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# Safety in Assisted Reproductive Technologies: Insights from Gene Expression Studies During Preimplantation Development

Daniela Bebbere, Luisa Bogliolo,
Federica Ariu, Irma Rosati and Sergio Ledda
*Department of Veterinary Clinics and Pathology,*
*University of Sassari,*
*Italy*

## 1. Introduction

The mammalian oocyte and embryo display considerable plasticity. Even in sub-optimal conditions, as in vitro environment may be certainly considered, the oocyte is able to mature, face fertilization and develop first into an embryo and finally to a live offspring. Such ability has encouraged, over the decades, the development of numerous in vitro assisted reproductive technologies (ART) in several species, included human. Nowadays, more than 30 years after its inception, human ART are routinely and successfully applied to solve fertility problems, which affect ~ 15% of reproductive age couples and have significant medical, social and financial implications.

The use of ART has increased steadily over the last years also because of its perceived safety. Worldwide, it is now estimated that more than 3 million babies have been born as a consequence of the application of ART (Grace & Sinclair, 2009). Although the majority of children born after ART are healthy, safety is still a cornerstone for reproductive technologies.

A number of studies have hypothesized that manipulation of conception may negatively affect embryonic and fetal development and possibly have lifelong consequences on the offspring (DeRyche et al., 2002; Thompson et al., 2002). Moreover, abundant evidence from animal species showed that in vitro manipulation during ART influences the genetics and physiologic development of the embryos.

These data support the idea that the earliest stages of life set the basis for the future health of the offspring (Barker, 1997) and highlight an inadequate understanding of the cellular and molecular basis of reproduction. A deeper knowledge about preimplantation development is a fundamental prerequisite for a safer application of reproductive in vitro technologies.

Because of the peculiar characteristics of the mammalian oocyte and pre-implantation embryo, the analysis of gene expression status during the very first phases of life is essential for the evaluation of ART safety. As a matter of fact, disruption in the regulation of gene expression has been often observed as a consequence of in vitro manipulation (Humpherys et al., 2001; Yang et al., 2005; Young et al., 2001).

Importantly, in several species ART has been associated with imprinting disruption. In vitro culture was seen to cause abnormal epigenetic modifications and subsequent deregulation of imprinted genes, in association with early embryonic losses and a variety of abnormal phenotypes (de Sousa et al., 2001; Wrenzycki & Niemann, 2003; Yang et al., 2005; Young et al., 2001). Faulty nuclear reprogramming is considered the primary cause of the reported defects.

Epidemiologic studies have revealed that the use of some reproductive technologies is associated with an increased frequency of imprinting defects in humans as well (De Rycke et al., 2002; Powell, 2003; Stromberg et al., 2002; Thompson et al., 2002). Disruption of imprinting has raised particular concern since it is involved in the etiology of severe developmental disorders in humans (Arnaud & Feil, 2005; Scarano et al., 2005), such as Beckwith-Wiedemann and Angelman syndromes.

Studies on preimplantation embryo development are crucial to gain insight into the molecular mechanisms correlated with an undisturbed embryonic and fetal development and to improve efficiency and safety of assisted reproductive biotechnologies.

Due to the obvious scarcity of human oocytes and embryos for research, the use of appropriate animals models (reliable, cost-effective and featuring the characteristics of human fertilization) is of irreplaceable support.

In this chapter, we will discuss the effect of ART on early embryonic development, focusing on gene expression. The characteristics of the transcriptome of mammalian oocytes and pre-implantation embryos will be described, as well as the reprogramming events that take place during oocyte-to-embryo transition. The effects of ART on regulation of gene expression and on imprinting will be examined, together with short- and long-term consequences for the embryo/fetus/offspring. The benefits of using animal models will be addressed, highlighting the peculiarities and advantages of different mammalian species.

