# Preimplantation embryo-endometrial signalling

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Cover: Light microscopic view of a day 8 human embryo developing on a monolayer of decidual cells.



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# Preimplantation embryo-endometrial signalling

Pre-implantatie embryo-endometrium communicatie
(met een samenvatting in het Nederlands)

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Voor mijn ouders

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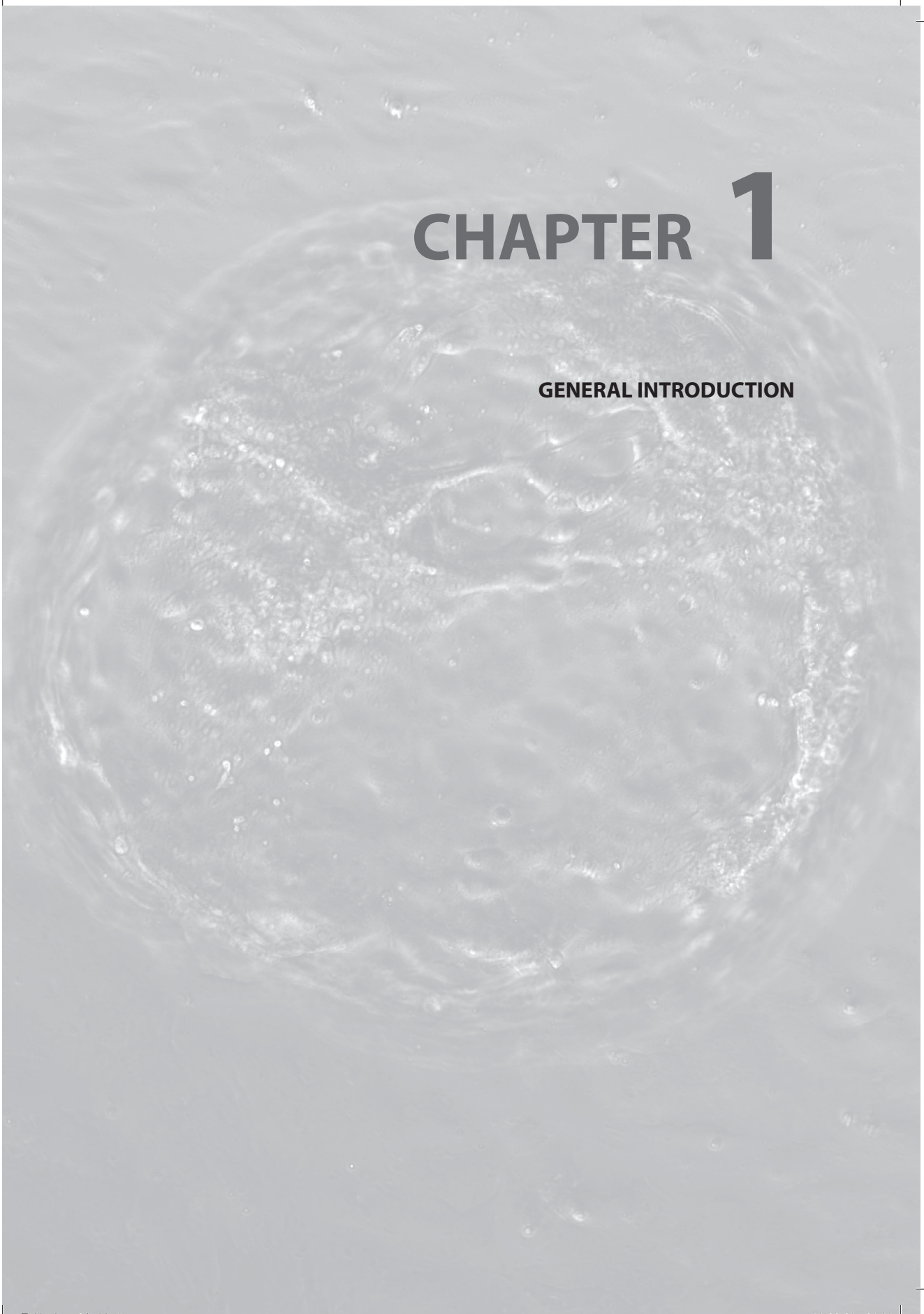

# CHAPTER 1

## GENERAL INTRODUCTION

## Human reproduction

The continuing, and for some, worrying growth in the world population suggests that humans are a highly fertile species. However, human reproduction is relatively inefficient. Fertility can be measured by the time taken to achieve pregnancy after stopping contraception in order to conceive. Time-to-pregnancy (TTP) is expressed in monthly fecundity rates (MFR), i.e. the probability of achieving pregnancy within one menstrual cycle. The average MFR in humans is, compared to other mammalian species[1,2] relatively low at approximately 20%[3]. Furthermore, embryo wastage and pregnancy loss in humans is common, estimated to be 30% prior to implantation (preimplantation loss) and another 30% before 6 weeks gestation (early pregnancy loss or EPL)[4,5]. Finally, clinical miscarriage is estimated to be the outcome in 10% of all conceptions[5]. With the age of women at first childbirth increasing, the cumulative probability of spontaneous conception will continue to decline. To date, one in six couples in developed countries have an unwanted delay in conception and half of these couples eventually turn to assisted reproductive technologies (ART) such as in vitro fertilisation (IVF). The International Committee for Monitoring Assisted Reproductive Technologies (ICMART) has reported that more than 3 million babies have been born using ART[6]. Although this would appear to indicate the success of IVF, pregnancy success rates using assisted reproductive technologies remain disappointingly low, largely due to failure of the transferred embryo to implant.

## Human embryo implantation

Fertilisation or conception rates do not necessarily equate to pregnancy rates. While the majority of initiated IVF treatments result in viable embryos that can be transferred into the uterus, ongoing pregnancy appears to be impeded at a crucial step towards successful reproduction; implantation. In women conceiving spontaneously, the situation appears to be similar. Implantation is a complex, species-specific biological process dependent on two-way communication between the embryo and the endometrium[7]. This embryo-maternal dialogue requires highly orchestrated, progesterone-dependent changes in the endometrium to render it responsive to embryonic signals. In humans, the period of endometrial receptivity, sometimes termed the 'implantation window' starts approximately 6 days after ovulation and normally lasts no longer than 4 or 5 days[5,8]. During this period, the endometrium achieves a receptive phenotype[9]. The end of the implantation window, approximately 10 days after ovulation, coincides with the extraordinary transformation of endometrial fibroblasts into secretory, epitheloid decidual cells[10]. Analysis of uterine fluid and data from immunohistochemical studies indicate that regulatory molecules including LIF[11], CX3CL1 and CCL14[12-14] are released into the uterine lumen where they can affect the blastocyst even prior to attachment. Other changes to the luminal epithelium include alterations to cell surface glycoconjugates such as mucins[15,16], and to adhesion molecules on both apical and lateral membranes[17,18]. At the same time, the peri-implantation blastocyst reaches a stage of attachment competence, at around day 6[19]. The blastocyst attaches to the luminal epithelium of the endometrium and penetrates the epithelial layer and the underlying basement membrane.

Next, the trophoblast invades through the stroma, interacting with the extracellular matrix (ECM) of decidualized stromal cells to complete the process of implantation to form a functional placenta.

## Recurrent pregnancy loss

Miscarriage, the most common complication of pregnancy, is the spontaneous loss of a pregnancy before the fetus has reached viability. Two types of miscarriage can be recognized: sporadic and recurrent pregnancy loss (RPL). Recurrent miscarriage affects about 1-3% of couples and is defined as two or more pregnancy losses that don't necessarily have to be consecutive losses[20]. RPL can be attributed to either maternal factors or chromosomal errors in the conceptus. On the maternal side, pregnancy loss has been attributed to genetic, structural, infective, endocrine, immune or unexplained causes. Conversely, at least 50-60% of all miscarriages are associated with cytogenetic abnormalities in the fetus, the most frequent being trisomy[21,22]. The current paradigm of RPL and its management are firmly anchored in the conjecture that pre-existent disease, often much more relevant to subfertility, also underpins RPL. However, clinical experience is quite different. Many women with RPL report exceptionally high pregnancy rates and may belong to the 3% of the population classified as superfertile according to the Tietze model[3,23]. As such, the clinical phenotype of RPL can be considered to be a separate entity with higher percentages of implantation and subsequent pregnancy loss.

## Human embryo-endometrial signalling

The process of human implantation is far from being fully understood. A highly coordinated series of events involves both embryonic and maternal active participation. First, apposition and attachment to the endometrium occurs. Next, the endometrial compartment undergoes adaptations that involve proliferation, growth, and differentiation of resident stromal cells to form the decidua. The decidua produces endocrine and paracrine signalling molecules and provides a nutrient source for the expanding trophectoderm[24,25]. The two major steroid hormones estradiol and progesterone are crucial for the acquisition of receptivity and the change in transcription activity of target genes[26,27]. There is an accumulating body of evidence suggesting that uterine receptivity is controlled primarily, although not exclusively, by locally acting trophic factors and cytokines[7,28,29]. The process is facilitated through a well-balanced network of mediators in epithelial, stromal and immune cells. It has been demonstrated that implantation triggers an inflammatory response, likely to be mediated through pro-inflammatory cytokines, which may be secreted either by the embryo or endometrium or both[30].

Mass spectrometry and protein-array studies have shown that factors from the IL-6 family and its receptors (e.g. IL-1, Leukemia Inhibitory Factor (LIF) and IL-6R) are being expressed or secreted by the preimplantation embryo[31,32]. Other factors imposed are the TNF receptor 1, IL-10 and HB-EGF[31,32]. Metabolic profiling has been employed to determine amino acid turnover in embryo

culture medium[33,34]. A quiet metabolism, or "silent embryo", is suggested to be associated with high cell viability in embryos, whereas embryos of poor quality operate at higher metabolite or nutrient turnover rates[35]. In this perspective, developmentally impaired embryos can be considered "noisy", as opposed to the developmentally competent "silent embryos". On the endometrial side, global gene expression analysis has been applied in several studies to identify genes and their networks associated with receptivity[36,37]. Amongst upregulated genes upon trophoblast invasion are CXCL1 (GRO1), IL-8, CXCR4, pentraxin 3 (PTX3), IL-6 and metalloproteases (MMP1, 10, 14)[38], whereas IGF1, FGF1, TGF1 and genes involved in Wnt signalling are downregulated[38]. However, to fully understand the reciprocal communication, more emphasis should be made on the dialogue that is initiated by the arrival of the embryo within the uterine cavity. Both in mice and in humans, endometrial gene expression is considered to be regulated by the embryo[39,40].

## Investigating human embryo-endometrial interactions

Although the later stages of human implantation in which the trophoblast invades the decidua and maternal vasculature have been studied in first-trimester placental tissue[41], analysis of the early stages of implantation remains difficult, largely because early implantation sites in the human are inaccessible to experimental manipulation in vivo. Because of this, many of the existing data are derived from animal models, particularly those employing knockout mice[42,43]. While these models provide important clues to the processes governing human implantation, the degree to which these data can be extrapolated to humans is limited. For instance, while LIF has been shown in the mouse to be crucial for implantation[42,44], it is not clear that it shares a similar key role in the human. Of all animal models, the baboon is probably the most representative and valuable for extrapolation to the human[45]. However, ethical and technical issues and the apparent differences between the various species, continue to hinder our understanding of the causes of implantation defects in humans. Therefore, the study of human embryo implantation relies largely on *in vitro* models of implantation. Several *in vitro* models of embryo-endometrial interactions have been established in the field of reproductive medicine. The different stages of implantation encompassing apposition, adhesion and consequent invasion into the endometrium, can thus be observed in an environment designed to simulate the *in-vivo* uterine environment. Molecular techniques such as microarray, PCR and ELISA/Multiplex Assay analysis are promising methods to decipher the complex interactions that take place during conceptus-endometrial contact.

## Aims and outline of this thesis

In this thesis we attempt to address some key questions that underlie embryo development and preimplantation embryo-endometrial signalling. For this we made use of an *in vitro* model, which has the ability to give insight into these topics in the human.

Models that attempt to reconstruct the human implantation site *in vitro* are based on creating an artificial endometrium in which the embryo is able to implant. **Chapter 2** reviews the literature regarding the available *in vitro* models for the study of early human embryo-endometrium interactions.

