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The role of the endometrium and embryo in human implantation

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Despite many advances in assisted reproductive technologies (ART), implantation rates are still low. The process of implantation requires a reciprocal interaction between blastocyst and endometrium, culminating in a small window of opportunity during which implantation can occur. This interaction involves the embryo, with its inherent molecular programme of cell growth and differentiation, and the temporal differentiation of endometrial cells to attain uterine receptivity. Implantation itself is governed by an array of endocrine, paracrine and autocrine modulators, of embryonic and maternal origin. Implantation failure is thought to occur as a consequence of impairment of embryo developmental potential and/or impairment of uterine receptivity and the embryo–uterine dialogue. Therefore a better comprehension of implantation, and the relative importance of the factors involved, is warranted. New techniques for monitoring changes in the endometrium and/or the embryo at the level of gene regulation and protein expression may lead to the identification of better markers for implantation. Moreover, the use of predictive sets of markers may prove to be more reliable than a single marker. Continuing refinements to ART protocols, such as optimizing ovarian stimulation regimens, the timing of human chorionic gonadotrophin injection, or the timing of embryo transfer, should help to increase implantation rates further.

Keywords: endometrium; embryo development; implantation; ovarian stimulation

Introduction

The techniques used in assisted reproductive technologies (ART) have advanced considerably since the first *in vitro* fertilization (IVF) birth in 1978. Tools are now available that enable the selection of high-quality embryos or assessment of endometrial status. Furthermore, ART protocols continue to evolve with the aim of achieving higher pregnancy rates, fewer multiple births and healthy babies from genetically affected progenitors. However, despite these advances, pregnancy rates are still relatively low and have not increased significantly in the last decade (Nygren and Andersen, 2001; Andersen *et al.*, 2005). This suggests that implantation rates in stimulated cycles remain suboptimal. Other factors, which are yet to be identified, must play a role.

Historically, both endometrial receptivity and embryo quality have been judged using morphological assessments, and the search for predictors of implantation has focused primarily on the analysis of single markers. There is now a movement towards more sophisticated, high-throughput technologies, such as DNA chips and proteomic arrays, capable of rapidly monitoring small changes in the levels of thousands of different genes or proteins, respectively. This not only enables the sampling of many more potential molecular candidates, but also the identification of characteristic molecular profiles (e.g. gene expression clusters or cytokine 'fingerprints') rather than single biomarkers. This strategy may be particularly relevant in the field of implantation because numerous factors are involved, many of these have multiple functions, and there is potentially a large amount of redundancy.

The aim of this review is to outline the current understanding of implantation in humans and to describe and critique the tools currently available for the study of the human preimplantation embryo, the receptivity of the endometrium and the embryo–uterine dialogue. In addition, this review will identify key areas in implantation research and methodology where efforts need to be focused in the future.

Implantation

On the basis of studies in rhesus monkeys, it is thought that human implantation involves a number of different stages (Enders *et al.*, 1986). Prior to implantation, the blastocyst shows evidence of polarity, assuming a particular orientation as it approaches the endometrium. Once the blastocyst is oriented correctly

(apposition), the zona pellucida is shed. The blastocyst then comes into contact with the epithelial layer and adheres to the endometrial surface (adhesion). Finally, the blastocyst penetrates the epithelial layer and invades the stroma (invasion).

Successful implantation requires the appropriately timed arrival of a viable blastocyst into a receptive endometrium. The endometrium is remodelled throughout the menstrual cycle, and exhibits only a short period of receptivity, known as the 'implantation window'. In humans, during a natural cycle, the embryo enters the uterine cavity ~4 days after ovulation (Croxatto *et al.*, 1978). The endometrium becomes receptive to blastocyst implantation 6–8 days after ovulation and remains receptive for ~4 days (cycle days 20–24) (Bergh and Navot, 1992). The importance of endometrial environment is highlighted by the observation that high-quality embryos transferred into women involved as embryo recipients in a surrogacy procedure have a higher probability of implanting than if they are transferred back into the donor women (Check *et al.*, 1992; Stafford-Bell and Copeland, 2001). Poor embryo quality has also been identified as a major cause of implantation failure (Urman *et al.*, 2005).

It is clear that to improve implantation rates in stimulated cycles, it is important to find ways to pinpoint the window of implantation, ensure that the best embryo is selected and synchronize embryo transfer with the time of optimal endometrial receptivity. Importantly, ways of evaluating and enhancing endometrial receptivity and embryo quality without disrupting the delicate process of implantation itself must be identified.

Implantation in humans is controlled by a complex and sophisticated interaction between embryo and endometrium, which begins at the early stages of oocyte maturation (Emiliani *et al.*, 2005). This dialogue enables synchronous development of the oocyte and maturation of the endometrium, followed by embryo orientation, apposition, adhesion and endometrial invasion by the blastocyst (Enders *et al.*, 1986). By understanding the activity and function of the hormones and factors involved in this dialogue, it may be possible to use them as predictors of endometrial receptivity or embryo quality to maximize implantation rates in hormonally stimulated ART cycles.

Epidemiology

The majority of spontaneous human conceptions fail to complete implantation and to achieve ongoing pregnancy. Evidence from sperm donation programmes have indicated that the maximal chance of achieving successful implantations under optimal conditions is ~40% per cycle, and this rate declines with age (Ferrara *et al.*, 2002; Achard *et al.*, 2005). The proportion of human conceptions that fail to implant remains uncertain, as data are limited. More is known about the fate of the embryo post-implantation. Using markers of early pregnancy, such as human chorionic gonadotrophin (hCG), it has been demonstrated that one-third of post-implantation early pregnancy losses occur during the pre-clinical stages of pregnancy in fertile women (Wilcox *et al.*, 1999). The situation is even worse for recipients in ovum donation programmes or in patients undergoing IVF, who show rates of early pregnancy loss as high as 37 and 48%, respectively (Simon *et al.*, 1999a).

A high incidence of chromosomal abnormalities has been reported for human embryos (Munné, 2001) and a significant proportion of pregnancy wastage is caused by numerical or structural chromosomal abnormalities (Hassold *et al.*, 1980). The frequency of embryonic genetic abnormality increases with maternal age (Hassold *et al.*, 1980) and is higher among infertile couples than in the general population (Munné, 2001). Therefore, genetic abnormalities are thought to be a major factor contributing to implantation failure in ART. Patients undergoing ART procedures often hold unrealistically high expectations of achieving pregnancy (Peddie *et al.*, 2005), and this may stem from a lack of awareness about the low implantation rates observed in natural cycles.

