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The Actors of Human Implantation: Gametes, Embryo, Endometrium

Virginie Gridelet et al.*
University of Liège (ULg)
Belgium

1. Introduction

The success of pregnancy depends on a receptive endometrium, a normal blastocyst, a synchronized cross-talk at the maternal–fetal interface at the time of implantation, and finally a successful placentation and remodeling of uterine vasculature. In routine, less than 5% of oocytes collected in *in vitro* fertilization (IVF) cycles and only 20 to 25% of embryos transferred lead to a birth. Implantation and placentation processes remain the black box of fertility, involving following steps: fertilization, endometrial receptivity, embryo implantation (apposition-adhesion-invasion), trophoblastic differentiation and invasion (Cartwright et al., 2010).

At the time of fertilization, a cascade of cytokines mediates the oocyte-sperm dialogue long time before embryo implantation in the endometrium. At the oocyte side, genomic and proteomic profiles of good follicle are under investigation. Markers of oocytes with high subsequent embryo implantation potential are one of the main goals of the actual research. For example, granulocyte colony stimulating factor (G-CSF or CSF-3) in individual follicular fluids (FF) appears to correlate with the birth potential of the corresponding embryo in two opposite models of ovarian monitoring, standard ovarian hyperstimulation and modified natural IVF/ICSI cycles (Ledee et al., 2008b; Ledee et al., 2011a). To achieve the fertilization step, a good quality oocyte must meet a normal sperm with low DNA damages, leading to the development of a functionally normal blastocyst able to dialogue with maternal endometrium. Sperm DNA damages include fragmentation, trouble of condensation and epigenetic modification that could impair implantation process and methylation of imprinted genes (Boitrelle et al., 2011; Tavalaee et al., 2009).

Endometrial receptivity is established during a limited period of time called the *implantation window*. During this 4-days window (d20-d24), endometrium is highly receptive to the different signals and ligands produced by embryo throughout apposition, adhesion and invasion steps of implantation. The complex signaling networks that regulate this tightly coordinated maternal-fetal crosstalk are clearer as studies on endometrial receptivity and early pregnancy are performed. Many target molecules have been identified in the receptive

* Olivier Gaspard, Barbara Polese, Philippe Ruggeri, Stephanie Ravet, Carine Munaut, Vincent Geenen, Jean-Michel Foidart, Nathalie Lédée and Sophie Perrier d'Hauterive
University of Liège (ULg)
Belgium

endometrium such as specific cytokines equilibrium, growth factors and angiogenic factors but also some specific immunological target cells. Despite research and technique progresses, despite a lot of publications defining profiles – normal or pathological- at the genomic and proteomic level, the molecular fingerprint of the receptive endometrium remains unknown (Berlanga et al., 2011; Rashid et al., 2011; Singh et al., 2011).

The progression of implantation and then pregnancy requires immunological tolerance which allows conceptus survival. It has been proposed that uterine natural killer cells (uNK) could exert, directly or indirectly, either positive or negative control over these early steps (Dosiou and Giudice, 2005). These cells secrete an array of cytokines important for adequate local immune regulation, angiogenesis, placental development, and establishment of pregnancy. Successful subsequent placentation and remodeling of the uterine vasculature is a fundamental step for a healthy pregnancy that requires also a highly orchestrated reciprocal signaling process. Deficiencies in this process are implicated in a number of dangerous pregnancy complications with excess (percreta/accreta placentation) or defective implantation (preeclampsia, intra uterine growth restriction). Implantation failure, recurrent miscarriage and preeclampsia have several recognized causes in common, but in most cases, the precise etiology remains obscure.

The most limiting and difficult issue to evaluate during the implantation process is the dialogue at the materno-fetal interface. Embryo is able to cross-talk with the endometrium through different molecules, cytokines and hormones. It is able to actively participate to its own implantation and to influence endometrial gene expression (Kashiwagi et al., 2007). Inversely, endometrium is competent to differently answer to the implanting embryo, to favor or reject implantation (Bauersachs et al., 2009). Moreover, the cytokine network acting in the female reproductive tract around implantation integrates environmental information to program the embryo and fine-tune the maternal immune response and endometrial remodeling to determine implantation success (Robertson et al., 2011). All these interactions are not accessible to the researcher for obvious ethical reasons that let understand why implantation remains the black box of reproduction, even in 2012.

Among the factors produced by the embryo, its specific signal chorionic gonadotropin hormone (hCG) and its hyperglycosylated form H-hCG are another example of target molecules at this crossroads of immune tolerance, angiogenesis, and invasive process at the maternal-fetal interface.

This chapter will overview the recent literature and personal data concerning impact of gametes, endometrium and embryo during implantation process.

2. The oocyte

Maternal factors play a predominant role during early embryo development. Oocyte supports indeed by itself the early cleavage of the zygote until the 4-8 cells stage. Unfortunately, oocyte morphology is poorly discriminative and allow mainly a negative selection (Balaban and Urman, 2006; Rienzi et al.) and oocyte quality remains one of the main limiting factors of success of Assisted Reproductive Technology (ART) in human. This is due not only to the prime impact during early embryo growth but also to the fragility of oocytes along the life time. Less than 5% of oocytes collected after ovarian hyperstimulation in IVF program lead to birth. The selection of oocytes able to give rise to implanting embryo is crucial to avoid such

wastage. Actual research focuses on the oocyte physiology and aim to evidence functional markers of good quality oocyte and competence to complete morphological observation of embryos which are insufficient to highly predict subsequent successful implantation.

Throughout the process of folliculogenesis and ovulation, the oocyte maturation from resting primary oocyte to secondary oocyte includes a complex sequence of nuclear and cytoplasmic events that prepare oocyte to fertilization and initiation of embryo development. During all their maturation, oocytes grow and develop in a highly coordinated and mutually dependent manner with the cumulus cells surrounding them. The formation of meiotic spindles during the two phases of the meiosis is essential for accurate chromosome segregation and for the highly asymmetric cell divisions necessary for the formation of small polar bodies and a large polarized oocyte (Schatten and Sun, 2011). The first polar body is extruded from the oocyte just before fertilization while the second one is expelled at the end of the second meiotic division which occurs after the fertilization. Chromosomal segregation errors occurs in approximately 15-20% of oocytes (Pellestor et al., 2005) and 5% of all pregnancies are aneuploidy (Hassold and Hunt, 2001).

During mostly IVF procedures, high doses of gonadotropins are administered to hyperstimulate the development of multiple oocytes to obtain a maximum of matures eggs in a single cycle. The aim of such an ovarian stimulation is the production of more than one embryo allowing the embryologist to select the best to transfer (while the others are cryopreserved for a possible later transfer). The assumption that ovarian stimulation could impair oogenesis, embryo quality and endometrial receptivity becomes more and more evident. The underlying mechanisms of these detrimental effects are still poorly understood, and further knowledge is needed in order to increase the safety of ovarian stimulation and to reduce potential effects on embryo development and implantation (Santos et al., 2010). A better understanding of oocyte oocyte-cumulus physiology/interactions, as well as, the improvement of oocyte selection based on its competence of giving rise to a healthy embryo, will ultimately increase the safety of ovarian stimulation and then pregnancy rates in IVF.

2.1 Oocyte quality and physiology

A “high quality” oocyte is an oocyte that is able of maturing, being fertilized, realizing a good implantation and giving rise to a healthy baby.

The poor quality of an oocyte seems to be characterized by a range of morphological defects or abnormalities, although there is still no precise quantification of the relative importance of each different anomaly. There is still not a comprehensive morphological oocyte grading scheme able to enough optimize the selection of normal oocytes (Lasiene et al., 2009; Patrizio et al., 2007; Rienzi et al., 2011) for fertilization in IVF or for oocyte cryopreservation. Criteria usually used to determine the grade of quality of oocytes morphology include the evaluation of the structure of oocyte: cumulus complex, oocyte cytoplasm, polar body, perivitelline space, zona pellucida and meiotic spindle (Lasiene et al., 2009). An oocyte with a large potential to give a competent embryo is describe with different characteristics:

- The oocyte is surrounded by a compact cumulus (at least five layers of cells)(Mayes and Sirard, 2001; Nagano et al., 2006; Warriach and Chohan, 2004).

- Ooplasm is almost transparent and homogeneous or a dark ring is seen around the cytoplasm. It is not granular (Balaban et al., 2008).
- There is none extracytoplasmic abnormalities (like dark zona pellucida, granular, vacuoles or cytoplasmic fragments) (Balaban et al., 2008; Rienzi et al., 2008).
- The perivitelline space is not too large and without grains (Hassan-Ali et al., 1998; Xia, 1997).
- It is estimated that oocytes observed with a polarization light microscope that shown birefringent spindle had higher developmental potential after fertilization than oocytes without birefringent spindle (Fang et al., 2007; Moon et al., 2003; Rienzi et al., 2004; Shen et al., 2006; Wang et al., 2001).