## 2. Controversy on safety in assisted reproduction

Since the birth of the first baby conceived by in vitro fertilization, in 1978 (Steptoe & Edwards, 1978), more than 3 million babies have been born as a consequence of ART application (Grace & Sinclair, 2009). The original IVF technique, which involved mixing oocytes and sperm in vitro and then transferring the embryo into the womb, was evaluated as "safe" in follow-up studies of IVF children (Friedler et al., 1992; Saunders & Lancaster, 1989). Progressively more interventionist and daring techniques have been set up to treat infertility. Women were hormonally treated to stimulate ovulation, oocytes and sperm have been manipulated in various way to achieve fertilization, microassisted techniques, such as Intracytoplasmic Sperm Injection (ICSI) and pre-implantation genetic diagnosis (PGD), were developed. However, unlike most therapeutic procedures used in medicine, assisted reproductive technologies never underwent rigorous safety testing before clinical use. Consequently, safety concerns arise for at least two major reasons:

i. treatments for infertility overcome natural barriers that prevent fertilization. (i.e. as these technologies are used to overcome infertility phenotypes that may have a genetic basis, unwanted genetic traits may be possibly transmitted to offspring)
ii. the reproductive technology itself may exert adverse consequences on the health of the offspring.

The application of IVF/ICSI to treat infertility is a clear example of this phenomenon. ICSI is an in vitro fertilization procedure in which a single sperm is injected directly into an oocyte. It was developed in 1992 (Palermo et al., 1992) and was quickly undertaken as allows men not producing healthy mobile sperm to become fathers. Nowadays, ICSI is often the method of choice for ART, and accounts for more than half of all assisted reproductive treatment in the western countries (USA 57.5%, Australia/New Zealand 58.6%, Europe 59.3%; Andersen et al., 2008). The use of such an aggressive technique has raised heavy concerns. The injection of the sperm directly inside the oocyte bypasses natural selection mechanisms, possibly passing infertility problems to the next generation or, even worse, overcoming natural barriers that are meant to stop genetic abnormalities carried out by faulty sperms. Moreover, ICSI may physically impair molecular mechanisms needed for proper fertilization and further development.

The concern about ART safety is supported by several studies that assessed the risk of birth defects in children conceived by ART compared to naturally conceived infants.

Higher risk of birth defects was reported in children conceived by IVF or ICSI compared to controls (Allen et al., 2006; Hansen et al., 2002; Kurinczuk & Bower, 1997), while singletons conceived after assisted fertilization consistently showed higher risk of low birthweight, preterm delivery and perinatal death than spontaneously conceived singletons (Allen et al., 2006; Bergh et al., 1999; Jackson et al., 2004; McDonald et al., 2005, 2009; McGovern et al., 2004; Schieve et al., 2002; Sutcliff & Ludwig, 2007;). Noticeably, even after adjusting for factors such as multiple birth, mother's age and preterm deliveries, the risks of birth defects highlighted in these studies remain higher for assisted reproduction-groups.

Over the years, this scientific evidence on ART safety has attracted considerable attention and has been highly criticized, especially by practitioners of assisted reproduction. The studies were accused of design flows, including the retrospective nature of most of them. In addition, the debate was enlivened by several studies clashing with the evidence of safety risks associated with ART procedures: no evidence for worries in IVF/ICSI babies was in fact repeatedly reported (Bonduelle et al., 1996; Romundstad et al., 2008).

It has been argued that some of the morbidity associated with ART does not result from the techniques, but from the underlying health risks of being subfertile. A large population-based cohort study was carried out using sibling-relationship comparisons (women conceiving at least one child spontaneously and one after ART; Romundstad et al., 2008). Results suggested that the adverse outcomes of assisted fertilisation compared with those in the general population could be due to factors leading to infertility, rather than factors related to the reproductive technology. Accordingly, studies of couples with reduced fertility, who eventually conceived spontaneously, showed higher risk of adverse perinatal outcomes than those without fertility problems (Basso & Baird, 2003; Draper et al., 1999; Ghazi et al., 1991; Henriksen et al., 1997; Williams et al., 1991).

In the bargain, the disagreement among studies is often due to different definition of "birth defects", leading to an inconsistent classification of congenital abnormalities and other adverse outcomes.

In summary, despite the large effort to study the effects of reproductive technologies, there is still only an incomplete picture of the risks associated with the use of ART. The existing

worries on potential unpleasant outcomes on a short- and long-term call for increased studies on the basic biology of fertilization and pre-implantation development, and on the effect of in vitro manipulation of oocytes and embryos.

Research in animal models, which is by its nature free of the biases that make the studies in human defective, should be broadened to identify the potential risks of ART application on short and long-term.