**Chapter 3** describes the use of an *in-vitro* co-culture model to investigate the chromosomal constitution and development of embryos from compaction to the peri-implantation stage *in vitro*. **Chapter 4** identifies soluble factors involved in implantation, using the same model consisting of decidualizing endometrial stromal cells and single hatched human blastocysts. The data presented in this chapter lead to the hypothesis being proposed that the endometrium serves as a sensor of embryo quality. To further test this hypothesis the study presented in **chapter 5** aims to identify genes differentially expressed in decidual cells cultured with human embryo-conditioned medium from a good quality developing embryo or a developmentally incompetent embryo, using mRNA expression microarray analysis.

The aim of **chapter 6** was to characterize patients with a history of recurrent miscarriage. This study demonstrates that this clinical phenotype is associated with abnormal cyclic differentiation of endometrial stromal cells into specialized decidual cells and an aberrant response to soluble embryo signals. **Chapter 7** discusses the experimental and clinical findings in the previous chapters and proposes the hypothesis that recurrent miscarriage is caused by impaired natural embryo selection and can be considered a separate entity of superfertility.

Finally, **chapter 8** summarizes the most important conclusions from the conducted studies and discusses the implications for clinical practice and future research.

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# Chapter 2

## IN VITRO MODELS FOR THE STUDY OF EARLY HUMAN EMBRYO-ENDOMETRIUM INTERACTIONS

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## Abstract

The molecular interactions at the embryo-endometrial interface during the period of blastocyst adhesion and subsequent invasion into the endometrial stroma are not fully understood. Current knowledge is primarily based on evidence from implantation studies in the mouse. The degree to which data derived from animal studies mirror human implantation is limited. The ethical and technical challenges studying implantation in the human can partly be overcome by designing in vitro models of embryo-endometrium interactions. In this review the principal models in current use are described. Basic models using tissue explants and monolayers are distinguished from complex models using multilayer isolated cells, and embryo-endometrium co-culture systems used therapeutically. While there are limitations to current approaches, a number of research questions that could be addressed using these techniques are identified.

## Introduction

Implantation of the embryo into the uterus is an important limiting step in human reproduction[1]. The process commences with molecular signalling by the embryo, followed by apposition, attachment and subsequent invasion into the endometrium. As the preimplantation embryo develops into the blastocyst on the fifth day post-conception, it becomes capacitated for implantation[2,3]. Simultaneously, ovarian steroid hormones and downstream factors for growth- and differentiation transform the endometrium to a state of receptiveness[4]. Reciprocal interaction of the embryo and the endometrium is essential for successful implantation and the establishment of a functional fetal-maternal interface capable of supporting pregnancy to term. This molecular dialogue involves cell-cell and cell-extracellular matrix interactions, mediated by integrins, matrix-degrading enzymes and their inhibitors. Classical studies and the common clinical condition of ectopic pregnancy indicate that the embryo is capable of initiating implantation into many tissue types[5]. However, the majority of human embryos fail to successfully implant into the endometrium, despite its specialised role for this purpose[1]. The endometrium demonstrates cyclical changes in receptivity to the embryo, and appears to be amenable to implantation for a limited period, known as the putative 'window of implantation'[4,6-8].

The molecular interactions at the embryo–maternal interface during the period of blastocyst adhesion and subsequent invasion through the endometrial stroma are not fully understood. Techniques used to study the molecular profile of the receptive endometrium include analysis of gene- and protein expression in endometrial biopsy material[9-11] and endometrial flushings and secretions[12-15]. At the same time, mapping the metabolic products secreted and consumed by the preimplantation embryo during in vitro culture in IVF/ICSI treatments is providing new insights into the molecular profile of the embryo with high implantation potential[16-18]. Limitations of these approaches include their static snapshot analysis of a dynamic process, and their unilateral focus on either the embryo or the endometrium.

The technical and ethical obstacles to study human implantation *in vivo* mean that many of the existing data are derived from animal studies, particularly those employing knockout mice. While animal models provide important clues to the processes governing human implantation, the degree to which these data can be extrapolated is limited. For instance, while LIF has been shown in the mouse to be crucial for implantation[19,20]. It is not clear that it shares a similar key role in the human. Of all animal models, the baboon is probably the most representative and valuable for extrapolation to the human[21]. However, ethical and technical issues and the apparent differences between the various species, continue to hinder our understanding of the causes of implantation defects in humans. Therefore, for the study of human embryo implantation *in vitro* models are potentially of great value.

Several *in vitro* models of embryo-endometrium interactions have been established in the field of reproductive medicine. The different stages of implantation encompassing apposition, adhesion and consequent invasion into the endometrium, can thus be observed in an environment designed to simulate the in-vivo uterine environment. Molecular techniques such as microarray, PCR and ELISA/Multiplex Assay analysis promise to decipher the complex interactions that take place during conceptus-endometrial contact. In addition to providing improved understanding of the embryo implantation process, *in vitro* embryo co-culture with endometrial tissue may have clinical uses.

This review describes the principal *in vitro* model systems of human embryo implantation that have been designed to date. Models using tissue explants are distinguished from models using (multilayer) isolated cells their pros and cons are described. The criteria an ideal model of implantation should meet to answer some key research questions are proposed and a number of research questions that could be addressed using these techniques are described.

## Models of embryo implantation

Models that attempt to reconstruct the human implantation site *in vitro* are based on creating an artificial endometrium in which the embryo is able to implant. The means of simulating the receptive endometrium differ widely and have led to several experimental models. The first study of in vitro mammalian implantation was reported almost 50 years ago[22]. The model used strips of rabbit endometrium derived from the uterine horn 6-7 days after mating. These were stretched out on strips of cellulose-acetate fabric placed on a solid medium in a watch glass. 6-7 days post-coitum, rabbit blastocysts were placed on the endometrial explants and co-cultured for up to six days. One of the important observations of this pioneering study was the necessity of removal of the zona pellucida for implantation to take place successfully extracorporeally. Furthermore it was demonstrated that for endometrial explants to function in such a model, they should be obtained from progestational phase of development, which indicates that endometrial differentiation is of vital importance for successful implantation[22]. However, in this system, there was clear evidence of tissue damage and necrosis in the cultured endometrium. Therefore, while the use of uterine organ culture had the advantage of simulating the in-vivo state, maintenance of the cultured organ and visual observations of blastocyst attachment itself proved to be difficult. These challenges led investigators to develop monolayer systems using mouse uterine cells[23,24].

The first successful IVF procedure in 1978 changed the field of reproductive medicine and provided a new impulse to research in human implantation[25]. Instead of relying on the ethically challenging and invasive technique of flushing out concepti from post-conception women[26], supernumerary embryos created by IVF generated an important and ready source of material for study. Secondly, it became quickly evident that implantation failure was a major obstacle to improving success rates from Assisted Reproductive Techniques (ART)[27]. The scene was therefore set for the development

of human models of embryo-endometrial interactions. Models used today are not only used to describe the process of embryo implantation, but also to dissect the molecular cross talk between the conceptus and endometrium. Newly available techniques such as multiplex assays, proteomics and micro-arrays have proved to be effective tools for the investigation of different processes during implantation, monitoring embryo-derived signals, the endometrial response and the soluble factors produced during interaction. Ultimately, implantation can be experimentally modulated by the specific removal or addition of putative factors involved in embryo-endometrial interaction.

**Basic and monolayer co-culture models**

Organ explants would appear to provide perfect models for mimicking the in vivo environment, as the three-dimensional structure and integrity of the endometrium is preserved and all layers of the endometrium are included. Landgren et al (1996) developed a model using endometrial biopsies taken 4, 5 and 6 days after the LH peak from healthy women with normal regular menstrual cycles. Histological dating confirmed the tissue to be mid-secretory phase material. Biopsies were incubated in culture medium RPMI 1640 and co-cultured with one to three embryos fertilized in vitro. The embryo was placed on the lining epithelium of the endometrium within 3 hours of the biopsy being taken. Before and after co-culture, the biopsies were analysed by morphologic means using light microscopy. Embryos obtained 4 days after IVF penetrated the lining of the endometrial epithelium obtained from a biopsy obtained 4 days after the LH peak[28].

An alternative approach utilizing trophoblast cells to study interactions with endometrium was the in vitro suspension culture system described by Kliman et al (1990). In this model, first, second and third trimester villous tissue was dispersed and purified by Percoll gradient to be subsequently co-cultured with endometrial tissue. The endometrial tissue was derived after hysterectomy or curettage. Tissue was minced into 1-2mm cubes and transferred to medium supplemented with 20% FCS. Co-cultures were performed in polypropylene tubes and gyrated on an angled rotator. Endometrium cultured alone remained viable for up to 3 days, before tissue necrosis presented centrally in the minced cubes. hCG positive staining confirmed the attachment and, in some cases, penetration of trophoblast cells into the explants. Trophoblasts adhered to the exposed stromal surfaces of the tissue fragments, but interestingly the surface epithelium was not receptive to trophoblast attachment unless the tissue had been derived during the "window of implantation" on day 19[29]. Furthermore, upon contact of the trophoblast cells with the stroma a "contact-necrosis" was observed to occur. This peripheral necrosis was ascribed to secretion of proteases by the trophoblast or the activation of proteases in the endometrium. Remarkably, no difference in behaviour was seen between first, second and third trimester trophoblast cells. An important finding in these experiments was that the trophoblast cells were shown to be capable of inducing secretory changes in proliferative endometrium, as confirmed by ultrastructural imaging.

Though attractive in theory, models using endometrial explants also present specific challenges. Tissue necrosis remains a major problem, and the timing of the biopsies and the handling of the specimens is labour intensive and susceptible to faults. Therefore, models developed later mostly use a purified cell population grown in optimal conditions.

Employing a system in which endometrial stromal were co-cultured with human trophoblast cells, Popovici et al (2006) studied the impact of trophoblast contact on stromal cell gene expression. Trophoblast tissue was obtained from early pregnancy terminations of uncomplicated, unwanted pregnancies. Endometrial stromal cells were derived form women undergoing diagnostic hysteroscopy for benign reasons. Stromal cells were all derived form proliferative endometrium and passaged once. In this study, the stromal cells were not decidualized. In an earlier study by the same group they used this model to investigate the effect of decidualization on stromal cells, again by microarray[30]. Trophoblast explants attached approximately 2 -3 hours, which were then kept in culture for a total time of 24 hours. Affymetrix GeneChip Expression was performed and 171 genes were upregulated upon contact with the trophoblast tissue, mostly involved in inflammatory response and signal transduction. 119 genes were decreased, with a pronounced role for cytoskeletal anchoring, proteolysis and the humoral immune response. Validation of microarray data showed significantly increased levels of TIMP-3, DKK-1, IGFBP-1, PTX-3 and IL-8 (InterLeukin-8). PTX-3 is particularly interesting because women with preeclampsia or IUGR and thus possibly impaired implantation, have elevated levels of PTX-3 in their serum[31]. Significant downregulation of MMP-11 (Matrix MetalloProtease) and SOX-4 (Sry-related high-mobility group - box 4) was also confirmed by PCR, which indicates decreased apoptotic stimuli in the endometrial stromal cells[32]. Using Ingenuity Pathways Analysis (Ingenuity® Systems, Redwood City, USA), the authors ascribed the major effects to a network related to "inflammatory, immune, haematological and development". This approach appeared to enable multiple different biological processes that are regulated concomitantly within stromal cells by contact with trophoblast to be analysed[32]. This work is one of the few published papers that address the later phase of human implantation.

