European Society of Human Reproduction and Embryology

COURSE 3

Epigenetic risks on ART conceptus

Special Interest Group Embryology

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Contents

Course 3: Special Interest Group "Embryology"
"Epigenetics risks on ART conceptus"

PROGRAM

Course co-ordinator(s): D. Royere (F), E. Van den Abbeel (B), K. Lundin (S), M.C. Magli (I)

Course description: The aim of this Course is to stress some of the epigenetic events that may alter early embryo development, based on animal experimental data and clinical human data.

09.00 - 09.30: What is epigenetics? - *M. De Rijcke (B)*
09.30 - 09.45: Discussion

09.45 - 10.15: In vitro culture and methylation. - *L. Young (UK)*
10.15 - 10.30: Discussion

10.30 - 11.00: Coffee break

11.00 - 11.30: Influence of vitro environment on fetal growth in animal models. - *J.P. Renard (F)*
11.30 - 11.45: Discussion

11.45 - 12.15: Imprinting pattern during male and female germ cell development - *D. Lucifero (CND)*
12.15 - 12.30: Discussion

12.30 - 13.30: Lunch

13.30 - 14.00: Business meeting Special Interest Group "Embryology"
14.00 - 14.15: Discussion

14.15 - 14.45: Beckwith - Wiedemann syndrome in ART conceived children. – *S. Rossignol (F)*
14.45 - 15.00: Discussion

15.00 - 15.30: Coffee break

15.30 - 16.00: Critical analysis of epidemiological data on epigenetic risk in ART conceived children. – *M. Bonduelle (B)*
16.00 - 16.15: Discussion

16.15 - 16.45: How far long-term follow up of children could be improved? – *K.G. Nygren (S)*
16.45 - 17.00: Discussion

What is epigenetics?

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Learning objectives

The aim of this manuscript is
- to discuss the etymology and the current definition of epigenetics
- to learn about the key enzymes of DNA methylation
- to outline the various histone modifications and explain the concept of the “histone code”
- to gain an insight into the epigenetic mechanisms of genomic imprinting
- to describe epigenetic reprogramming events during the mammalian life cycle
- to have a broader understanding of epigenetic diseases

Epigenetics, a historical view

The term “epigenetics” has over time been used in various senses. The root term “epigenesis” goes back to Aristotle and describes embryonic development as a series of events where new structures and functions appear with increasing complexity. The developmental biologist Conrad Waddington introduced the term “epigenetics” in 1942 as the study of the processes by which genotype gives rise to phenotype. In his book “Organisers and genes” published in 1940, he proposed the idea of “the epigenetic landscape”, in which he visualised embryonic development as a ball (the embryo) rolling down a sloping surface with branching valleys representing the choices of developmental pathways for an embryonic cell.

Since researchers started to learn the molecular basis of epigenetics in the 1990s, the definition has often been narrowed to a molecular one, defining epigenetics as the study of mechanisms of gene-expression.

What is epigenetics?

In biology today, epigenetics can be defined as the study of reversible and heritable (through mitosis and meiosis) changes in gene expression that occur without changes in the DNA code and that do not follow Mendelian laws.

The Greek prefix “epi” means “above” or ”in addition”, indicating that epigenetics extends the information content of the genetic code.

Epigenetic mechanisms involve DNA methylation at CpG dinucleotides, the covalent modification of histones (phosphorylation, acetylation, methylation, ubiquitination) and chromatin remodelling,

which are all linked and cooperate at the transcriptional level, whereas non-coding RNAs seem to function predominantly at the posttranscriptional level. Eukaryotic DNA is compacted with histones into chromatin. The basis unit of chromatin, the nucleosome, consists of an octamer of core histones (two copies of each histone H2A, H2B, H3 and H4) around which 147 bp of DNA is wrapped. Further folding of this nucleosomal array gives higher-order structures. Chromatin is organized into either highly condensed or open domains, called 'heterochromatin' and 'euchromatin' respectively. Heterochromatin is transcriptionally inactive and late replicating while euchromatin is transcriptionally active and early replicating. Epigenetic modifications will alter chromatin structure and regulate chromatin function. Modifications of DNA or associated histones will modulate the interactions between histones, DNA, RNA and non-histones proteins whereas chromatin remodelling complexes may displace nucleosomes. This will change the overall chromatin structure and influence DNA accessibility, thereby regulating gene expression as well as other cellular processes such as replication, recombination, repair and chromosome segregation.

Epigenetic patterns are imposed on the genome during embryonic development and differentiation through predetermined programmes (genetic or intrinsic factors). The establishment of the appropriate patterns in space and time is essential for further development. Epigenetic systems also allow the early as well as the mature organism to adapt gene expression patterns to environmental changes (hormones, growth factors, etc.). In addition to intrinsic and environmental factors, it seems that the epigenetic state of some genomic regions is determined stochastically. Normally, all epigenetic modifications are erased in the germ line to ensure totipotency. Incomplete erasure may lead to epigenetic inheritance.

DNA methylation

DNA methylation can be found in almost all living organisms. In eukaryotes, it mainly involves the covalent addition of a methyl group to a cytosine at CpG dinucleotides. The establishment and the 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. Mice lacking either one of these methyltransferases die pre- or postnatally.

It has been well established that mammalian DNA methylation is essential for embryonic development, genome stability, X chromosome inactivation and genomic imprinting. DNA methylation plays a pivotal role in gene silencing. Some genomic regions like the repetitive sequences and transposons are hypermethylated and silenced; other regions such as the CpG islands in the promoters of house-keeping genes are hypomethylated and are transcriptionally active. The methylation levels of differentiated cells are relatively stable through mitosis. Nevertheless, with age, repeated sequences become hypomethylated whereas specific genes become hypermethylated.

Histone modifications and chromatin remodelling

The post-translational modifications of specific amino acids (AA) within the NH2-terminal histone tails have been known for a long time. Modifications within the globular histone cores at the histone-DNA interface have been mapped only recently. Some modifications like acetylation and phosphorylation have a high turnover rate and are linked with inducible gene expression patterns; others like methylation are very stable and are linked with long-term expression patterns. Several classes of chromatin -modification and - remodelling complexes have been identified. Histone acetyl transferase enzymes (HATs) transfer an acetyl group from the cofactor acetyl CoA to lysines. The reverse reaction is catalysed by histone deacetylases (HDACs). Both HATs and HDACs are

often part of transcription regulatory protein complexes. Histone methylation is catalysed by histone methyl transferases (HMTs) which use S-adenosylmethionine as a cofactor in a relatively irreversible reaction. Another family of protein complexes are the ATP-dependent chromatin remodelling complexes that are capable of (re)positioning nucleosomes using the energy of ATP hydrolysis.
The idea of the histone code hypothesis is that histone modifications, alone or in various combinations, will influence chromatin structure and determine whether the underlying genetic information is active or not. Modifications at the histone-DNA interface may have a direct impact on chromatin whereas modifications in the histone tails may alter chromatin structure indirectly by recruitment of chromatin-associated proteins that will modulate chromatin structure. The methyl modification of Lys9 in H3 has been shown to recruit chromodomain proteins that will induce heterochromatin assembly and epigenetic silencing. Proteins with bromodomains are attracted to acetylated lysines and this has been linked with transcriptional activation. The acetyl modification reduces the histone-DNA interactions within nucleosomes and recruits transcriptional co-activators. Similarly, phosphorylation and ubiquitination 'marks' form binding sites for specific chromatin-associated proteins. The findings that the various histone modifications can work antagonistically or synergistically and that the core histones can be replaced with specialized histone variants add further complexity to the system.

What is genomic imprinting?