## 3. Focus on preimplantation embryo development

It is nowadays accepted that the earliest stages of life set the basis for the future health of the offspring (Barker, 1997). From this perspective, it is foreseeable that any disturbance during the very early development, as well as sub-optimal conditions (i.e. in vitro environment), may adversely affect the future offspring. Accordingly, abundant evidence in several species assessed that manipulation of gametes and embryo through ART may exert undesirable consequences on the offspring on a short- and long-term (DeRyche et al., 2002; Thompson et al., 2002).

Although the use of fertility treatment is increasing all over the world and in vitro reproductive technologies are routinely applied in several species for both research and commercial purposes, the technologies are still far from being perfect. Even in the best cases, in vitro embryo production achieves success rates hardly comparable to the in vivo ones, indicating that current in vitro procedures do not sufficiently resemble the reproductive physiology. At the same time, ART conceived offspring was seen to be different from naturally conceived individuals. Further research on gametes and preimplantation embryos is therefore fundamental to ameliorate the technologies and to evaluate the effect on the offspring.

As transcription is the first biologic/adaptive response to a perturbation or to an external stimulus, an adequate understanding of the gene expression status and regulation during the very first phases of development is an essential approach for the evaluation of ART safety. For this reason, in recent years gene expression studies have been increasing and integrated the numerous experimental approaches used in the past. Although the birth of a live and healthy offspring is considered the best parameter to evaluate the fitness of an embryo, gene expression studies during pre-implantation development have the advantage of being cost- and time-effective and, most importantly, they highlight differences at the molecular level that may be undetectable at birth, but affect the health of the adult.

### 3.1 Transcriptome of mammalian oocytes and pre-implantation embryos

In mammals, oogenesis is characterized by alternating active meiotic progression to long times of meiotic arrest. Resumption of meiosis occurs in fully grown oocytes (FGO) that complete first meiosis and then mature to metaphase II (MII). Completion of meiosis is dependent on fertilization, that leads to anaphase II and the formation of the first mitotic interphase. The time from fertilization to implantation of the embryo in the uterus is called pre-implantation embryonic development (PED).

The regulation of gene expression in oocytes and pre-implantation embryos shows peculiar characteristics. Oocyte maturation and early pre-implantation development are essentially under "maternal command" from factors deposited in the cytoplasm during oocyte growth,

independent of de novo transcription from the mature oocyte and the nascent embryo. The FGO contains all the maternal RNAs and proteins necessary to activate the molecular pathways required for fertilization and early embryogenesis (Cui and Kim, 2007; Pennetier et al., 2004). As a consequence, just after fertilization, the transcriptome of the embryo consists only in the maternally deposited transcripts. After several cell divisions, these maternal transcripts are specifically degraded and are replaced by embryonic transcripts produced by the new diploid cells, containing both maternal and paternal genes. This transition is termed embryo genome activation (EGA). The timing of EGA varies among species: in humans and bovines it occurs between the four- and eight- cell stages (Telford et al., 1990), in ovine between the eight-and the sixteen-cell stage (Kopecny, 1989), while in mouse between the one- and two-cell stage of development (Schultz, 1993).

Precise control of the dynamic changes that occur in the ooplasm during the phases of oocyte-to-embryo transition (OET), until EGA, is crucial for a proper development of the nascent embryo. The transcriptional quiescence requires extensive post-transcriptional and post-translational activities (Seydoux, 1996; Solter et al., 2002). Three major mechanisms take place, commencing at oocyte maturation and during the subsequent transcriptionally silent stages of development: (i) timely translation of stored maternal transcripts (ii) post-translational modification of existing and/or newly synthesized proteins (iii) degradation of no longer needed proteins and mRNAs.

These mechanisms exploit the differential stability of the maternal mRNAs stored in the ooplasm (Oh et al., 2000). During oocyte growth, many transcribed mRNAs are de-adenylated and stored in the ooplasm in a stable dormant form for subsequent translation. During translational activation, their limited 3′ poly(A) tails lengthen (Bachvarova, 1992), a sign that active translation is occurring (Richter, 1999). Half of the poly(A) mRNAs found in the fully-grown oocyte is de-adenylated during maturation, and by the 2-cell stage, the embryo contains less than 30% original amount of adenylated mRNAs found in the egg (Piko & Clegg, 1982).