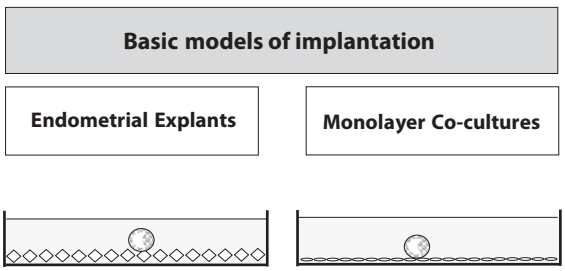
Regarding earlier stages of implantation, endometrial stromal cells have also been shown to offer a ready means of studying the initial intimate contact of the embryo with the uterine lining. Carver et al (2003) have described a model of implantation that focuses particularly on the invasion phase of implantation. In this experimental model human preimplantation blastocysts are co-cultured with primary human endometrial stromal cells in a monolayer[33]. Endometrial stromal cells are derived form biopsies, purified and decidualized by cAMP before co-culture with a day six human blastocyst. This group showed that blastocysts attach to the stromal cells, and the trophectoderm subsequently undergoes outgrowth on, and invasion into, the stromal cell layer, thus penetrating under the stromal cells. These observations were supported by immunohistochemical analysis, which allows investigation of ligand-receptor interactions. Furthermore they showed that the embryo-

endometrial microenvironment holds significant amounts of hCG, which indicates trophoblastic outgrowth. More recently, the Oxford Group has used this model to demonstrate a major role for Rho GTPases Rac1 and RhoA as modulators of implantation[34]. Silencing Rac1 was shown to inhibit trophoblast invasion, whereas RhoA silencing resulted an opposite effect, potentiating invasion. Rac1 expression is associated with cell motility and embryo-induced upregulation at the site of implantation suggests increased stromal cell motility during trophoblast invasion[35].

This type of model offers the means of studying the impact on implantation of the selective silencing of genes or blocking factors in the co-culture medium. Analysis of this co-culture promises to offer clearer insights in the molecular interactions between the conceptus and the stromal fraction. There are however a number of potential pitfalls using this type of model. Clearly, only a single fraction of the cells that consitiute the receptive endometrium is present in this model, and the stromal cells themselves are not modulated by these other cell types when in vitro. Another downside is the fact that the blastocyst loses its original three-dimensional structure upon inverted application of the coverslip onto a glass slide.

However, the practical and simple nature of this model type has led others to establish similar systems. Wetzels et al employed human fibroblasts to study embryo development in vitro and implantation[36]. After successfully using this cell culture in co-culture with mouse embryos[37,38], human cells were obtained from the skin of the chest area of a normal male fetus, and passaged in cell culture (CCD-27sk). Using this system for clinical co-culture in IVF, they found no difference in pregnancy rates, rate of implantation and embryo quality. The model could provide new insights in embryo development. The major ´weakness´ of course is the absence of endometrial-derived tissue.

**Figure 1.** Approaches to investigate embryo-endometrium interaction in vitro – Basic models of implantation.




**Complex and Multilayer co-culture models**

The co-culture of both endometrial epithelial cells (EEC) and stromal cells (ESC) with an embryo is attractive, because ESC and EEC not only interact with the embryo directly, but also through each other. Pierro and co-workers developed an in vitro co-culture system for studying interactions

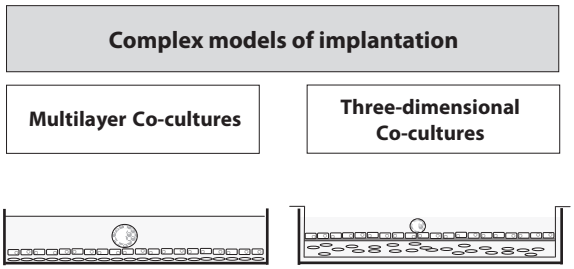
between ESC and EEC via soluble signals. The purpose of the study was to investigate whether 17ß-estradiol and insulin can induce proliferation of EEC through ESC-secreted factors, but the model can also be applied to study implantation[39]. Endometrial tissue specimens from the late proliferative phase were processed for separation of EEC and ESC in suspensions by an extensive protocol.[40] The cellular suspension containing EEC was applied to a Matrigel-coated insert and co-cultured with the subcultured ESC in a 24-well plate. The model proved useful to study the interactions between the two endometrial cell populations.

Rather than growing dissociated epithelial cells on a Matrigel surface as in the model described above, a similar model embeds isolated fragments of epithelium directly inside the matrix. This model, described by Bläuer et al (2005) was used to investigate the effects of estradiol ($E_2$) and medroxyprogesterone acetate (MPA) on cell proliferation and the expression of estrogen receptor $\alpha$ (ER$\alpha$) and progesterone receptor (PR) in epithelial organoids[41].

In order to overcome the limitations of models which employ just one of the cell types which constitute the human endometrium, and to address the spatial relationship between the different layers of the endometrium, a number of groups have worked on the development of three dimensional co-culture models.

In the model described by Bentin-Ley and co-workers (1994), endometrial epithelial cells were embedded onto an artificial basement membrane of Matrigel with endometrial stromal cell matrix underneath[42]. This model has been shown to closely simulate the ultrastructural architecture of the endometrium[43,44] and resulted in some stunning SEM (scanning electro-microscopic) pictures that revealed the presence of pinopode formation around the implantation site[45]. Attachment of the embryo was seen between 16 and 42 hours after onset of co-culture. After penetration of the epithelial cells, a penetration cone invaded into the stromal cell fraction below. Interestingly, they found the inner cell mass situated in this cone and thus, on the endometrial side of the invading embryo. Recently, the same group has used the model to study the effect of several contraceptives on the endometrium and consequently embryo attachment[46-48]. Classen-Linke et al established a similar model using a dual chambered system[49], which although not actually constituting an in vitro model for implantation, did allow physiological and morphological processes within the endometrium to be studied in-vitro.

**Figure 2.** Approaches to investigate embryo-endometrium interaction in vitro – Complex models of implantation.



**Limitations of current approaches**

Clearly, no in vitro model has yet been developed which can completely mimic the in-vivo conditions it tries to represent. The major limitations of current approaches are summarized in Table 1. However, each approach described offers the means of opening windows onto specific phases or processes involved when the human embryo engages with the endometrium. Such models also provide a unique opportunity to study human embryo development beyond the day 6 blastocyst stage and the role and means of endometrial interaction with this stage of the developing embryo. In the first few days of development, the cell count of an embryo increases exponentially. Simultaneously, embryonic differentiation in both trophectoderm and inner cell mass is constantly evolving. In vitro models of implantation open a window on to these previously unobservable phases of human development.

**Table 1.** Limitations of current models to study human embryo implantation

| |
|---|
| - The only source of human embryos is from in vitro fertilization (IVF) and intracytoplasmic sperm injection of eggs (ICSI) treatments, which may not be representative of the natural embryo pool |
| - Endometrial tissue sampling from "healthy" women demonstrates biological differences between primary cell lines and different passages |
| - Culture systems are generally unable to support human embryo development beyond day 9 of development |
| - Because of the differential intrinsic quality of human embryos, it becomes difficult to relate unsuccessful implantation in vitro to either embryonic or endometrial factors |
| - No proven convincing beneficial effect of coculture on embryo development before embryo transfer |

**Therapeutic co-culture models**

As a strategy for overcoming the clinical problem of recurrent implantation failure, endometrial co-culture systems have been advocated as a means of improving the implantation potential of embryos generated by IVF. Barmat et al (1999) developed a system combining both autologous endometrial epithelial and stromal cells[50]. Endometrial biopsies were taken from luteal phase endometrium in the cycle prior to IVF treatment. Tissue was then dissociated in four enzymatic digestion steps, with separation of glands and stromal cells by differential sedimentation at unit gravity. An equal mixture of stromal cells and glands were combined in a four-well dish on the estimated day before hCG administration in 1ml of Ham's F-10 medium, supplemented with 15% patient serum. Approximately 50-75% confluence was achieved when pre-embryos were placed into the co-culture system, and at transfer close to a monolayer existed. In their study, they found a higher number of blastomeres per pre-embryo on co-culture compared to the previous non co-culture cycle. No significant differences in implantation and clinical pregnancy rates were demonstrated[50]. Moreover, although offering the

clinical advantages of autologous culture, this model proved to be highly labour intensive, requiring to be set up at short notice depending on the timing of the oocyte pick up.

An alternative model initially developed for similar clinical purposes, also involves co-culture of autologous endometrium with human embryos that were transferred into the uterus afterwards. This model, however, only includes endometrial epithelial cells in the co-culture system. They found their approach to be safe and effective in improving implantation in patients with implantation failure undergoing ovum donation, but not in IVF patients[51]. Spandorfer et al used the same model for clinical purposes and reported a role for this technique in endometrial dating[52]. Endometrial cells from biopsies that were out of phase did not show any improvement in embryos, while endometrial epithelial cells from in-phase biopsies did. They showed that production of granulocyte macrophage-colony stimulating factor (GM-CSF) by the autologous endometrial fraction in co-culture is associated with pregnancy outcome in a population with multiple implantation failure[53].

The same group also reported that cultured endometrial epithelial cells showed regulation and polarization of CXCR1, CXCR4 and CCR5 receptors in the presence of a human blastocyst. In the same study they performed endometrial biopsies throughout the menstrual cycle and found increased mRNA levels of CXCR4 during the window of implantation in vivo. CXCR4 was showed moderate-to-strong staining in luminal, glandular and stromal cells in the late follicular phase. These data suggest an important role for chemotaxis by the human blastocyst and endometrial regulation of chemokine receptors.

## Conclusions and future perspectives

In vitro models provide a means of addressing a number of challenges that have hindered progress in understanding human implantation, and provide a unique insight in the multiple interactions that occur during for apposition, attachment and invasion to succeed. Moreover, the relative scarcity of human embryos for research and the moral imperative for their judicious use requires effective models for the study of their development and interaction with the endometrium. From monolayer co-cultures to complex multilayer and three-dimensional models; all have proven to be useful tools to illuminate a specific process during embryo-endometrium interaction.

More complex models may not necessarily be ´better´ models for the investigation of embryo-endometrial molecular cross talk. While they may come closer to mimicking the in vivo situation, their complexity increases the risk of introducing artefactual errors, and renders them less robust and reproducible. On the other hand, complex models are more likely to come closer to representing in vivo conditions for descriptive analysis of implantation and studying early blastocyst development. Monolayer co-culture models have the role of specific factors using gene silencing and blocking experiments to be determined. Though these models are restricted and don't mirror the whole population of endometrial cells in vivo, this limitation also offers the advantage of allowing a simpler, more robust model set-up allowing extensive study of the specific endometrial cellular components in the model.

The models described here are not only informative to study the implantation process, but also provide the opportunity to study the human peri-implantation embryo at a stage of development which normal in-vitro conditions have difficulty in achieving. Until now, the study of human embryology beyond day 6 of development has been very difficult. As a result very little is understood about the regulation of gene expression in human embryos beyond day 3-4. In vitro models thus also provide potentially useful tools to investigate embryo development beyond the preimplantation stage.

Clinically, co-culture systems may provide means of developing novel culture media, overcoming recurrent implantation failure while on the other hand providing a tool for developing new contraceptive agents[51,54]. Because ovarian hormones not only have a direct effect on the endometrium, but induce many downstream factors which may have indirect effects on epithelial, stromal and immune cells in the uterine lining, it has been proposed that non-hormonal substrates may be good candidates for novel, local contraceptive therapies. The effect of these possible agents is best explored in a controllable ex vivo way, so that dose and effect relations can be evaluated.
Finally, it remains unclear which player; the embryo or the endometrium, initiates the preimplantation dialogue. Does the viable embryo actively signal to the endometrium to prepare for implantation or is the receptive endometrium by default available for implantation, unless it recognises the presence of an abnormal embryo? In vitro models may also have an important role in elucidating the answers to such questions, and providing more insights into the pathophysiology of implantation failure and recurrent pregnancy loss.

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# Chapter 3

## THE FATE OF THE MOSAIC EMBRYO: CHROMOSOMAL CONSTITUTION AND DEVELOPMENT OF DAY 4, 5 AND 8 HUMAN EMBRYOS

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## Abstract

Background: Post-zygotic chromosome segregation errors are very common in human cleavage stage embryos after *in vitro* fertilisation, resulting in chromosomally mosaic embryos. Little is known regarding the significance of mosaicism for the developmental potential of early embryos. We assessed chromosomal constitution and development of embryos from compaction to the peri-implantation stage at day 8 after fertilisation. Methods: From 112 cryopreserved day 4 human embryos donated for research, 21 were immediately fixed and all cells analysed by fluorescent in situ hybridisation (FISH). The remaining 91 embryos were thawed, with 54 embryos suitable for biopsy of one or two cells. Biopsied cells were fixed and analysed by FISH for chromosomes 1, 7, 13, 15, 16, 18, 21, 22, X and Y. Biopsied embryos were kept in standard culture conditions for 24h. Embryos arrested before cavitation (n=24) were fixed. Developing day 5 blastocysts (n=24) were submitted to co-culture for a further 72 h on a monolayer of decidualised endometrial stromal cells, followed by fixation. Cell numbers were counted and all nuclei analysed by FISH. Data derived from a previous FISH analysis done by our group on cryopreserved good quality day 5 blastocysts (n=36) were included in the present study. Results: FISH analysis was successfully performed on 18 day 4 embryos. According to our definition, 80% of embryos were mosaic and 11% showed a chaotic chromosomal constitution. FISH analysis of two blastomeres from morula-stage embryos showed that 54% of the embryos were mosaic, 40% normal, and 6% abnormal. Analysis of day 4, 5 and day 8 embryos showed a decrease in incidence of mosaic embryos over time, from 83% on day 4 to 42% on day 8. A significant positive correlation was observed between the total cell number and the percentage of normal cells in developing day 5 and day 8 embryos but not in day 4 morulas or embryos arrested before cavitation. Conclusions: These data suggest that both the developmental arrest of a significant proportion of mosaic embryos on day 4, and the cell death or reduced proliferation of aneuploid cells within an embryo may be responsible for the observed decrease of aneuploid blastomeres from compaction to the peri-implantation stage.