The morphology of the first polar body expulsed can also be studied to determine oocyte quality. Some morphological criteria such as the shape, the size, the surface and the integrity of cytoplasm of the polar body can be used to determine oocyte quality (Fancsovits et al., 2006; Navarro et al., 2009; Rienzi et al., 2008).

The contribution of each of these characteristics for the selection of a good quality embryo are largely discuss in the literature but they are still unclear (Lasiene et al., 2009; Rienzi et al., 2011; Setti et al., 2011). The selection of good quality oocyte is primordial in order to help during the management of ART but also to cryopreserve them to realize a bank of oocytes able to support thawing. Recently pregnancies were obtained with oocyte that has been cryopreserved or vitrified (Cobo and Diaz, 2011). Cryopreservation of oocytes is a desired tool for the possibility of extending the reproductive capability of young women with malignant diseases in cases where the treatment may compromise the ovarian reserve (Herrero et al., 2011; Saragusty and Arav, 2011).

Chromosomally abnormal oocyte is morphologically impossible to distinguish with traditional observation by the biologist, while it is an important cause of development or implantation failure of human embryo produced *in vitro*. Beside the necessity to optimize oocytes observation in order to fertilize them, there is a great need to better characterize a good quality oocytes with the purpose of cryopreserve them during fertility saving and egg donation programmes.

The transcriptomic profiling of human oocytes has been studied in normal oocytes and in aneuploidy oocyte to find a non-invasive biomarker of aneuploidy (Fragouli et al., 2010). Furthermore, the dialogue between the oocyte and its cumulus could be a key factor for the development of a competent embryo (Royere et al., 2009). Individual oocytes and their associated cumulus cells has been analysed by microarray technologies to provide potential viability markers related to oocyte competence. Increasing number of papers have shown correlation between cumulus gene expression and oocyte maturation, fertilization rate and pregnancy outcome. (Assou et al., 2010; Feuerstein et al., 2007; Hamel et al., 2008; van Montfoort et al., 2008; Yerushalmi et al., 2011). The study of Assou et al.(Assou et al., 2010), have demonstrated a good correlation between the expression profile of 45 genes from cumulus cells analysed by microarray and pregnancy outcome without relationship to the morphological grade of the embryo. The transcription of genes of cumulus cells has also been studied by Feuerstein et al., the genes were chosen because their expression was induced by the LH peak. Expression levels of all genes investigated, except one, were increased after resumption of meiosis (Feuerstein et al., 2007).

Furthermore, the transcriptome of the first polar body (extruded from the oocyte before fertilization) was analysis with the assumption that the polar body transcriptome is representative of that of the oocyte. The results show that the transcriptome of the human polar body reflect the one of the oocyte. This study could lead at the first molecular diagnostic for gene expression in oocyte ready for the fertilization, using mRNA detection and quantification (Reich et al., 2011).

Currently, the selection of the embryo to transfer is based on a morphological base such as kinetic of growth, number/size/form of blastomeres, early cleavage, fragmentation rate, aspect of ooplasm, aspect of pronuclei, and absence of multinuclear blastomeres. These usual morphological criteria are judged not sufficient for selecting the ideal embryo able to develop until the blastocyst stage and to transfer and to cryopreserve (Guerif et al., 2007; Guerif et al., 2009). Identification of non-invasive test of oocyte competence would undoubtedly improve the efficiency of assisted reproductive technology in selecting competent embryos.

The fluid that is in the follicular antrum, accumulated from the early stage of follicle development is rich of components and ease of access for studies that may contribute to a better understanding of the mechanisms underlying follicular development, oocyte quality or even ovarian hyperstimulation (Fahiminiya et al., 2011; Jarkovska et al., 2011). The aim of the study realize by Jarkovska *et al*. (Jarkovska et al., 2011) was to identify candidate proteins in follicular fluid (FF) which may help to identify patient at risk of ovarian hyperstimulation syndrome of women undergoing *in vitro* fertilization. Three proteins were found as potential markers among which kininogen-1 was highlighted by computer modeling as a potential key factor for mediated inflammation and angiogenesis. In the study realize Fahiminiya *et al.* (Fahiminiya et al., 2011), FF were collected from ovaries at three different stages of follicle development (early dominant, late dominant and preovulatory) and were analyzed by 2D-PAGE, 1D-Page and mass spectrometry to observe the proteomic expression in crude, depleted and enriched FF. They demonstrate that the enrichment method could be used to visualize and further identify the low-abundance proteins in FF, which could reflect the physiological status of the follicle.

A new marker measurable in FF has been proposed to select embryo with a high potential of implantation: the granular colony stimulating factor (G-CSF) (Ledee et al., 2008b).

2.2 Biomarker of the competence of the oocyte: G-CSF in follicular fluid

Research performed by Lédée team has extensively explored the follicular Granulocyte – Colony Stimulating Factor (G-CSF or CSF-3) properties as non-invasive immune biomarker of the oocyte competence able to predict subsequent birth (Ledee et al., 2008b). In a first study, FF were collected individually and the traceability of each fluid was ensured until birth or failure of the attempt was known. Twenty seven cytokines and chemokines were simultaneously measured in each FF collected from 132 individual follicles of oocyte subsequently fertilized and transferred after conventional ovarian hyperstimulation. The conclusion of this study was that the level of G-CSF in individual FF samples correlates with the implantation potential of the corresponding embryo. These data were reproduced in a cohort of 200 embryos while detailing the adequacy of distinct methods for measuring follicular fluid G-CSF (Ledee et al., 2010).

A third study was subsequently conducted including 83 patients undergoing a modified natural IVF/ICSI cycle to measured 26 soluble factors in the FF of these patients. The aim of this study was to provide an experimental model where the traceability was complete: only one oocyte was recovered and therefore only one embryo was transferred. Each of the 26 factors was evaluated as a potential biomarker of subsequent birth and G-CSF was found to be the best predictor of birth in this study. The combination of FF G-CSF and morphological embryo scoring on day 2 has been suggested as a possible prognostic value before starting the embryo transfer (Ledee et al., 2011a). Through these 3 studies, selection of follicular fluids over thousand fluids was performed to select the ones corresponding to an embryo successfully transferred with the traceability of each sample until birth. Each fluid was analysed through multiplex bead based technology. In all experiments, FF G-CSF appears as an excellent non-invasive biomarker of oocyte competence in regard to its significant strong power of discrimination, independently so adding value, to our daily embryo morphology based-selection.

We postulate that G-CSF factor in individual FF appears to correlate with the birth potential of the corresponding embryo in two opposite models of ovarian monitoring: standard ovarian hyperstimulation and modified natural IVF/ICSI cycles. Prospective randomized studies are needed to confirm the hypothesis and evaluation of adding value when correlated with the embryologist morphological choice is currently studied by our team.

The presence of G-CSF in the female genital tract and its possible role in reproduction have already been studied in the past. G-CSF has been shown to be secreted by granulosa cells at ovulation (Salmassi et al., 2004), then during the luteal phase within the endometrium and finally during gestation in the placenta (Duan, 1990). FF G-CSF may promote local maternofetal tolerance (Rutella et al., 2005) or influence the oocyte's own mRNA levels or its potential for self-repair (Yannaki et al., 2005). It might also interact with environmental cells to produce cytokines and growth factors which are necessary for the embryo's development and implantation.

It has been suggested key interactions within the follicle involving immune cells such as dendritic cells and regulatory T (Treg) cells)(Ledee et al., 2011b). Local maternal-foetal immunotolerance could potentially be promoted by G-CSF: almost all the miscarriages observed in our cohort were found in the group with a low follicular fluid G-CSF level and a recent study reported that G-CSF administration significantly increases live-birth rates in patients with unexplained recurrent miscarriages (Scarpellini and Sbracia, 2009).

The measure of the FF G-CSF is a non-invasive method for the assessment of the potentiality of the embryo to implant, and is cover by a patent. This method could be quickly implemented with a short time response compatible with the selection of the embryo before transfer. FF G-CSF may also help to promote the single embryo policy by helping to distinguish even before the fertilization of the oocyte with a good or a bad potential of implantation. It may also be helpful to evaluate individually the oocyte (for women with a low ovarian reserve), to identify the best protocol of ovarian stimulation to apply (specific indication of minimal stimulation) and to choose in a more powerful way the embryo to cryopreserve.

