REVIEW

Epigenetic Reprogramming and Assisted Reproduction

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Abstract

Since the birth of the first IVF baby in 1978, assisted reproductive technologies (ART) have revolutionized the treatment of infertility and a broad spectrum of ART had become available so far. Despite the safety record of ART on perinatal outcomes, like birth weight alterations, aneuploidies and fetal malformations, it is important to monitor children born of ART for longer time, few of whom have yet reached adulthood. In this review, we will analyze the safety aspect of assisted reproduction at the epigenetic level based on brief overview of epigenetic reprogramming in the gamete and early embryo. Furthermore, interference of ART with epigenetic reprogramming as well as the possible epigenetic inheritance will be discussed. The phenotypes associated with epigenetic defects, is difficult to recognize in short-term studies. Moreover, a complete safety evaluation may even require studies from a two-generation view.

Keywords: reproductive techniques, assisted, genomic imprinting, imprinted genes

Özet

Epigenetik Yeniden Programlama ve Yardımcı Üreme Teknikleri

1978'de ilk yardımla üreme teknikleri (YÜT) bebeğinin doğumundan bu yana, YÜT infertilite tedavisinde devrim yaratmış ve bugüne kadar geniş bir spektruma ulaşmıştır. YÜT'nin, doğum ağırlığı, anöploidi ve fetal malformasyonlar gibi perinatal sonuçlarda güvenilirliğini bildiren yayınlara rağmen, henüz erişkin dönemine ulaşabilmiş olan bu YÜT çocuklarının daha uzun dönem izlemi önem kazanmıştır. Bu derlemede, yardımla üremenin epigenetik düzeyde güvenilirliği, gamet ve erken embryo döneminde epigenetik yeniden programlama üzerine genel bir bakıştan sonra analiz edilecektir. Ayrıca, YÜT'nin epigenetik yeniden programlama üzerine etkileri, olası epigenetik kalıtımla birlikte tartışılacaktır. Epigenetik defektlerle ilişkili fenotiplerin kısa dönem çalışmalarda tanınması zor olduğu gibi, tam bir güvenilirlik profili, iki jenerasyon içeren çalışmaları gerektirmektedir.

Anahtar sözcükler: yardımla üreme teknikleri, genomik imprinting, imprinted genler

Introduction

Assisted reproduction, with its widespread use and success, allowed conceivement of one in ten people of reproductive age who had fertility problems (1). Although follow-up studies of children conceived with assisted reproduction have revealed normal birth and development, potential long-term risks associated with the possible disturbance of epigenetic phenomena brought about by embryo culture and manipulation in ART have not been studied well because these techniques are relatively new for these long-term consequences. In particular, publications over the last year have argued about the possibility of an increased incidence of rare genomic imprinting disease such as Angelman Syndrome and Beckwith-Wiedemann syndrome in children conceived with ART (2-6). Despite acceptation of the epigenetic dysregulation for somatic cell nuclear transfer technology (7), debate surrounds the idea that ART may be susceptible to similar yet less stated impact. The mechanisms and timing of critical events of genomic imprinting occur in gametes and an embryo coincides with the use of ART that bypasses essential reprogramming steps in gametogenesis and early embryogenesis. These fundamental steps of the genomic imprinting are erasure, establishment, and maintenance. This review intends not to describe overall outcomes of ART pregnancies but to focus on possible alterations at the epigenetic level.

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Genomic imprinting

The expression of a few genes in the human genome depends on whether they are located on the maternal or on the paternal chromosome. This phenomenon is called genomic imprinting. In contrast to the Mendelian view of gene action, a cohort of our genes, at present more than 60, is known to be subject to genomic imprinting. Genomic imprinting is not random as

inactivation of one X chromosome, but depends on the parental origin of the chromosome. For certain genes, only the maternal allele is active, whereas for others the paternal allele is active (maternal allele is imprinted or silenced). Two parental alleles maintaining different epigenetic profiles result in the monoallelic expression of imprinted genes. Epigenetic describes a hereditary process, which regulates gene activity without affecting the genetic code. The genetic information of a DNA sequence cannot be complemented without epigenetic modifications. Epigenetic patterns are instituted on the genome during differentiation through predetermined programs. Additional epigenetic changes allow cells to defense to environmental factors by modifying the expression level of the gene without having to change the DNA code itself. Something other than DNA sequence must distinguish the parental alleles and determine sex-specific gene expression. Therefore, imprinting is an epigenetically controlled phenomenon.

Evolutionary role of genomic imprinting:
A complementary role of the parental genomes

Nuclear transplantation experiments have proved the fact that both maternal and paternal genomes are required for a normal mammalian development and growth by presenting the non-viability of uniparental embryos (8-10).