Morphology and cellular composition of the endometrium

The endometrium is a multilayered, dynamic organ overlaying the myometrium and comprises a functional layer and a basal layer. Each month, cells in the functional layer are separated from the basal layer during menstruation. The basal layer is attached to the myometrium and remains intact during menstruation, serving as a base for endometrial regeneration. The endometrium is composed of several different cell types, including luminal and glandular epithelial cells, stroma with stromal fibroblastic cells, immunocompetent cells and blood vessels. The numbers, activity, structure and function of these cells change throughout the menstrual cycle and change again during pregnancy.

In the early 1950s, Noyes and co-workers (Noyes *et al.*, 1950; Noyes and Haman, 1953) examined the histological features of endometrial biopsies taken during 8000 spontaneous cycles in 300 women. By associating histological changes with natural changes in basal body temperature, they were able to link distinct histological patterns to particular time points during the menstrual cycle. The criteria for endometrial dating that resulted from this work have since remained the gold standard approach for evaluating endometrial responsiveness and detecting endometrial abnormalities.

Endometrial biopsy is known to disrupt normal anatomical layering. Biopsies may contain different parts of the endometrial layers, low uterine segment fragments and variable amounts of glands or stroma. Nevertheless, compared with other biopsy techniques, analysis of samples using the Noyes method generally allows evaluation of the cellular architecture. Other key advantages of the Noyes method are that it enables differential component analysis and both the morphology and function of the cells can be assessed (Table 1) (Bourgain *et al.*, 1994).

As well as these benefits, a number of weaknesses in Noyes' approach have been identified. Biopsies can only provide a snapshot of the real situation in the endometrium, and sample bias is

Table 1: Strengths and limitations of morphological and immunohistochemistry assessments of endometrial receptivity

Advantages	Limitations
Established technique	Subjective interpretation (high intraobserver variability)
Widely used and accepted	Snapshot analysis
Architecture preserved	Sample bias
Provides information about morphology and function	Inter-cycle association poor
Differential component analysis	Disregards embryo interaction

unavoidable because it is not applicable to take large numbers of samples. Histological interpretation is inherently subjective, both intra- and interobserver variability are high and intraobserver variability has been shown to be highest among infertile women during the implantation window (intraclass correlation coefficient = 0.65) (Murray *et al.*, 2004; Myers *et al.*, 2004). Variability is also introduced because of the differences between women and the differences between cycles in the same woman (Murray *et al.*, 2004). Furthermore, ovarian stimulation in artificial cycles may lead to differences in the timing of endometrial maturation compared with natural cycles (Papanikolaou *et al.*, 2005).

The issue of timing based on endometrial dating is critical (Fig. 1). During the 2 days following ovulation, the morphological features of the endometrium do not change significantly. Therefore, an error of 2 days is introduced into endometrial dating for biopsies taken during this period. A similar situation is evident for biopsies taken during the mid-luteal phase, where there is lack of positive morphology criteria for a period of 4–5 days (stromal oedema is the only feature that changes significantly during this period). It is clear that more stringent criteria are needed to improve the precision of timing with endometrial dating.

Using the luteinizing hormone (LH) surge to predict ovulation is one approach that has been investigated, although there would still remain a degree of uncertainty in timing, as the LH surge occurs over a period of 30 h (Acosta *et al.*, 2000). Electron microscopy allows the examination of endometrial ultrastructures present during the implantation window, such as pinopodes and nucleolar channels, which may prove useful markers of endometrial receptivity (Bentin-Ley *et al.*, 1999; Isaac *et al.*, 2001). Methods that associate morphology and function (e.g. immunohistochemistry, molecular markers) may help to improve the precision of endometrial dating. Unfortunately, immunohistochemistry suffers from the same problems as morphological assessments. Furthermore, the most promising molecular candidates for markers of the implantation window have, so far, failed as predictors of endometrial status (Acosta *et al.*, 2000). However, other markers may be more successful (e.g. mucin (MUC-1), integrins) (Lessey *et al.*, 1996; DeLoia *et al.*, 1998). In the future, laser capture microdissection may be coupled with gene expression analysis, providing another useful tool that could be used to link endometrial morphology and function (Yanaihara *et al.*, 2004).

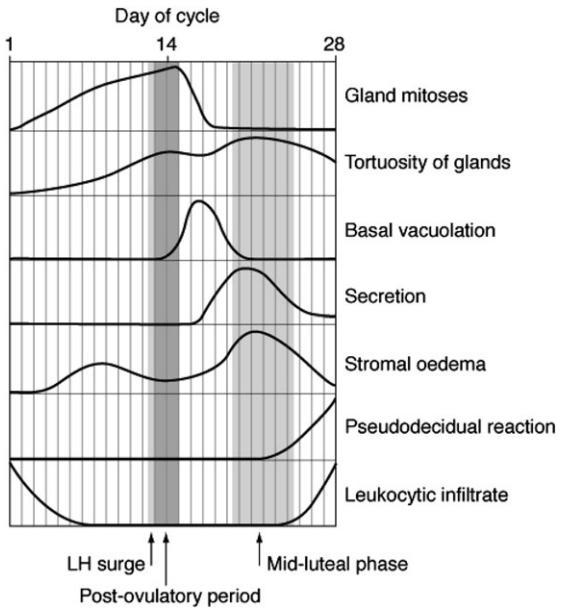

Figure 1: Graph illustrating the Noyes method of endometrial dating, which highlights the uncertainty in timing introduced during the post-ovulatory period, the mid-luteal phase and by measuring the LH surge. The distribution over time of many observed changes is too diffuse to allow precise endometrial dating, for example 2 days of the post-ovulatory period and 4–5 days in the mid-luteal phase

Another key consideration when using endometrial dating is that it disregards the status of the embryo. Ensuring the endometrium is receptive is of little use if a poor-quality embryo is introduced. Therefore, to ensure optimal conditions for implantation, endometrial dating should not be used in isolation, but should be combined with other techniques that provide information about embryo quality.

Endocrinological aspects

Progesterone and estrogen are the dominant hormonal modulators of endometrial development. Ovarian estrogen and progesterone condition the uterus for implantation, and knowledge about the precise temporal action of these hormones within the menstrual cycle has allowed the development of hormone-based contraception. Both the epithelial and stromal compartments express progesterone and estrogen receptors, and the response depends on the levels of these receptors as well as on the concentration of the hormones themselves. The interactions of progesterone and estrogen with estrogen receptors (ER) during endometrial development are illustrated in Fig. 2. In recent years, a better understanding has been gained in terms of the types of receptors involved (ERα, ERβ, PRA, PRB) and the dynamics of receptor expression (Fig. 3) (Cooke *et al.*, 1997; Mote *et al.*, 1999). It is apparent that the appropriate cyclical pattern of receptor expression is crucial for achieving endometrial receptivity and successful implantation (Lessey, 2003; Ma *et al.*, 2003).