## 4. Animal models: Peculiarities and advantages of different mammalian species

Due to the obvious scarcity of human oocytes and embryos for research, the use of appropriate animals models is of irreplaceable support for studying the basic biology of fertilization and pre-implantation development and for ART optimization. This is possible because the molecular mechanisms contributing to oogenesis and to PED progression are highly conserved among mammals (Gilbert, 2000). Although EGA occurs at different stages of development in different mammalian species (Kopecny, 1989; Schultz, 1993; Telford et al., 1990), the mechanisms of activation of the embryonic genome are in fact similar. All mammalian species progress through the same morphologic stages; perhaps, the most marked difference is the amount of time spent at each step, while the other notable interspecies differences appear after the blastocyst stage.

The comparison between maternally-deposited and EGA-activated transcripts in humans, cattle and mice indicates that maternal transcripts are generally more conserved than transcripts newly synthesized by the embryo (Xie et al., 2010). The conservation of the first phases of PED among mammals has encouraged the use of animal models for studying

meiotic progression and early pre-implantation development. Indeed, most knowledge about maternal translation and embryonic transcription reprogramming is based on mouse (Hamatani et al., 2004; Pangas et al., 2006; Xie et al., 2010) or ruminant models (Misirlioglu et al., 2006; Vigneault et al., 2009a, 2009b; Xie et al., 2010), while limited data are available from primates and humans (Nyholt de Prada et al., 2010; Xie et al., 2010; Zhang et al., 2009a, 2009b).

The main features sought in animal models are reliability, cost-effectiveness and biologic similarity to human fertilization. Each mammalian species exhibits specific characteristics and advantages for reproductive studies. Combining information related to a specific reproductive issue in different animal models has proved to be a useful approach to identify conserved mechanisms (Xie et al., 2010). Moreover, unveiling the basis of species-specific responses to certain technologies contributes to identifying the molecular or cellular mechanisms solicited by the manipulation.

Rodents, such as rats and mice, have been widely used and have yielded fundamental contributions to biomedical research. Over the past century, the mouse has developed into the premier mammalian model system for genetic research. Genetic and physiological similarities to humans allowed the generation of disease and treatment models and the creation of specific mutant lineages. The advantages of mouse as a model for human medicine include the genetic and physiological similarities to humans, the relatively low cost of maintenance, its ability to quickly multiply, as well as the ease with which its genome can be manipulated and analyzed.

The contributions of mouse model to understanding reproductive processes are numerous. Functional studies in mouse paved the way to the discovery of maternal effect genes (MEG) in mammals (Dade et al., 2004; Dean, 2002; Rajkovic & Matzuk, 2002). These oocyte specific genes, stored in the growing oocyte, are involved in the regulation of early cleavage, and their knockout often results in the inability of the embryo to develop beyond the first cleavage. *Zar1, MATER, NPM2,* are among the MEG that were later seen to be conserved in all the studied mammalian species, as bovine (Pennetier et al., 2004; Thelie et al., 2007; Uzbekova et al., 2006), human (Tong et al., 2002; Uzbekova et al., 2006; Wu et al., 2003b), swine (Uzbekova et al., 2006) and sheep (Bebbere et al., 2008).

While the mouse is an ideal model to study knockout effects and to create specific mutant lineages, large domestic animals are more suitable to study different aspects of reproduction. Sheep and cattle are mono-ovular and have similar reproductive endocrinology and ovarian biology (Gosden et al., 1994). They display biparentally contributed assembly of the zygotic centrosome during fertilization, like humans, while rodents show maternal inheritance. In ruminants, EGA occurs during the period from 4- to -16 cell stage (Camous, 1986; Kopecny, 1989), encompassing the period when it occurs in humans (Telford et al., 1990). Conversely, in mice EGA occurs very early and abruptly at the 2-cell stage, thus narrowing the window of opportunity for analysis of the reprogramming events during this delicate phase. The spreading of EGA over 3 to 4 cell cycles in large domestic animals allows a better analysis of the progressive phases leading to the major wave of transcriptional activation (Bensaude & Morange, 1983). This feature allowed the identification of functions specific to different points of PED, and was exploited in the analysis of MEG expression in sheep (Bebbere et al., 2008) and cattle (Bettegowda et al., 2007; Pennetier et al., 2004, 2006).