Genomic imprinting is an epigenetic form of gene regulation which involves the mono-allelic expression of one of the two parental alleles in a parent-of-origin specific manner. Imprinting is only found in eutherian mammals, marsupials and higher plants. Imprinted genes have a crucial role in embryonic growth and behavioural development. They are also involved in carcinogenesis. Several human syndromes are known to be associated with imprinted genes such as the Beckwith-Wiedemann syndrome on chromosome 11 and the Prader-Willi/Angelman syndrome on chromosome 15. The parental alleles are differentially marked or "imprinted" in the parental germ lines when the two genomes are separate. The imprints of the gametes are maintained in the zygote and further through somatic cell divisions. They will lead to mono-allelic expression in the embryo and in the adult. The imprints are reset in the germ line according to the sex of the embryo. The differential marking at imprinted loci involves allele-specific epigenetic modifications of which DNA methylation is the best studied so far.

Epigenetic reprogramming during the mammalian life cycle

Epigenetic reprogramming takes place during gametogenesis and another period of major reprogramming occurs in the early embryo. Epigenetic reprogramming in the germ line starts with complete erasure of existing modifications in imprinted and non-imprinted genes to ensure genetic totipotency. Later during gametogenesis, de novo methylation and chromatin remodelling take place in the whole genome, whereas allele-specific marks are established at imprinted loci (imprint resetting). This reprogramming provides the genome in the gametes with molecular programs for oocyte activation, zygotic gene activation and embryonic development.

The gametes that come together at fertilisation are epigenetically quite different. Both genomes are highly methylated and transcriptionally inactive. The oocyte chromatin structure which is packed with highly methylated histones is much more repressive than that of sperm which is packed with protamines. After fertilisation, stored ooplasmic factors as well as other factors modify the epigenetic status of the parental genomes. The paternal chromosomes decondense and a rapid remodelling occurs: protamines are substituted with maternal, highly acetylated histones and the DNA, except for the paternally methylated imprinted genes, undergoes active demethylation before replication.

The maternal genome except for the maternally methylated imprinted genes is passively demethylated after the first cell division. This passive loss of methylation relates to the lack of Dnmt1 in oocytes and in the preimplantation stage. Paternal and maternal genomes reach about equivalent DNA methylation levels in the 8-cell stage. At the time of implantation a genome-wide DNA and histone methylation takes place in the inner cell mass but not in the trophectoderm. Imprinted genes resist this global wave of de-novo methylation.

Epigenetic diseases

Mutations in the genes coding for the epigenetic machinery will lead to the deregulation of developmental processes and to various diseases. In humans, several diseases have been associated with epigenetic defects. Mutations in the methyltransferase DNMT3b gene underlie the rare ICF (Immunodeficiency, Centromeric instability and Facial anomalies) syndrome characterised by hypomethylation of pericentromeric regions. Rett syndrome (RTT) is a neurodevelopmental disorder in girls in which initial normal development is followed by progressive degeneration of acquired motor skills, loss of speech and mental retardation. Mutations in the methyl-binding protein MECP2 underlie RTT, whereas distinct mutations in the same gene underlie non-specific X-linked mental retardation. Mutations in the ATRX protein, that is part of a chromatin remodelling complex, result in the ATR-X (X-linked a-thalassaemia/mental retardation) syndrome which is characterised by mental retardation, facial dysmorphism, a-thalassaemia and urogenital abnormalities. These diseases all show genome-wide changes in gene expression.

Aberrant DNA methylation patterns have been associated with cancer, where methylation at transposons is lost and tumour suppressor genes are inactivated because of CpG island hypermethylation, leading to an overall picture of changes in gene expression and chromatin structure along with genome instability.

Deregulation of genomic imprinting has also been associated with several human syndromes and with cancer. Imprinting diseases such as Beckwith-Wiedemann syndrome (BWS) and Prader-Willi/ Angelman (PWS/AS) syndrome are characterised by non-mendelian inheritance patterns that exhibit parental origin effects. Features of imprinting disorders are pre-and postnatal growth defects, behavioural changes and neurological defects. Imprinted genes are located in clusters. Regulation of imprinting within these clusters is coordinated by long-range cis-acting Imprint Control Elements (ICE) which are associated with differentially methylated regions. Genetic as well as epigenetic defects in these clusters underlie imprinting disorders. Sixty per cent of sporadic BWS cases are caused by epigenetic defects, whereas in AS less than 5 % of cases are due to epigenetic defects. Concern raised after recent reports about a possible higher incidence of imprinting syndromes BWS and AS, in children born after assisted reproductive technologies. Remarkably, further molecular analysis showed that the underlying defect in both AS and BWS cases was loss of methylation at the maternal allele. It has been suggested that in vitro embryo culture or hormonal stimulation of the ovaries may interfere with imprint establishment in the gametes or imprint maintenance in the preimplantation embryo.

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excellent review of genomic imprinting and its relevance in reproduction and ART.

In vitro culture and methylation

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Learning objectives

- To describe epigenetic reprogramming in the preimplantation embryo
- To list the types of sequence that undergo methylation reprogramming
- To describe epigenetic variation within sperm and oocytes
- To describe the process of genomic imprinting
- To review imprinting disruptions in embryos of various species
- to diagram the major components of the methyl cycle
- To list the concentrations of methyl cycle components in human embryo culture media
- To describe potential methods for methylation analysis
- To discuss the potential of human embryonic stem cells to model epigenetic change

Lecture summary

Epigenetic regulation of transcription

Epigenetics is the study of modifications to DNA that control gene expression, without changing DNA sequence. There are a variety of known epigenetic modifications both to DNA and to the histone proteins that package DNA into nucleosomes (reviewed in first presentation this session. This presentation will focus on DNA methylation, the addition of methyl groups to cytosine residues in DNA. DNA methylation is the best studies epigenetic modification in early development and is of interest in ART since early embryos undergo major changes throughout the genome in their DNA methylation status and this modification is stably inherited, indicating long-lasting consequences of any early perturbation.

Epigenetic reprogramming in the embryo

In most of our cells, DNA is packaged into chromatin around proteins that can also have regulatory modifications. The nuclei of sperm and oocytes exist in very different chromatin states that need to be remodeled shortly after fertilization to allow activation of the embryonic programme of gene expression. Recent studies have shown that this remodeling process involves dynamic global changes in epigenetic modifications in the preimplantation embryo that do not occur in differentiated cell lineages. Another active period of epigenetic reprogramming occurs during gametogenesis. Thus there may be a high propensity for epigenetic perturbation by ART during these key phases of development.

Species differences occur in epigenetic reprogramming. Initial studies using an antibody which

detects global changes in DNA methylation suggested a two phase loss of methylation, with the male pronucleus demethylating in the oocyte cytoplasm and then the female genome losing most of it's methylation in the cleavage stages. The human male pronucleus also demethylates rapidly after fertilization. Further varying degrees of embryonic demethylation then follows in most species studied in the cleavage-stage embryo, with remethylation often evident by the blastocyst stage.

Genomic regions regulated by methylation

Most methylation in mammalian genomes is concentrated in repeat regions in pericentromeric regions of chromosomes and reduced methylation of these regions causes chromosomal instability in human ICF syndrome. Other repeat sequences inherited throughout the course of evolution from viral infections are interspersed between genes and can effect ectopic gene expression or gene deletion when demethylated. Genes can also be methylated often around the promoter regions and this can occur in a single gene locus in a tissue-specific or developmental stage-specific manner. Finally, a small subset of the 30,000 genes in the human genome are subject to genomic imprinting, often involving silencing of only one of the parentally-inherited alleles by methylation-dependent mechanisms. Thus demethylation of different regions of the human genome could theoretically occur during gametogenesis and preimplantation development, with a wide range of region-dependent effects on the resulting conceptus.

Sources of epigenetic variation in embryos

Evidence from a range of mammals has implicated superovulation, embryo culture, IVF, ICSI, sperm defects and maternal nutrition in perturbing embryo development, often with phenotypic consequences in the offspring. Recently, epigenetic variation has been demonstrated in both human sperm and human embryos, with the oocyte contribution still to be examined.