Although progesterone and estrogen are the key modulators of endometrial maturation, their roles in this process are complex and sophisticated (Punyadeera *et al.*, 2003). Hormonal activity depends on not only the levels of progesterone, estrogen and their receptors, but also on the rates of progesterone and estrogen metabolism (e.g. up-regulation of enzymes that convert estradiol (E_2) to estrone or estrone sulphate or remove sulphate from E_2 and estrone) (Punyadeera *et al.*, 2003). The activities of progesterone and estrogen are also influenced by the effects of co-activators and repressors (Punyadeera *et al.*, 2003). Furthermore, both hormones regulate the expression of numerous endometrial proteins (paracrine signalling) (Cooke *et al.*, 1997).

In addition to progesterone and estrogen, a number of other endocrinological factors are known to mediate endometrial function (Kodaman and Taylor, 2004). In rodents, prostaglandins (PGs) are thought to facilitate increased vascular permeability during implantation (Kennedy, 1979), and enzymes involved in PG production (COX-1 and COX-2) shown cyclical changes in expression (Chakraborty *et al.*, 1996; Das *et al.*, 1999). hCG is thought to have direct effects on the endometrium and also mediates cross-talk between the embryo and the endometrium, through chorionic gonadotrophin receptors present on epithelial cells (Srisuparp *et al.*, 2003). The effects of androgens are often

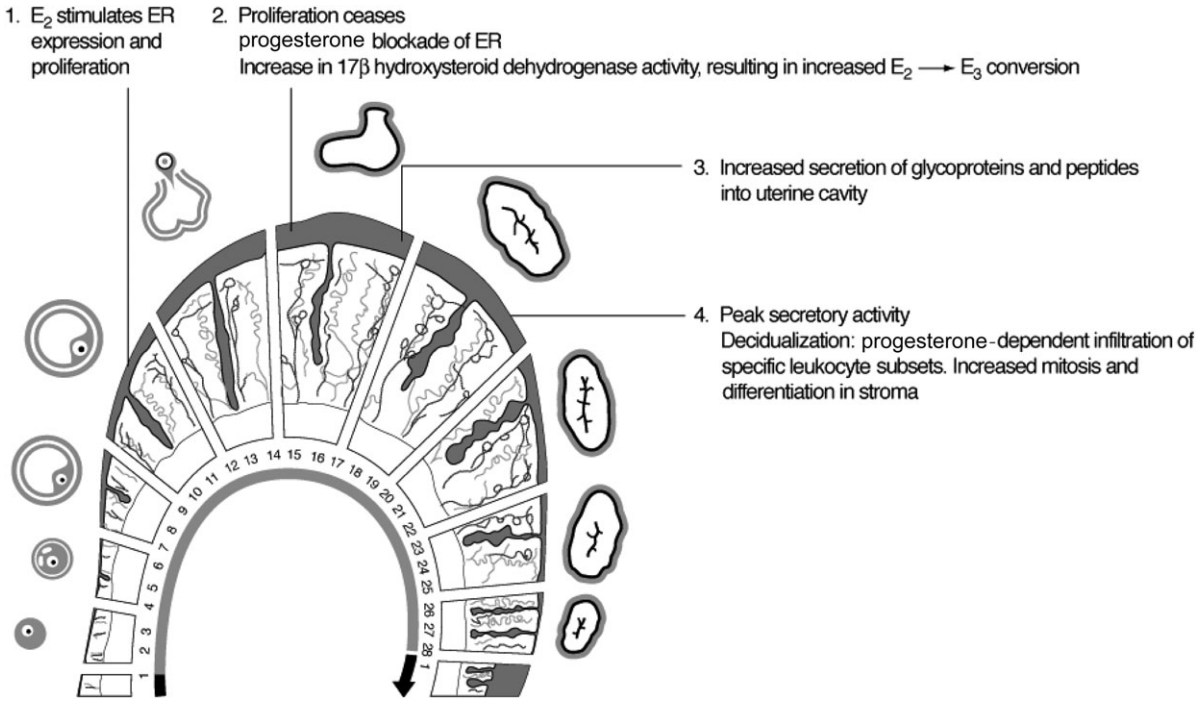

Figure 2: The roles of progesterone and estrogen (E_2; E_3, estriol) and estrogen receptors (ER) during endometrial development

overlooked in the female reproductive cycle. However, androgen receptors are present on stromal and epithelial cells in the endometrium, and both androstenedione and testosterone induce changes in endometrial function that may be important during implantation (Kodaman and Taylor, 2004).

Endometrial modulators of implantation

Endometrial factors are critical mediators of all phases of the implantation process (Fig. 4). Once the embryo reaches the uterus, the first cells it encounters are the epithelial cells of the endometrium. These cells secrete a range of factors into the uterine lumen, which may affect embryonic attachment as well as further development of the early placenta and embryo. However, the precise roles of individual factors as well as the molecular interactions involved have mostly not been elucidated for humans, and the current understanding of these processes stems primarily from research in rodents (reviewed in Dimitriadis *et al.*, 2005; Tranguch *et al.*, 2005; Wang and Dey, 2005).