Research on ruminants is favored by the large numbers of gametes that can be easily obtained from farms and slaughterhouses. Moreover, the detailed information on ruminant fertilization is strengthened by years of research and well-defined reproductive technology aimed at increasing the productivity of farm animals. Several reproductive technologies have been developed in farm species, but have then contributed to human reproductive medicine and to understanding reproductive processes. Artificial insemination (Herman, 1981), semen cryopreservation (Foote, 1982; Polge, 1949), superovulation, in vitro fertilization and embryo transfer are among the many techniques that were mainly developed in farm animals (Roberts, 2001).

## 5. The effects of ART on regulation of gene expression: Pros or cons?

Embryonic development in vitro may be compromised by inappropriate in vitro culture systems designed to induce oocyte maturation or to sustain fertilization and further development. Media used for in vitro oocyte and embryo culture, being so far based more on empiricism than on precise knowledge of embryo needs, inevitably provide a variety of discordant biochemical signals that confound the reprogramming of at least some embryos. The sub-acute nature of some alterations induced by in vitro embryo production may remain undetected in the short term. Embryos are often capable to reach the blastocyst stage, a frequently used hallmark for the efficiency of in vitro embryo culture systems, in spite of a sub-optimal culture environment. Such ability, however, may be inconvenient for their postnatal health. Studies in several mammalian species have indeed shown that in vitro conditions during PED do affect the quality of the embryo, albeit being compatible with full-term development.

Abundant evidence based on different systems to evaluate embryo quality showed that in vitro-produced embryos are not necessarily similar to naturally conceived ones and it is now widely accepted that embryos derived from in vitro culture are of inferior quality to those derived in vivo. Compared to their in vivo counterparts, IVP embryos display a number of marked differences, e.g., gross morphology (color, density, cell number and size), timing of development (Greve et al., 1995), zona pellucida stability and resistance to criopreservation (Leibo & Loskutoff, 1993; Niemann et al., 1995).

The proliferation of molecular technologies has given a boost to experimentation and confirmed that in vitro environment does alter the oocyte and early embryo molecular structure (Ecker et al., 2004; Gutiérrez-Adán et al., 2004; Lonergan et al., 2003a, 2003b; McEvoy et al., 2001; Niemann et al., 2000; Summers & Biggers, 2003). In particular, the analysis of gene expression evidenced that the differences in phenotype correspond to altered transcriptomes in the developing embryos, and gave for the first time the opportunity to identify the single molecules solicited by in vitro environment.

Many studies examined the differences in the expression of specific genes, selected on a functional basis, between in vitro- and in vivo produced embryos of several species (Corcoran et al., 2007; Knijn et al., 2005; Rizos et al., 2002a). The expression of laminin chain-specific genes (Shim et al., 1996) and of gap junction gene Cx43 (Wrenzycki et al., 1996) was seen to decrease in in vitro produced blastocysts, while expression of stress-related genes, such as Heat Shock Protein 70.1, was seen to increase in pre-implantation embryos due to in vitro exposure (Christians et al., 1995). Genes related to glucose metabolism were seen to be down-regulated

in IVP embryos (Knijn et al., 2005; Uechi et al., 1997; Wrenzycki et al., 1998a). A temporal variation in several transcript abundance was observed in embryos at different stages of PED cultured in vitro or in vivo (Corcoran et al., 2007; Tesfaye et al., 2004).

The advent of microarray technologies offered the opportunity to gain an insight into the transcriptional response of a whole genome to a particular event or environmental insult, giving an overall picture that was unthinkable before the advent of large scale studies. Information on entire gene regulatory networks were made available. Several studies examined the global gene expression profile of IVP embryos compared with their in vivo counterparts (McHughes et al., 2009; Mohan et al., 2004; Smith et al., 2005, 2009,). The observation of global gene expression profiles made the burden of a non-physiological environment on the early embryo transcriptome even clearer (Ecker et al., 2004.; Wrenzycki et al., 2001).

A culture-induced change in the transcriptome was observed not only between in vivo or in vitro produced embryos, but also between embryos produced in different in vitro systems. The specific composition of culture media was indeed seen to have profound effects on the relative abundance of gene transcripts in the embryo that, in turn, can have serious implications for the normality of the blastocyst (Corcoran et al., 2007; Lonergan et al., 2003a, 2006; Rizos et al., 2002a, 2003; Walker et al., 2000; Wrenzycki et al., 1998b; Wrenzycki et al., 1999; Wrenzycki et al., 2005). Even subtle changes were seen to alter the patterns of gene expression in pre-implantation embryos, such as the concentration of a single constituent (NaCl) in the culture medium (Ho et al., 1994), or the culture under suboptimal conditions for as little as 1 day (Lonergan et al., 2003a).