## Introduction

Most of our current knowledge concerning the chromosomal constitution of human preimplantation embryos comes from the analysis of cleavage stage embryos by preimplantation genetic screening (PGS) performed 3 days after fertilisation, when embryos are usually composed of 6-10 cells (blastomeres). Molecular cytogenetic analysis of interphase nuclei by fluorescence *in situ* hybridisation (FISH) has been the most frequently used technique for the analysis of chromosomal abnormalities in human embryos. Data obtained by such studies have indicated that more than 50% of human cleavage stage embryos generated by *in vitro* fertilisation (IVF) contain chromosomally abnormal cells[1]. These abnormalities may arise from an error during meiosis, resulting in a uniform abnormality present in all cells, or from segregation errors occurring during the first mitotic divisions. The latter event results in chromosomal mosaicism, defined as the coexistence of karyotypically distinct cell lineages derived from a single zygote. Mosaic embryos can be composed of a mixture of chromosomally normal and abnormal cells or of abnormal cells with different abnormalities. Mosaicism has been reported to affect up to 91% of human preimplantation embryos if all cells are investigated[2-8]. Studies using comparative genomic hybridisation (CGH) and array CGH in human preimplantation embryos, allowing the screening of all chromosomes, have confirmed the high prevalence of chromosomal mosaicism at this early stage of development and also demonstrated the high incidence of structural abnormalities[2,3,8]. These recent findings have changed our understanding of the cytogenetic processes occurring at the cleavage stage. However, the implications of chromosomal mosaicism for further embryonic development and implantation remain unclear. So far, the majority of the studies investigating the chromosomal constitution of human blastocysts have suggested no definite selection against most of the chromosomal abnormalities observed at the cleavage stage[4,6,9-15]. Although there are reports of an increase in the proportion of blastocysts showing chromosomal mosaicism, compared to early cleavage-stage embryos,[4,13] the proportion of aneuploid cells within an embryo seems to decline towards the blastocyst stage[4,12]. In spite of the high frequency observed in preimplantation embryos, a low percentage (0.3%) of aneuploidy is found at term birth[16]. Up to 91% of preimplantation embryos are mosaic[8]. However, the incidence of mosaicism in spontaneous abortion specimens is significantly lower (<10%). First trimester diagnoses in chorionic villi from viable pregnancies show an even lower incidence of mosaicism (1 to 2%)[17]. It seems that the majority of mosaic embryos disappear prior to the period of first trimester, either due to a selection against mosaic embryos, or "normalization", due to selection against abnormal cells within the embryo[17]. Therefore, a "black box" remains concerning events surrounding implantation[18]. At present, our knowledge of the fate of the mosaic human embryo is limited to the blastocyst stage at day 6 post-fertilisation. Clearly, if the significance of mosaicism for subsequent development is to be understood, events subsequent to this stage need to be elucidated. The aim of the present study was therefore to assess the prevalence of mosaic embryos and how the chromosomal constitution of human embryonic cells evolves from compaction to the peri-implantation period at day 8 post-fertilisation, using an *in vitro* implantation model.

## Results

### Control lymphocytes

Home-labelled probes used were the same as previously described[19]. These probes were tested on 10 metaphase and 100 interphase nuclei of lymphocytes to check localisation and test for FISH efficiency. The hybridisation efficiencies for the individual probes were calculated as the percentage of nuclei showing the expected number of signals and ranged between 94-100%, depending on the individual DNA probe. This was comparable to what was described previously[19].

### Analysis of non-biopsied day 4 embryos

In order to assess the chromosomal constitution and incidence of mosaicism of compacting embryos, cryopreserved day 4 embryos were thawed (n=21) and fixed within 10 minutes after removal from liquid nitrogen. None of the day 4 embryos successfully analysed by FISH (n=18) consisted of only normal cells. Only one (6%) embryo was classified as normal (embryo 1) according to our definition, with 3/15 (20%) differently abnormal cells (Figure 1 and Table 1). Fifteen (83%) of the thawed day 4 embryos were diagnosed as mosaic and two (11%) as chaotic. The average percentage of chromosomally normal cells per embryo was 55% (range 0-83%).

**Figure 1 Incidence of mosaicism on days 4, 5 and 8 post-fertilisation.** Percentage of day 4 morulas, embryos arrested before cavitation, developing day 5 blastocysts and day 8 peri-implantation embryos, diagnosed as normal, mosaic, chaotic, and uniformly abnormal after FISH analysis of chromosomes 1, 7, 13, 15, 16, 18, 21, 22, X and Y.

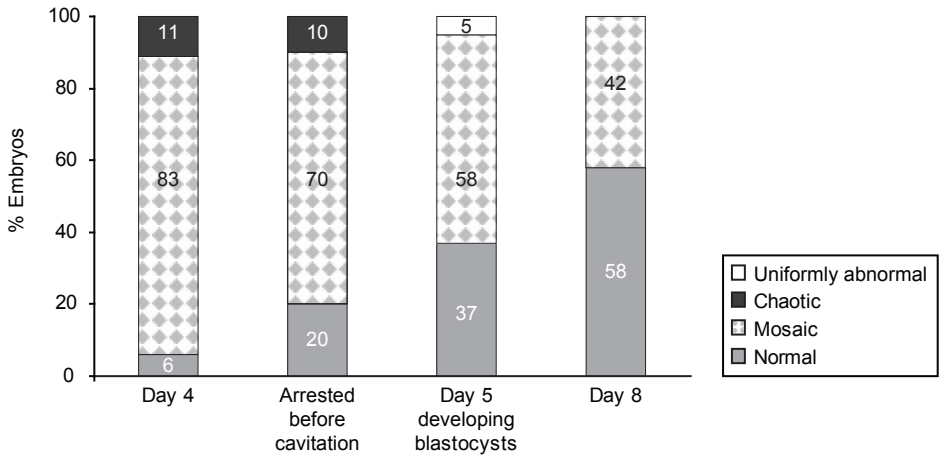


**Table 1:** FISH analysis of non-biopsied frozen-thawed day 4 embryos using probes for chromosomes 1, 7, 13, 15, 16, 21, 22, X and Y.

| NON-BIOPSIED DAY 4 | | | |
|---|---|---|---|
| Embryo no. | No. cells analysed | % normal cells | FISH interpretation |
| 1 | 15 | 80 | Normal |
| 2 | 8 | 88 | Mos +1 / 2N |
| 3 | 15 | 67 | Mos -7 / 2N |
| 4 | 13 | 69 | Mos -13 / 2N |
| 5 | 11 | 45 | Mos -15 / 2N |
| 6[3] | 10 | 30 | Mos +15 / 2N |
| 7 | 19 | 53 | Mos -16 / 2N |
| 8 | 6 | 83 | Mos -18 / 2N |
| 9 | 13 | 69 | Mos -X / 2N |
| 10 | 9 | 78 | Mos -1 / +15 / 2N |
| 11 | 14 | 57 | Mos -7 / -21 / 2N |
| 12 | 14 | 57 | Mos -15 / -13 / 2N |
| 13 | 9 | 56 | Mos -15 / -1 / -16 / 2N |
| 14 | 13 | 31 | Mos -22 / -15 / -18 / 2N |
| 15 | 8 | 38 | Mos -22 / -18 / -15 / +21,+22,+22 / 2N |
| 16 | 8 | 63 | Mos +21 / -7,+22 / +1,+1,+13,+15,+15,+16,+18,+22 / 2N |
| 17 | 9 | 22 | Chaotic[1] |
| 18 | 12 | 0 | Chaotic[2] |

[1] Mos -1,-13,-X,null 21 [3] / -18,-Y,null 22 [2] / -1,-13,-18,-21 [1] / +13,+22 [1] / 2N [2].
[2] Chaotic embryo, where all the cells had different combinations of abnormalities. However, 58% of the cells showed errors in common: +1,+7,+15,+X.
2N = normal copy number for the chromosomes investigated; Mos = mosaicism; null = nullisomy. In the mosaic cases the different abnormal cell lines are presented according to their size with the largest first. A normal diploid cell line is always listed last (ISCN 2009)[20].
[3] Case where the chromosomal abnormality might have had a meiotic origin followed by a subsequent mitotic error.

**FISH analysis of blastomeres and biopsied embryos**

In total, 91 blastomeres from 53 compacting embryos were analysed by FISH. From 18 embryos, one cell was available for analysis and for 33 embryos the analysis was based on two blastomeres. Embryos 38 and 50 showed partial compaction by the time of biopsy, and therefore 3 cells were inadvertently biopsied. From the group of embryos where a single blastomere was biopsied, 39% were normal. In the group of embryos with two or three blastomeres biopsied 40% were normal, 54% mosaic and 6% abnormal (Tables 2 and 3). FISH analysis of two blastomeres is consistent with the analysis of all cells from non-biopsied morulas, revealing a high rate of mosaicism, with more

than half (54%) of the embryos identified as mosaic. These findings are also comparable to our earlier report on day 3 biopsy of cryopreserved embryos, where 24% of embryos with two cells biopsied (n=21) were chromosomally normal, 57% mosaic and 19% abnormal[19].

**Reanalysis of biopsied day 5 and day 8 embryos**

After embryo biopsy and FISH analysis, embryos were reanalysed at day 5 if arrested at that time or at day 8 and the diagnosis compared to the day 4 analysis. From the 24 embryos arrested before cavitation that were fixed on day 5, 20 were successfully analysed after two rounds of FISH, showing that 14 (70%) were mosaic, 4 (20%) normal, and 2 (10%) chaotic (Figure 1 and Table 2). From the 24 blastocysts put into co-culture, 8 had degenerated by day 8 and could not be retrieved. The remaining 16 embryos were successfully fixed on that same day. However, 4 embryos were excluded from further analysis due to failed hybridisation for the second round of FISH. In total, 2859 blastomere nuclei from 12 embryos were analyzed for 10 chromosomes. In 8% of the nuclei, some overlap was observed and these were excluded from the FISH analysis. We diagnosed seven day 8 blastocysts (58%) as normal and 5 (42%) as mosaic. No day 8 embryo consisted of only normal cells, nor did we find an embryo with uniform aneuploidy. In each embryo, a range of cells with different abnormalities was found, each abnormality present in cell numbers not reaching the 10% threshold of our definition for a mosaic cell line. For instance in embryo 43, several cells with either a monosomy 1, monosomy 15, monosomy 16, monosomy 18, trisomy 13, monosomy 13, trisomy 21, monosomy 21 or monosomy 22 were present. These nine different abnormalities cannot all have originated from errors arising during the first cleavage divisions. This may therefore indicate that new segregation errors have occurred in this embryo after day 4. The average percentage of chromosomally normal cells per embryo was 66% (range 35-84%).