Mouse androgenetic embryos, created from a zygote with two male pronuclei lacking of any maternal genome demonstrated proliferation of extraembryonic tissues and indigent embryonic development. In contrast, parthenogenetically activated oocytes gave rise to relatively normal embryos that survive to an early embryonic stage with failure of extra embryonic tissues. These observations propose that genes expressed by the maternal genome appears to be adjusted towards expressing genes that contribute to proper embryo development while paternal genome are directed towards the development of extra embryonic tissues essential to support the growth of the embryo.

Several theories of imprinting have been suggested so far. The "parental conflict'' hypothesis also known as "the battle of the sexes'' suggested that the paternal genome has evolved to express genes that favor the extensive use of maternal resources and lead to optimal fetal development and growth, thus ensuring transmission of the father's genes to the next generation (11,12). On the other hand, genes expressed by the maternal genome serve to counteract the attempt made by paternally expressed genes, and limit investments in embryo development and growth in favor of rescuing resources for future pregnancies. The "parental conflict theory'' has been extended to postnatal effects as well, including effects on maternal behavior. Mothers homozygous for targeted mutations in the paternally expressed Peg3 and Peg1 genes have defects in nurturing behavior (13,14). Besides the "conflict theory,'' a more "cooperative'' theory proposes that genomic imprinting makes sex necessary since both genomes are complementary. Because of sex, genetic variation increases, that is advantageous for the species. Alternate and additional theories have also been proposed (15-19).

In the human, similar observations have been made respectively in teratoma and in the complete hydatiform mole (20). Teratoma may arise from parthenogenetically activated oocytes with duplication of the maternal genome, and thus contain only maternal chromosomes. This leads to a tumor consisting of different tissues from all three germ layers, but without extra embryonic components, indicating that maternal genes are necessary for the development of the embryo itself. In contrast, in the complete hydatiform mole, there is an abundance of placental tissue, but absence of embryo and fetal circulation. The most frequent mechanism leading to a complete mole is the fertilization of an oocyte devoid of a maternal pronucleus, followed by a duplication of the paternal chromosomes. Briefly, genes on the paternal genome are required for development of the placenta whereas certain maternal genes are essential for the development of the embryo itself.

In conclusion, the functional and sex-specific non-equivalence of imprinted alleles explains the developmental failure of uniparental embryos and confirms the requirement of both parental genomes for normal development.

Mechanisms of genomic imprinting

Epigenetics covers a broad spectrum of effects: allele-specific DNA methylation, antisense transcripts, noncoding RNA including micro RNA, covalent modifications of histones and remodeling by other chromatin-associated complexes. The observation that DNA methyltransferase complexes associate with histone deacetylases propose co-operation between overall chromatin state in the regulation of imprinted gene allele-specific expression.

The role of DNA methylation in genomic imprinting has been extensively investigated, and numerous studies have confirmed its crucial role in the epigenetic process (21). In general, the two parental alleles have different levels of DNA methylation, and in many cases, the methylation is concentrated in a single area, called a differentially methylated domain, within or near the imprinted gene.

There are at least two critical periods in which epigenetic reprogramming occurs, one during gametogenesis and another during the preimplantation embryonic stage (22). Imprints established in the gametes must be faithfully maintained during preimplantation development while the methylation status of non-imprinted genes undergoes dynamic changes.

Experiments on mice suggests that upon every reproductive cycle, genomic imprints in the parental gametes are erased, reestablished in the immature germ cells of the developing embryo according to their fate as either male or female gametes, and maintained through both the preimplantation period as well as postimplantation development. As such, imprints are dynamically changing during both germ cell and embryo development. As imprinted alleles are differentially marked to allow for their sex-specific expression, gametogenesis and the zygotic stage of embryogenesis periods during which

they are uniquely separate must be when the marking event occurs. Imprints are erased, prior to the establishment of these sex-specific marks in the germ line. Following this erasure, the timing of acquisition of genomic imprints between the two germ lines is significantly different.

Although several enzymes have been presumed to be involved in either erasing imprints or marking imprinted genes for parental allele-specific expression with the exception of the DNA methyltransferase (DNMT), little is known.

Erasure of imprints may take place as little as 24 h, at about the time when the germ cells initially enter the gonad. This observation suggests an active erasure process, although the identity of the enzymes or the molecular complex that is responsible for this demethylation is unknown. A complete defect of imprint erasure would result in half of the gametes maintaining an inappropriate imprint and carrying the opposite sex's epigenotype at certain imprinted loci.