Detailed gene expression studies on the effect of culture media during development of the human preimplantation embryo are still missing. A paucity of material and obvious ethical restrictions make such studies difficult to undertake. It seems likely, however, that patterns of gene expression are culture-dependent also in human (Summers & Biggers, 2003). This point is of particular concern when considering the recent proliferation in media for the extended culture of human preimplantation embryos. A recent microarray analysis compared the relative transcript abundance values for blastocysts produced in ten in vitro systems, differing primarily in culture medium formulation (Côté et al., 2011). A panel of novel uncharacterized transcripts were variably expressed depending on the medium in which the blastocysts were produced. Hierarchical clustering of microarray data indicated that the closest treatment to the in vivo reference produced also one of the best blastocyst yields. Notably, the differences in transcript abundance were affected by the conditions of oocyte maturation as well.

There is considerable opportunity for the disruption of gene activity when embryos are removed from their natural environment and manipulated in vitro. Several variables during in vitro culture were seen to affect the success rate and the quality of the developing embryos. Embryo density during in vitro, for instance, significantly affected the expression of stress-related genes (de Oliveira et al., 2005), the developmental competence to blastocyst stage, as well as the gene expression patterns on a large-scale (Hoelker et al., 2009).

Evidence from studies utilizing the sheep oviduct for the post-fertilization culture of in vitro derived zygotes (Enright et al., 2000; Rizos et al., 2002a, 2002b) indicated that the period of culture after fertilization is the key part of the process responsible for suboptimal embryo

quality. In vivo culture (in the ewe oviduct) of in vitro produced bovine zygotes markedly increases the quality of the resulting blastocysts, in terms of cryotolerance, to a level similar to embryos produced entirely in vivo (Enright et al., 2000; Rizos et al., 2002a, 2002b). At the transcript level, such in vivo cultured embryos showed gene expression patterns similar to true in vivo embryos (Lazzari et al., 2002; Lonergan et al., 2003a, 2003b). Similarly, the in vitro or in vivo post-fertilization environment affected the gene expression patterns of ovine embryos produced by IVM and IVF of oocytes deriving from prepubertal (Bebbere et al., 2008) and adult donors (Bogliolo et al., 2009).

While efforts are being spent to improve the efficiency of existing reproductive biotechnologies and to develop new approaches, attention should always be paid to the effects exerted by new treatment on the nascent embryos. Among the new methods proposed to improve the efficiency of in vitro embryo production systems, the exposure to sub-lethal hydrostatic pressure (HP) treatment is emerging as an approach to improve the general resistance of gametes and embryos to suboptimal conditions. While treatment with HP was seen to improve the quality of in vitro-produced ovine blastocysts by increasing their cell number and reducing the proportion of nuclear picnosis, it was also seen to alter the expression status of the blastocysts (Bogliolo et al., 2011). Whereas the change in mRNA content may give the HP exposed blastocysts a temporary higher gear, the effect on a longer term should be examined.

The observation of altered gene expression patterns in in vitro manipulated embryos with increased developmental competence raises a point on the interpretation of gene expression profiles during PED. The numerous studies that evaluated the impact of in vitro environment have mostly compared the treatment to in vivo produced embryos, which are considered to be the gold standard of quality. However, it is unlikely that embryos produced in an artificial system exhibit the same profiles as the ones that have been grown in vivo, especially because culture systems do not perfectly mimic the in vivo conditions. As such, some perturbations in the gene expression profiles should be considered normal. The question then is rather to define to what extent these perturbations are acceptable, not compromising embryonic viability or leading to deleterious long-term effects (Seli et al., 2010). Studies should be addressed to clarify the characteristics of developmental competence for an embryo cultured in vitro: is the "best" embryo the one offering the largest plasticity level and thus being able to adapt and cope with more intense environmental insults? Or is it the one that more resembles the embryo developed in vivo, maintaining its characteristics despite a non-physiological environment?