Tables 2 and 3 summarize the interpretation of the FISH analysis on days 5 (embryos arrested before cavitation) and 8 (developing blastocysts) and compare observations with the results from the biopsy on day 4. The day 4 diagnosis was not predictive of the potential of the embryo to develop until day 8. Reanalysis of biopsied embryos that either arrested before cavitation or developed until day 8 showed similar cytogenetic confirmation rates (40% for arrested embryos and 36% for day 8 embryos). However, the proportion of embryos with a false positive diagnosis on day 4 (i.e. embryos that were diagnosed as abnormal or mosaic on day 4 but classified as normal after reanalysis) was higher on day 8 embryos (4/7=57%) than on arrested embryos analysed at day 5 (2/12=17%) (Tables 2 and 3). Conversely, the incidence of a false negative diagnosis was higher on day 5, where 6/8 (75%) embryos diagnosed as normal on day 4 were found to be mosaic on day 5, whereas 1/4 (25%) day 8 embryos were falsely diagnosed as normal. This was largely due to the increased incidence of chromosomally normal embryos on day 8, with the percentage of embryos diagnosed as mosaics falling from 70% on day 5 to 42% on day 8 (Figure 1). Reanalysis of the FISH results of day 5 good-quality blastocysts analysed in a study by Baart *et al.* (2007) showed

**Table 2:** FISH analysis of blastomeres from frozen-thawed day 4 embryos and reanalysis of corresponding embryos on day 5, using probes for chromosomes 1, 7, 13, 15, 16, 21, 22, X and Y.

| DAY 4 | | | | ARRESTED BEFORE CAVITATION (DAY 5) | | | Confirmation |
|---|---|---|---|---|---|---|---|
| Embryo no. | No. biopsied cells | FISH results (nucleus 1/ nucleus 2) | FISH interpretation | No. cells analysed | % normal cells | FISH interpretation | |
| 19 | 1 | 2N | Normal | 18 | 67 | Mos -7 / -18 / 2N | - |
| 20 | 1 | 2N | Normal | 21 | 24 | Mos -13 / +13 / +13,+16 / 2N | - |
| 21[5] | 1 | -7,-22 | Monosomy 7, 22 | 5 | 0 | Mos -22 / -21,-22 / -7,-15,-18,-22 | + |
| 22[5] | 1 | +21 | Trisomy 21 | 12 | 50 | Mos +21 / 2N | + |
| 23 | 2 | 2N / NA | Normal | 15 | 80 | Normal | + |
| 24 | 2 | 2N / 2N | Normal | 5 | 100 | Normal | + |
| 25 | 2 | 2N / 2N | Normal | 15 | 80 | Mos -16 / 2N | - |
| 26 | 2 | 2N / 2N | Normal | 8 | 88 | Mos +1 / 2N | - |
| 27 | 2 | 2N / 2N | Normal | 13 | 54 | Mos -18 / +13 / +16 / 2N | - |
| 28[1] | 2 | 2N / 2N / 2N | Normal | 21 | 29 | Mos -18 / -15 / 2N | - |
| 29 | 2 | 2N / -18 | Mosaic | 21 | 76 | Normal | - |
| 30 | 2 | 2N / -13 | Mosaic | 16 | 81 | Normal | - |
| 31 | 2 | 2N / -18,+21 | Mosaic | 24 | 67 | Mos +13 / 2N | - |
| 32 | 2 | 2N / 4N | Mosaic | 8 | 75 | Mos -X / -7, -18 / 2N | - |
| 33[5] | 2 | 3N,-7,-22 / 3N,-1,-7,-22 | Mosaic | 15 | 0 | Mos 3N / -13, -22 / -22 | + |
| 34 | 2 | 2N / -18 | Mosaic | 8 | 50 | Mos -13,-18,-21 / -18 / XYY / 2N | + |
| 35 | 2 | -1,-7,-13 / -1,-7 | Mosaic | 6 | 0 | Chaotic[2] | - |
| 36[5] | 2 | -18 / NA | Monosomy 18 | 32 | 47 | Mos -18 / -1 / 2N | + |
| 37[5] | 2 | -16,+7 / -16,+7 | Monosomy 16, Trisomy 7 | 10 | 30 | Chaotic[3] | + |
| 38[4] | 3 | -13 / 2N / 2N / 2N | Mosaic | 10 | 60 | Mos -18 / -16,-18/ XYY / 2N | - |

[1] Two blastomeres biopsied, with one of them being binucleated.

[2] +13,-18 / +17,+13,-18 / +7 / +13 / +7,+13,+18 / +1,+1,+7,+7,+13.

[3] Confirmation of the trisomy 7 in two cells: +1 [2] / -1,-22,+7,+7,XYY [2] / -4,-7,+13,+15,+22 [1] / +1,+15,+15 [1] / +13,+1,+1,+15,+15,XYY [1] / 2N [3].

[4] Three blastomeres biopsied, with one of them being binucleated.

2N = normal copy number for the chromosomes investigated; Mos = mosaicism; NA = not analysed. In the mosaic cases the different abnormal cell lines are presented according to their size with the largest first. A normal diploid cell line is always listed last (ISCN, 2009)[20].

[5] Cases where the chromosomal abnormality might have had a meiotic origin followed by subsequent mitotic errors.

a lower percentage of chromosomally mosaic day 5 blastocysts (58%) than observed in the present study (70%), where embryos arrested before cavitation were analysed at day 5. Overall, we identified seven cases (7/50=14%) where at least one of the abnormalities observed likely originated during meiosis.

For each reanalysed embryo, the proportion of chromosomally normal cells was determined and correlated to the total number of cells. We found a significant positive correlation (p=0.034) between the total number of cells and the percentage of normal cells of day 8 embryos (Figure 2-D). However, non-biopsied day 4 embryos and embryos arrested at day 5 showed no such correlation (Figure 2 – A,B). In contrast to the embryos arrested before cavitation analysed at day 5, data derived from our earlier study on good-quality day 5 blastocysts[19] did show a significant (p=0.002) positive correlation between the total number of cells and the percentage of normal cells (Figure 2-C).

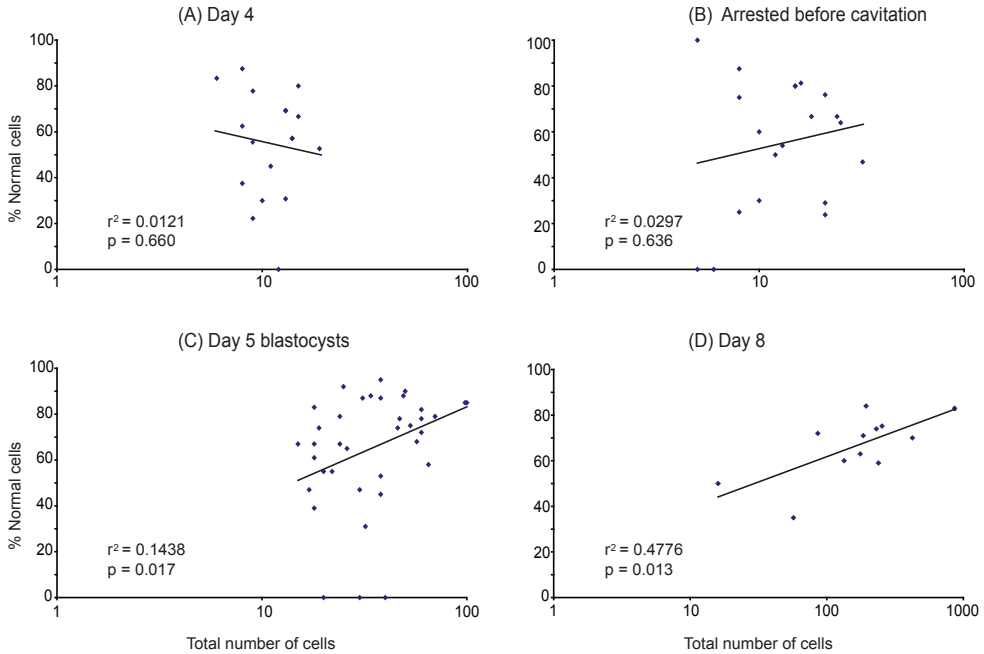
**Table 3:** FISH analysis of blastomeres from day 4 embryos and reanalysis of corresponding embryos on day 8.

| | DAY 4 | | | DAY 8 | | | |
|---|---|---|---|---|---|---|---|
| Embryo no. | No. biopsied cells | FISH results (nucleus 1/ nucleus 2) | FISH interpretation | No. cells analysed | % normal cells | FISH interpretation | Confirmation |
| 39 | 1 | 2N | Normal | 231 | 74 | Normal | + |
| 40 | 1 | +16 | Trisomy 16 | 185 | 71 | Normal | - |
| 41 | 1 | +13 | Trisomy 13 | 424 | 70 | Mos +13 / 2N | + |
| 42 | 2 | 2N / 2N | Normal | 194 | 84 | Normal | + |
| 43 | 2 | 2N / 2N | Normal | 863 | 83 | Normal | + |
| 44 | 2 | 2N / NA | Normal | 134 | 61 | Mos +13 / 2N | - |
| 45 | 2 | -15,-X / -1,-15,-X | Mosaic | 86 | 72 | Normal | - |
| 46 | 2 | 2N XYY / 2N | Mosaic | 176 | 63 | Normal | - |
| 47 | 2 | 2N / +13 | Mosaic | 239 | 59 | Mos -18 / 2N | - |
| 48 | 2 | 2N / -15 | Mosaic | 16 | 50 | Mos 6N / -13 / +13 / 2N | - |
| 49 | 2 | +16 / NA | Trisomy 16 | 254 | 75 | Normal | - |
| 50[1] | 3 | NA | NA | 57 | 35 | Mos -15 / 2N | NA |

2N = normal copy number, and 6N = six copies for each of the chromosomes investigated; Mos = mosaicism; NA = not analysed. In the mosaic cases the different abnormal cell lines are presented according to their size with the largest first. A normal diploid cell line is always listed last [20].

[1] Case where the chromosomal abnormality might have had a meiotic origin followed by a subsequent mitotic error.

**Figure 2 Correlation between the percentage of chromosomally normal cells and the total number of cells per embryo at different stages of development. (A)** Day 4 morulas **(B)** embryos arrested before cavitation **(C)** Day 5 blastocysts **(D)** Day 8 peri-implanted embryos. A and B: non-significant correlation (p>0.05). C and D: significant correlation (p<0.05).




## Discussion

In the present study we performed FISH analysis of human embryos in order to assess the frequency of chromosomal mosaicism at three different stages of development (morula, preimplantation blastocyst and peri-implantation blastocyst). Good quality, frozen-thawed morula stage embryos were either fixed and all cells analysed, or biopsied and allowed to develop *in vitro*. Reanalysis was performed either at day 5, in case of developmental arrest, or at day 8. Data derived from a previous study done by our group on cryopreserved good quality day 5 blastocysts were included. This approach allowed evaluating the chromosomal constitution of human embryos during different stages of development and determining how chromosomal constitution may influence the developmental capacity of embryos.

Consistent with previous findings[19], we found almost all embryos to be mosaic at the morula stage. This high rate of mosaicism is reflected in our results obtained after biopsy and FISH analysis of one or two cells from day 4 embryos. We found that the incidence of mosaic embryos decreased over

time, with a significant decrease between day 4 and day 8 blastocyst stage (Figure 1). Moreover, we found a positive correlation between the total number of cells in the embryo and the proportion of chromosomally normal cells in developing day 5 and day 8 blastocysts, but not in day 4 morulas and embryos arrested before cavitation. Finally, we observed that FISH diagnosis on 1 or 2 blastomeres of day 4 embryos was not predictive of subsequent developmental potential.