Genomic imprinting

Of particular relevance to clinical ART are associations of increased incidence of imprinting disorders with IVF and/ or ICSI. Furthermore, the increased use of blastocyst culture has raised concerns of developmental consequences, including through epigenetic means. It is now timely to investigate and quantify the risks to children conceived by ART through epigenetic perturbation, particularly when new protocols are continually been introduced to clinical practice. However, technologies for detecting epigenetic changes in embryos are currently very limited.

Imprinting disruptions in embryos

This presentation will outline current knowledge of imprinted genes regulating normal embryonic development and review the known disruptions. Whilst speculating on mechanisms of inducing epigenetic perturbation, this presentation will suggest that the only way to safely evaluate changing procedures in ART is to begin to unravel the molecular mechanisms that are perturbed and to develop reliable screening methods.

Environmental Influences on DNA Methylation

Some nutrients are known to influence DNA methylation in adult tissues and in the developing conceptus via maternal nutritional effects. Our laboratory focuses on whether nutrients influencing the methionine metabolic cycle, which provides the methyl groups for DNA methylation, can affect DNA methylation either in vivo or in embryo culture environments. Relevant nutrients include methionine, folate, vitamin B12, vitamin B6 and selenium.

Analysing methylation in the embryo

Many classical techniques for analyzing DNA methylation are difficult to apply to the analysis of oocytes or embryos due to the paucity of genetic material. The most widely techniques used at present are bisulphate sequencing and methylation-sensitive PCR, both of which require a range of careful controls to ensure validity.

Using human embryonic stem cells to model embryo culture

Direct studies on environmental factors that may induce epigenetic perturbation are difficult in spare human embryos due to their heterogeneity, limited availability and lack of "normal" controls. Our laboratory is beginning to investigate the utility of human embryonic stem cells, derived from the blastocyst stage embryo, to test specific hypotheses and model epigenetic programming event sin the human embryo. The advantages and challenges of this system are reviewed by Allegrucci et al., (2005), copies of which will be available at the course.

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Influence of vitro environment on fetal growth in animal models

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NOTES:

Imprinting patterns during male and female germ cell development

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Learning objectives

After this session, participants should have an understanding of:

- the molecular basis of genomic imprinting;
- the 'life-cycle' of genomic imprints; and
- the DNA methylation dynamics that underlie imprint establishment during male and female germ cell development.

Our understanding of the importance of both maternal and paternal contributions to normal development began just over twenty years ago with elegant pronuclear transfer experiments conducted by McGrath and Solter (1984) and Barton et al. (1984). By reconstructing diploid mouse embryos with two female or male pronuclei, the authors showed that both biparental gynogenones and androgenones were unable to complete normal embryogenesis and thus revealed the parental genomes to be functionally different. Because the genetic contribution by the egg and sperm to the embryo is identical at the level of DNA sequence, their functional asymmetry points to the existence of epigenetic differences between the parental genomes. Epigenetic inheritance refers to phenomena or modifications which are heritable through cell division and can influence gene activity without affecting the DNA code. In the past two decades since the initial pronuclear transfer experiments described in 1984, our understanding of parental specific gene regulation and the important role of epigenetic modifications in this process has continued to advance at an impressive pace.

This summary will provide a general overview of imprinted genes, the role of DNA methylation in genomic imprinting, as well as the timing of imprint establishment in germ cells. For a more thorough and in-depth discussion of these topics please refer to the references cited at the end of this summary.

Genomic imprinting

Genomic imprinting is defined as the parent-of-origin dependent mono-allelic expression of a gene. Although roughly 60 imprinted genes have been described, it is estimated that the number of genes subject to imprinting will total roughly 100. The table below provides a summary of several maternally and paternally expressed imprinted genes. The majority of these genes play important developmental roles in regulating embryo growth and placental function. Others appear to be involved in postnatal behavioural processing and cognition. Loss of imprinting has been implicated in the development of certain cancers and is also the cause of childhood diseases such as Angelman, Prader-Willi and Beckwith-Wiedemann syndromes.

Genomic imprinting is thought to be restricted to mammalian genomes but has also been described in marsupials. The 'parental conflict' theory is the most widely accepted theory to date that has been described to explain the existence of imprinted genes. In keeping with the phenotypes of gynogenones and androgenones as well as the relationship between two of the first imprinted genes characterized, Igf2 and Igf2r, the parental conflict theory proposes that the paternal genome has evolved to express genes that support embryo growth and development of extra-embryonic tissues thereby ensuring that the father's genes are passed on to healthy offspring. In response, the maternal genome has been programmed to counteract by expressing genes which limit embryo growth and conserve resources for future pregnancies.

Table 1. Examples of imprinted genes

Gene	Function	Role			Human chromosomal location
		Fetal growth	Placenta	Brain	
Paternally expressed genes					
Peg1	a/b-hydrolase family	+	+	+	7q32
Igf2	Fetal-specific growth factor, trophoblast-specific isoform	+	+		11p15
Kcnq1ot1	Non-translated RNA, antagonize expression of Cdkn1c	+			11p15
Snrpn	RNA processing, splicing factor protein			+	15q11
Peg3	Zinc finger DNA binding protein, TNF-NFkB regulation	+	+	+	19q13
Maternally expressed genes					
Igf2r	Mannose-6-phosphate receptor, clearance receptor for Igf2	+			6q25
H19	Abundant non-translated RNA, Igf2 regulation	+	+		11p15
Cdkn1c	Cyclin-dependent kinase inhibitor, growth inhibitor	+			11p15
Kcnq1	Potassium channel			+	11p15
Gnas	Gs protein a subunit	+		+	20q13

DNA methylation

DNA methylation occurs at the fifth position of the cytosine ring within the context of 5'-CpG-3' dinucleotides. The reaction is catalyzed by a family of DNA methyltransferases (DNMTs) which use S-adenosylmethionine (AdoMet) as a methyl donor (Figure 1). DNA methylation occurs at approximately 3% to 8% of all cytosine residues in mammalian genomes and is sometimes referred to as a fifth DNA base which allows for an additional layer of information to be carried by the DNA molecule. DNA methylation is associated with transcriptional repression in that methylation within gene promoters can prevent the binding of transcription factors and machinery and thus impede the expression of a gene. DNA methyltransferases are found in complex with other proteins involved in transcriptional repression and may act in concert with these other enzymes (e.g. histone deacetylases) to lead to stable silencing effects.

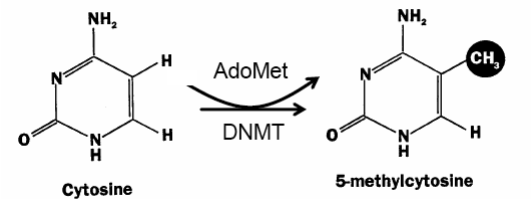

Figure 1. DNA methylation
The transfer of a methyl group to the fifth position of the cytosine ring is depicted. The reaction is catalyzed by a group of enzymes known as DNA methyltransferases and AdoMet, a cofactor in the reaction, serves as the methyl donor.

The allele-specific expression of imprinted genes requires that some differential epigenetic mark distinguish the parental alleles from one another. To date, DNA methylation remains the best characterized epigenetic modification known to be involved in genomic imprinting. Mouse gene targeting experiments directed at several DNA methyltransferase enzymes (Dnmt1, Dnmt1o, Dnmt3a, Dnmt3L) have shown DNA methylation to be essential for normal imprinted gene expression. The majority of imprinted genes that have been characterized harbour differentially methylated regions or DMRs which, if deleted, can result in the aberrant expression of both the genes themselves and in some cases their flanking genes as well. By definition, the molecular feature of the underlying imprint is required to have a number of characteristics. It must be heritable yet reversible and able to influence gene transcription. The properties of DNA methylation satisfy each of these requirements. 5-methylcytosine patterns are reversible and also heritable as maintenance methyltransferase activity allows for DNA methylation profiles to be faithfully copied to daughter strands after cell division.