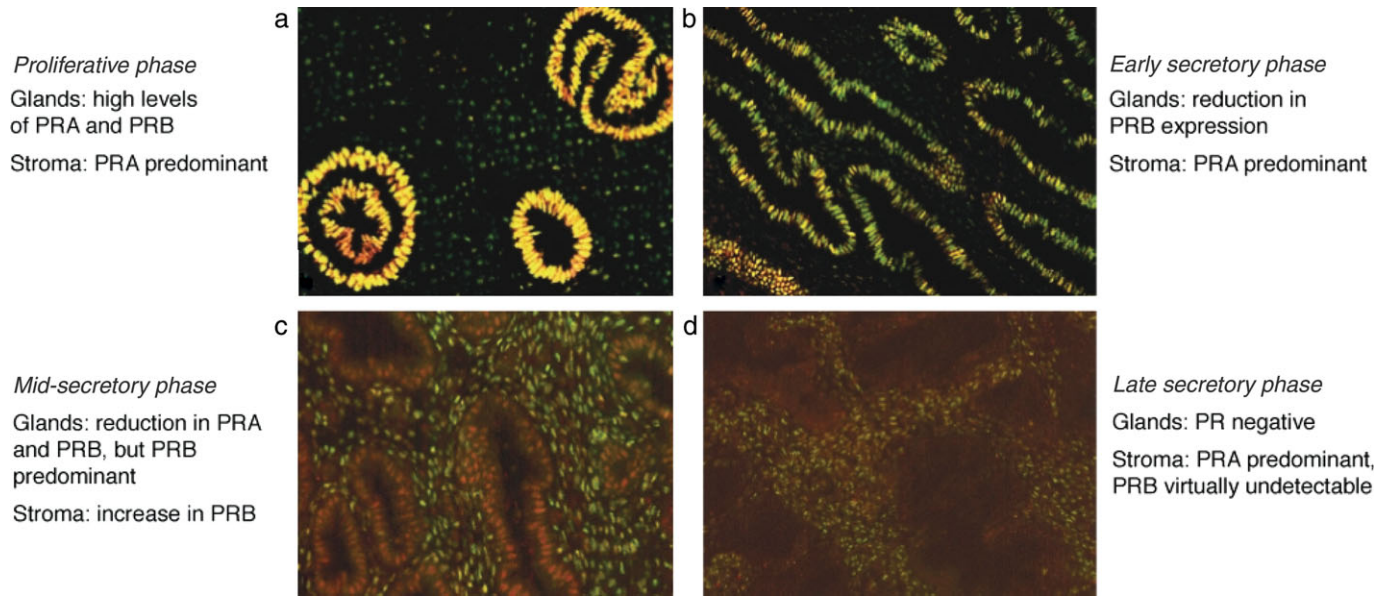

Figure 3: Changes in the expression of progesterone receptors (PRA, PRB) in glandular epithelial cells and stromal cells during the different phases of the menstrual cycle. Adapted with permission from Mote *et al.* (1999)

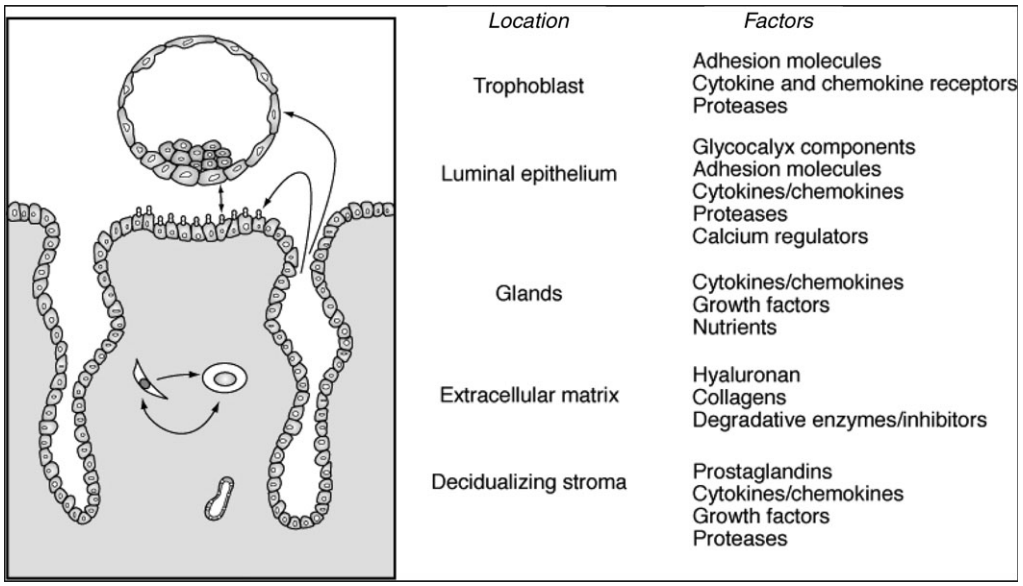

Figure 4: Factors regulated during the early stages of implantation. Adapted with permission from Dimitriadis *et al.* (2005)

In humans, one factor that has attracted particular interest is leukaemia-inhibitory factor (LIF), which is an interleukin (IL)-6 cytokine expressed in endometrial epithelial cells at the appropriate time for which receptors are present on preimplantation embryos. Studies in mice demonstrate that LIF plays a role in implantation and may also promote embryonic development. Observational studies in humans are suggestive of a possible role for LIF in humans (Robb *et al.*, 2002; Dimitriadis *et al.*, 2005). Indeed, infertility in some women has been associated with the dysregulation of LIF, and also of IL-11, which is produced in the same manner in the endometrial glands during the receptive phase (Dimitriadis *et al.*, 2006). However, the importance of LIF in implantation is still under debate, as promising results in animal models have failed to translate to humans (Kimber, 2005).

Embryonic factors and the impact of ART

With the increasing trend towards single embryo transfer in ART (Vilska *et al.*, 1999; Hamberger *et al.*, 2005), the selection of viable embryos is becoming more and more important. Morphological assessment is currently the standard tool for embryo selection in ART (Table 2) (Borini *et al.*, 2004). Over the years, with an improving understanding of embryonic development and advances in *in vitro* culture techniques, the developmental stage at which an embryo can be transferred has become more advanced, and embryo selection criteria have evolved accordingly. However, no single method of embryo selection has emerged, with some groups selecting blastocyst stage embryos, and others still opting to select at the 2PN stage or cleavage stage (De Neubourg *et al.*, 2002). Furthermore, it is recognized that morphological assessment of embryo quality is still highly subjective and, therefore, a number of alternative approaches are currently being explored, such as assessment of the embryo culture medium to detect nutrient uptake or metabolite secretion (Sakkas and Gardner, 2005).

Table 2: Strengths and limitations of morphological assessments of embryo viability

Advantages	Limitations
Established technique	Subjective approach
Widely used and accepted	There is no consensus on the timing of selection (2PN versus cleavage stage versus blastocyst)
Non-invasive	Selection criteria cannot yet guarantee an embryo with developmental potential is transferred

Preimplantation genetic diagnosis (PGD) was initially developed as a preconception test for couples carrying genetic disorders who were at risk of having a child affected by the disorder (Thornhill *et al.*, 2005). However, more recently, the technique has been used extensively in the context of optimizing IVF outcomes in infertile patients who are not carriers of a heritable disease (Sermon *et al.*, 2007). Chromosomal analysis of human gametes and embryos has revealed that chromosome aberrations occur at high frequency in the early preimplantation embryo. Preimplantation genetic screening (PGS) enables the testing of gametes and embryos for numerical chromosomal aberrations commonly found in early pregnancy loss, with the aim of replacing only euploid embryos and increasing pregnancy rates after IVF in groups of women who have poor IVF success rates (Munné, 2003; Verlinsky *et al.*, 2004; Caglar *et al.*, 2005; Kearns *et al.*, 2005). Genetic analysis can be performed on polar bodies extracted from the oocyte before fertilization (first polar body) and/or after fertilization (second polar body) (Verlinsky *et al.*, 2001). At later stages, genetic testing can be performed on one or two blastomeres from the cleavage stage embryo (day 3), or on trophectoderm tissue of the blastocyst (day 5) (Staessen *et al.*, 2004; McArthur *et al.*, 2005). Although data are emerging from clinical studies investigating the use of PGS in cleavage stage embryos and blastocysts (Staessen *et al.*, 2004; McArthur *et al.*, 2005; Platteau *et al.*, 2005a,b; Twisk *et al.*, 2006), it remains to be established whether advantages from genetic selection are counteracted by a detrimental effect of the biopsy procedure and the removal of embryonic cells, respectively.