The high incidence of chromosomal abnormalities in cleavage stage embryos has been brought to light by several studies employing PGS-FISH over the past years[1]. However, it is still unclear which mechanisms lead to such high aneuploidy rates. The inefficiency of the cell cycle checkpoints during the first cleavage divisions (before embryonic genome activation at around the 8-cell stage)[21,22] has been suggested as a possible cause for improper chromosome segregation[17]. According to this hypothesis, activation of the embryonic genome and initiation of compaction could lead to the establishment of functional cell cycle checkpoints, resulting in prevention of new errors and the developmental arrest of chromosomally abnormal cells and/ or the entire embryo. In the present study we investigated the frequency of chromosomal abnormalities after compaction, to ascertain whether the incidence of chromosomal abnormalities decreases after presumable activation of the embryonic genome. FISH analysis of morula-stage embryos revealed that the great majority of day 4 embryos (83%) are mosaic according to our definition, and no embryo consisted of normal cells only. Thus, the compaction stage does not provide a developmental barrier for chromosomally abnormal or mosaic embryos. These results are concordant with data from a previous study by our group, where FISH analysis of 15 chromosomes in cryopreserved compacted morulas (n=12) revealed all embryos to be mosaic[19]. Cryopreservation has been suggested to possibly induce chromosomal abnormalities after thawing and subsequent culture[23-25]. Therefore, both in the present and in the previous study by Baart *et al.* (2007), embryos were fixed immediately after thawing to avoid changes in chromosome constitution. Work done by Bielanska *et al*. (2002) using fresh embryos showed that more than half of morula-stage embryos are mosaic (58%), when screening for 9 chromosomes. We therefore believe our results to be a good representation of the chromosomal constitution of morula-stage embryos. When comparing the percentage of mosaic embryos at day 4 *vs* day 5 blastocysts, we observed a significant decrease by day 5 of development (Figure 1). This suggests that a proportion of mosaic day 4 embryos do not reach the blastocyst stage. We report a decrease in the incidence of mosaic embryos over time, with the lowest incidence observed at day 8. The decrease of mosaic cases over time may be partially caused by our definition of mosaicism, which requires at least 10% of the cells to carry the same chromosome abnormality. This criterion is currently the best available method to distinguish true aneuploidy from FISH artefacts, when control material is lacking. However, it may lead us to underestimate the proportion of mosaic embryos at day 8 and overestimate at day 4 and day 5. An example is embryo 46 (Table 3), where only 63% of the cells were found to be normal, but none of the abnormalities reached the 10% threshold. It is currently not known if this embryo could be diagnosed as normal, since the minimal proportion of normal cells needed for further normal development is yet unknown.

The presence of chromosomally abnormal cells does not exclude blastocyst development, as seen in this and previous studies[13-15,26]. However, our data also suggest that a significant proportion of mosaic embryos undergo developmental arrest before reaching the blastocyst stage. According to the model proposed by Evsikov and Verlinsky (1998), if the number of aneuploid cells at the morula stage reaches a certain threshold level, there is self-elimination (arrest) of the whole embryo. However, embryos with a number of aneuploid cells below the threshold level develop further and reach the blastocyst stage. So far this hypothesis of a threshold has not been directly investigated, but mouse knockout models have shown that up to 30% of aneuploid cells can be tolerated in apparently healthy animals[27]. Contrary to somatic cells, human and mouse embryonic stem cells (ESCs) containing chromosomal abnormalities do not initiate apoptosis. However, upon differentiation of ESCs, there is apoptosis of chromosomally abnormal cells[28]. A similar mechanism has been suggested to be involved in human preimplantation embryos, where differentiation is initiated with the formation of the blastocyst. Thus, selection against chromosomally abnormal cells may be initiated at the blastocyst stage, via the elimination and/or non-proliferation of such cells[29]. In this study we aimed at finding indirect evidence supporting the model where apoptosis of chromosomally abnormal cells is initiated upon differentiation at the blastocyst stage, but not before this stage of embryonic development. Therefore, we tested for a correlation between the total cell number and the percentage of normal cells within an embryo at day 4, day 5 (arrested and blastocyst), and day 8 (Figure 2). We found a significant positive correlation between the total number of cells and the percentage of chromosomally normal cells per embryo in day 5 blastocysts and day 8 peri-implanted embryos, but not in day 4 morulas or embryos arrested before cavitation. The difference in the results for the two groups analysed at day 5 (arrested embryos that failed to initiate cavitation and blastocysts) support the model. Thus, our data provide indirect evidence that cavitation may be critical for the onset of a negative selection against abnormal cells[10] and/or for the establishment of a growth advantage of the normal over the abnormal cells[3,14]. Surprisingly, analysis of day 8 embryos showed the persistence of high numbers of cells with different chromosomal abnormalities until this stage of development, although not falling within the range of our definition for mosaicism. The identification of numerous different segregation errors at day 8 indicates that new abnormalities can arise after cavitation. This may explain the reported poor predictive value of FISH diagnosis on 1 or 2 blastomeres of day 4 embryos. Out of the 50 embryos analysed, we identified seven embryos (14%) where at least one of the abnormalities was likely to have been caused by a meiotic error. All of these cases were combined with additional post-meiotic errors.

This study is the first to provide insight in the fate of chromosomally abnormal cells in human embryos during development from the morula stage up to the peri-implantation stage of day 8. It is however important to note that the embryos studied have been cultured *in vitro* for an extended time period. *In vitro* culture conditions may affect the chromosomal competence of embryos, as demonstrated in a mouse model[30]. It is also important to note that the possible effect of embryo-endometrial stromal cell co-culture is unknown. However, co-culture of human embryos and stromal endometrial cells

has been established into the routine practice of some IVF centres and associated with increased pregnancy and implantation rates[31]. No increased risk of congenital birth defects associated with the co-culture technique have been reported. Furthermore, embryos have been selected (first for cryopreservation and later for co-culture from day 5 to day 8) and therefore may not reflect the general blastocyst population. Additionally, we only analysed the copy number of 10 of the 23 pairs of chromosomes and we have no information on the incidence of structural abnormalities. These have recently been demonstrated to be highly prevalent in human IVF embryos (Vanneste et al., 2009). However, the embryos used in this study represent the best currently available model accessible for research.

Tetraploidy has been described as a normal phenomenon in embryonic trophoblast cells[32]. Furthermore, the occurrence of some tetraploid cells has been considered as a normal phenomenon of *in vitro* cultured embryos[10,33]. Therefore, we included tetraploid and near tetraploid cells into the group of chromosomally normal cells. However, our definition may lead to an underestimation of the incidence of chromosomal abnormalities, as tetraploid cells can arise after aberrant cell division[34]. We have at the moment no method of distinguishing between these possibilities. In conclusion, our data suggests that a proportion of mosaic embryos undergo developmental arrest between compaction and cavitation. If the embryo continues to develop, reduced proliferation or cell death of aneuploid cells may be responsible for the increased proportion of chromosomally normal cells throughout development of human embryos. Although the biological implications of chromosomal mosaicism has not been well explored yet, emerging evidence illustrate that we may currently underestimate the impact on embryonic development and disease in later life[35].

## Materials and Methods

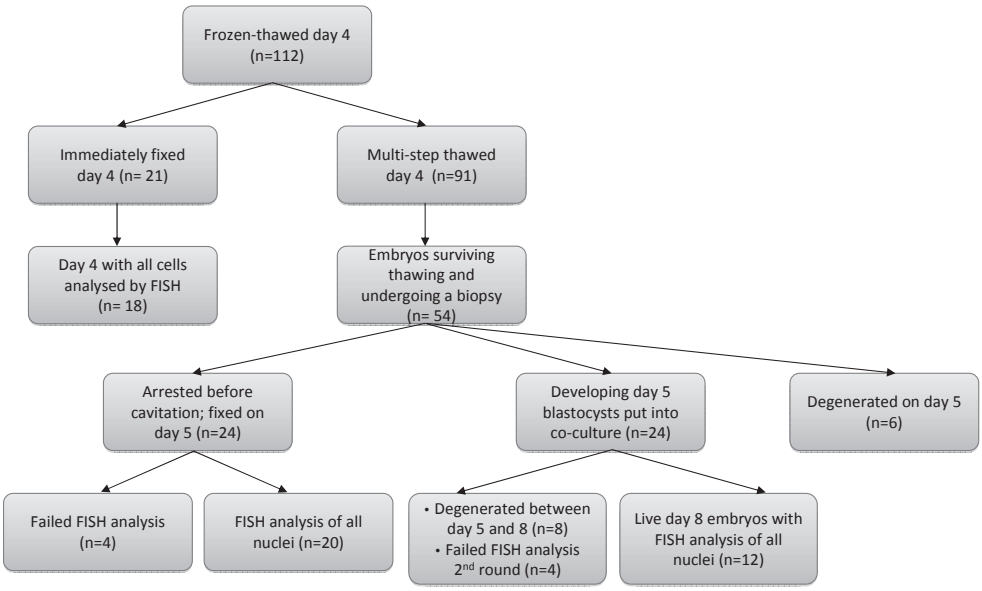
### Embryos

Surplus cryopreserved preimplantation embryos were donated with written consent by 25 couples undergoing routine IVF at the University Medical Centre Utrecht in the period between April 1997 and October 2003. Women participating in this study were aged between 29-41 years-old and all became pregnant in the fresh cycle. The use of these embryos for this study was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO – NL12481.000.06) and the local institutional ethics committee.

Ovarian stimulation, oocyte retrieval and IVF procedures, including assessment of embryo morphology, were performed as described previously[36]. A maximum of two day 4 embryos were transferred per cycle. Supernumerary good quality embryos showing some degree of compaction and <20% fragmentation were cryopreserved. Cryopreservation was performed in straws using a slow freezing standard protocol of 1.5 M dimethyl sulfoxide (DMSO) in HTF medium containing 10% GPO (human plasma solution, CLB, Amsterdam, The Netherlands). The embryos were cooled to -6°C for seeding and subsequently slowly cooled to -40°C (rate 0.3°C/min). Finally, they were cooled rapidly at -25°C/min to -140°C, before immersion in liquid nitrogen.

Figure 3 summarizes the experimental setup of this study. A total of 112 day 4 cryopreserved embryos were used for this study, after storage for an average of 8.6 years (range: 4.7-11.9). Twenty-one randomly selected day 4 embryos were thawed and all the cells fixed within 10 minutes after removal from liquid nitrogen. Whole embryos were fixed on slides as described for blastomere spreading (see section "Biopsy and fixation of blastomeres and embryos"). From the remaining 91 embryos, a total of 57 survived the thawing procedure, consisting of consecutive washes in decreasing DMSO concentrations. These embryos were then transferred to culture medium supplemented with 14.2% GPO and biopsied within 2h, making use of the spontaneous decompaction embryos demonstrated shortly after thawing and when washed in calcium/magnesium-free medium (G-PGD medium, Vitrolife, Sweden). Before the biopsy procedure, the embryos were scored for quality and number of blastomeres, according to previously described criteria[37,38]. Embryos at an advanced and irreversible stage of compaction (n=3) were not biopsied and therefore excluded from this study.

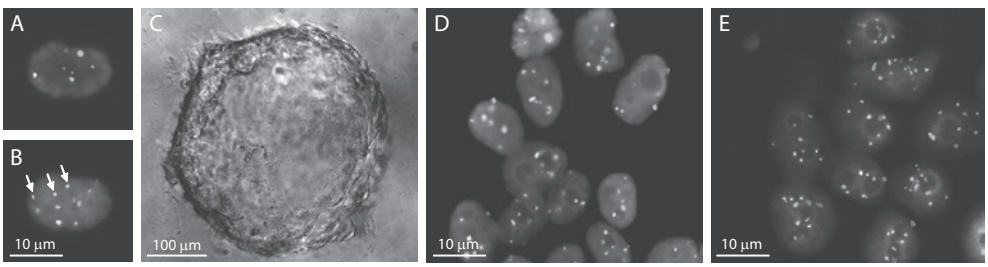
**Figure 3** Flow chart summarizing the experimental design of the study.



**Embryo-endometrial stromal cell co-culture**

Biopsied embryos (n=54) were cultured for a further 24h under standard embryo culture conditions[36]. Six of these had degenerated and were therefore not analysed. On day 5, embryos arrested before cavitation (n=24) were fixed, whereas developing blastocysts (n=24) were co-cultured on a monolayer of decidualised endometrial stromal cells for a further 72h, as described previously[39]. In short, endometrial tissues at different stages of the menstrual cycle were obtained from fertile patients undergoing hysterectomy for benign conditions. Samples of surplus endometrial tissue were collected from the Pathology department of the UMCU, with consent from the patients. Endometrial stromal cells were isolated as described previously[39] and decidualisation was induced by culturing confluent endometrial stromal cells in the presence of 0.5 mM 8-Br-cAMP and 1 µM MPA (both Sigma, UK) for 3-5 days. Day 5 embryos showing signs of cavitation were subjected to 0.1% Pronase in HEPES buffered HTF to remove the zona pellucida and co-cultured with confluent monolayers of decidualised endometrial stromal cells for 72h in the presence of DMEM/F-12 complete medium (Invitrogen, USA). At the end of the co-culture period, embryos were photographed by phase-contrast microscopy, using a Zeiss Axio Observer inverted microscope and the AxioVision imaging system (Zeiss, Germany) (Figure 4-C). Finally, day 8 embryos were removed from the co-culture and fixed on a slide as described below, the number of blastomeres counted, and all nuclei analysed by FISH (Figure 4-D,E). The proportion of chromosomally normal cells per embryo was calculated. Statistical analysis using Pearson correlation coefficient allowed testing for statistical significance, with $p<0.05$ considered significant.

