the multiple genome parts (nuclear DNA, recipient mtDNA, donor mtDNA) and lead to unpredictable outcomes (Cummins, 2001).

Cloning involves nuclear transfer (NT) into an enucleated oocyte. The nucleus may come from an undifferentiated cell such as an embryonic stem cell (ES) or from a differentiated somatic cell. So far, the efficiency of the technique is low and most clones die before birth. The few clones that develop to term have often developmental abnormalities (LOS). Successful cloning requires that the epigenetic state of the donor nucleus is reprogrammed to that of the zygote (Rideout *et al*., 2001). The donor nuclei are not silent and their chromatin organization is likely to be different from that of the gametes. Moreover, there is a time limit to this reprogramming as it must be completed by the time that the embryonic genome is activated. The available data clearly demonstrate that reprogramming is incomplete in most NT embryos. A comparison of global methylation patterns in cloned and normal bovine preimplantation embryos showed disturbances in methylation dynamics in the cloned embryos (Bourc'his *et al*., 2001).

The epigenetic state of undifferentiated ES cells may resemble that of the early embryo better and require less reprogramming. On this basis, a higher cloning efficiency is expected for ES cell nuclear transfer. The percentage of ES cell NT blastocysts that develop to term is 10–20-fold higher than with somatic nucleus transfer. However, the percentage of initial clones that reach blastocyst stage is much lower with ES cell NT. This is probably related to the cell stage as well as to the epigenetic instability of the ES cells. Donor nuclei should preferentially be in the G_0/G_1 or G_2 phase, and many ES cells are in the S phase. Examination of imprinted gene expression in cloned mice and in donor ES cell lines from which they were derived showed that the expression of the imprinted *H19* and *Peg1* genes varied between ES cell subclones and even among mice derived from cells of the same ES cell subclone. These results indicate an unstable epigenetic state of ES cells (Humpherys *et al*., 2001).

Epigenetic inheritance

Failure or incomplete epigenetic reprogramming at the gamete and preimplantation embryo may lead to developmental problems and early mortality. Some epigenetic disturbances will be tolerated during development and these will result in phenotype changes which will show at a later stage. An important characteristic that distinguishes epigenetic modifications from genetic modifications or mutations is their reversibility. Epigenetic modifications are normally erased in the germ line. Incomplete erasure results in epigenetic inheritance. Another way for epigenetic modifications to be transmitted to the next generation is when they occur after fertilization but before specification of the germ line (John and Surani, 1999). At present, not many examples are known of epigenetic inheritance in mammals: methylation blue prints in humans have been shown to be inherited to some degree (Silva and White, 1988) and incomplete erasure in the maternal germ line has been shown to be responsible for the epigenetic inheritance of the A^{vy} allele in mice (Morgan *et al*., 1999). Nuclear transplantation experiments with early mice embryos have induced altered patterns of gene expression which have resulted in phenotype changes (reduced body weight) later in development. Such epigenetic modifications were transmitted to most of the offspring of the manipulated parent mice (Roemer *et al*., 1997). The last example is derived from epidemiological studies: starvation during the third trimester of pregnancy led to low birthweight during the Dutch Winter of Famine (1944–45). Unexpectedly, increased perinatal mortality and low birthweight were observed in the children of the females, who were not underweight at birth, but were malnourished in the first and second trimester of their own fetal development (Lumey, 1992). This may be explained by epigenetic deregulation, in response to the malnutrition, at the level of fetal germ cells. Failure to erase epigenetic modifications at certain alleles in the fetal germ cells will give rise to no effects in the children themselves, but will be transmitted to the next generation. An intriguing question that is raised in this context is whether the reduced birthweight of the IVF and ICSI children will be transmitted to their offspring. In a worst-case scenario, where low birthweight results from incomplete epigenetic erasure in the preimplantation embryo during in-vitro culture, both somatic and germ cells will be affected, as they are not yet separated. This would affect the children as well as their offspring.

Conclusions

Concern about birth defects and health problems in children born as a result of assisted reproduction has led to the initiation of risk assessment studies to evaluate the safety of these techniques. Prospective and retrospective follow-up studies of children born as a result of assisted reproduction have shown that development is normal and that malformation rates are similar to those in the general population. Neonatal data have indicated a lower birthweight in the IVF and ICSI singletons as compared with naturally conceived children.