Furthermore, mosaicism (presence of both aneuploid and euploid cells in an embryo) is commonly found in cleavage-stage embryos, although the clinical relevance of this phenomenon remains unclear (Bielanska *et al.*, 2005; Baart *et al.*, 2006).

Another key issue in ART protocols that is still under debate is the timing of embryo transfer. Whereas in an unselected patient population a clinical benefit of day-5 transfer (blastocyst transfer) with respect to live-birth rate and multiple-pregnancy rate has not been shown (Blake *et al.*, 2005), in patients with a good prognosis (young patients, minimum number of good quality embryos on day 3) blastocyst transfer yields a significantly higher live-birth rate (Papanikolaou *et al.*, 2006a,b; The Practice Committee of the American Society for Reproductive Medicine and the Practice Committee of the Society for Assisted Reproductive Technology, 2006). Blastocyst transfer gives the option to select the morphologically best embryo, whereas it has also been indicated that good-quality blastocysts have a decreased incidence of aneuploidy (Fig. 5) (Staessen *et al.*, 2004).

Failure of the blastocyst to release from the zona pellucida has been identified as a potential cause of implantation failure in assisted cycles, particularly in older women (Seif *et al.*, 2006). A potential solution to this is artificial disruption of the zona pellucida or assisted hatching. A systematic review of studies investigating the effects of this technique on conception found that assisted hatching significantly improved pregnancy rates, but had no effect on live-birth rates or spontaneous abortion rates, and multiple-pregnancy rates were significantly increased (Seif *et al.*, 2006). Unfortunately, there were insufficient data for this analysis to investigate the impact of assisted hatching on a number of other important outcomes, such as monozygotic

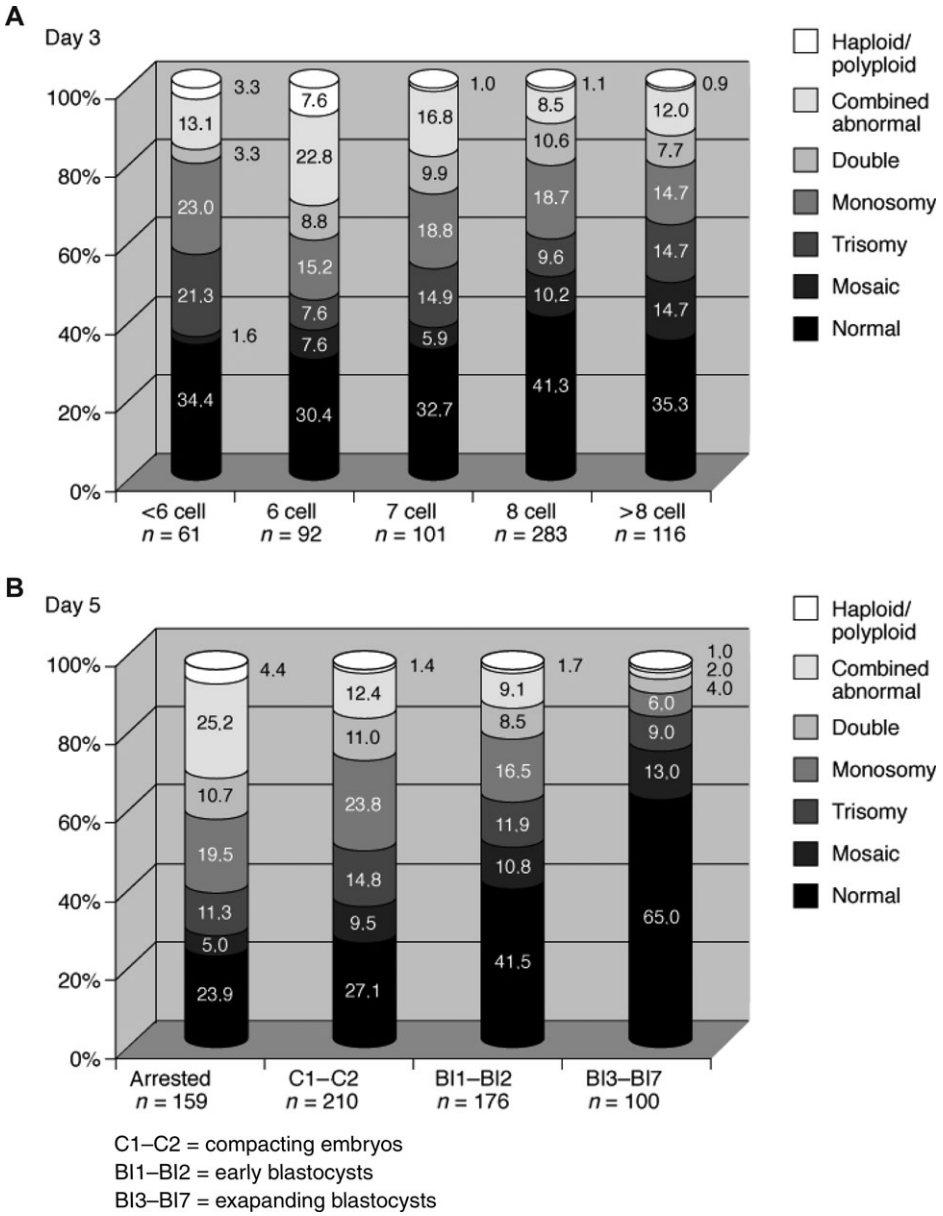

Figure 5: Relationship between chromosomal abnormalities and developmental stage on (**A**) day 3 or (**B**) day 5 of embryonic development. Figure reproduced with permission from Staessen *et al.* (2004)

twinning, embryo damage, congenital and chromosomal abnormalities and *in vitro* blastocyst development.

When optimizing ART procedures to mimic nature as closely as possible, it is important to remember that controlled ovarian stimulation itself interrupts natural physiological processes and is likely to alter key parameters such as the rate of embryonic development and the extent and timing of endometrial receptivity. Artificial stimulation affects the levels of progesterone and estrogen, the ratio between these two hormones and endometrial expression of their receptors (Beckers *et al.*, 2000; Papanikolaou *et al.*, 2005). There is evidence that supraphysiological steroid levels impair the luteal phase, and this is true, even when stimulation is started in the late follicular phase (Bourgain *et al.*, 1994; Ubaldi *et al.*, 1996; Macklon and Fauser, 2000a; Kolibianakis *et al.*, 2003). Therefore, if the luteal phase is not supplemented, premature luteolysis can occur and pregnancy may not be achieved (Beckers *et al.*, 2000, 2003). In ART cycles, the aim is to produce multiple mature follicles, which leads to elevated levels of progesterone and estrogen compared with natural cycles, and this can induce changes in the endometrium (Bourgain and Devroey, 2003) that can be detected using standard histological techniques (Garcia *et al.*, 1984) and scanning electron microscopy (Kolb *et al.*, 1997).