2.3 Oocyte influence on embryo development

First days of embryo development are characterized by a sequence of cells divisions of the fertilized oocyte into smaller and smaller cells. These divisions will transform the zygote

into an implantation-competent blastocyst. The size of the whole embryo doesn't change until the blastocyst formation, capsuled into the zona pellucida.

The preimplantation embryo is able to realize a form of autonomous development firstly fueled by products provided by the oocyte and then from products coming from the activation of its own genome (Schultz, 2005). At the beginning of its life, right after the fertilization, the developmental program is initially directed by the maternally inherited protein and transcripts. Thereafter the embryonic cells will need to become totipotents. This totipotency is acquired by the reset of the epigenetic states of the DNA of the germ cells (the oocyte and the spermatozoa) and then by the activation of the embryo's genome and its own epigenetics arrangements. This activation will influence gene expression patterning to produce two specific cell types necessary for the survival of the embryo: the trophoblast and the internal cellular mass. It will exhibit correct spatiotemporal activity of the sequence of events necessary for the embryo development achievement (Corry et al., 2009). The next step will be the progressive replacement of maternal derived transcripts located in the cytoplasm with the transcripts specifics newly formed from the embryo (Schultz, 2002). A variety of epigenetic mechanisms underlies each step of the development to allow the specific identity of each cell type. These epigenetic mechanisms may change depending on environmental and temporal conditions (Corry et al., 2009).

Compared to spermatozoa, the DNA methylation status in oocyte prior to fertilization is little known. It is difficult to obtain mature, ready-to fertilize oocyte in large enough quantities (compared to spermatozoa).

About the embryo, the organization and the introduction of different epigenetic marks are done early during the development of the embryo and are essential for the development of the new organism. These epigenetic marks, DNA methylation, histone modifications and noncoding RNAs, have a critical role in the cell memory during the development. During gametogenesis, spermatozoa and oocyte are produced with distinctive chromatin resulting from a epigenetic reprogramming from the original cells (Hales et al., 2011).

2.4 Environmental influences on oocyte

- Oocyte aging

Females who show a progressive decline in fecundity can have oocyte aging. It is known that after a certain age the ovarian reserve diminish more quickly. Treatments have been proposed to increase the maintenance of the ovarian reserve. Dehydroepiandrosterone (DHEA) has been reported to improve pregnancy chances in case of diminished ovarian reserve. Currently best available evidence suggests that DHEA improves ovarian function, increases pregnancy chances and, by reducing aneuploidy, lowers miscarriage rates. DHEA over time also appears to improve ovarian reserve (Gleicher and Barad, 2011; Gleicher et al., 2010a, b; Sonmezer et al., 2009). Although levels of DHEA produced locally in FF have been measured and levels correlate negatively with *in vitro* fertilization outcomes (Li et al., 2011). But age has also an influence on the oocyte itself. When oocyte is getting older, cellular and molecular abnormalities have a greater probability to occur. A lot of functional changes associated with oocyte aging have been observed, including furthermore: decreased fertilization rate, polyspermy, parthenogenesis, chromosomal anomalies, apoptosis and abnormal and/or retarded development of embryo (Miao et al., 2009). A study realized by

Wilcox et al., showed that oocyte aging increase significantly the risk of early pregnancy loss (Wilcox et al., 1998). It has moreover been showed that embryos resulting from aged oocyte fertilized with ICSI showed low implantation rates and low developmental potential after transfer (Esfandiari et al., 2005; Javed et al., 2010; Liu et al., 1995; Nagy et al., 1993; Van Steirteghem et al., 1993). In a healthy body, reactive oxygen species (ROS) and antioxidants remain in balance. When there is an unbalanced towards an overabundance of ROS, oxidative stress occurs and can do damages. It has been suggested that oxidative stress modulates the age-related decline in fertility (Agarwal et al., 2008; Agarwal et al., 2005) . Antioxidants are actually used to enhance female and male fertility, although benefits on fertility are still controverted (Ruder et al., 2008; Ruder et al., 2009; Visioli and Hagen, 2011).

- Obesity

Obesity is known as a cause of increased risk of infertility, mostly because of ovulatory dysfunction, but also because obese women have increased risks for miscarriage and stillbirth. In case of ART, implantation and pregnancy rates are lower in obese subjects versus subjects with normal weight (Maheshwari et al., 2007). A retrospective study using oocyte donation model analyse 97-first cycles recipients of oocyte donation under conditions of controlled hormonal stimulation and embryos quality was evaluated. The conclusion of this paper said that they found no correlation between the BMI of the women receiving the embryo (given by another woman with a high Body Mass Index) in their uterus and the implantation rates for the same grade of embryo morphology quality. These results could mean that uterine receptivity is not negatively influenced by obesity and that the oocyte or the embryo is the cause of decreased pregnancy rate in obese women (Wattanakumtornkul et al., 2003). Another retrospective study on 536 first-cycle recipients with donor oocytes had confirmed these results (Styne-Gross et al., 2005). Metabolic syndrome and polycystic ovary syndrome are two diseases related to obesity; they can also have a negative impact on the women fertility. In mice, it has been recently shown that the negatives effects of obesity and insulin resistance persist beyond the pre- and the peri-conception period, affecting embryonic development and reproductive outcomes (Cardozo et al., 2011). The mechanisms leading to an altered oocyte and embryo quality in obese women could result from an altered maternal metabolic environment in FF. A study has explored different molecules present in FF from obese women compared to women with a moderate BMI and have found that obese women have an increased level of C-reactive protein in FF. This molecule might indicate increased oxidative stress in the oocyte's microenvironment, which impairs its development (Robker et al., 2009).

- Smoking

In a study realize by Gruber et al., in 2008, smokers presented a higher number of non-fertilized oocytes than nonsmokers (20.1% vs. 10.8% of fertilization failure)(Gruber et al., 2008). Another study realize in 2006 in a cohort of twenty-seven patients undergoing IVF classified as smokers and 32 as non-smokers showed that smokers had decreased number of retrieved oocytes compared with non-smokers ($p < 0.05$). This study demonstrates that active cigarette smoking increases the zona pellucida thickness of oocytes and decreases the quality of oocytes (Depa-Martynow et al., 2006). Tobacco compounds exert a deleterious effect on the process of ovarian follicle maturation. This effect is expressed by decreased pregnancy rate, increased early spontaneous abortions and altered ovarian reserve. (Sepaniak et al., 2006; Soares and Melo, 2008).

3. The sperm

The role of the sperm has long been reduced to its progression through the female genital tract, and the penetration of the oocyte in order to provide the paternal genetic material. However, the quality of this genetic inherited material is of great importance for embryo development and early pregnancy. In fact, it has little influence on fertilization and early embryo cleavage until the passage of 4 to 8 cells-stage, because the embryo uses almost exclusively the maternal RNA and proteins produced and stored during oocyte growth and maturation. By the third day of development, the embryonic genome begins to be expressed and embryonic RNAs and proteins gradually replace maternal ones. In other words, even if ART allows *in vitro* production of embryos, these embryos can be unable to implant and give rise to an ongoing pregnancy and/or a healthy offspring. Their development will depend on the activation and quality of its genome, half of which being of paternal origin.

3.1 The role of spermatozoa and the classical evaluation of semen

The different roles of the sperm during reproductive process are classified according to the stage at which embryo development fails (Tesarik et al., 2004):

- Early paternal effects includes inability to go through the female genital tract (problems of concentration, mobility, classic morphology), to spontaneously or in an assisted way (IVF, ICSI) fertilize the oocyte (inability to fix the zona pellucida glycoproteins ZP 1, 2 or 3, or to perform the acrosome reaction, …), to activate the oocyte (deficiency in phospholipase zeta (Saunders et al., 2002) ...)
- Late paternal effects will become visible during (or after) the activation of the embryonic genome, particularly the paternal genome, resulting in the development of apparently normal early embryos (until the third day of development), but which are unable to implant or to continue a long-term development after implantation.
- Very late paternal effects are suspected when faced with recurrent miscarriages or with pathologies of imprinted genes (Lucifero et al., 2004; Zini, 2011b) leading potentially to inherited diseases.