The 'life-cycle' of imprints

The 'life-cycle' of genomic imprints can generally be divided into three windows or phases: erasure, establishment and maintenance, as depicted in Figure 2. Prior to the establishment of imprints during germ cell development, somatic imprints inherited by the primordial germ cells (PGCs) must be erased. This erasure event must occur so that imprints can be 're-set' according to the sex of the developing embryo. Studies investigating imprint erasure have shown that this occurs in PGCs at roughly 12.5 days post-coitum (dpc) and is accompanied by a dramatic loss of DNA methylation.

Imprint establishment in the germline

The monoallelic expression of imprinted genes requires that the parental alleles be distinguished from each other by some epigenetic mark. As previously mentioned, DNA methylation remains the best characterised modification that distinguishes imprinted alleles. This does not exclude the possibility that other epigenetic mechanisms, particularly those that involve chromatin modifications, are also involved in differentially marking imprinted genes. For example, the parental alleles of a number of genes, including Snrpn and Igf2r, are marked by different posttranslational modifications of histone tails.

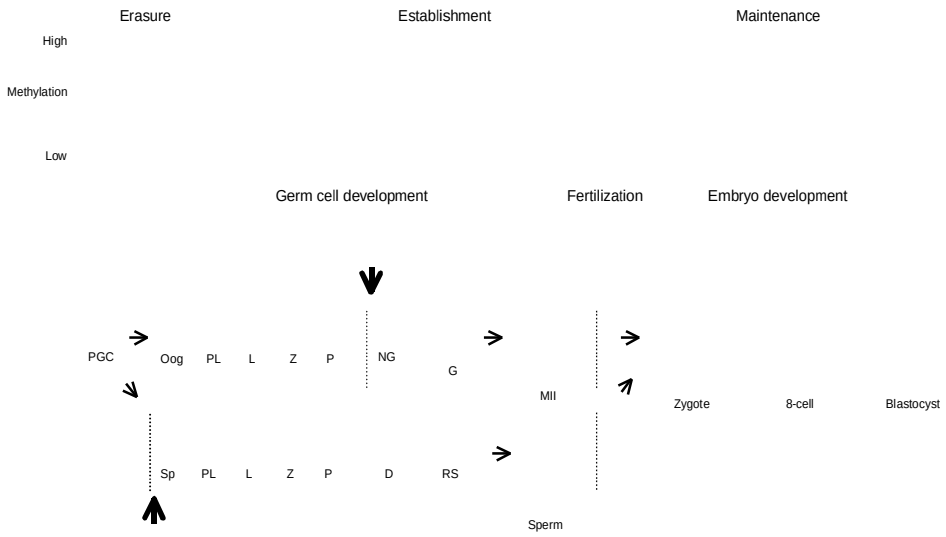

Figure 2. Developmental dynamics of DNA methylation profiles on imprinted genes
The top pa*nel depicts the DNA methylation dynamics of both maternally and paternally methylated imprinted genes, shown by the dark and lighter lines respectively. During germ cell development the methylation profiles of non-imprinted genes is similar to that of imprinted genes. After fertilization the DNA methylation status of the parental genomes does not resemble that of imprinted genes, as illustrated by the lighter lines. The bottom panel depicts the progression of methylation imprint establishment during female and male germ cell development. The shading above and below the female and male germ cells respectively is meant to represent the timing of methylation imprint acquisition. The shading above the preimplantation embryos depicts the maintenance of maternal and paternal methylation imprints after fertilization. Arrows illustrate the stage of germ cell development at birth in the mouse. PGC = primordial germ cell; Oog = oogonia; Sp = spermatogonia; PL = preleptotene; L = leptotene; Z = zygotene; P = pachytene; D = diplotene; NG = non-growing oocyte; G = growing oocyte; RS = round spermatid; MII = metaphase II oocyte.*

While the precise nature and components of the imprinting mark remains the subject of much interest and research, the time window during which these differential imprints are established has been more clearly defined. Gametogenesis is the only period when the maternal and paternal genomes are separate. Therefore, during this time DNA carried by the male and female germ cells can be modified in distinct ways so that when the parental genomes come together upon fertilization the different marks carried by the alleles can be read in a parent-of-origin specific manner. Though unlikely, it remains possible that imprints can also be established differentially after fertilization and before syngamy in the zygote.

Spermatogenesis

Much of our understanding of imprint establishment in the male germ line comes from experiments by Davis et al. (2000) on the acquisition of DNA methylation patterns at the H19 locus during mouse spermatogenesis. H19 is a maternally expressed gene with a germ-line DMR that inherits a DNA methylation mark from the paternal genome; therefore, it is unmethylated in oocytes but methylated in sperm. The DNA methylation status of the H19 DMR was examined in male germ cells at multiple prenatal time points after 12.5 dpc to determine when methylation becomes established at this locus. Detailed bisulfite sequencing indicated that this region was largely unmethylated until 14.5 dpc when analysis of prospermatogonia showed a number of alleles to have acquired methylation. While the level of DNA methylation in spermatogonia was significant, the area of H19 analyzed showed complete methylation in spermatocytes. Interestingly, parental allele-specific analysis allowed the authors to determine the origin of the alleles in the male germ cells and showed that the methylation imprint is acquired at different times for the parental alleles with the paternally inherited alleles being methylated prior to alleles of maternal origin.

Other imprinted genes which have been shown to inherit a paternal methylation imprint in murine germ cells include Rasgrf1 as well as the intergenic DMR between Dlk1 and Gtl2. Human studies investigating DNA methylation imprints in male germ cells have shown conservation of H19 methylation with profiles that have been described in mice.

Oogenesis

Our knowledge on the subject of imprint acquisition is incomplete without an understanding of when imprints become established in both germ lines. Nuclear transfer experiments carried out by Kono and colleagues using nuclei from mouse oocytes at various stages of development were

instrumental in providing a timeframe for when maternal imprints were likely to be established. These experiments pointed to the post-natal oocyte growth phase as the period during which imprints were acquired. Subsequent studies by other groups have furthered this knowledge by investigating the molecular mechanisms underlying maternal imprint establishment.

To complement experiments carried out at the H19 locus in male germ cells, we undertook studies to characterize the DNA methylation status of a number of imprinted genes in postnatal mouse oocytes. An example of our findings for the Snrpn DMR is depicted in Figure 3. Our results showed that maternal methylation imprints for Snrpn, Igf2r, Peg1 and Peg3 are acquired during the postnatal growth phase of oogenesis. Comparative studies indicated that the precise timing of methylation acquisition for each of the genes was somewhat different thereby suggesting gene-specific regulation of methylation imprint establishment. Analysis of Snrpn methylation in growing oocytes of different diameters indicated that the setting down of methylation imprints is related to oocyte diameter in that mouse oocytes isolated from 15 day old juvenile mice that were greater than 50 _m in diameter were hypermethylated compared to oocytes isolated from mice of the same age that measured less than 50 _m in diameter. Using inter-specific hybrids, we also examined allele-specific profiles and compared the DNA methylation status of alleles of maternal vs. paternal origin. In a manner similar to H19, DNA methylation of the Snrpn DMR appeared to be targeted to alleles of maternal origin prior to being established on paternally derived alleles. This observation, which confirmed that in both germ lines methylation gets targeted to alleles that were previously methylated, suggests the existence of some mechanism of epigenetic memory.

Understandably, investigations into the DNA methylation status of imprinted genes in human oocytes have been very few. A study by Geuns et al. (2003) provided evidence that the SNRPN DMR is also methylated in fully grown human oocytes as is the case in the mouse. Given that maternal methylation imprints appear to be established relatively late during oogenesis and because the molecular defect underlying the imprinting diseases associated with assisted reproductive technologies appears to be targeted to the maternally derived methylation imprint, a more thorough understanding of methylation imprint establishment during oogenesis will be important for the continued safeguarding of these technologies.