To overcome some of the effects potentially associated with hormonal stimulation, various modified stimulation protocols have been investigated. For example, milder stimulation regimens have been studied, in which gonadotrophins were administered at a lower dose, or later in the cycle, or that used gonadotrophin-releasing hormone antagonists for pituitary downregulation (Macklon and Fauser, 2000b; Hohmann *et al.*, 2001, 2003). Early administration of hCG for final oocyte maturation (as soon as three follicles $\geq$17 mm are present) appears beneficial in terms of pregnancy rates, especially when day 3 embryo transfers are performed (Kolibianakis *et al.*, 2005).

Animal models and human in vitro systems

The implantation process itself has never been observed directly *in vivo* in humans (Lee and DeMayo, 2004). However, studies in animals, primarily rodents, sheep and primates, have provided clues about the hormonal and morphological changes that might occur in women prior to and during implantation (Lee and DeMayo, 2004). Indeed, the three stages of endometrial development observed in animals (endometrial neutrality, receptivity and refractoriness) are also thought to occur in humans (Rogers, 1995). It is recognized that different species show a wide variety of mechanisms by which implantation occurs (Ringler and Strauss, 1990) and, therefore, different animals may be more suited as models for particular steps in the human implantation process. For example, pigs and sheep are potential candidates for the study of the early stages of implantation, as they have extended apposition and attachment phases (Lee and DeMayo, 2004). Conversely, macaques and humans have similar mechanisms for trophoblast invasion and, therefore, macaques are a suitable model for studying the later phases of implantation (Lee and DeMayo, 2004).

Although information about the physiology of implantation has been gained from a range of different animal models, the current understanding about this process on a molecular level results largely from studies in mice (Lee and DeMayo, 2004). However, the mechanisms of implantation in mice and humans are quite distinct. During implantation in mice, the luminal epithelium forms an invagination that surrounds the trophoblast (eccentric mechanism) and is subsequently shed by apoptosis, whereas in humans, the trophoblast invades the stroma by penetrating the luminal epithelium (interstitial mechanism) (Wimsatt, 1975).

Studies on LIF illustrate how promising findings in mice have translated to disappointing results in humans. Targeted mutagenesis studies in mice clearly established an essential role for LIF in mouse implantation, prompting intensive investigation into its role in humans. However, LIF expression varies widely in humans, and although putative LIF mutations have been identified (Giess *et al.*, 1999; Kralickova *et al.*, 2006), their functional significance is unclear. Moreover, low LIF levels have been associated with increased success in IVF/embryo transfer programmes in some studies (Ledee-Bataille *et al.*, 2002), whereas others have found no association (Olivennes *et al.*, 2003). Collectively, these data question an essential role for LIF in human implantation and are cause for reflection as to the translatability of animal studies to human biology.

This issue of translatability has important implications for future research, as rodent models are best suited for testing the functional role of genes and proteins. Consequently, animal studies should be validated using alternative *in vivo* models, including primates, and *in vitro* systems that can reproduce critical stages of the implantation process with fidelity, prior to the initiation of large-scale clinical trials or development of methods to assess endometrial receptivity or improve implantation rates. To address this need, a number of *in vitro* models using human cell culture systems have been developed to study various aspects of embryo–endometrial interaction.

Bentin-Ley *et al.* (1994) constructed a complex 3D endometrial cell culture system containing stromal cells embedded in a collagen matrix and separated from an epithelial monolayer by basement membrane material ('Matrigel': Becton and Dickinson Biosciences, San Jose, CA, USA). Using this model, they demonstrated that human blastocysts attach preferentially to pinopode-presenting areas on the endometrial surface (Bentin-Ley *et al.*, 1999). Another group cultured a complete endometrial biopsy of the upper functional layer of the endometrium onto collagen gel (Landgren *et al.*, 1996). Although they were able to observe human blastocyst adhesion of the stromal layer in this 'miniorgan', there was evidence of tissue degeneration after 48 h.

Simon *et al.* have developed *in vitro* models to specifically study the apposition and adhesion phases of implantation (Simon *et al.*, 1999b; Mercader *et al.*, 2003). In the apposition model, embryos obtained after ovarian superovulation and insemination (IVF or intracytoplasmic sperm injection) were co-cultured with luteal phase endometrial epithelial cells. This model resulted in a clinical programme where embryos could be co-cultured with epithelial cells until blastocyst stage and transferred back to the mother (Mercader *et al.*, 2003). For the adhesion model, a 3D culture was prepared, comprising epithelial and stromal cells cultured from endometrial biopsies. Blastocysts cultured on these endometrial epithelial cells attached to the epithelial surface and could be immunologically localized using anti-β-hCG staining (Galan *et al.*, 2000; Meseguer *et al.*, 2001). These models have provided information about the embryonic regulation of endometrial epithelial molecules such as anti-adhesion molecules (Meseguer *et al.*, 2001), cytoskeletal proteins (Martin *et al.*,

Table 3: Strengths and limitations of measuring changes in protein or gene expression to assess endometrial receptivity and/or embryo viability

Advantages	Limitations
Objective approach	Not accessible to many groups (expensive and high level of technical skill required to analyse and interpret results)
Provides information about related groups of molecules (clusters)	New technology, methodology needs to be standardized
Large amounts of information generated in a small amount of time	Poor reproducibility between experiments in different groups
May be more representative of biological phases than morphological methods	Correct sample preparation is essential and should be consistent

2000), chemokines (Dominguez *et al.*, 2003) and the leptin system (Cervero *et al.*, 2004) during the apposition and adhesion phases of human implantation.

An *in vitro* model has also been developed to study the process of blastocyst invasion (Carver *et al.*, 2003). Carver *et al.* (2003) were able to observe structural and hormonal changes occurring during blastocyst invasion using time-lapse photography, immunostaining and measurement of hCG levels for human hatched blastocysts co-cultured with human endometrial stromal cell monolayers.

Molecular approaches

Advances in biotechnology have lead to the development of new techniques that allow the examination of changes in the endometrium and embryo at the molecular level. DNA microarrays enable analysis of the simultaneous expression of thousands of genes in a single sample. Bioinformatic tools have been developed, which can quantify and link such molecular changes (Table 3). These genomic and proteomic techniques have been used to study changes occurring throughout the cycle, examine the impact of artificial stimulation and determine the patterns of gene expression in different cell types.