It is expected that, as it is the case in all living cells, adaptation can be stretched to a certain limit, beyond which irreversible damage will occur. The definition of embryonic competence should therefore include the level of plasticity and should be seen as an interval of acceptance rather than a clearly defined threshold value.

## 6. The effects of ART on genomic imprinting

While some patterns of gene expression observed as a consequence of in vitro environment seem to be compatible with a proper development of the embryo, other alterations in the embryo transcriptome consistently result in reduced quality associated with fetal and neonatal abnormalities. A substantial amount of evidence demonstrates that the culture

conditions to which the embryo is exposed may perturb the epigenetic status of the embryo genome, with potentially important long-term consequences. Although linking the variations in gene expression with the observed phenotypes has been extremely challenging, it is now generally accepted that assisted reproductive technologies are associated with genomic imprinting disorders.

In mammals, genomic imprinting is an epigenetic process by which certain genes are expressed in a parent-of-origin-specific manner. It involves methylation and histone modifications in order to achieve monoallelic gene expression without altering the genetic sequence. These epigenetic marks are established in the germline, rearranged during embryonic reprogramming, and then maintained throughout all somatic cells of an organism. During PED, reprogramming involves extensive epigenetic modifications of the differentiated gamete nuclei by the ooplasm that transforms them to a totipotent embryonic nucleus. The changes in the embryo epigenome regulates the transition from maternal to embryonic control of transcription. A correct epigenetic reprogramming is needed for totipotency, correct initiation of embryonic gene expression, early lineage development, and is essential for a proper establishment of genomic imprinting in the new embryo.

Germ cell development and early embryogenesis are crucial windows in the erasure, acquisition and maintenance of genomic imprints. ART include isolation, handling and culture of gametes and early embryos at times when imprinted genes are likely to be particularly vulnerable to external influences. It is therefore predictable that in vitro manipulation during these early phases influences the epigenetic marking of the embryonic genome, and consequently its gene expression.

In recent years, concern has grown on the occurrence of disorders linked to imprinting problems in ART conceived children. Several epidemiologic studies have reported an increased frequency of imprinting defects in association with ART application (Arnaud & Feil, 2005; De Rycke et al., 2002; Owen & Segars 2009; Powell, 2003; Scarano et al., 2005; Stromberg et al., 2002; Thompson et al., 2002). Being involved in the etiology of severe developmental disorders (Arnaud & Feil, 2005; Scarano et al., 2005), such as Beckwith-Wiedemann (BWS) and Angelman Syndrome (AS), disruption of imprinting has raised particular alarm. Studies reported that the risk of AS may be increased by the use of ICSI (Cox et al., 2002; Orstavik et al., 2003), and that ART results in a three-to-six-fold-increase in the incidence of the normally rare BWS (DeBaun et al., 2003; Gicquel et al., 2003, Maher et al., 2003).

Abundant evidence on the connection between ART and altered expression of imprinted genes originates from animal models: studies performed in the mouse, sheep, and bovine species showed that the epigenetic and genetic programming of the embryo may be severely affected by in vitro environment (Lonergan et al., 2003a; Khosla et al., 2001; Young et al., 2001). Association between in vitro embryo production and disrupted imprinting resulted in a variety of abnormal phenotypes, early embryonic losses, and perinatal deaths in several mammalian species (de Sousa et al., 2001; Yang et al., 2005; Young et al., 2001; Wrenzycki & Niemann, 2003). Faulty nuclear reprogramming due to artificial manipulation is considered the primary cause of the reported defects.

In vitro culture was seen to cause abnormal epigenetic modifications and subsequent deregulation of several imprinted genes, many of which are involved in the control of pre- and postnatal growth (Walker et al., 2000). Others play important roles in regulating

resource acquisition of the embryo and fetus (Isles and Wilkinson, 2000), and therefore it has been proposed that, in mammals, imprinting co-evolved with the placenta.