Since the beginning of ART, the evaluation of male fertility has often been restricted to a conventional semen analysis as recommended by World Health Organization (WHO) (World Health Organization., 2010). In the last edition of the WHO manual for the semen analysis and processing, limit thresholds have been modified according to the results published by Cooper (Cooper et al., 2010). Actually, 1953 men from 5 studies in 8 countries have been included in this review. The studies have analyzed semen from men who recently become parent with a known TTP (time-to-pregnancy: time between the beginning of unprotected intercourse and occurrence of a pregnancy) of maximum 12 months. Percentiles 95 for the different results were then defined as the new limit thresholds. But in daily ART practice, it appears that these tests are not sufficient to permit the diagnostic of possible late or very late paternal effects. Some men first judged as infertile succeed to obtain a pregnancy with their partner (Haugen et al., 2006), while other men judged above limit thresholds as defined by WHO failed to obtain a pregnancy (Bonde et al., 1998) or achieved it with miscarriage. Despite these limitations, conventional sperm analysis has permit to propose adapted ART treatments in a majority of patients, and the Intracytoplasmic Sperm Injection (ICSI) technique (Palermo et al., 1992) used since the early nineties has given the chance for a part of these subfertile or infertile men to become parents.

3.2 Sperm DNA particular organization

The first studies on the genetic material carried by sperm have been published at the end of the 70s with the establishment of the first human sperm karyotypes by Rudak (Rudak et al., 1978). In the 90s, the technique of Fluorescent in situ hybridization (FISH) permits to study aneuploidy, abnormalities such as translocations, and recombinations during meiosis (Shi et al., 2001). Over the past decade, a growing number of papers have been published on the organization of sperm chromatin and the integrity of the DNA double helix. It appears that different problems in the sperm genetic material - called DNA damages - can affect male fertility (Shen et al., 1999; Spano et al., 2000). These DNA damages include abnormalities in chromatin condensation and organization, in fragmentation of the double helix (single and/or double strand), in DNA bases modifications, in epigenetic damages, etc... (Aitken et al., 2009).

During spermiogenesis, the final phase of spermatogenesis after meiosis, chromatin is radically reorganized and undergoes an extreme condensation resulting in a shift from a nucleosome-based genome organization to the sperm-specific, highly compacted nucleoprotamine structure. The DNA double helix of somatic cells is indeed organized into chains of nucleosomes by a family of nuclear proteins, the histones. At spermiogenesis, histones are mostly replaced by transition proteins, which are themselves replaced by two types of specific nuclear proteins of the male gametes, protamines 1 and 2. These proteins are particularly rich in arginines (which help to neutralize negative charges of DNA phosphate groups), in cysteines (which allow the formation of disulfide bonds intra and inter –protamines), and in histidines (which, in combination with cysteines, permit the establishment of zinc bridges). Because of this particular composition, the sperm chromatin fibers are strongly compacted, and have almost a crystalline organization (Bjorndahl and Kvist, 2010). In human sperm, about 85% of histones are replaced by protamines, while the other 15% remain. So sperm chromatin is organized predominantly in toroids (by protamines), the rest being organized in nucleosomes (by histones) or attached to the nuclear matrix (MARs: Matrix attached regions) (Govin et al., 2011; Jonge and Barratt, 2006; Ward, 2010). Recent data support the idea that region-specific programming of the haploid male genome is of high importance for the post-fertilization events and for successful embryo development. The molecular basis of post-meiotic male genome reorganization and compaction constitutes one of the last black boxes in modern biology of reproduction. Although the successive transitions in DNA packaging have been well described, the molecular factors driving these near genome-wide reorganizations remain obscure (Rousseaux et al., 2011).

This particular organization has several roles. Firstly sperm DNA occupies about 10 times less space than somatic cell DNA, which reduces the volume of the head and facilitates the movement of sperm. Secondly, this extreme condensation makes the DNA inaccessible to molecules such as free radicals (ROS produced by sperm mitochondria and by leukocytes), providing a protection against oxidative attacks. Finally, the distribution of remaining nucleosomes is not random but concerns gene regions involved in the early embryonic development. Moreover it seems increasingly clear that this organization and the inheritance of paternal histones have a very important role in epigenetic control (Miller et al., 2010; Tavalaee et al., 2009; Ward, 2010). Firstly remaining histones preferentially bind to DNA regions involved in early embryo development. As they are less compacted than if

organized by protamines, these regions can be easily decondensed and transcripted. Secondly, sperm histones undergo different modifications (methylation, acetylation,...) that impact expression of the linked DNA domains (Rousseaux et al., 2008), and that can be perturbed in in infertile patients. Similarly, the ratio of protamines 1/2, which should normally be close to 1, can have a significant negative impact on fertility when disturbed (Hammoud et al., 2011). So it is clear that bad condensation of sperm DNA have an impact on male fertility (Venkatesh et al., 2011), and a recent study demonstrates the great differences between a fertile control group and a patient group presenting repeated spontaneous abortions in terms of chromatin condensation and stability (Talebi et al., 2011).

3.3 Sperm DNA damages and oxidative stress

Damages to the DNA double helix are quite varied (Bennetts et al., 2008). The most common is the fragmentation of the DNA double helix, which involves the breaking of a bond between a phosphate group and the neighboring deoxyribose on one or both strands: this is called single- or double-strand fragmentation. Another damage consists of base modifications, becoming oxidized or alkylated. Covalent bonds between a strand and another molecule or the other strand may also appear. DNA adducts are formed from xenobiotics and their metabolites, or from the oxidation of membrane lipids of the sperm, such as ethenonucleosides. These adducts can be either stable or result in the loss of a base. All these changes interfere with DNA replication or transcription. Similarly, an aberrant base repair may cause the appearance of a mutation that can be transmitted to the offspring (Aitken and Roman, 2008).

Another important group of damages to sperm genetic material concerns the epigenetic control. One of the most studied epigenetic marks is DNA methylation. Knowing that the epigenetic environment of the sperm, especially histones and their modifications, has an important role in the establishment and maintenance of epigenetic marks in the embryo, it appears that aberrant epigenetic regulation during spermatogenesis has a major impact on male fertility and embryonic development (Rajender et al., 2011). For example, poor sperm quality has been shown to be associated with hypermethylation of sperm genome (Houshdaran et al., 2007). This is probably due to a failure in epigenetic marks erasure that normally occurs during spermatogenesis. Promoter methylation aberrations of the gene encoding the enzyme methylenetetrahydrofolate reductase (MTHFR, which plays a key role in maintaining the bioavailability of methyl groups) are associated with secretory azoospermia (Khazamipour et al., 2009). Moreover, abnormal methylation can affect gene imprinting, and have an impact on embryonic development. Similarly, methylation aberrations were detected in imprinted genes in men with idiopathic infertility (Poplinski et al., 2010). Finally, many syndromes (Prader-Willi, Angelman, Silver-Russell and Beckwith-Wiedemann syndromes) are known to be in a number of cases due to imprinting defects (Rajender et al., 2011). All these methylation abnormalities observed in patients are fortunately not directly transmitted to the embryo: the question is how far they can be hereditary? In the epigenetic reprogramming of sperm DNA, these marks are erased and then re-established differentially in men and women. Chromatin organization plays a key role in the establishment and maintenance of the methylation profile.

An important matter about the DNA damages concerns their origin. A theory has been proposed by Aitken (Aitken et al., 2009): DNA damages can be considered as a process

taking place in two stages. First of all, spermatogenesis, especially spermiogenesis, leads to inadequate sperm production, with an improperly compacted DNA. In a second step, these badly compacted spermatozoa are vulnerable to outside attacks, mainly of oxidative type (ROS). There is indeed a significant correlation between DNA fragmentation and the level of 8-hydroxy-2'-deoxyguanosine [8OHdG], a DNA adduct derived from the oxidation of DNA (Aitken and De Iuliis, 2010). Apoptosis also appears to play an important role since the sperm cell is unable to perform a complete process of programmed cell death (Aitken and De Iuliis, 2010). The compacted DNA, mainly silent, contained in a cytosol-depleted cell, and physically separated from mitochondria limit the action of classical apoptosis effectors. The problem is that these defective sperm cells, normally silently eliminated in the female genital tract, remain capable of fertilization, especially in ARTs (Barratt et al., 2010; Kurosaka et al., 2003; Sakkas et al., 2004; Weng et al., 2002).

In a normal sperm, there is a balance between antioxidant processes and ROS production, the latter being normal below a certain level, since it plays a role in sperm capacitation, acrosome reaction, and fertilization (Griveau and Le Lannou, 1997). However, oxidative stress remains controlled, and the presence of many antioxidants in seminal fluid (taurin, vitamin C, vitamin E, glutathione, uric acid, thioredoxin, glutathione peroxidase) limits the damaging effects (Tremellen, 2008). However, the balance between ROS and antioxidants may be disturbed by various external factors or diseases, which reduce the production of antioxidants, increase production of ROS, and / or influence the senescence of spermatozoa. All this may lead, especially in cases of inadequate chromatin compaction, to DNA damages (Koppers et al., 2008).