It has been suggested that the accumulation of epigenetic defects during embryo culture may lead to aberrant phenotypes. Several lines of evidence support this hypothesis. The accumulation of severe epigenetic disturbances above a certain threshold may lead to early mortality. Embryos that develop to term may still have epigenetic defects and these may result in obvious aberrant phenotypes or in subtle changes in gene expression that can be easily overlooked.

Epigenetic disturbances induced by in-vitro culture may underlie the phenotype of lower birthweight. It is difficult on the basis of the available data to deduce whether there are other aberrations with an epigenetic origin in the population of children as a result of assisted reproduction. This is because the evaluation of epigenetic risks in the follow-up studies has been inadequate so far. Data on imprinting and methylation defects have often been collected in relatively small study groups. Given that the incidence of Prader–Willi or Angelman syndrome is 1/15 000, a large study population is needed to detect increased risks with sufficient power. Data on the incidence of imprinting disorders in IVF and ICSI children should therefore be collected preferentially from multiple centres. Another reason why epigenetic risks have been studied insufficiently is that the phenotypes associated with epigenetic defects may be difficult to recognize in short-term studies. Phenotypes due to imprinting defects may be obvious and show up early in life while clinical findings of neurodevelopmental delays and mental retardation may point in the direction of phenotypes caused by methylation defects. Other phenotypes may be subtler, such as the phenotypes of cancer predisposition that can be measured only later in life.

In conclusion, continuous follow-up studies are necessary in larger series of children in order to be able to assess long term risks linked to epigenetic disturbances after assisted reproduction. A complete safety evaluation may even require studies from a two-generation perspective.

Meanwhile, additional information on epigenetic reprogramming may be derived from molecular studies on preimplantation embryos. Preferentially and where possible, studies should be done on human embryos, as results from animal studies cannot always be extrapolated to humans.

Finally, the further elucidation of the mechanisms and factors involved in epigenetic regulation in fundamental research programmes will yield valuable information and identify elements that can be used to evaluate the safety of assisted reproductive technologies.

As the impact of epigenetic disturbances on later life of humans born as a result of assisted reproduction is not known, a definite answer about the safety of assisted reproduction cannot be given. Long-term clinical follow-up studies of the children born as a result of assisted reproduction as well as further molecular research are recommended.

Acknowledgements

We would like to thank W.Lissens, K.Sermon and H.Van de Velde for critical comments and helpful discussions. We also thank F.Winter for reviewing the manuscript. This work was supported by the Fund for Scientific Research, Flanders, Belgium.

References

Amir, R.E. and Zoghbi, H.Y. (2000) Rett Syndrome: methyl-CpG-binding protein 2 mutations and phenotype-genotype correlations. *Am. J. Med. Genet*., **97**, 147–152.

Ariel, M., Cedar, H. and McCarrey J. (1994) Developmental changes in methylation of spermatogenesis-specific genes include reprogramming in the epididymis. *Nature Genet*., **7**, 59–63.

Barritt, J.A., Willadsen, S., Brenner, C. and Cohen, J. (2001) Cytoplasmic transfer in assisted reproduction. *Hum. Reprod*., *Update*, **7**, 428–435.

Battaglia, D.E., Goodwin, P., Klein, N.A. and Soules, M.R. (1996) Influence of maternal age on meiotic spindle assembly in oocytes from naturally cycling women. *Hum. Reprod*., **11**, 2247–2222.

Bonduelle, M., Joris, H., Hofmans, K., Liebaers, I. and Van Steirteghem, A. (1998) Mental development of 201 ICSI children at 2 years of age. *Lancet*, **351**, 1553.

Bonduelle, M., Camus, M., De Vos, A., Staessen, C. Tournaye, H., Van Assche, E., Verheyen, G., Devroey, P., Liebaers, I. and Van Steirteghem, A. (1999) Seven years of intracytoplasmic sperm injection and follow-up of 1987 subsequent children. *Hum. Reprod*., **14** (Suppl. 1), 243–264.

Bonduelle, M., Liebaers, I., Deketelaere, V., Derde, M., Camus, M., Devroey, P. and Van Steirteghem, A. (2002) Neonatal data on a cohort of 2889 infants born after ICSI) (1991–1999 and of 2995 infants born after IVF (1983–1999). *Hum. Reprod*., **17**, 671–694.