In ruminants, a faulty imprinting is linked to early embryonic losses, perinatal deaths, and a variety of pathological symptoms that are summarized under the term "large offspring syndrome" (LOS) (Yang et al., 2005; Young et al., 2001). Being among the best described adverse impacts of IVP, LOS comprise a series of abnormal phenotypes, such as increased gestational duration and birthweight, abnormal physiology, organ, placenta and skeletal development (McEvoy et al., 2000; Sinclair et al., 1999). It is associated with imprinting disruption (McEvoy et al., 2000; Sinclair et al., 1999; Young et al., 1998) and seems to result from the exposure of in vitro produced embryos to fetal calf serum (Farin et al., 2001; Sinclair et al., 1999, 2000). Although most pronounced in cloned embryos, LOS was reported following other types of ART, including IVF (de Sousa et al., 2001; Tilghman, 1999; Yang et al., 2005). Studies on LOS in sheep have identified altered expression level of the IGF2R imprinted gene, due to epigenetic changes (Young et al., 2001). Similar overgrowth problems seen in mice and humans are caused by errors in Igf2 and H19 imprinted genes (Eggenschwiler et al., 1997), suggesting that several genes responsible for fetal growth and development could be involved in LOS.

Notably, specific characteristics of ART-associated LOS in ruminants resemble the clinical phenotypes due to imprinting disruptions typically observed in human, such as BWS and AS (Arnaud & Feil, 2005; Scarano et al., 2005).

In rodents, studies on the preimplantation embryo suggested that particular in vitro culture conditions can alter the transcription pattern of imprinted genes and produce long-term neuro-developmental and behavioral disorders (Doherty et al., 2000; Ecker et al., 2004; Fernandez-Gonzalez et al., 2004; Humpherys et al., 2001; Mann et al., 2004; Sjoblom et al., 2005; Toppings et al., 2008). In particular, inclusion of serum in culture media was seen to alter the expression of imprinted genes and reduce the developmental potential after embryo transfer (Khosla et al., 2001). A microarray-based assessment of genomic methylation showed evidence of generalized hypermethylation, as well as greater locus-to-locus variability, in in vitro murine embryos when compared with in vivo control (Wright et al., 2011).

The alterations seen in mice, sheep and cattle in consequence of the application of ART procedures are probably relevant to most eutherian mammals, including humans. They may result from embryo exposure to suboptimal in vitro culture environments, which are incapable to supply the right signaling cues, and can lead to the deregulation of genes and aberrant epigenetic modifications (Fernandez-Gonzales et al., 2007). The sub-acute nature of some of these disruptions allows them to remain undetected in the short term, so that blastocyst production can often be achieved despite the detrimental environmental effects. However, undesirable postnatal phenotypic consequences may arise during the future development of the fetus or of the offspring, due to alteration of long-term gene expression programs (Gluckman & Hanson, 2004).

## 7. Conclusion

Epidemiological studies on ART conceived children and molecular analysis in several mammals yield yet contrasting results. The incomplete picture on the safety of ART

demands for further studies on the basic biology of fertilization and pre-implantation development. Only a deeper understanding of the cellular and molecular mechanisms ruling life early phases will enable a proper evaluation of the severity of ART impact.

The knowledge on the regulation of gene expression is continuously advancing, unveiling a process that involves a wide range of molecules and mechanisms, and is conspicuously more complex than expected. Several classes of non coding RNAs (i.e. long non coding RNAs and microRNAs) are being recognized as crucial for the control of the transcriptome activity. Most probably, the advances in this field will transform our current concept of developmental competence and plasticity and will renovate the ideas to improve the technologies and their safety.

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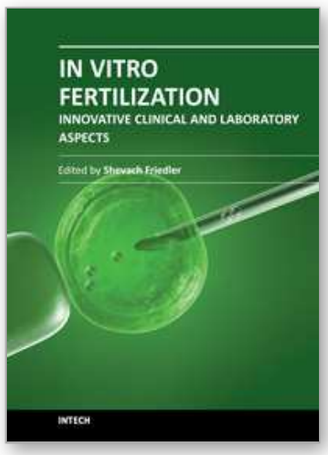


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The field of In Vitro Fertilization is a relatively new field in medicine, constantly on the move. This field is an exquisite example of the vast power in the complementary use of basic research with clinical practice and opened a new route of great basic and clinical research possibilities. The knowledge base that allowed the accomplishment of the idea of in vitro fertilization and embryo transfer has much developed since. The vast body of research pertaining to this field allowed deepening our understanding in the processes related to reproduction. In this book on in vitro fertilization we present new and interesting updated information in various aspects of this field. This work is a result of collaborative work of an international group of professionals dedicated to contribute to the advancement of our knowledge.



**InTech Europe**
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
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