3.4 Impact of sperm DNA damages on fertility and ART

DNA damages have been studied in different populations of patients or sperm donors. In patients with an elevated production of seminal ROS, DNA fragmentation is also increased (Mahfouz et al., 2009). Avendano *et al*. (Avendano et al., 2009) analyzed simultaneously sperm morphology and DNA fragmentation, and showed an increased fragmentation in the infertile patient group, despite the selection of morphologically normal spermatozoa for the DNA analysis. Correlations exist between fragmentation and classical sperm features, but not always those expected: in a population of 1633 patients, an inverse correlation between sperm count, fast progressive motility and DNA fragmentation has been found (Cohen-Bacrie et al., 2009). Altogether, these results allow us to conclude that the study of fragmentation gives further additional information about male fertility to those provided by conventional analysis of semen.

In a great part of all the published data concerning fragmentation, authors try to assess the predictive value of different tests in terms of chances for achieving pregnancy, spontaneous or by different ARTs. Zini showed a strong association between DNA damage and failure to obtain a pregnancy naturally or by intrauterine insemination (Zini, 2011a; Zini and Sigman, 2009). This association, although weaker, exists also with the clinical pregnancy rate in IVF, and in a lesser extent for ICSI. On the other hand, fragmentation increases the risk of miscarriage for IVF and ICSI. The impact of DNA damages is also more significant for the IVF and ICSI in terms of take-home baby rate than clinical pregnancy rate (Avendano et al., 2010; Benchaib et al., 2007; Boe-Hansen et al., 2006; Borini et al., 2006; Bungum et al., 2007; Check et al., 2005; Duran et al., 2002; Evenson et al., 1999; Evenson and Wixon, 2008;

Frydman et al., 2008; Gandini et al., 2004; Henkel et al., 2003; Host et al., 2000; Huang et al., 2005; Lin et al., 2008; Loft et al., 2003; Micinski et al., 2009; Muriel et al., 2006; Simon et al., 2010; Spano et al., 2000; Speyer et al., 2010; Tarozzi et al., 2009; Zini et al., 2005).

However, the sensitivity of fragmentation tests remains low. The explanation for this observation is that the predictive value of DNA fragmentation tests depends on many factors (Sakkas and Alvarez, 2010): the type of DNA damage (single or double strand), the method of analysis, the site of injury (introns or exons), the percentage of fragmented cells, the extent of damage per cell, or the presence of other damage such as DNA adducts. On the other hand, it is clear that the sperm DNA quality is not the only explanation for the failure to obtain a pregnancy: oocyte quality and in particular its ability to repair sperm DNA (Meseguer et al., 2011), the number of oocytes available in the case of IVF, and the quality of the endometrium have a direct impact on the chances of pregnancy.

Another important point is to avoid a negative impact of ARTs – and in particular of the semen processing – on oxidative stress and fragmentation. It is therefore important to separate safe sperm from leucocytes and dead cells. Gradient centrifugation or swim-up allows the separation of different cell types, while the centrifugation of semen without cell separation should be rejected (Jackson et al., 2010; Marchesi et al., 2010; Monqaut et al., 2011). Anyway, it seems important to limit the number of centrifugations, which stimulate the ROS production by spermatozoa (Aitken and Clarkson, 1988; Aitken et al., 2010). The elimination of apoptotic sperm by Magnetic Activated Cell Sorting (MACS) could also improve pregnancy rates (Dirican et al., 2008; Lee et al., 2010; Polak de Fried and Denaday, 2010; Rawe et al., 2010). Another issue is to avoid transition metals (which may increase the damages caused by ROS) and to add antioxidants to the sperm preparation medium (Aitken et al., 2010). Finally, the addition of antioxidants in the cryopreservation medium seems to decrease the oxidative stress caused by the cryopreservation itself (Thomson et al., 2009).

Concerning the technique to fertilize the oocyte when sperm DNA damage is present, it seems that ICSI is the least influenced by the DNA fragmentation, although the impact of the latter remains significant on the risk of miscarriage (Zini and Sigman, 2009). Several techniques derived to improve ICSI aim to add new sperm selection criteria in relation to the quality of their genetic material and / or their fertilizing ability.

The use of polarized light highlights the well-organized structures such as the oocyte meiotic spindle, but also the well-organized sperm chromatin which then appears bright, in contrast to the improperly condensed one. Used in ICSI, this technique seems to improve implantation and clinical pregnancy rates (Gianaroli et al., 2008; Gianaroli et al., 2010). Sperm selection based on its ability to recognize and bind hyaluronic acid (HA), a major component of cumulus-oocyte complexes extracellular matrix, has been also proposed. Using HA-coated Petri dishes, this technique, called PICSI or HA-ICSI, allows the selection of sperm with a less fragmented DNA and a better head morphology assessed by high magnification (X6500-X10000) living sperm observation (Motile Sperm Morphology Examination organelle or MSOME) (Parmegiani et al., 2010a). It also appears to improve embryo quality and implantation rate (Parmegiani et al., 2010b).

Finally, the technique of IMSI (Intracytoplasmic Morphologically Selected Sperm Injection), which has been developed 10 years ago, looks promising in particular for the selection of

sperm without problems of chromatin organization. In 2001 Bartoov and his team have associated their MSOME technique with ICSI to give birth to IMSI. Sperm is then observed and selected on a morphology basis at a 10000 times magnification, as compared to a conventional ICSI magnification of 200 to 400 times. In IMSI, a Nomarski differential interference contrast is used and allows a more precise evaluation of the classical strict criteria of sperm morphology (shape of the head, midpiece...), and of the presence at the head of surface abnormalities called vacuoles. These vacuoles vary in size and number between sperm an also between patients. Several teams have shown a correlation between a higher rate of vacuolated sperm and higher DNA damages (Franco et al., 2008; Garolla et al., 2008; Oliveira et al., 2010), and IMSI was presented as a potential solution for the selection of non-fragmented sperm in order to increase the implantation rate and to reduce the miscarriage rate (Antinori et al., 2008; Bartoov et al., 2003; Bartoov et al., 2002; Berkovitz et al., 2006a; Berkovitz et al., 2006b; Berkovitz et al., 2005). These date are however controversial and the question about the origin – fragmentation or decondensation – of the vacuoles remain open. Recently, different teams aimed to answer this question. They studied a specific population of sperm with large vacuoles (more than 13% of the head surface)(Boitrelle et al., 2011; Franco Jr et al., 2011; Perdrix et al., 2011) and came to the conclusion that their presence is correlated with abnormal DNA condensation. In this particular population, the surface depressions observed in MSOME reflects the presence of a nuclear vacuole containing a badly condensed chromatin, as confirmed by aniline blue staining (Perdrix et al., 2011). On the other side, no positive correlation was demonstrated between these large vacuoles and DNA fragmentation (Boitrelle et al., 2011; Perdrix et al., 2011). So it seems now clear that the presence of large vacuoles reflects a disruption of sperm chromatin condensation. Other types of vacuoles also exist (particularly small vacuoles, sometimes numerous, with an area <4% of the surface of the head). Further studies are still needed to understand if these vacuoles also reflect DNA damages.

3.5 Impact of environment on sperm DNA damages

Numerous factors affect semen oxidative balance, sperm quality and DNA damages. Tobacco has an impact on oxidative stress, by increasing the presence of leukocytes in semen (Saleh et al., 2002; Soares and Melo, 2008). Pesticides provoke sperm DNA damages, especially in cases of occupational exposure, as well as pollutants such as heavy metals or polycyclic aromatic hydrocarbons in the air (Delbes et al., 2010; Perry, 2008; Somers and Cooper, 2009). Occupational exposure of testicles to heat also has deleterious effects on male fertility (Mieusset et al., 1987; Paul et al., 2008) as well as sporadic exposure (Rockett et al., 2001). The effects of obesity on semen quality are marked not only on the concentration of sperm, but also on DNA fragmentation (Kort et al., 2006), probably as a result of a high scrotal temperature and of an important hormonal disruption (Du Plessis et al., 2010). Mobile phone port to the belt, especially when using a hands-free kit (the emission of electromagnetic waves is more important during a call) could have an adverse effect on male fertility (Desai et al., 2009). Age has also an effect on sperm quality and in particular on the quality of genetic material (Sartorius and Nieschlag, 2010). It has been demonstrated by Singh (Singh et al., 2003) that the double-strand fragmentation increases with age. Finally, other factors such as high consumption of alcohol, dietary exposure to plasticizers, stress, poor diet, are potentially impacting the maintenance of the balance ROS / antioxidants in the semen (Tremellen, 2008).