**Figure 4 (A)** Nucleus of a blastomere after the first round of FISH showing chromosomes 1 (aqua), 7 (blue), 15 (green), X (red) and Y (yellow). DNA is stained with DAPI. **(B)** Same nucleus after the second round of FISH showing chromosomes 13 (red), 16 (aqua), 18 (blue), 21 (green) and 22 (yellow). An extra signal for chromosome 16 is shown (arrows). **(C)** Day 8 embryo attached to a monolayer of stromal decidualised cells. **(D)** Spread of nuclei of a day 8 embryo after the first round of FISH (DNA stained with DAPI) and **(E)** after the second round of FISH.



**Biopsy and fixation of blastomeres and embryos**

Prior to biopsy, embryos were washed twice in calcium/magnesium-free medium (G-PGD medium) and then incubated in the same medium for 5 min at 37°C, allowing decompaction. The biopsy was performed on the heated stage of a Nikon IX-70 microscope, equipped with micromanipulation tools. An infrared diode laser system (OCTAX Laser Shot, OCTAX Microscience GmbH, Germany) with appropriate software (OCTAX EyeWare) was used for dissection of the zona pellucida prior to biopsy. The retrieved blastomeres were dissolved in lysis buffer (0.01 N HCl, 0.1% Tween 20) and the nuclei fixed on poly-L-lysine coated slides using methanol:acetic acid (3:1), as described before[23,40].

Non-biopsied day 4 and biopsied embryos that either were arrested at day 5 or developed until day 8 were also dissolved using lysis buffer (0.01 N HCl, 0.1% Tween 20) to remove the zona pellucida and the cytoplasm. Nuclei were washed by gentle agitation of the lysis solution until clear and good spreading of nuclei was evident to minimize overlapping. Finally, a drop of methanol:acetic acid (3:1) was added for fixation. Fixed nuclei from biopsied blastomeres and whole embryos were viewed using a phase contrast microscope and their location marked with a diamond pen. Preparations were air-dried and stored at -20°C for up to 6 months prior to FISH analysis.

**FISH**

Two rounds of five colour FISH were applied to single blastomeres or embryos. In the first round, FISH was performed for chromosomes 1, 7, 15, X and Y and in the second round for chromosomes 13, 16, 18, 21 and 22. The DNA probes used in the first round were centromere probes for chromosomes 1 (pUC 1.77[41], 7 (pa7t1[42]), 15 (pTRA-20[43]), X (pBamX5[44]) and a Y chromosome heterochromatin probe (RPN1305[45]). These were labelled as described previously[19], but using a BioPrime DNA labelling kit (Invitrogen), according to the instructions of the manufacturers. The second round of FISH was performed using a commercial ready-to-use probe mix containing centromere probes (Multivysion PB kit; Vysis, USA). The efficiency of the FISH probes was tested on cultured and uncultured peripheral lymphocyte spreads from two men and two women with normal karyotypes. Slides were prepared according to standard protocols and hybridised using the same protocol as for the embryonic cells. Chromosome localization of the probes was verified on metaphase spreads and FISH signals were counted in 100 interphase nuclei. In addition, the positions of 10 individual nuclei were recorded and images were obtained after each round to check for persisting signals from the first round.

Hybridisation was performed as described previously[19]. Per slide, 0.2 µl (single blastomeres) or 0.4 µl (whole embryos) of hybridisation mixture was applied. Slides were examined with a Zeiss Axio Observer epifluorescence-equipped inverted microscope, using appropriate filters. An "embryo map" was drawn for whole embryos, marking the location and attributing a number to the individual nuclei. Images of representative nuclei were captured with AxioVision imaging system. After the second round, images of the same nuclei were recorded and compared with those from the first round to ensure these had not persisted. Overlapping nuclei were excluded from the FISH analysis.

**FISH signal analysis and interpretation**

For both rounds, we used the scoring criteria previously published[46]. Based on the analysis of two blastomeres per embryo, we classified day 4 embryos as normal (both nuclei showing the normal amount of signals for the chromosomes investigated), mosaic (one normal nucleus and one abnormal or each nucleus showing a different abnormality) (Figure 4-A,B) or abnormal (both nuclei carrying the same abnormality). Day 4 embryos in which only one blastomere was analysed were classified as chromosomally normal or abnormal. After analysis of all the cells from each embryo on days 4, 5 or 8, we used the following definitions on the basis of the results obtained. To distinguish between true aneuploidy and FISH artefact, an abnormal cell line was defined as at least 10% of the nuclei showing the same chromosome abnormality. This threshold is frequently used in cytogenetics, if control material is lacking. Applying this criterion resulted in embryos being classified as normal if at least 60% of nuclei showed a normal chromosome constitution, and more importantly, if less than 10% of the nuclei showed the same chromosome abnormality. Embryos were classified as aneuploid if >90% of nuclei showed the same abnormality and if <10% of the nuclei showed a normal or different abnormal chromosome constitution. Embryos were classified as mosaic, if composed of cells with either a normal or an abnormal chromosomal constitution, with between 10 and 90% of the cells showing the same chromosomal abnormality. Chaotic embryos were classified as such when almost all the cells showed different and complex chromosomal abnormalities. Embryos with >90% haploid, tetraploid or triploid nuclei were classed as such. However, we considered the occurrence of some tetraploid cells as a normal phenomenon of *in vitro* cultured embryos[4,10] and counted them as normal cells. After reanalysis on day 5 and 8, confirmation rates were calculated to investigate the reliability of the day 4 diagnosis. The chromosomal constitution diagnosed at day 4 was considered to be cytogenetically confirmed when the chromosome constitution of the investigated blastomeres was reflected in at least 10% of the cells within the embryo analysed on day 5 or 8[6].

**Reanalysis of data collected in a previous study**

In the present study, the FISH analysis performed at day 5 post-fertilisation was done on selected embryos showing signs of developmental arrest before reaching the blastocyst stage. Therefore, this group of embryos is unlikely to be representative of the chromosomal constitution of good-quality day 5 embryos (blastocysts). In a study previously published by our group[19], cryopreserved good quality day 5 blastocysts (n=36) were analysed by FISH for 15 chromosomes. In order to gain insight into the changing chromosomal constitution of embryos between days 4, 5 and 8, we included in the analysis data from Baart *et al.* (2007), excluding the information regarding 5 extra chromosomes not analysed in the present study. The proportion of normal cells was then calculated. Statistical analysis using Pearson correlation coefficient allowed testing for statistical significance, with $p<0.05$ considered significant.

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# Chapter 4

## NATURAL SELECTION OF HUMAN EMBRYOS: DECIDUALIZING ENDOMETRIAL STROMAL CELLS SERVE AS SENSORS OF EMBRYO QUALITY UPON IMPLANTATION


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## Abstract

Background: Pregnancy is widely viewed as dependent upon an intimate dialogue, mediated by locally secreted factors between a developmentally competent embryo and a receptive endometrium. Reproductive success in humans is however limited, largely because of the high prevalence of chromosomally abnormal preimplantation embryos. Moreover, the transient period of endometrial receptivity in humans uniquely coincides with differentiation of endometrial stromal cells (ESCs) into highly specialized decidual cells, which in the absence of pregnancy invariably triggers menstruation. The role of cyclic decidualization of the endometrium in the implantation process, and the nature of the decidual cytokines and growth factors that mediate the crosstalk with the embryo, are unknown.

Methodology/Principal Findings: We employed a human co-culture model, consisting of decidualizing ESCs and single hatched blastocysts, to identify the soluble factors involved in implantation. Over the 3-day co-culture period, approximately 75% of embryos arrested whereas the remainder showed normal development. The levels of 14 implantation factors secreted by the stromal cells were determined by multiplex immunoassay. Surprisingly, the presence of a developing embryo had no significant effect on decidual secretions, apart from a modest reduction in IL-5 levels. In contrast, arresting embryos triggered a strong response, characterized by selective inhibition of IL-1b, -6, -10, -17, -18, eotaxin, and HB-EGF secretion. Co-cultures were repeated with undifferentiated ESCs but none of the secreted cytokines were affected by the presence of a developing or arresting embryo.

Conclusions: Human ESCs become biosensors of embryo quality upon differentiation into decidual cells. In view of the high incidence of gross chromosomal errors in human preimplantation embryos, cyclic decidualization followed by menstrual shedding may represent a mechanism of natural embryo selection that limits maternal investment in developmentally impaired pregnancies.

## Introduction

Pregnancy depends on intimate interactions between a developmentally competent embryo and a receptive endometrium. The endometrial luminal epithelium, the primary barrier for implantation, is not normally receptive and must transiently acquire this phenotype to allow first apposition and then attachment of the blastocyst[1]. In humans, the putative 'implantation window' opens 6 days after the postovulatory progesterone surge and is thought to last no longer than 2-4 days[2]. Once the luminal epithelium is breached, most invading mammalian embryos elicit a decidual response, characterised by transformation of stromal fibroblasts into secretory, epitheloid-like decidual cells, influx of specialized uterine immune cells and vascular remodelling[3,4]. In the human situation, however, this decidual response is primarily under maternal control and initiated in the mid-secretory phase of each cycle, irrespective of whether pregnancy has occurred or not.

Spontaneous decidualization of the stromal compartment in the absence of pregnancy invariably causes menstruation, a rare biological phenomenon confined to a handful of species[5]. The reason is that once the endometrium undergoes a decidual response, the integrity of the tissue becomes inextricably dependent upon continuous progesterone signalling. In the absence of pregnancy, declining progesterone levels triggers a switch in the secretory repertoire of decidual stromal cells, now characterized by expression of pro-inflammatory cytokines, chemokines and matrix metalloproteinases, which activates a sequence of events leading to tissue breakdown and menstrual shedding[6,7].

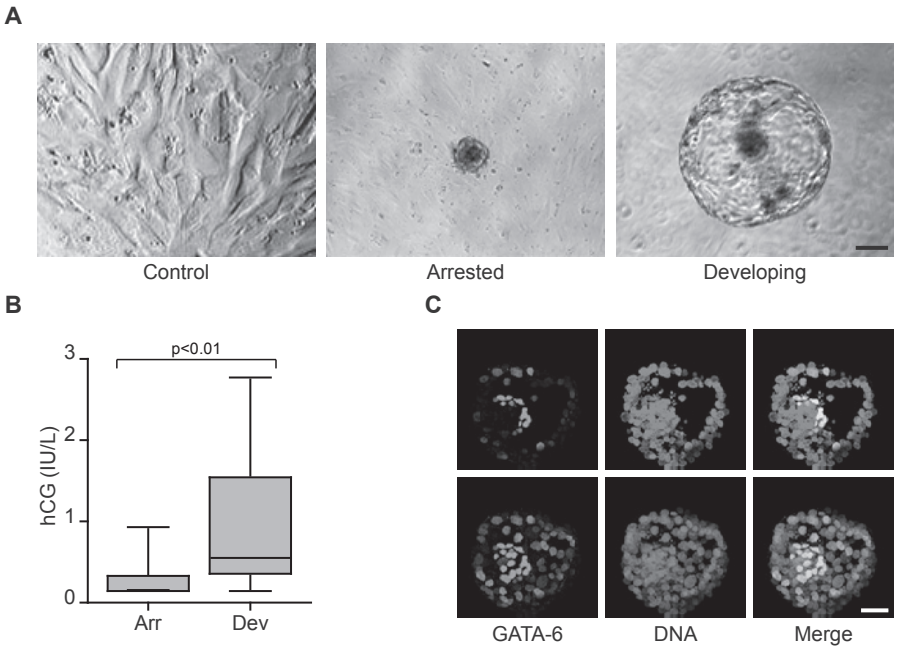
Another feature of human reproduction is that it is remarkable inefficient[8]. Monthly fecundity rates in fertile couples average around 20%, which is disappointingly low compared to many species[5]. While this lack of intrinsic reproductive efficacy may reflect a multitude of complex social and biological factors, for example the loss of estrous behaviour and concealed ovulation, it is foremost attributable to the high prevalence of chromosomal abnormalities in human embryos, which limits their developmental potential and accounts for the age-dependent decline in fertility[9,10]. The emergence of array-based technologies that allow genome-wide screening of individual cells has revealed that the frequency and complexity of chromosomal aberrations are much higher in early human embryos than hitherto appreciated[11]. Using this approach, a recent study demonstrated that less than 10% of cleavage-stage IVF embryos, obtained from fertile women under the age of 35, have a normal karyotype in all blastomeres, approximately half have no normal cells at all, and the remainder are mosaic for large-scale structural chromosomal imbalances, caused predominantly by mitotic non-disjunction[12]. While these observations suggest that not all chromosomal rearrangements in human preimplantation embryos compromise subsequent viability, they also imply that selection mechanisms must exist that limit maternal investment in a defective conceptus.