Acquisition of imprints, The DNMT involved in the acquisition of methylation imprints in the male germ line are currently unknown; however, DNMT3A and DNMT3L are presumed to be involved since male mice with targeted knockouts of the genes encoding these enzymes have abnormalities in spermatogenesis. DNMT1 is not required for the acquisition of imprints in female germ cells. Moreover, DNMT3L is supposed to be a regulator of maternal imprint, which may interact with known (DNMT3A or DNMT3B) or unknown DNA methyltransferases. Establishment defects could result in absence of an imprint at a specific locus and again lead to the gametes concealing the opposite sex's imprint epigenotype.

Maintenance of imprints, Gene-targeting studies revealed that DNMT1 is required for the maintenance of DNA methylation patterns on imprinted and non-imprinted genes in the postimplantation period (23) with other possible unknown DNMT involvements. Defects in imprint maintenance could occur at any stage of pre- or postimplantation embryo development.

Genomic imprinting defects may occur at any of the described stages. Defects at any of these stages may arise because of problems with the enzymes responsible for erasing, establishing, or maintaining imprints. Alternatively, epigenetic damages may cause changes in the chromatin conformation or methylation status within imprinted genes, leading to abnormal expression patterns.

Additionally, besides ART, imprinting defects may occur sporadically in normal embryos and that the processes of imprint erasure, establishment, and maintenance are exposed to errors (24).

There appears to be little experimental evidence that defects in genomic imprinting can be repaired. Due to the parental allele-specific nature of imprints, it is difficult to contemplate a mechanism that would allow damaged imprints to be repaired post-zygotically in the embryo.

Fetal development, placental function, human disease, and imprinted genes

It is estimated that the total number of imprinted genes in the mouse and human genomes may range between 100 and 200. Of those that have been described to date, a significant number appear to have important roles in fetal development. Moreover, imprinted genes play important roles in the placenta by regulating the growth of the placenta and/or the activity of transplacental transport systems to control the balance between supply and demand for nutrients. Therefore, defects in imprinted genes expressed in the placenta may be associated with clinical syndromes such as intrauterine growth retardation.

Loss of function of several imprinted genes has been found to be associated with human genetic diseases, the progression of certain cancers, and number of neurological disorders.

Angelman syndrome is caused by a loss of function of the maternal allele or duplication of the paternal allele within a region that spans UBE3A, is characterized by ataxia, hypotonia, severe mental and motor retardation, epilepsy, and absence of speech (25).

Prader-Willi syndrome is associated with a loss of function of the paternal allele or maternal duplication at the SNRPN locus, which is harbored within the same 15q11-q13 region of the Angelman Syndrome. Patients with PWS are generally obese, mentally retarded, of short stature, suffer from muscular hypotonia, hypogonadotropic hypogonadism and have characteristic reduced fetal activity.

Another disease that exhibits parent-of-origin effects in its inheritance is BWS. This disorder is linked to a loss of function of the maternal allele at 11p15 where the imprinting cluster that includes H19, IGF2, CDKN1C, KCNQ1 and KCNQ1OT1 resides BWS is an overgrowth disorder. Main features of the disease are exomphalos, macroglossia, visceromegaly, neonatal hypoglycaemia, umbilical and abdominal wall abnormalities, as well as characteristic indentations of the ear. Children with BWS are predisposed to developing embryonic and childhood cancers.

Silver-Russell syndrome is a disorder characterized by low birth weight, dwarfism, and lateral asymmetry and has been linked to the loss of function of genes within a less well-described imprinted cluster (26). About 7-10% of patients with Silver-Russell syndrome show maternal uniparental disomy for a region on chromosome 7, while patients with paternal uniparental disomy of the same region are unaffected; these findings implicate imprinted genes in the etiology of the disease in a subset of patients.

Tumors that show imprinting effects include Wilms' tumor where, in a subset of cases, loss of imprinting occurs at chromosomal region 11p15, which is in close proximity to the region involved in the pathogenesis of Beckwith-Wiedemann Syndrome. Other cancers suggesting loss of imprinting are hepatoblastoma, neuroblastoma, sporadic osteosarcoma,

rhabdomyosarcoma and choriocarcinoma (25). Furthermore, some imprinted genes act as tumor suppressor genes, the best-characterized being IGF2R and WT1.

Studies of Peg1, Peg3, Ube3a, Grf1 and Gabrb3 knockout mice, as well as mice carrying a uniparental disomy at chromosome 2, suggest a functional role of imprinted genes in cognition and behavior. Many neurological disorders also appear to be inherited in a parent-of-origin-dependent manner. Some examples include bipolar affective disorder, autism, epilepsy, schizophrenia, Tourette syndrome, Turner's syndrome, and late onset Alzheimer's disease (27).