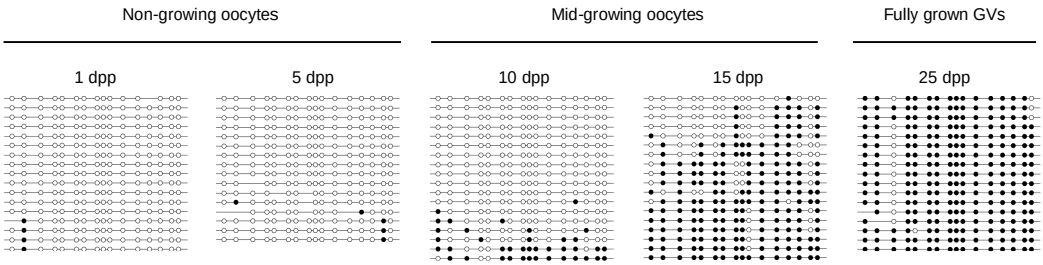

Figure 3. Snrpn methylation profiling during oocyte development in the mouse
Representative bisulfite sequencing results for the imprinted gene Snrpn are depicted. Circles represent CpG sites; white circles depict an unmethylated site; black circles represent a methylated CpG; missing circles depict sites where sequencing data was unclear. Snrpn alleles gradually become methylated during the postnatal oocyte growth phase in the mouse. Dpp = days post-partum; GVs = germinal vesicle stage oocytes.

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Beckwith - Wiedemann syndrome in art conceived children

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Learning objectives :

- to describe 11p15 region organization as a model of imprinted region
- to list the clinical and molecular abnormalities associated with Beckwith-Wiedemann syndrome
- to list the arguments in favour of a link between ART procedures and imprinting defects

Introduction

Assisted reproductive technologies (ART) have dramatically changed the treatment of infertility and account now for 1-1.5% of births in Western countries. Moreover, the popularity of some technologies such as intracytoplasmic sperm injection (ICSI) is increasing. Although most studies of children born after ART were reassuring, large-scale case-control studies of ART outcomes, including long-term follow-up, are lacking.

Epigenetic modifications are important for genome function and are involved in various physiological and pathological processes. Epigenetic mechanisms do not affect the DNA sequence but involve DNA methylation and chromatin modifications such as histone acetylation, methylation, and phosphorylation. Genomic imprinting is an example of epigenetic inheritance in Eutherian mammals and results in monoallelic expression of genes depending on the parental origin of the allele. Epigenetic marks, including genomic imprinting, are reprogrammed during normal gametogenesis. After fertilization, imprinting marks are maintained through somatic cell divisions and read by the transcription machinery.

Imprinted genes play a crucial role in mammalian development and particularly in the regulation of fetal growth, placental function and cell differentiation. Aberrant imprinting results in numerous human disorders, including birth defects, behavioural disorders and cancer.

Recently, syndromes involving epigenetic alterations have been reported to occur in animals and humans conceived by ART techniques. These include Large Offspring Syndrome (LOS) in sheep and cattle, Beckwith-Wiedemann syndrome (BWS), and Angelman syndrome (AS) in human.

Beckwith-Wiedemann syndrome: a model imprinting disorder

BWS is a model imprinting disorder characterized by pre-and/or postnatal overgrowth, macroglossia, abdominal wall defects, hemihyperplasia, hypoglycaemia, ear abnormalities and an increased risk

of childhood tumours. BWS is caused by dysregulation of the expression of genes within the imprinted 11p15 region. The 11p15 region includes two imprinted subdomains controlled by two different imprinting centers (IC1/DMR1 and IC2/DMR2 for the telomeric and the centromeric domains respectively). The telomeric domain includes H19 and IGF2 and the centromeric domain includes CDKN1C (p5KIP2), KCNQ1, KCNQ1OT (LIT1), TSSC5 and TSSC3 (Figure 1).

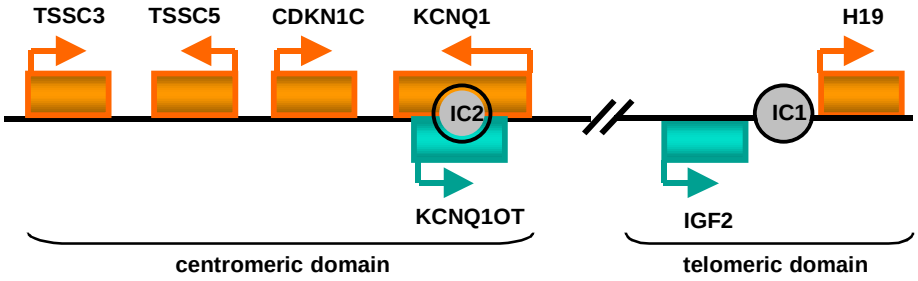
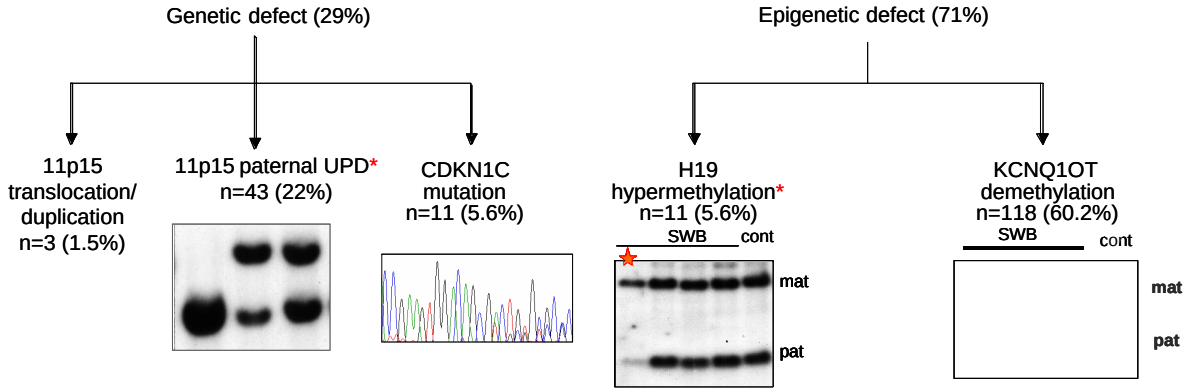


Figure 1: *Schematic representation of the imprinted 11p15 region. Maternally expressed genes are shown in orange and paternally expressed genes are shown in blue. IC1/DMR1 is methylated on the paternal allele and IC2/DMR2 is methylated on the maternal allele.*

BWS has been associated with a variety of genetic and epigenetic alterations on chromosome 11p15 (1 and Figure 2). Approximately 1-2% of patients have maternal translocations or paternal duplications of 11p15. About 20% of patients exhibit somatic mosaicism for paternal uniparental disomy (UPD) of both the centromeric and telomeric domains. Another 5% of BWS cases involve germline null mutations in the maternal allele of CDKN1C, a maternally expressed gene encoding a cyclin-dependent kinase inhibitor. CDKN1C mutations account for most familial cases. Ten percent of BWS patients involve imprinting defects of the maternal alleles of the H19 and IGF2 genes within the telomeric domain. The normally unmethylated maternal allele is hypermethylated at IC1/DMR1. This defect is responsible for the silencing of the maternal H19 gene whereas the IGF2 gene is overexpressed. Finally, the most common epigenetic alteration associated with BWS (50-60% cases) is the loss of methylation at IC2/DMR2 associated with loss of imprinting of the KCNQ1OT and CDKN1C genes. The KCNQ1OT gene encodes a noncoding antisense transcript within intron 10 of the KCNQ1 gene and might be involved in the regulation of parental imprinting of the centromeric domain of the 11p15 region. Very recently, it was shown that microdeletions within IC1 or IC2 account for a low percentage of BWS cases with hypermethylation of H19 or demethylation of KCNQ1OT1. However, the exact frequency of these microdeletions is still unknown. Hypermethylation of the H19 gene and 11p15 UPD are associated with predisposition to childhood tumours in BWS. CDKN1C mutation and demethylation of KCNQ1OT are associated with abdominal wall defects, such as exomphalos.