Bourc'his, D., Le Bourhis, D., Patin, D., Niveleau, A., Comizzoli, P., Renard, J.-P. and Viegas-Péquignot, E. (2001) Delayed and incomplete reprogramming of chromosome methylation patterns in bovine cloned embryos. *Curr. Biol*., **11**, 1542–1546.

Bowen, J.R., Gibson, F.L., Leslie, G.I. and Saunders, D.M. (1998) Medical and developmental outcome at 1 year for children conceived by intracytoplasmic sperm injection. *Lancet*, **351**, 1529–1535.

Brenner, C.A., Barritt, J.A., Willadsen, S. and Cohen, J. (2000) Mitochondrial DNA heteroplasmy after human ooplasmic transplantation. *Fertil. Steril*., **74**, 573–578.

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

Carlone, D.L. and Skalnik, D.G. (2001) CpG binding protein is crucial for early embryonic development. *Mol. Cell. Biol*., **21**, 7601–7606.

Couvert, P., Bienvenu, T., Aquaviva, C., Poirier, K., Moraine, C., Gendrot, C., Verloes, A., Andrès, C., Le Fevre, A.C., Souville, I. *et al.* (2001) MECP2 is highly mutated in X-linked mental retardation. *Hum. Mol. Genet*., **10**, 941–946.

Cummins, J.M. (2001) Mitochondria: potential roles in embryogenesis and nucleocytoplasmic transfer. *Hum. Reprod. Update*, **7**, 217–228.

Dean, W. and Ferguson-Smith, A. (2001) Mother maintains methylation marks. *Curr. Biol*., **11**, 527–530.

Devroey, P., Nagy, P., Tournaye, H., Liu, J., Silber, S. and Van Steirteghem, A. (1996) Outcome of intracytoplasmic sperm injection with testicular spermatozoa in obstructive and non-obstructive azoospermia. *Hum. Reprod*., **11**, 1015–1018.

Doherty, A.S., Mann, M.R., Tremblay, K, D., Bartolomei, M. S. and Schultz, R.M. (2000) Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. *Biol. Reprod*., **62**, 1526–1535.

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.

Falls, J.G., Pulford, D.J., Wylie, A.A. and Jirtle, R.L. (1999) Genomic imprinting: implications for human disease. *Am. J. Path*., **154**, 635–641.

Gibbons, R.J. and Higgs, D.R. (2000) Molecular-clinical spectrum of the ATR-X syndrome. *Am. J. Med. Genet*., **97**, 204–212.

Gibbons, R.J., McDowell, T.L., Raman, S., O'Rourke, D.M., Garrick, D., Ayyub, H. and Higgs, D.R. (2000) Mutations in ATRX, encoding a SWI/SNF-like protein, cause diverse changes in the pattern of DNA methylation. *Nature Genet*., **24**, 368–371.

Handyside, A.H., Kontogianni, E.H., Hardy, K. and Winston, R.M. (1990) Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature*, **344**, 768–770.

Howell, C.Y., Bestor, T.H., Ding, F., Latham, K.E., Mertineit, C., Trasier, J.M. and Chaillet, J.R. (2001) Genomic imprinting disrupted by a maternal effect mutation in the Dnmt1 gene. *Cell*, **104**, 829–838.

Humpherys, D., Eggan, K., Akutsu, H., Hochedlinger, K., Rideout, W.M. 3rd, Binszkiewicz, D., Yanagimachi, R. and Jaenisch, R. (2001) Epigenetic instability in ES cells and cloned mice. *Science*, **293**, 95–97.

International Working Group on Preimplantation Genetics (2001) Tenth anniversary of preimplantation genetic diagnosis. *J. Assist. Reprod. Genet*., **18**, 64–70.

Isles, A.R. and Wilkinson, L.S. (2000) Imprinted genes, cognition and behaviour. *Trends Cogn. Sciences*, **4**, 309–318.

Jirtle, R.L. (1999) Genomic imprinting and cancer. *Exp. Cell Res*., **248**, 18–24.

John, R.M. and Surani, M.A. (1999) Agouti germ line gets acquisitive. *Nature Genet*., **23**, 254–256.

Jones, P.A. and Takai, D. (2001) The role of DNA methylation in mammalian epigenetics. *Science*, **293**, 1068–1069.