Infection of the genitourinary tract is an important medical cause of DNA damages, as the influx of activated leukocytes provokes an increased production of free radicals. Systemic infections may also increase oxidative stress in sperm through an increase in the concentration of leukocytes in semen (HIV) (Umapathy et al., 2001), or via a systemic oxidative stress (Tremellen, 2008). Patients who underwent surgery to treat cryptorchidism still have a production of ROS and a sperm DNA fragmentation higher than those of fertile men (Smith et al., 2007). The presence of varicoceles also increases oxidative stress and DNA damage (Chen et al., 2004; Smith et al., 2006). Finally, there is a variety of iatrogenic origins of DNA damages. Cancer treatments (chemotherapy, pelvic radiotherapy) are generally detrimental to the integrity of sperm DNA (Tremellen, 2008). Concerning ARTs, various semen manipulations may affect sperm DNA. Centrifugation and cryopreservation increased oxidative stress, and consequently the DNA damage (Aitken et al., 2010; Thomson et al., 2009).

Several therapeutic strategies have been proposed (Hazout et al., 2008). The most effective is of course to treat infections of the urogenital tract. The patient must also be aware of the harmful effects of lifestyle (smoking, obesity, exposure to heat ...) on the quality of his sperm. If possible, it may be beneficial to limit exposure to pollutants. In the presence of varicoceles, embolization or surgery improve sperm quality, not really in term of numeration or morphology but clearly in terms of fragmentation of DNA (Smit et al., 2010). Finally there are several studies that have examined the effect of antioxidant treatment semen quality and sperm DNA. While it is necessary to have new controlled-randomized studies, many preliminary publications suggest beneficial effects (Agarwal and Sekhon, 2010).

In conclusion, the study of sperm DNA has highlighted abnormalities in chromatin organization, in DNA double helix and in epigenetic marks that impact male fertility and sperm role during reproduction. Sperm DNA analyses enable new diagnosis in infertilities previously classified as of idiopathic origin. These problems of DNA damages appear to influence fertility in early pregnancy by causing failure of implantation or repeated miscarriages. New treatments begin to be developed, but need to be well evaluated in prospective controlled-randomized studies. Improved lifestyle, environment and antioxidant treatments could improve semen and sperm DNA quality. However the newly developed sperm selection techniques remain limited to the quality of semen used: if all the sperm have an abnormal DNA, it becomes impossible to make a selection.

Finally, in accordance with the conclusions of the Position Report published by the Working Group of the ESHRE Andrology (Barratt et al., 2010), several recommendations can be made. First, it is necessary to continue to develop basic research on the chromatin organization to better understand the causes and the nature of DNA damages. It is also important to standardize analysis techniques to allow a correct interpretation of results, and also to be able to compare the different studies. The development of animal models allowing studies on the long-term effects of sperm DNA damages and ARTs, and well conducted studies paralleling DNA damages and results in ARTs (with neonatal data) will help the clinician in the guidance of patients in treatment options. Finally, the long-term monitoring of children from ARTs should be systematized.

4. Embryo

Successful implantation requires a competent blastocyst able to cross-talk with the receptive endometrium. This process includes dynamic and coordinated changes at the intricate

crossroad between endocrinology, immunity and angiogenesis. Implantation process is not easily accessible *in vivo* to the research for obvious ethical and technical reasons. The only way to evaluate it is to extend data derived from animal studies, from *in vivo* studies about endometrium and embryo separately or from *in vitro* endometrium-early blastocyst co-culture. Despite extensive research in this field, the implantation process remains the black box of the reproduction, even in 2012. An important wastage of embryos is observed since the majority of blastocysts will perish before or around implantation (Macklon et al., 2002; Robertson et al., 2011; Teklenburg et al., 2010a). Implantation failure would come from an inadequate gamete/embryo quality, an erroneous cross-talk at the implantation site or an inadequate peri-conceptual environment. As recently demonstrated by Robertson et al., the peri-conceptual period and environment are critical for pregnancy success: the local cytokine network of the reproductive tract integrates environmental information and provides a signaling system programming both maternal receptivity and embryo development (Robertson et al., 2011). On the other hand, data from Kashiwagi (Kashiwagi et al., 2007) and from Bauersachs (Bauersachs et al., 2009) evidence that both embryo and endometrium are closely related and are actively influenced by each other, in order to favor pregnancy or, at contrary, to reject it.

Implantation is a 3-steps process including free-floating apposition of the blastocyst then adhesion between two epitheliums: endometrium and trophoblast, and finally, the fine-tuned process of trophoblastic invasion and differentiation. This materno-fetal dialogue is mediated through a broad array of molecules released at the implantation site both by trophoblast and/or endometrium. During implantation process, embryo and maternal tissues talk about immunity and angiogenesis, on an autocrine, paracrine and/or juxtacrine mood (Singh et al., 2011). In definitive, implantation process is the best example of a successfully tolerated graft and controlled tumor invasion.

4.1 Trophoblast differentiation

Placentation in Humans is characterized by the formation of a highly invasive hemochorial placenta accompanied with dramatical changes in the vasculature of the uterus. Differentiation of cytotrophoblast includes villous trophoblast ensuring exchanges at the maternal-fetal interface, and extravillous cytotrophoblast (EVCT) anchoring the placenta and participating to the vascular remodeling of the wall of spiral arteries. This deep trophoblastic invasion through the entire depth of the endometrium and the inner third of the myometrium is considered the hallmark of human pregnancy. Trophoblast deriving from trophectoderm, clothes the terminal villi (the outermost branches of the villous trees) that descend from the chorionic plate and fix to the basal plate by anchoring villi (Carter, 2011). The chorionic villi are then the structural and functional unit of the placenta, floating within the maternal blood present in the intervillous chambers. Villous cytotrophoblast that mantles the chorionic villi, aggregates and fuses to form the syncytiotrophoblast (ST), allowing efficient communication and signal exchanges. It ensures the endocrine function of placenta, releasing hormones involved in the homeostasis of pregnancy such as chorionic gonadotropin and placental lactogen. In the other hand, EVCT refers to the invading trophoblast that exits the villi and colonizes the uterine wall and spiral arteries, the lumen of which is plugged by trophoblastic cells during the first 8 weeks of gestation (Fournier et al., 2011).

Subsequent transformation of spiral arteries is a 2-steps process: the first seems to be mediated to factors secreted by uNK and covers endothelial swelling and vascular smooth muscle cells (VSMC) loss of coherence (Harris, 2011); the second is linked to the loss of endothelium, the beak down of VSMC/elastic fibers and finally the incorporation into the vessel wall of extravillous trophoblast cells, embed in a fibrinoid-rich matrix (Carter and Pijnenborg, 2011). The consequences of these changes are the widening of the vessel lumen, highly increasing the supplying blood to the intervillous space. In the placenta formation as during implantation process, immunity and angiogenesis are closely related.

Successful invasion is mediated through both maternal and embryo factors. Differentiation of cytotrophoblast to ST or EVCT is accurately controlled by transcription factors, hormones, growth factors, cytokines, and O2 level. Implantation, early embryo development and placentation processes take indeed place under a low oxygen environment during the first trimester of gestation. Many pregnancy disorders such as fetal growth restriction or pre-eclampsia are associated to the loss of invasiveness, with the extravillous trophoblast that does not reach the myometrium and the corresponding arterial segments retaining therefore their endothelium; whereas an excessive trophoblast invasion is associated with invasive mola, placenta accrete or choriocarcinoma (Lunghi et al., 2007). Pre-eclampsia is a pregnancy-specific syndrome characterized by hypertension, proteinuria and edema. It resolves on placenta delivery. Placental hypoxia is likely to be responsible for the maternal vascular dysfunction, through the increased placental release of anti-angiogenic factors such as soluble receptors flt1 and endoglin, both binding vascular endothelium growth factor (VEGF), placental growth factor (PLGF) and transforming growth factor (TGF)beta1/3 in the maternal circulation and causing endothelial dysfunctions (Lorquet et al., 2010).