Figure 2: *Genetic and epigenetic 11p15 abnormalities associated with Beckwith-Wiedemann syndrome (French series; n=196). *: Genotypes associated with tumour risk. Cont: control Becwith-Wiedemann syndrome and art*



Recently, a series of reports have suggested that ART may increase the risk of BWS (2-5). These reports were issued from BWS registries of four different countries (USA, UK, France, and Australia) and compared the prevalence of ART in a group of BWS patients with the prevalence in the general populations of these countries. De Baun et al. (2) identified 7 BWS patients born following ART (four from an initial registry and three from a prospective study). From their prospective study, they reported a sixfold increase in prevalence of ART (4.6% vs. 0.76% in the general population). Four of the six patients whom DNA was available exhibited an isolated demethylation of the KCNQ1OT gene. Maher et al. (3) reported a fourfold increase in prevalence of ART (4% vs. 0.997% in the general population) and demonstrated that two of the six patients whom molecular analysis could be done, exhibited an isolated demethylation of KCNQ1OT. In the French study (4), we used a registry of 149 patients molecularly diagnosed as BWS, since all of them exhibited genetic or epigenetic defects at the 11p15 locus. We reported the same numbers (six of 149) as Maher et al. and showed that representation of ART (4%) in our series of BWS patients was three times higher than that in the general population (1.3%). The six BWS patients born following ART exhibited the same epigenetic abnormality (isolated demethylation of KCNQ1OT). Because the British and French studies were retrospective studies and a detailed reproductive history was not available for all BWS patients, the frequency of ART cases may have been underestimated. More recently, in an Australian case-control study, Halliday et al. found that nearly 11% (4/37) of the BWS patients but less than 1% (1/148) of the matched controls were born following ART, giving an odds ratio of nearly 18 (5). Furthermore, they estimated the risk of BWS after ART procedures to be 9 times greater than in the general population. Once again, demethylation of KCNQ1OT was the only detected abnormality (3/3 molecular analysis). These studies are summarized in table 1.

Table 1: Summary of studies of BWS after ART

Series	Gicquel et al 2003	Maher et al 2003	Debaun et al (2003) 2 registries		Halliday et al 2004
Nature of the study	retrospective	retrospective	retrospective	prospective	case-control
BWS definition	molecular	clinical	clinical		clinical
N° in registry	149	149	-	65	37 BWS
N° of ART cases	6	6	4	3	4
Odds ratio	3.2	3.5	?	5.7	17.8
(95% CI)	(1.7-7.3)	(1.5-8.8)	?	?	(1.8-432.9)
Molecular analysis					
N° analyzed	6/6	2/6	6/7		3/4
Demethylation of KCNQ1OT	6/6	2/2	4/6		3/3
ART procedures					
Sperm	6/6 ejaculated	?	6 ejaculated/1testicular		?
ICSI	2	3	5		3
IVF	4	3	2		1
Frozen embryo	1/6	?	?		3/4
Transfer day 2/3/5	4/1/1	?	?		4/0/0

Children with BWS conceived after ART had similar clinical features as children conceived naturally. As shown in table I, these children were issued from various ART procedures: classical IVF, ICSI, embryo cryopreservation, transfer on day 2, day 3 and day 5. More recent procedures like ICSI or blastocyst transfer did not prevail over other techniques. In a series of 19 BWS patients born after ART, no specific ART method, specific in vitro media, or timing of embryo transfer was identified (6). As in the french studies, the cause of infertility varied from primary male or female infertility to mechanical problems (e.g., tubal disease or uterine factor).

Numerous genetic and epigenetic mechanisms are involved in BWS but all BWS patients born after ART [except one from (1)] exhibit isolated partial demethylation of IC2/DMR2 involving loss of imprinting of the KCNQ1OT gene. Notably, this epigenetic alteration in BWS patients born after ART is the same as the alteration exhibited by BWS monozygotic twins. The prevalence of monozygotic twinning is highly increased in BWS patients and BWS is nearly always discordant in pairs of identical twins (7). The affected and unaffected twins always exhibit a demethylation of the KCNQ1OT gene, suggesting that the KCNQ1OT locus is vulnerable to demethylation at a critical stage of preimplantation development and that this loss of imprinting could predispose to twinning. A model involving failure of Dnmt1o to maintain the methylation imprint at KCNQ1OT at the 8 cell-stage has been proposed by Bestor et al. (8).

It is remarkable that in the different imprinting disorders (LOS, BWS and AS) described following ART, the epimutation involves a loss of maternal allele methylation (demethylation of KCNQ1OT in BWS, demethylation of SNRPN in AS and demethylation of DMR2 of the IGF2R gene in LOS). Nevertheless, the methylation status of imprinted regions other than the 11p15 or 15q11-13 regions, has not been investigated in ART-conceived BWS or AS patients.

At this stage, the underlying mechanism for the association of imprinted disorders and ART is unknown. Gamete and embryo manipulations used in ART might interfere with genomic imprinting, by altering the erasure, the acquisition or the maintenance of imprints during germ cell maturation or early embryogenesis. The acquisition of maternal methylation marks occurs during oocyte growth. Procedures such as superovulation might alter imprint acquisition in oocytes, and more particularly, imprint acquisition of genes that acquire their imprints late during oocyte growth. To our knowledge, the timing of acquisition of imprint on the KCNQ1OT maternal allele has not been studied. Alternatively, ART might alter the maintenance of methylation imprints in preimplantation embryos. Studies in the aetiology of LOS and the BWS twinning model suggest that in vitro culture conditions might be implicated in the pathogenesis of the epimutations in ART-associated BWS and AS children. To better approach the cause of the association between imprinting disorders and ART, it is now very important to precisely record the different procedures used at each step of the stimulation protocol and the in vitro treatment, including the underlying cause of infertility.

Conclusions

Although the published data in humans (2--5) are case reports or small studies, an association between ART and epigenetic alterations is likely. There is now an urgent need for large-scale and long-term outcome studies of children born after ART and more basic research on animal models (9-11). These studies will allow to estimate the exact risk of imprinting disorders after ART and to identify the underlying cause of this association. These prospective studies will also allow to test if some disorders more frequent in ART-conceived children, such as intrauterine growth retardation, are caused by epigenetic alterations and if other epigenetic alterations that have not yet been recognized (involving imprinted or not imprinted regions) are favoured by ART. Moreover, in regards to the role of epigenetic mechanisms in cancer and the report of a risk of retinoblastoma

following ART (12), a particular interest should be given to the assessment of cancer in ART-conceived subjects.

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Critical analysis of epidemiological data on epigenetic risk in ART conceived children

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Learning objective

- A series of case reports and small studies have suggested that ART technology might induce an increased risk of imprinting disorders such as Beckwith-Wiedemann syndrome.
- The significance and implications of these findings are discussed.
- It is speculated that epigenetic errors might account for a wider spectrum of ART related complications
- Further research priorities are discussed.

Introduction

More then 1 million children have now been born worldwide after reproductive technologies (ART), and expansion of newer techniques such as ICSI and embryo biopsy is still continuing. In the meanwhile, evidence has accumulated through a number of studies on the outcome of ART that there is an increased risk of major birth defects: a risk that's approximately 30-40% higher than in the general population (Hansen et al. 2005). In addition, compared to babies conceived naturally, there is an increased risk of intra-uterine growth retardation and a higher percentage of children with low birthweight and very low birthweight even in singleton births (Shieve et al. 2002; Jackson et al. 2004). As epidemiological data suggest, there is a link between intra-uterine growth retardation and insulin insensivity and cardiovascular disease in later life (Barker et al.1993), so these findings may have implications for the lifelong health of ART children.