Kausche, A., Jones, G.M., Trounson, A.O., Figueiredo, F., MacLachlan, V. and Lolatgis, N. (2001) Sex ratio and birth weights of infants born as a result of blastocyst transfers compared with early cleavage stage embryo transfers. *Fertil. Steril*., **79**, 688–693.

Kerjean, A., Dupont, J., Vasseur, C., Le Tessier, D., Cuisset, L., Paldi, A., Jouannet, P. and Jeanpierre, M. (2000) Establishment of the paternal methylation imprint of the human H19 and MEST/PEG1 genes during spermatogenesis. *Hum. Mol. Genet*., **9**, 2183–2187.

Killian, J.K., Nolan, C.M., Wylie, A.A., Li, T., Vu, T.H., Hoffman, A.R. and Jirtle, R.L. (2001) Divergent evolution in M6P/IGF2R imprinting from the Jurassic to the Quaternary. *Hum. Mol. Genet*., **10**, 1721–1728.

Khosla, S., Dean, W., Reik, W. and Feil, R. (2001) Culture of preimplantation embryos and its long-term effects on gene expression and phenotype. *Hum. Reprod. Update*, **7**, 419–427.

Leslie, G.I., Gibson, F.L., McMahon, C., Cohen, J. and Saunders, D.M. (2001) Sperm quality as a predictor of child health and development in children conceived using ICSI. *Hum. Reprod*., **16** (Abstract Bk.1), p.38.

Lumey, L.H. (1992) Decreased birthweights in infants after maternal *in utero* exposure to the Dutch famine of 1944–1945. *Paediatr. Perinat. Epidemiol*., **6**, 240–253.

Manning, M., Lissens, W., Bonduelle, M., Camus, M., De Rycke, 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.

Mayer, W., Niveleau, A., Walter, J., Fundele, R. and Haaf, T. (2000) Demethylation of the zygotic paternal genome. *Nature*, **403**, 501–502.

Ménézo, Y.J.R., Chouteau, J., Torello, M.J., Girard, A. and Veiga, A. (1999) Birthweight and sex ratio after transfer at the blastocyst stage in humans. *Fertil. Steril*., **72**, 221–224.

Morgan, H.D., Sutherland, H.G.E., Martin, D.I.K. and Whitelaw, E. (1999) Epigenetic inheritance at the agouti locus in the mouse. *Nature Genet*., **23**, 314–318.

Niemann, H. and Wrenzycki, C. (2000) Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: implications for subsequent development. *Theriogenology*, **53**, 21–34.

Oberlé, I., Rousseau, F., Heitz, D., Kretz, C., Devys, D., Hanauer, A., Boue, J., Bertheas, M.F. and Mandel, J.L. (1991) Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science*, **252**, 1097–1102.

Okano, M., Bell, D.W., Haber, D.A. and Li, E. (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell*, **99**, 247–257.

Olivennes, F., Mannaerts, B., Struijs, M., Bonduelle, M. and Devroey, P. (2001) Perinatal outcome of pregnancy after GnRH antagonist (ganirelix) treatment during ovarian stimulation for conventional IVF: a preliminary report. *Hum. Reprod*., **16**, 1588–1591.

Palermo, G., Joris, H., Devroey, P. and Van Steirteghem, A.C. (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*, **340**, 17–18.

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

Reik, W., Dean, W. and Walter, J. (2001) Epigenetic reprogramming in mammalian development. *Science*, **293**, 1089–1093.

Rideout, W.M. III, Eggan, K. and Jaenisch, R. (2001) Nuclear cloning and epigenetic reprogramming of the genome. *Science*, **293**, 1093–1097.

Robertson, K.D. and Wolffe, A. P. (2000) DNA methylation in health and disease. *Nat. Rev. Genet*., **1**, 11–19.

Roemer, I., Reik, W., Dean, W. and Klose, J. (1997) Epigenetic inheritance in the mouse. *Curr. Biol*., **7**, 277–280.

Salpekar, A., Huntriss, J., Bolton, V. and Monk, M. (2001) The use of amplified cDNA to investigate the expression of seven imprinted genes in human oocytes and preimplantation embryos. *Mol. Hum. Reprod*., **7**, 839–844.

Shamanski, F.L., Kimura, Y., Lavoir, M., Pedersen, R.A. and Yanagimachi, R. (1999) Status of genomic imprinting in mouse spermatids. *Hum. Reprod*., **14**, 1050–1056.