4.2 Embryonic signals: Example of the embryo-specific hCG

The first known human embryo specific signal is hCG which is produced by the embryo before implantation, since it has been detected in embryo supernatants as soon as day 2 post-fertilization (Ramu et al., 2011). Human chorionic gonadotropin is the major pregnancy glycoprotein hormone, from the cystine knot cytokines superfamily, and is specifically secreted by trophoblast. It is classically well known from it action as corpus luteum progesterone production rescuer but many recent studies has evidenced more and more extra-gonadal actions. HCG is a non-covalently linked heterodimer composed of 2 subunits α (produced by cytotrophoblast) and β (produced by syncytium). Maternal concentration and glycosylation of hCG change throughout the pregnancy since 3 majors isoforms are described, all of which sharing the hCG β amino acide sequence (Banerjee and Fazleabas, 2011):

- **native hCG**: produced by syncytium, its concentration reach a peak around week 10 then drops. Its main gonadal action is to rescue corpus luteum and support progesterone production but many extragonadal actions on uterine receptivity, trophoblast functions, immunity and angiogenesis are described (see below). It signals through the LH/hCG receptor (LHR), which has been described on different extra-gonadal human tissues (Berndt et al., 2006; Rao, 2006). Recent data concerning impact of hCG on uNK cells suggest that hCG may also bind to the mannose receptor (Kane et al., 2009).
- **hyperglycosylated hCG (H-hCG):** produced by EVCT, it accounts for 90% of total hCG production with a peak in the week following implantation. It is an autocrine factor

modulating its own production and highly promoting invasiveness of trophoblast. H-hCG 2D structure is different from native hCG, appears to be independent in numerous biological functions, overall promoting placental implantation/invasiveness and is likely to signal through a different receptor than LHR (TGF-beta Receptor?). H-hCG is also critical for choriocarcinoma growth and malignancy (Cole, 2010; Fournier et al., 2011; Guibourdenche et al., 2010).

- **free beta subunit of H-hCG (hCG free β):** produced by all non-trophoblastic malignancies, it inhibits apoptosis and promotes malignant transformation of cancer cells.

Data from literature and from our own work show that through native hCG (and H-hCG concerning invasiveness of EVCT), embryo profoundly intervenes in its own implantation and favors immunological tolerance and active angiogenesis that are crucial for successful implantation (Banerjee and Fazleabas, 2011; Tsampalas et al., 2010).

At the first steps of implantation, hCG is able to prolong the WOI, by inhibiting decidual Insulin like Growth Factor (IGF)-Binding Protein and IGFI (both markers of complete decidualization) (Fluhr et al., 2008b), and in association with interleukin 1 (IL-1), to favor adhesion via the increase in trophinin expression on endometrial epithelium (Sugihara et al., 2008). Concerning trophoblast functions hCG (and its hyperglycosylated variant) has been demonstrated to promote trophoblastic cells migration, and to reduce endometrial barrier, through its action on matrix metalloprotease protein 9 (MMP9, increased), TIMP-1, -2, -3 (reduced), GM-CSF, IL-11 (Chen et al., 2011; Fluhr et al., 2008a; Paiva et al., 2011). We also observed that hCG is able to increase leukemia inhibitory factor (LIF) production by endometrial epithelial cells *in vitro*, a cytokine known to be crucial for implantation in mice (Perrier d'Hauterive et al., 2004; Stewart, 1994). These data has been confirmed by different teams (Licht et al., 2002; Sherwin et al., 2007).

HCG levels coincide with the development of trophoblast tolerance. Indeed, it offers many immunological properties. For example, hCG increases the number of uterine natural killer cells that play a key role in the establishment of pregnancy (Kane et al., 2009). HCG also intervenes in the development of local immune tolerance through apoptosis via Fas/Fas-Ligand (Kayisli et al., 2003). It also modulates the Th1/Th2 balance toward the Th2 pathway (Koldehoff et al., 2011). During pregnancy, the balance of Th1 (cell-mediated immunity) and Th2 (humoral immunity) cytokines is characterized by an initial prevalence (but not an exclusivity) of Th2 cytokines, followed by a progressive shift towards Th1 predominance late in gestation, that when is abnormal, may initiate and intensify the cascade of inflammatory cytokine production involved in adverse pregnancy outcomes (Challis et al., 2009). In a more general way, Khan *et al.* showed that the administration of hCG to nonobese diabetic mice (NOD) before the beginning of the clinical symptoms reduced the increase in glycaemia, reversed establishment of insulitis, and inhibited the development of Th1 autoimmune diabetes (Khan et al., 2001). A recent large proteomic study demonstrated the influence of several molecules produced by the trophoblast that regulate the mother's immune tolerance. Among these molecules, hCG inhibits T lymphocytes (Dong et al., 2008).

The transient tolerance, evident during gestation is at least partially achieved via the presence of regulatory T cells that are expended during pregnancy (Dimova et al., 2011; Ernerudh et al., 2011; Fainboim and Arruvito, 2011; Nevers et al., 2011; Xiong et al., 2010) at

the maternal-fetal interface, showing a suppressive phenotype, whereas Treg cells are not increase in the circulation of pregnant women. In part, they are attracted by hCG at the fetal–maternal interface during early pregnancy, via a LHR signalling (Leber et al., 2010; Schumacher et al., 2009). Finally, hCG treatment of activated dendritic cells results in an up-regulation of MHC class II, IL-10 and IDO expression, reducing the ability to stimulate T cell proliferation. Impact of dendritic cells during implantation has recently been review by Blois (Blois et al., 2011).

Interestingly, immunologic properties of hCG are likely to differ as far as urinary versus recombinant molecules are concerned. These data are particularly important to keep in mind if immunomodulation of hCG would apply in clinical practice (Carbone et al., 2010; Kajihara et al., 2011).

Beside immune tolerance, a successful implantation requires also an extensive vascular remodeling of maternal arteries at the placental bed. Recent data demonstrate angiogenic effects of hCG via its interaction with endometrial epithelial cells which are able to produce angiogenic VEGF under hCG-LHR binding. Moreover, hCG highly increases angiogenesis of new mature vessels, via the stimulation endothelial cells proliferation as well as the migration of smooth muscle cells leading to the maturation of vessels, an important step for placentation (Berndt et al., 2009; Berndt et al., 2006).

Taken together the immune and vascular roles of hCG during early pregnancy, it is not surprising that this hormone has been studied during pregnancy disorders such as pre-eclampsia (Kalinderis et al., 2011; Norris et al., 2011).

5. The endometrium

Implantation of the embryo into the maternal endometrium represents a unique biological process example of an immunological (tolerance of an allograft) and biological (adhesion of two epithelia) paradox (Perrier D'hauterive et al., 2002). The success of implantation depends on a receptive endometrium, a functionally normal blastocyst and a synchronized cross-talk between embryonic and maternal tissues. Though sexual steroids control the process (Paulson, 2011), a cascade of cytokines, growth factors and adhesion molecules are the private paracrine mediators of the uterine receptivity. Particularly, progesterone is crucial to establishment of endometrial receptivity, modulating subsequently the appearance or disappearance of a wide network of molecules responsible of the uterine receptivity.

5.1 The window of implantation and the decidua

Endometrium undergoes cyclic morphological and functional changes, including growth, differentiation and desquamation. Altogether, these cyclic physiological modifications lead to the preparation of a receptive endometrium able to tolerate the foetal allograft and to control invasiveness of trophoblastic cells, allowing implantation process during the mid-secretory phase. Control of gene expression is crucial to this process, and inappropriate epigenetic modifications occasioning an altered chromatin structure and transcriptional activity may result in aberrant expression of receptive endometrial pattern. Epigenetics is likely to be associated with the initial regeneration and then proliferation of endometrium, angiogenesis, decidual reaction and angiogenesis (Munro et al., 2010).

Whereas the implantation can occur in any human tissue, the endometrium is one of rare in which the embryo cannot implant, except during one limited period called the *window of implantation* (WOI), at the time when progesterone reaches peak serum concentrations (day 20-day24), allowing embryo to implant only under optimal circumstances. During this period, it offers a high receptivity to the embryo, resulting in a successful pregnancy (Lessey, 2011). This physiological state of the endometrium at the mid-secretory phase allows blastocyst 3-steps implantation process: apposition, then adhesion followed by trophoblast invasion and then induction of localized changes in the stroma called decidualization (Rashid et al., 2011).

WOI ends with the decidual transformation, where endometrium becomes a well vascularized tissue and stromal cell differentiate into specialized decidual cells (Teklenburg et al., 2010b). Factors secreted by decidual cells (such as LEFTY-A) compromise endometrial receptivity, ending the WOI. Oedema, increased vascular permeability, proliferation and transformation of fibroblast into cuboid secreting cells, invasion of leucocytes and angiogenesis are the principal mechanisms enabling decidua to resist to oxidative stress, to allow interaction within immune cells and to restrain the invasiveness of trophoblasts.

Apprehending molecular mechanisms of endometrial receptivity and then decidual transformation would provide a better understanding implantation process and its pathological implications. Failure to express adequate decidualization pattern is likely to induce early pregnancy lost or predispose to obstetrical complications such as implantation failure, recurrent miscarriages, pre-eclampsia, fetal growth restriction... (Blois et al., 2011; Brosens, 2011; Plaisier, 2011).