The expression of many endometrial genes has been shown to change over the course of the menstrual cycle (Ponnampalam *et al.*, 2004; Talbi *et al.*, 2006). However, some of these expression patterns do not appear to associate with histopathological changes occurring in the endometrium (Ponnampalam *et al.*, 2004). Possibly, gene expression may be a better marker of the biological phases and may be a more reliable predictor of endometrial receptivity than morphology.

To date, five studies have examined changes in endometrial gene expression during the receptive phase and all have reported genes that are strongly up- or down-regulated when the endometrium is receptive (Carson *et al.*, 2002; Kao *et al.*, 2002; Borthwick *et al.*, 2003; Riesewijk *et al.*, 2003; Mirkin *et al.*, 2005). One striking observation is that only a single gene (osteopontin) was differentially expressed (up-regulated) in all five of these studies (Carson *et al.*, 2002; Kao *et al.*, 2002; Borthwick *et al.*, 2003; Riesewijk *et al.*, 2003; Mirkin *et al.*, 2005). The divergent results from different studies have been attributed to differences in study design and the software/statistics used in the analysis of the data (Riesewijk *et al.*, 2003). This finding highlights the need for standardization of methodology if meaningful conclusions are to be made from genomic and proteomic studies.

Microarray studies comparing natural and stimulated cycles indicate that controlled ovarian stimulation has a profound effect on endometrial gene expression during the window of implantation (7 days after the LH surge compared with 2 days; Fig. 6) (Horcajadas *et al.*, 2005; Simon *et al.*, 2005). Over 200 genes were differentially expressed in stimulated cycles (Horcajadas *et al.*, 2005), and the pattern of expression depended upon the type of down-regulation protocol used (agonist or antagonist) (Simon *et al.*, 2005). Studies have also examined gene expression changes in human endometrial cells *in vitro* during decidualization (Popovici *et al.*, 2000; Brar *et al.*, 2001; Tierney *et al.*, 2003), in response to progesterone (Okada *et al.*, 2003), or in endometrial biopsies taken at different phases of the menstrual cycle (Ponnampalam *et al.*, 2004; Punyadeera *et al.*, 2005; Talbi *et al.*, 2006).

Laser capture microdissection coupled with gene expression analysis enables accurate comparison of gene expression patterns between different cell types from the same tissue. To date, one study has used this technique to examine differences in normal human endometrial tissues from the secretory phase (Yanaihara *et al.*, 2004). A total of 28 genes were found to be differentially expressed in epithelial and stromal cells, and a number of these genes have known immunological functions (Yanaihara *et al.*, 2004).

As well as array technologies being used to study gene expression, methods are also being developed to study proteomic changes occurring during implantation. Endometrial secretion aspiration is one such approach and enables the measurement of protein changes in the uterine lumen during treatment cycles (van der Gaast *et al.*, 2003). A key advantage of this approach is that the technique itself does not appear to have an adverse effect on implantation (van der Gaast *et al.*, 2003). However, it is important to note that secretion aspirations may contain cellular contaminants, such as leukocytes, stromal cells or epithelial cells, which must be removed prior to analysis, or taken into consideration when interpreting the results.

As well as studying the molecular changes occurring in the endometrium, it is equally important to conduct molecular studies on oocytes and embryos, but unfortunately these have been few (Neilson *et al.*, 2000; Stanton *et al.*, 2002; Dobson *et al.*, 2004; Katz-Jaffe *et al.*, 2006). Two of these studies have investigated differential gene or protein expression in human embryos (Dobson *et al.*, 2004; Katz-Jaffe *et al.*, 2006). Dobson *et al.* (2004) characterized global changes in gene expression during the first 3 days of embryonic development and found that embryonic transcriptional programmes were already established within 3 days of fertilization. Katz-Jaffe *et al.* (2006) examined changes in the proteome of individual human blastocysts and observed characteristic expression profiles that associated with changes in morphology or embryo degeneration. Such studies could reveal molecular signatures that are consistent with high-quality gametes and embryos and, more importantly, identify candidate secreted molecules that could be assessed non-invasively for association with implantation success. However, when assessing oocytes and embryos, much smaller amounts of material can be obtained compared with endometrial samples. The fewer cells that are removed from the embryo (or blastocyst), the less disruption is likely to be caused. Therefore, highly accurate methods of amplification and detection are required.

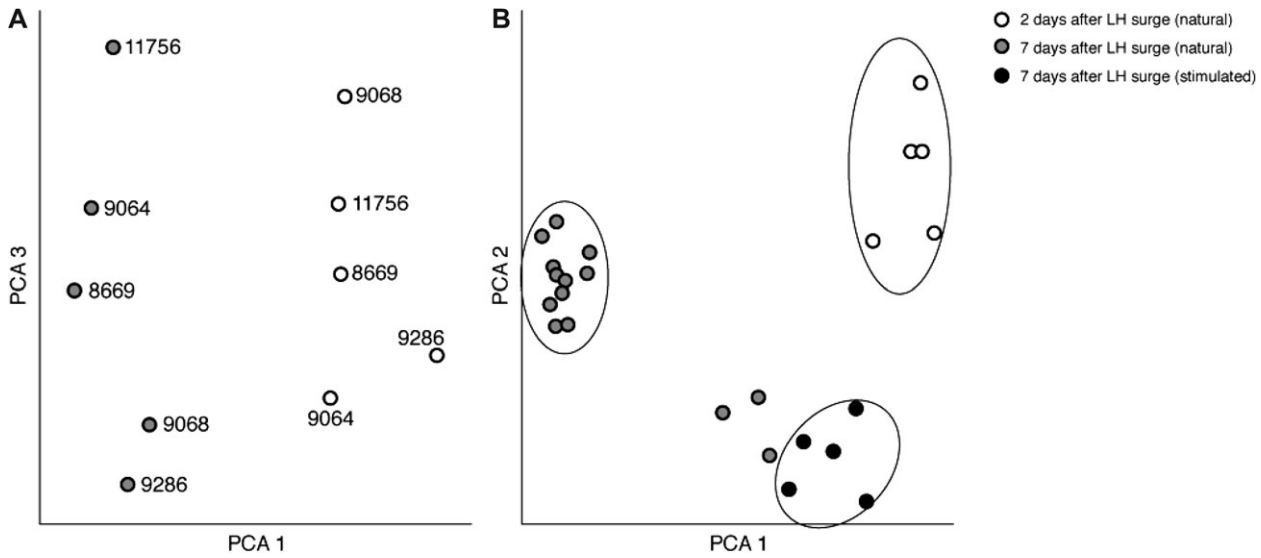

Figure 6: Principle component analyses (PCA) of endometrial gene expression showing clustering of samples from (**A**) 2 versus 7 days after the LH surge or (**B**) natural versus stimulated cycles. Adapted with permission from Riesewijk *et al.* (2003) and Horcajadas *et al.* (2005)