In the previous studies no cause was identified to explain the findings of growth retardation and increased malformation rate, and it was unclear whether the technology used, the hormonal stimulation or the underlying infertility could account for them. Recently, a possible link was made between ART and malformations through the publications of imprinting disorders in ART children.

Imprinted genes in development and in human disease

Imprinted genes are differentially expressed according to their origin in oocyte or spermatozoon. Only a minority of the human genes (approximately 50) are imprinted genes, representing 0.1-1% of all genes but they play a key role in embryonic and placental growth and postnatal development. Paternally expressed genes enhance growth, maternally expressed genes repress growth. Besides their important role in growth regulation, imprinted genes also influence brain function and behaviour. In addition to their contribution to development, imprinted genes are also frequently misexpressed in cancer.

Although imprinting disorders were rarely described as complications in ART until recently, epigenetic errors (methylation, chromatin structure) might account for a wider spectrum of ART related complications such as embryonic and placental growth and development (eg IUGR described in ART) as well as tumor suppression (eg retinoblastoma reported in Dutch population by Moll et al. 2003).

Animal data

Imprinting-related problems were already reported in animal research more then 10 years ago without creating too much concern in the medical world of human ART. In the meanwhile, however, there has been accumulating evidence from animal research.

Already in 1993, Reik et al intriguingly demonstrated that the frequency of epigenetic changes can be influenced by the constituents of the culture medium (Reik et al.1993; Doherty et al. 2000; Kosla et al.2001). A direct link between ART, imprinting disorders and fetal growth in sheep was provided by the demonstration of Large Offspring Syndrome (LOS) by Young et al. in 1998. LOS is associated with loss of methylation at an imprinting control site in IGF2R (Young et al. 2001, 2002). However, whether IGF2R is imprinted in humans is still controversial and so its relevance to clinical practice remains unclear.

Khosla et al. (2001) have shown that the culture of preimplantation mouse embryos affects expression of imprinted genes and fetal development.

Reasons for concern

The risk of imprinting related disorders is thus founded by animal data suggesting that in vitro culture conditions may be associated with disorders in genomic imprinting and produce alterations in intra-uterine growth. Special caution should also be given to the use of immature gametes in ART since their imprinting processes may be incomplete. This could be the cause in particular in case of in vitro maturation of immature human oocytes.
There are several suggestions in the literature that the increased risk of imprinting disorders might be related to the ovarian stimulation procedure or the infertility per se, apart from the in vitro culture conditions itself. Infertility might be associated with a certain genetic predisposition for increasing the transmission of imprint errors to offspring.

Literature data

1. Angelman syndrome

Angelman syndrome located on chromosome 15q11-q13 SNRPN locus ‡ loss of mat UBE3A expression Incidence 1/30 000 Symptoms : neurogenetic disorder with severe MR, absence of speech, delayed motor development, poor balance and jerky movements, outbursts of laughter

A number of case reports have sounded the alarm bell. So far, two children with Angelman syndrome (a neurogenetic disorder) due to a sporadic imprinting defect have been reported after use of the ICSI procedure (Cox et al., 2002). The lack of an imprinting centre mutation and the detection of a mosaic methylation pattern in one of the two patients made an inherited defect unlikely and pointed

to a postzygotic epigenetic defect (De Rycke et al., 2002). The authors put forward the hypothesis that ICSI disrupts the production or function of transacting factors necessary for imprinting of the maternal chromosome 15. Another report on Angelman syndrome was published by Orstavik et al. (2002) which presented one ICSI patient with a sporadic imprint defect.

2. Beckwith-Wiedemann syndrome (BWS)

Beckwith-Wiedemann syndrome Cluster of imprinted genes, maternally and paternally expressed on chrom 11p15.5 Incidence 1/15 000 Symptoms : overgrowth (pre-/postnatal), viscero-megaly, macroglossia, abdominal wall defects, ear anomalies, hemi hyperplasia and neonatal hyperglycemia predisposition to embryonal cancers (Wilms in 11% of the children before 4 years, hepatoblastoma, neuroblastoma…)

A few case reports were mentioned on a series of ART children. In a small case control series of ART offspring after cryopreservation, a single case of Beckwith-Wiedemann Syndrome (BWS) (a human overgrowth syndrome) was reported among 91 cases (Sutcliffe et al., 1995). One case of BWS was reported in a study by Olivennes et al. (2001) on ART children -where no further genetic origin (de novo or inherited) could be determined - reported on a total of 73 children. In the series of Bonduelle et al (2002), 2 ICSI patients with BWS were reported on a total of 5835 ART children (2840 ICSI + 2995 IVF).

Once the scientific interest had grown, a number of studies were published based on BWS registers, calculating the relative frequency of BWS in the ART population in an indirect way. Three retrospective analyses of BWS patients databases were carried out with similar results. DeBaun et al. (2002) reports a total of 7 children with BWS born after ART, of whom 4 were born after ICSI with ejaculated sperm and one after ICSI with testicular spermatozoa. Molecular studies indicated that 5 of the 6 children studied had specific epigenetic alterations associated with BWS (5/6 had imprinting mutations of the LIT1 and/or H19 genes, 2 distinct methylation alterations specific for BWS). Relative risk was evaluated in a prospective part of the study, leading to a frequency of BWS in ART of 6 times the expected frequency in the general population. Another retrospective study by Maher et al. (2003) describes 6 more patients (3 ICSI and 3 IVF) in a BWS register in the UK, which represents a 4-fold increase of the expected number of BWS patients. Loss of methylation was found in 2. Gicquel et al (2003) reported a similar retrospective series of BWS patients in a French BWS register and demonstrated loss of methylation of a maternally methylated BWS allele in all the patients. Finally Halliday et al. (2005) designed a case control study with a control group of 4 matched children (from the Victorian Perinatal Data Collection Unit) with the BWS patients from a single genetic clinic. They calculated the OR for BWS in ART compared to BWS in the general population and found an 18-fold increase of ART (OR 18 ; 95% CI 1.8-432.9) (4 BWS children from ART compared to 37 from natural conception)

In order to determine which factors contributed to the epigenetic modifications Chang et al (2005) studied the influence of different culture media in ART in a retrospective case analysis of mothers of 12 BWS children after ART. Analysis of treatment type: all had some type of ovarian stimulation. No increase in risk due to a specific type of culture medium, type of stimulation, type of ART or type of infertility was found. No phenotypic differences in BWS type between ART en general population (cancer risk not evaluated) were identified.

3. Prader Willi syndrome

Prader-Willi syndrome located on chromosome 15q11-q13 SNRPN gene ‡ loss of paternal gene expression Incidence 1/15 000 Symptoms : hypotonia, respiratory distress, failure to thrive (post neonatal) hyperphagia in early childhood, obesity

Manning et al. did not find any abnormal methylation pattern on chromosome 15 in 92 cases of ICSI children (Manning et al., 2000), but such a small sample size does not exclude a small but increased risk of imprinting disorders.

4. Retinoblastoma

Retinoblastoma located on chromosome 13q14 RB1 tumor suppressor gene Incidence 1/17000 livebirths (uni and bilat) Most new mutations in paternal germline (methylated)

Moll et al. (2003), reported 5 patients (4 IVF and 1 ICSI) in a Dutch Retinoblastoma Register and calculated a Risk Ratio of 4.9 (1.6-11.3) –to RR 7.2 (2.4-17.0) (based on the assumption of 1.5% or 1% IVF in the general population) for retinoblastoma in ART patients.

Critical analysis of literature data

The initial reports linking ART with Angelman syndrome seemed to suggest that ICSI might play a predominant role (Cox et al. 2002; Orstavik et al. 2003). However, of 19 ART related BWS cases reported in the three studies, only 9 have involved ICSI, whereas this technique accounts for up to 80 % of the ART procedure. It is therefore not ICSI per se that is the major determinant of the observed association between ART and imprinting disorders.