Epigenetic reprogramming and assisted reproduction

ART necessitate gamete, zygote and embryo incubation in synthetic culture medium. *In vitro* culture together with embryo micromanipulation has been accused with abnormal fetal development. It has been proposed that at least some of the problems may result from an accumulation of epigenetic alterations during embryo culture (28). In cattle and sheep, several reports have described an enhancement in fetal growth, which is described as, large offspring syndrome, whereas in mice and humans, there seems to be a reduction in birth weight. It has been shown that imprinting may vary between species, tissues, cells, and stage of embryonic development. It is therefore possible that this contradictory growth disturbances in humans and cattle result from similar epigenetic disturbances.

Although large offspring syndrome phenotype has not been observed in humans born of assisted reproduction so far, this phenomenon has also been described on cloned animals.

The precise mechanisms 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 perturbed by the synthetic culture medium and that this interferes with epigenetic reprogramming and gene expression. The use of prolonged culture systems may deregulate epigenetic mechanisms to a further extent, which is used for selected patients and in preimplantation genetic diagnosis cycles. Follow-up studies comparing blastocyst transfers versus early cleavage-stage embryo transfers showed no differences in birth weight (29,30).

Because of the imprinting status, sperm cells in different stage of maturation are epigenetically quite different. It is not obvious whether imprinting has been completed in immature gametes. From the limited data supposing that the resetting mechanism is similar in mice and humans, it appears that imprint acquisition is completed by the time the spermatid stage is reached. Moreover, spermatid chromatin has not yet been so densely packed in immature sperm cells. The genome may therefore be more vulnerable to events of delayed oocyte activation, such as the delayed inactivation of the metaphase-promoting factor that can cause aneuploidy in the embryo (31). 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 (32). However, there is a case report of two major malformations out of four pregnancies obtained after ICSI with elongated spermatids (33). Another study on a larger series did not detect an increased incidence of malformations (34). 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 higher rate of developmental arrest has indeed been found in embryos derived from round spermatid injection compared with embryos obtained after standard ICSI (35).

Epigenetic inheritance

Epigenetic modifications differ from genetic modifications or mutations in their reversibility. Incomplete erasure of the epigenetic modifications in the germ line results in epigenetic inheritance. Additionally, when epigenetic modifications occur after fertilization but before specification of the germ line, they are transmitted to the next generation. In mammals, the example is derived from epidemiological studies: starvation during the third trimester of pregnancy led to low birth weight during the Dutch Winter of Famine (1944–45). Unexpectedly, increased perinatal mortality and low birth weight were observed in the children of the females, who were not under-weight at birth, but were malnourished in the first and second trimester of their own fetal development (36). This may be explained by epigenetic dysregulation, at the level of fetal germ cells, in response to the malnourishment. 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 birth weight of the IVF and ICSI children will be transmitted to their offspring. In a worst case scenario, where low birth weight results from incomplete epigenetic erasure in the preimplantation embryo during in-vitro culture, both somatic and germ cells will be influenced, as they are not yet separated. This would affect the children as well as their offspring.

Conclusion

Risk assessment studies to evaluate the safety of ART, have risen from interest about birth defects and health problems in children born as a result of these techniques. Timing of the critical events of genomic imprinting, erasure, establishment, and maintenance, coincides with the use of ART. It has been suggested that the accumulation of epigenetic defects during embryo culture may lead to abnormal phenotypes. Furthermore, the accumulation of severe epigenetic disturbances above a certain threshold may lead to early mortality. It is difficult to draw conclusions based on the available data, whether there are other aberrations with an epigenetic origin in ART children. 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. In light of these results, there should be stricter and comprehensive animal testing of existing techniques used in ART for effects on imprinting and such testing should also precede the introduction of new embryo manipulations and technologies into the clinic. A greater organized operation to include more subjects from multiple registries is needed to reach enough power to detect a difference in an unlikely outcome (37). Because an increased cancer risk or neurodevelopmental problems may only manifest themselves in later years, detecting imprinting defects in children conceived by ART is likely to require long-term follow-up.

In conclusion, long-term follow-up studies are necessary in larger series of children in order to be able to estimate long-term risks linked to epigenetic disturbances after ART. A complete safety evaluation may even require studies from a two-generation perspective. Preferentially, studies should be done on human embryos, as results from animal studies cannot always be extrapolated to humans.

Techniques such as bisulphate genomic sequencing and PCR-based expression assays (38,39) now permit imprinting abnormalities (deviation from monoallelic expression or alterations in methylation) to be assessed in single blastocysts. These advances may enable critical human studies to be performed using single embryos.

As the impact of epigenetic disturbances on later life of humans born as a result of ART 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 ART as well as further molecular research are recommended.

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