Investigators at Serono International SA (Geneva, Switzerland) have begun to develop tools and a strategy to enable molecular-based embryo selection (Fig. 7). Using this approach, RNA amplification from a single blastomere provided sufficient amplified RNA for microarray analysis. The expression of over 8300 genes was detected in day 3 human embryos and enzyme-linked immunosorbent assay (ELISA) is in progress to confirm the expression of these genes. In parallel, detection of proteins

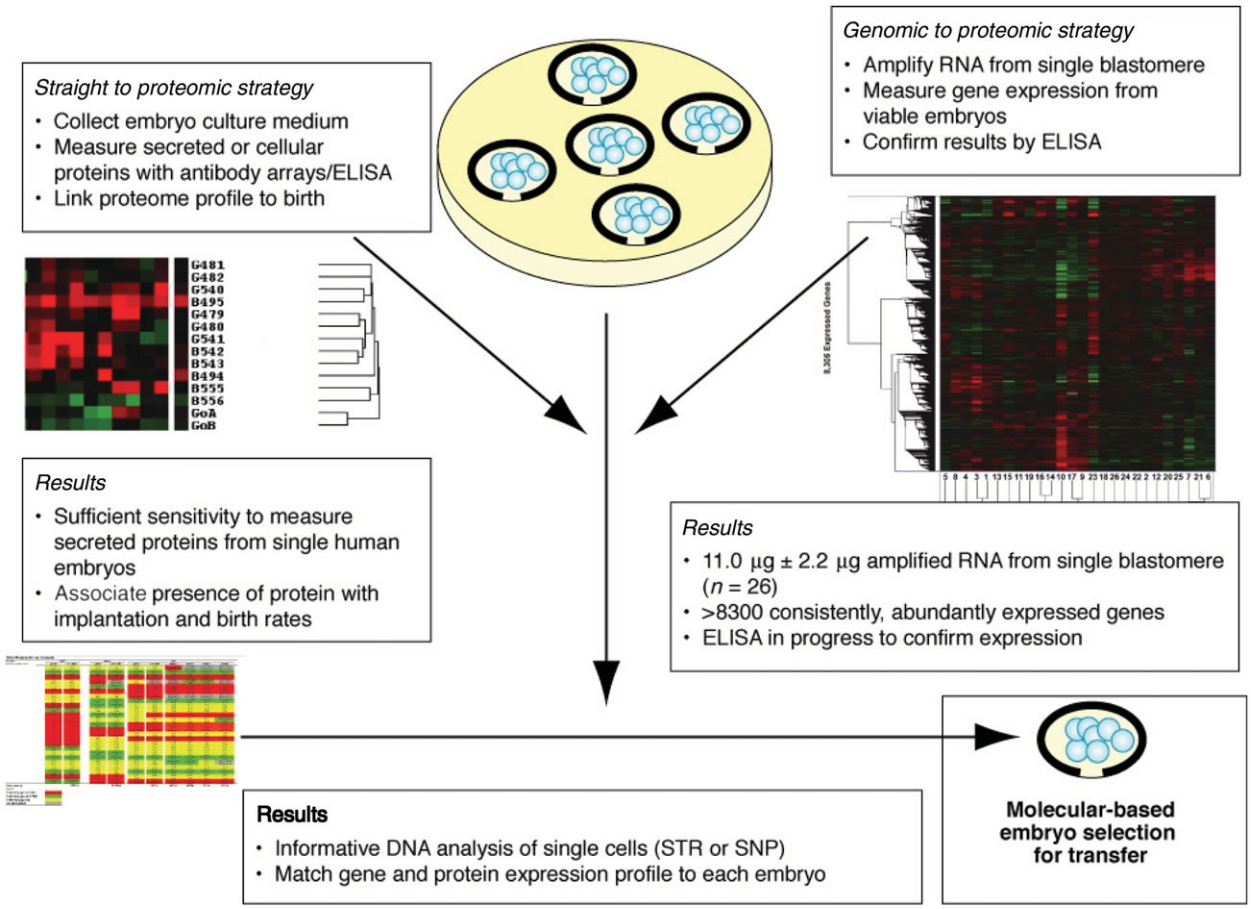

Figure 7: A molecular approach being developed to improve phenotypic selection of human embryos for transfer using genomic and proteomic tools. Some initial data from the 'straight to proteomics' and 'genomic to proteomic' approaches are also presented. RNA, ribonucleic acid; STR, short tandem repeat polymorphism

produced by embryos in culture using antibody arrays or ELISA provided sufficient sensitivity to identify secreted proteins from a single embryo, with the potential to assess embryo quality at day 3 of culture. Finally, DNA fingerprinting of embryos from single blastomere biopsy and later of amniocytes and fetal cord cells would enable matching of genomic and proteomic profiles to the embryo that successfully develops. Both microsatellite DNA analysis (short tandem repeat) and single-nucleotide polymorphism (SNP) analysis have demonstrated early potential for use with a single cell.

Moving beyond genomics and proteomics, metabolic profiles as well as embryonic signalling molecule profiles can be targeted as predictors of developmental potential ('metabolomics'). However, to date, most data on embryo metabolism stem from rodent research and need to be evaluated in humans.

The way forward

Animal studies and *in vitro* experiments have improved understanding about the hormonal and morphological changes that occur during implantation in natural cycles. In addition, numerous paracrine factors that mediate implantation processes have been identified, and a next step would be to associate this information with endocrinology and morphology. It is also clear that stimulated cycles behave differently to natural cycles, so establishing where these differences lie, in terms of both the endometrium and the embryo, is another important area of focus for future studies.

Current morphological markers of endometrial receptivity are poor predictors of pregnancy. Therefore, there is a need for non-disruptive *in vivo* methods to study endometrial receptivity and the implantation process itself, particularly in those women in whom pregnancy is achieved. Endometrial secretion aspiration may be one useful approach to this problem, providing aspiration does not affect implantation rates (van der Gaast *et al.*, 2003).

In the past, the focus for improving embryo selection has been morphological criteria and the detection of chromosomal abnormalities. However, while chromosomal abnormalities may be responsible for a large proportion of implantation failures, they are not the cause for all of them. Therefore, trials that examine the causes of implantation failure, particularly in older women, in whom levels of chromosomal abnormality are high, would be useful. Ideally, single embryo transfer would be used, although the policy of single embryo transfer may not be ethical in this particular group of patients.

New molecular techniques are becoming available for measuring both embryonic and endometrial changes prior to and during implantation. However, these approaches are still in their infancy, and although they hold much promise, it is important that standardized ways of working are devised at an early stage. Ultimately, the aim is to use these tools to increase implantation in artificial cycles and consequently improve live-birth rates.

Appendix

Evian Current Reproductive Medicine Workshop Group

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