Silber, S.J., Johnson, L., Verheyen, G. and Van Steirteghem, A. (2000) Round spermatid injection. *Fertil. Steril*., **73**, 897–900.

Silva, A.J. and White, R. (1988) Inheritance of allelic blueprints for methylation patterns. *Cell*, **54**, 145–152.

Skuse, D.H. (2000) Imprinting, the X-chromosome, and the male brain: explaining sex differences in the liability to autism. *Pediat. Res*., **47**, 9–16.

Solter D. (2001) Mammalian cloning: advances and limitations. *Nat. Rev. Genet*., **1**, 199–207.

Sousa, M., Barros, A., Takahashi, K. Liveira, C., Silva, J. and Tesarik, J. (1999) Clinical efficacy of spermatid conception: analysis using a new spermatid classification scheme. *Hum. Reprod*., **14**, 1279–1286.

Sousa, M., Fernandes, S. and Barros, A. (2000) Prognostic factors for successful testicle spermatid recover. *Mol. Cell. Endocrinol*., **166**, 37–43.

Sutcliffe, A.G., Taylor, B., Saunders, K., Thornton, S., Lieberman, B.A. and Grudzinskas, J.G. (2001) Outcome in the second year of life after in-vitro fertilisation by intracytoplasmic sperm injection: a UK case–control study. *Lancet*, **357**, 2080–2084.

Tarlatzis, B.C. and Bili, H. (1998) Survey on intracytoplasmic sperm injection: report from the ESHRE ICSI Task Force. *Hum. Reprod*., **13** (Suppl.), 165–177.

Tesarik, J. (1998) Oocyte activation after intracytoplasmic injection of mature and immature sperm cells. *Hum. Reprod*., **13** (Suppl.), 117–127.

Tesarik, J., Mendoza, C. and Testart, J. (1995) Viable embryos from injection of round spermatids into oocytes. *N. Engl. J. Med*., **333**, 525.

Van Steirteghem, A., Nagy, Z., Joris, H., Liu, J., Staessen, C., Smitz, J. Wisanto, A. and Devroey, P. (1993) High fertilization and implantation rates after intracytoplasmic sperm injection. *Hum. Reprod*., **8**, 1061–1066.

Verheyen, G., Crabbe, E., Joris, H. and Van Steirteghem, A. (1998) Simple and reliable identification of the human round spermatid by inverted phase-contrast microscopy. *Hum. Reprod*., **13**, 1570–1577.

Verkerk, A.J., Pieretti, M. and Sutcliffe, J.S. (1991) Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell*, **65**, 905–914.

Vicdan, K., Isik, A.Z. and Delilbasi, L. (2001) Development of blastocyst-stage embryos after round spermatid injection in patients with complete spermiogenesis failure. *J. Assist. Reprod. Genet*., **18**, 78–86.

Voo, K.S., Carlone, D.L., Britta, M., Flodin, A. and Skalnik, D.G. (2000) Cloning of a mammalian transcriptional activator that binds unmethylated CpG motifs and shares a CXXC domain with DNA methyltransferase, human trithorax, and methyl-CpG binding domain protein 1. *Mol. Cell. Biol*., **20**, 2108–2121.

Wang, M., Chen, D., Lui, J., Li, G. and Sun, Q. (2001) In vitro fertilisation of mouse oocytes reconstructed by transfer of metaphase II chromosomes results in live births. *Zygote*, **9**, 9–14.

Wennerholm, U.B. (2000) Cryopreservation of embryos and oocytes: obstetric outcome and health in children. *Hum. Reprod*., **15** (Suppl. 5), 18–25.

Young, L.E. and Fairburn, H.R. (2000) Improving the safety of embryo technologies: possible role of genomic imprinting. *Theriogenology*, **53**, 627–648.

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.

Yu, S., Pritchard, M. and Kremer, E. (1991) Fragile X genotype characterized by an unstable region of DNA. *Science*, **252**, 1179–1181.

Zech, H., Vanderzwalmen, P., Prapas, Y., Lejeune, B., Duba, E., and Schoysman, R. (2000) Congenital malformations after intracytoplasmic injection of spermatids. *Hum. Reprod*., **15**, 969–971.

Ziyyat, A. and Lefèvre, A. (2001) Differential gene expression in pre-implantation embryos from mouse oocytes injected with round spermatids or spermatozoa. *Hum. Reprod*., **16**, 1449–1456.