Actual research aims to dissect the molecular basis for the changes occurring during the WOI at the genomic and proteomic level (Berlanga et al., 2011; Diaz-Gimeno et al., 2011; Horcajadas et al., 2007). Despite a lot of experiments aiming to evidence relevant and selective biomarkers of uterine receptivity, there is to date no molecular fingerprint of the WOI. This is due partly to the discovery of an increasingly higher number of potential new markers according to the evolution of research, partly to the structural composition of endometrium, where the specialized epithelial cells that encounter the embryo are distinct from the glandular part. Finally, it is important to note the absence of consensus to which biomarkers to use for endometrial receptivity, and none of studied mediators (Mucine 1, L-selectin ligand, integrins, heparin-binding epidermal growth factor-like growth factor) has been explored in sufficient detail to validate its usefulness in clinical practice (Lessey, 2011).

The modification occurring in the endometrium during the WOI is closely related to angiogenesis and to immune system, allowing the tolerance of the fetal allograft and an adequate vascular remodelling during plancentation for a successful pregnancy until birth. Angiogenesis refers to the formation of new vessels from existing ones, by elongation, intussusception or sprouting of endothelial cells. One of the key local adaptations to pregnancy is the stimulation of maternal vessel network at the embryo implantation site. Normal fetal development requires extensive angiogenesis and important vascular remodelings allowing adequate supply of nutrients as well as gas and metabolite exchanges. Abnormal uterine blood supply is associated with higher perinatal morbidity and mortality caused by preterm delivery, preeclampsia, or intrauterine growth restriction. Stimulation of angiogenesis in many organs (including uterus) is mediated through enhanced expression

of VEGF, an angiogenic cytokine produced by epithelial endometrial and stromal cells (Berndt et al., 2006). As demonstrated at the next section, some other molecules from a trophoblastic origin, are able to actively stimulate maternal vascular remodelling as well.

In parallel, immune cells increasingly infiltrate endometrium from post-ovulation to menstruation in absence of pregnancy. In case of pregnancy, an higher increased number is observed after fertilisation up to mid gestation, since 30% of decidual cells are leukocytes, including 75% of uterine natural killers (uNK) (Plaisier, 2011; Zhang et al., 2011). It has been proposed that uNK could exert, directly or indirectly, either positive or negative control over implantation process. These cells secrete an array of cytokines important for adequate local immune regulation, angiogenesis, placental development, and establishment of pregnancy. Other immune cells or systems such as dendritic cells, T regulatory cells, macrophages, Th1/Th2 equilibrium orchestrate immune tolerance, trophoblast invasion and angiogenesis associated with embryo implantation (Blois et al., 2011; Denney et al., 2011).

ART programs, and particularly egg/embryo donation protocols, has clearly contributed to our knowledge of endometrial physiology. They gave us the opportunity to separately evaluate oocyte and endometrium, and to discover that endometrial receptivity can be controlled artificially with exogenous estrogen and progesterone, with a certain success (Paulson, 2011). Although it ensures obtaining a sufficient number of oocytes and thus embryos, ovarian hyper stimulation with exogenous gonadotropins is likely to be associated with modified endometrial development that may impact endometrial receptivity and success of implantation. These phenomenons would result from the inducing of an embryo-endometrium asynchrony (histologic endometrial advancement), the up-regulation of P-receptor expression, and a negative correlation between implantation and premature progesterone elevation, impacting fresh implantation rates in normal and high responders (Shapiro et al., 2011a, b), despite some other controversial results (Levi et al., 2001).

5.2 Clinical relevance of endometrial immunity

A better understanding of the uterine–embryo interaction and of the "seed and soil" regulations during embryo implantation is mandatory to increase the efficacy of ART. Implantation failure, recurrent miscarriage and preeclampsia have several recognized causes in common, but in most cases, the precise etiology remains obscure. Recent data in reproductive immunology identify the importance of the local immune environment and suggest to the clinician the need to develop tools to explore these endometrial perturbations (Tuckerman et al., 2010).

Indeed the semantic distinction between implantation failure, abortion and preeclampsia might in fact be more quantitative than qualitative (Chaouat, 2008). An important subset of implantation defects is the consequence of a deregulation of the interleukin systems, tumor necrosis factor (TNFα), interferon system as well as the uNK cell-mediated networks. Both deficient and excess expression of cytokines and immune cells number and activation play indeed detrimental key roles in implantation, since these actors can have both positive and negative effects.

For example, in human reproduction, a proper balance in the IL-12, IL-18, IL-15 and a correct uNK activation stat result in successful implantation and pregnancy. Conversely,

imbalances in these parameters correlate with implantation failure or early pregnancy loss (Ledee, 2005). Their expression controls the local uNK recruitment and subendometrial angiogenesis as reflected by the vascular flow index (VFI) determined by three-dimensional ultrasound (Ledee et al., 2008a; Ledee et al., 2011b).

The excellent correlation between IL-15 mRNA expression and the sub endometrial vascular flow index (VFI) suggests that this cytokine and the uNK cells that produce it participate to the local control of angiogenesis. Patients with a low sub-endometrial VFI and low IL-15 mRNA are patients with insufficient uNK recruitment and/or inadequate uNK-derived angiogenic-related proteins. In contrast, some patients with implantation failure exhibit very high VFI and, at the same time, high IL-15 and IL-18 mRNAs and CD^{56+} cell count. A Th-1 excess could possibly be involved in implantation failure (Kwak-Kim et al., 2005).

Abnormal subendometrial vascularization assessed by ultrasound may be the consequence of distinct cytokine dysregulation patterns. These may cause implantation failure, abortion or preeclampsia, through abnormal (insufficient or excessive) recruitment of uNK cells or through inadequate endothelial vascular remodeling before implantation. 3-D ultrasonography with vocal analysis may inform on the uterine preparation to a constructive dialogue with the conceptus through an adequate trophicity and angiogenesis. This point on echographic evaluation of endometrium opens up new horizons for the evaluation of the endometrium, well beyond the simple measurement of thickness that improperly correlate with the outcome of IVF, since without absolute cutoff (Noyes et al., 1995; Paulson, 2011). Nevertheless, focusing on vascularization at the time of implantation is absolutely mandatory from a physiological point of view (Ledee, 2005). In daily clinical practice, echographic evaluation of subendometrial VFI and measurement of the IL-15 and IL-18 mRNAs together with CD^{56+} counts may be useful to identify those women at risk of implantation failure (Ledee et al., 2011b).

Moreover, Tumor necrosis factor like WEAK inducer of apoptosis (TWEAK) is a transmembrane protein which, when cleaved, functions as a soluble cytokine. It is highly expressed by different immune cells and triggers multiple roles, including control of angiogenesis. TWEAK and IL-18 mRNA expression are correlated in patients with implantation failures. Basic TWEAK expression influences the IL-18 related uNK recruitment and local cytotoxicity. Actually, as demonstrated by Petitbarat et al., TWEAK doesn't act on IL-18 expression but is likely to control IL-18 related cytotoxicity on uNK cells when IL-18 is over-expressed (Petitbarat et al., 2010).

Using large-scale microarray analysis, endometrial gene expression at the time of the implantation window was compared in fertile control patients (FC) and women displaying previous IVF/ICSI repeated implantation failure (IF) or recurrent unexplained miscarriages (RM). Biological functions and gene networks were explored using the Ingenuity Pathways Analysis software. The number of differentially expressed genes revealed the extent of changes within the preconceptional endometrium as a function of either fertility or infertility. The main similarities of differentially expressed genes between IF and RM relate to immune and hematological system development abnormalities, especially deregulation of the differentiation and development of T lymphocytes and blood cells. As the endometrium is now thought to be a biosensor for the quality of the embryo, such differential expression may have direct consequences on the initial embryo-uterus dialogue (Chaouat et al., 2011; Ledee et al., 2011c).

6. Conclusion: Implantation process

Implantation process and placenta formation are closely associated to angiogenesis and immune tolerance, at the crossroad of endocrinology. Whereas embryo could implant in every human tissues and particularly in Fallopian tubes with sometimes a very huge morbidity (Shaw and Horne, 2011), uterus is tailored to tolerate the fetal allograft and to allow controlled invasiveness of trophoblast only during the WOI, ensuring the success of the intricate cascade of implantation. Blastocyst implantation requires a competent embryo-supported by the fertilization of a good quality oocyte by a top spermatozoa – able to cross-talk with a receptive endometrium, and the dialogue must talk about immunity and angiogenesis. Implantation failure and some pathologies of pregnancy are associated to defect of this close dialogue at one or many steps of the cascade.

Implantation rate in IVF treatment certainly benefits from research about all these fields in reproductive medicine, able to increase selection, diagnostic and treatment of the different actors of implantation: gametes, endometrium, embryo and the most difficult issue: the dialogue between each other.

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