From the epidemiological data on BWS there is evidence of a link between ART and imprinting disorders. Although the sample size of each of the 3 studies on BWS is small, with large confidence intervals, they all point in the direction of a higher risk for BWS in ART children, varying from 3 to 6 times more BWS in ART. Further interpretation of these results has been limited because of the reliance of these studies on case records and questionnaire data to determine the method of conception in BWS cases, a lack of appropriate controls and a statistical significance that was either borderline (Maher et al. 2003; Gicquel et al. 2003) or not mentioned (Debaun et al.2003). Furthermore, the frequency of IVF in the general population was estimated in the studies rather than really measured. In order to circumvent these problems, Halliday et al. (2005) made a case control study with the BWS patients from a single genetic clinic. Their calculations of OR for BWS in ART was even higher (OR 18; 95% CI 1.8-432), but with a large confidence interval due to the relative small size of the study.

Further evidence of a real association between BWS and ART is given by the genetic analysis. Thirteen out of 14 patients analysed demonstrate a loss of methylation at a differentially methylated region. (KvDMR) within the KCNQ1 gene. Normally the paternally inherited allele for this gene is unmethylated and the maternal is methylated. In 40-50% of the sporadic cases (non ART) there is loss of this maternal methylation so that the IGF2 (a gene within the same cluster) expression can

be altered. The frequency of KvDMR loss of methylation among ART-BWS children is significantly higher than in non-ART-BWS children (Engel at al. 2000), so that the association of BWS with ART seems to result predominantly from a increased risk of epimutations.

This risk of epimutations also seems to be confirmed by the Angelman data. The findings of epimutations in an Angelman patient (as was the case for all three) accounts for < 5% of all cases and is estimated to occur in 1/300,000 newborns. Therefore three children with epimutations would be expected to occur in 1/900,000 newborns. Since 1978 approximately 1million children have been born after ART and it is unlikely that all cases of Angelman have been reported. These 2 reports therefore raise the possibility of a specific imprinting disorder related to ART.

Conclusion

The epidemiological evidence associating BWS and Angelman syndrome with ART is still tentative and does not establish a causal link. Estimation of this risk is still low with an estimated risk of 1/ 3000 for BWS based on register data.

However, there is more evidence from animal data that in vitro culture might cause epigenetic effects. The fact that “large offspring syndrome” is reminiscent of the BWS syndrome reinforces this concern.

In both Angelman and BWS a loss of maternal imprint is described which makes it unlikely that sperm differentiation or ICSI itself is involved. Imprinting seems related to both ICSI and IVF.

There are also epidemiological data from the ART follow-up pointing to an adverse neonatal outcome. These findings could be related to impaired imprinting processes but are not yet understood. Mutations for IUGR are not yet defined and candidate genes need to be found. If this hypothesis is true imprinting disorders could play a more prominent role in the further development of ART children.

It is therefore time to organize better-designed prospective and multi-centre studies since “rare events” have to be researched. These studies should include a follow-up of physical as well as neurobehavioral development and an investigation on cancer incidence.

There is also a need for basic research on human embryos, for research on the imprinted gene function in the different ART procedures, and for studying risk in subgroups (eg non-obstructive azospermia, immature sperm, immature oocytes and outcome of frozen-thawed embryos)

Which factors are involved in the different steps of the fertility treatment has to be further clarified. Hormonal stimulations, culture conditions of the ovum and infertility-associated predisposition to epimutations will have to be analysed.

In the meanwhile, parents should be informed about a possible slight increase in the risk of imprinting disorders.

Suggested reading

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Epigenetic risks on art conceptus. How far long-term follow up of children could be improved?

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Learning objectives:

Participants will appreciate the difficulties to obtain reliable data on the individual risk of having a child with an epigenetic disorder of a very low incidence in the background population as well as in IVF-cohorts of children.

The ability to assess different options for data-collection will be developed.

Participants will be able to discuss also the possibility of a “limit of certainty” regarding the absence of any risk increase, the “zero- risk hypothesis”.

Search for data on different levels in society and with different techniques will be presented.

Background:

Recent observations on animals and on humans indicate the possibility of an increased risk of epigenetic disorders after assisted reproductive technologies, ART. The risk increase, if any, of these low frequency events is yet to be established.. The question on how best to tackle these problems needs to discussed. Data on risks with a higher incidence is being collected already at present, through different techniques, often utilizing IVF National and/or Regional and even Global IVF Registers.

Strategy options:

Data can be collected on at least four different levels: individual clinics, countries, regions and globally.

To give reliable indications of low frequency events, aggregation of data on the national level in the case of large population countries or else on a regional or even the global level is desirable or even necessary, to give researchers a big enough population from which to collect the data.

Large case-control studies is one option but several problems are attached to this technique: How to find large enough numbers of these rare cases? How to verify that the diagnosis is correct? When, in life, can the diagnosis be expected to be given to the person involved? How to establish the correct number treatments in the population at large?

Different solutions may be at hand for different settings. Some epigenetic syndromes are diagnosed or verified at genetic laboratories, who can be asked to report cases diagnosed in that laboratory. Other syndromes may rely only on clinical observation and therefore may be less well reported.

Some countries have an (almost) exact knowledge of the number of treatments in that particular country, whereas other countries do not.
Therefore, large international studies including national data from many countries are probably impossible to carry through. An example of the opposite may be at hand, however, with countries having a very similar organization in their data collection systems and where data collection actually covers the whole population. This is the situation among the five Nordic countries of Denmark, Iceland, Finland, Norway and Sweden. Plans are developed at present to aggregate safety data from all these countries to form a database that would include some 50.000 IVF-children born in these countries and with complete data-collection systems.

Another option, apart from large case-control studies, would be to utilize the existence in some countries of population based health registers (PBH-registers).

In the presence of a system with personal identification numbers (PIN) in a country, having PBH-registers running already, specific IVF-registers have been created, which are then cross-linked to PBH-registerss covering for example cancers,

malformations, causes of death, hospital discharge diagnoses and the outcome of births. Such a situation does exist for example in Sweden where, therefore, very reliable data on the safety of IVF children have been obtained.

The Swedish IVF register at present includes some 20.000 children, which number may be still to small to establish reliable risk data for epigenetic errors, but the plan to aggregate data from all the five Nordic countries (all with similar data resources) may be a solution. If this turns out to be too complicated or even legally impossible, we will have to wait for the Swedish register to grow larger, which it does by some 3000 IVF-children per year.

A third option, with data aggregated on a regional or global level, works well for IVF data on efficacy, the number of embryos replaced and multiple pregnany rates and possibly delivery rates, but not very well so for detailed safety data. Problems with definitions, coverage and validation etc are still major problems.

Data composition

Problems that need to be looked into, when considering epigenetic risks, involve also data on cancers and malformations.

Different types of, now, traditional IVF- techniques (standard-IVF and ICSI), as well as newer techniques (IVM, in-vitro maturation), need to be considered as well as the different infertility diagnoses.

Laboratory procedures, like different culture media, needs also to be documented.

Risk limits

To establish a zero-risk for low frequency events is difficult and indeed, in practice, not possible. At present, reliable cohort based data can be collected from some 20.000 children and soon, possibly, from some 50.000 children.

Case-control studies in large countries may be able to establish risk levels based on even larger

"theoretical" populations but data from such studies may always be less reliable.

In practice, and for the individual patient, it may be sufficient to state that the risk is not greater than 1:10.000, but that remains to be discussed.

Conclusion

Follow-up of children born after different types of ART and IVF techniques and different infertility backgrounds needs to be improved also for low frequency events like epigenetic errors.

Specific technical problems need to be tackled here.

Events to look for include different imprinting disorders, cancer and malformations.
Different strategic options have their pros- and cons- in different settings.

A "zero-limit" for risk estimation may need consideration.

Additional readings:

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