Human implantation sites are for ethical reasons inaccessible *in vivo*. Our understanding of early pregnancy events is therefore largely based on animal experiments, especially knockout studies in mice, and on gene expression studies in the human endometrium, aimed at identifying those factors that underpin the transient receptive phenotype[1,13]. These studies indicate that implantation

requires activation of evolutionarily conserved endometrial transcription factors (e.g. HOXA10, STAT3, p53)[14–17], expression of key cell adhesion molecules and their ligands (e.g. αvβ3 integrin, trophinin, L-selectin ligand)[18–20], and several growth factors and cytokines[1], all of which are essential for coordinated cross-talk with the implanting embryo, at least in mice.

In this study, we set out to characterize key soluble factors involved in crosstalk between endometrial stromal cells (ESCs) and an interacting embryo, using a validated human co-culture model[21]. Based on the current implantation paradigm, we anticipated that only developing embryos would express the appropriate molecular repertoire to engage with endometrial cells. Instead, we found that ESCs selectively recognize and respond to the presence of a developmentally impaired embryo but only upon differentiation into decidual cells.

**Fig. 1.** Human embryo development in co-culture. (A) Phase contrast images of decidualizing ESCs alone (control; left) or in the presence of an arrested (middle) or developing human embryo (right) (scale bar = 100 µm). (B) Arrested embryos (Arr) express significantly less hCG than developing embryos (Dev). Secreted hCG levels were assayed after 72 hours of co-culturing human embryos and decidualizing ESCs ($P < 0.01$). (C) Formation of primitive endoderm in developing human embryos co-cultured with decidual ESCs. Optical cross-sections through a day 8 embryo, cultured first on a decidualizing ESC monolayer for 72 hours, demonstrates the presence of GATA-6 positive cells aligning predominantly to the blastocoelic surface of the inner cell mass, which corresponds to the location of the primitive endoderm (top panel). The lower panel represents z-projection of all the images in the stack from a top to bottom scan through the embryo (scale bar = 50 µm).

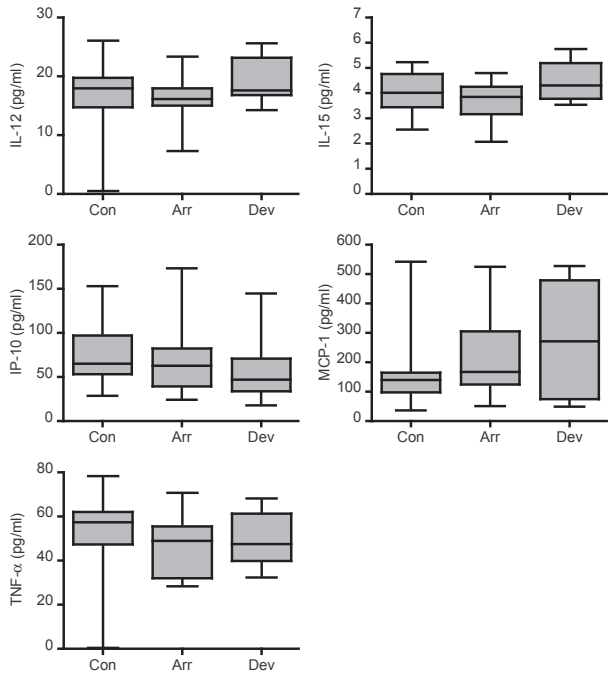



## Results

Implantation is thought to be dependent on an intimate dialogue between the blastocyst and uterine tissues, mediated by locally secreted factors[1], yet the nature of this crosstalk in humans is unknown. To examine embryo-endometrial interactions, we first established 41 co-cultures in 16 mm wells, each with a single hatched blastocyst seeded onto a confluent monolayer of ESCs decidualized for 5 days with 8-Br-cAMP and the progestin medroxyprogesterone acetate (MPA), a widely used protocol that elicits a differentiation response similar to that observed *in vivo*[22,23]. Control cultures received no blastocysts. Based on morphological criteria, 11 embryos developed normally beyond the expanding blastocyst stage over the 3-day co-culture period while 30 embryos arrested (Fig. 1A). Analysis of the culture supernatants further showed that developing embryos produced significantly higher human chorionic gonadotropin (hCG) levels (Fig. 1B). Moreover, confocal microscopy demonstrated that GATA-6, a transcription factor that marks the formation of the primitive endoderm[24], was selectively expressed at the blastocoelic side of the inner cell mass of co-cultured competent embryos, indicating that the *in vitro* conditions did not interfere with the normal embryonic developmental trajectory (Fig. 1C).



**Fig. 2.** Secreted decidual cytokines not regulated upon embryo co-culture. Primary ESCs were first decidualized for 5 days and then co-cultured with human embryos or not (control cultures, Con). Over the 72-hour co-culture period, 30 embryos arrested (Arr) whereas 11 continued to develop normally (Dev). Analysis of the culture supernatants revealed that the presence of an arresting or developing human embryo had no significant impact on the secretion of the indicated factors ($P > 0.05$).



Next, we used a multiplex immunoassay to determine the levels of 14 secreted cytokines, chemokines and growth factors, all of which have putatively been implicated in the process of implantation[25]. Decidualizing ESCs produced all mediators with the exception of IFN-g. The presence of an embryo had no discernable effect on IL-12, -15, TNF-a, MCP-1 or IP-10 production (Fig. 2). In contrast, decidual cells in co-culture secreted significantly lower levels of IL-1b, -6, -10, -17, -18, eotaxin, and HB-EGF but only in the presence of an arresting embryo (Fig. 3). IL-5 secretion, however, was inhibited irrespective of embryo quality, although more so in response to a developmentally impaired embryo. The co-cultures were repeated with undifferentiated ESCs, which lack the pronounced secretory phenotype of decidual cells. Nevertheless, 8 cytokines, including IL-1b, -10, and HB-EGF, accumulated in the culture supernatants over 3 days but none were affected by the presence of a developing or arresting embryo (Fig. 4). These data suggest that abnormal human embryos engage in intense signalling upon implantation, which in turn triggers a profound regional endometrial response but only following adequate decidualization of the stromal compartment.

**Fig. 3.** Developmentally impaired human embryos inhibit the secretion of selective implantation modulators by decidualizing ESCs. Primary ESCs were first decidualized for 5 days and then co-cultured with human embryos or not (control cultures, Con). Over the 72-hour co-culture period, 30 embryos arrested (Arr) whereas 11 continued to develop normally (Dev). Analysis of the culture supernatants revealed that the presence of an arresting embryo inhibited the secretion of the indicated factors. The letters above the box plots indicate significant differences between groups. $P < 0.01$ for all comparisons except for IL-6 and IL-17 ($P < 0.05$).

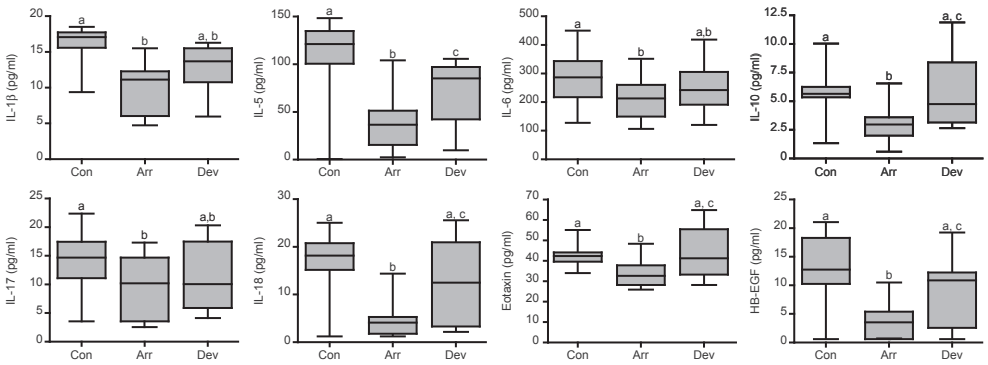
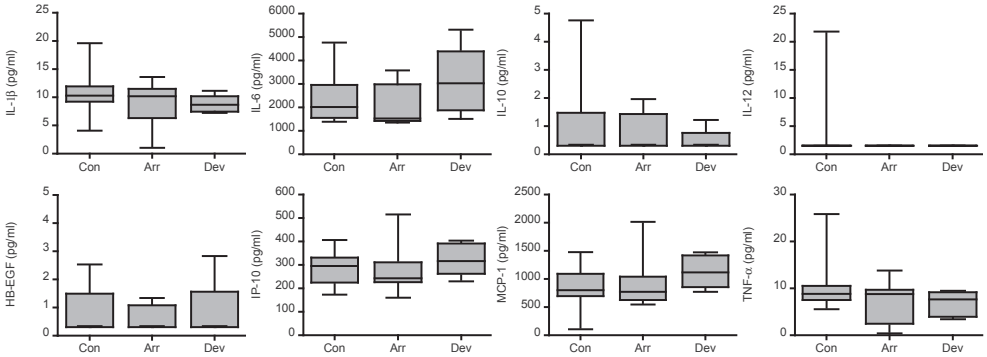


**Fig. 4.** The human embryo does not elicit a secretory response in undifferentiated endometrium. Undifferentiated primary ESCs were co-cultured with embryos or not (control cultures, Con). Over the 72-hour co-culture period, 15 embryos arrested (Arr) whereas 6 continued to develop normally (Dev). Co-culture with either an arrested or developing embryo had no impact on the secreted levels of the indicated factors ($P > 0.05$). The concentrations of IL-5, -12, -15, -17, -18, and eotaxin in culture supernatants of undifferentiated ESCs were below the level of detection.




## Discussion

Unravelling the mechanisms that control implantation is paramount to improving reproductive outcome, especially in couples suffering from infertility or recurrent pregnancy loss. Gene deletion studies leading to reproductive failure in mice have been hugely important in defining factors essential for embryo-endometrial interactions[26–28]. While it seems reasonable to assume that many of these implantation factors will be evolutionary conserved, especially in mammals where pregnancy depends on invasion of maternal tissues, it should also be acknowledged that there are important interspecies differences. For example, a large number of mammals, including mice, are capable of delaying implantation by temporarily suspending embryo development[29]. This process, termed 'diapause', is reversed when the endometrium signals an optimal metabolic and hormonal intrauterine milieu. There is, however, no evidence that human embryos are capable of delaying implantation while awaiting the right maternal signals. Conversely, and as mentioned before, it is the embryo that triggers a decidual response in mice and most other species, yet initiation of this process is uncoupled from embryonic signals in humans[3]. Finally, gross embryonic chromosomal abnormalities are rare in most mice strains, which means that little if anything is known about the fate of developmentally impaired embryos. Our co-culture experiments suggest that spontaneous decidualization of human endometrium, which in the absence of a viable pregnancy inevitably leads to menstruation, serves as a mechanism for embryo quality control. This supposition is based on the observation that decidualized but not undifferentiated ESCs selectively recognize the presence of a developmentally impaired embryo and respond by inhibiting the secretion of key implantation

mediators (e.g. IL-1b and HB-EGF) and immunomodulators (e.g. IL-5, -6, -10, -11, -17, and eotaxin). Considering that single human embryos were co-cultured with at least 50,000 ESCs, the magnitude of the maternal response is formidable. Apart from a modest reduction in IL-5 secretion, ESCs, decidualized or not, appear largely indifferent to the presence of a developing embryo. It is possible of course that the molecular dialogue with a competent conceptus is either more selective or confined to decidual cells in the immediate vicinity and, hence, insufficient to significantly impact on the total secreted levels of these factors.

The importance of the endometrial stromal compartment in regulating early human implantation events is further illustrated by recent studies, demonstrating that differentiating ESCs acquire a motile and invasive phenotype in response to embryonic trophoblast signals[21,30]. In other words, rather than being passively invaded, these observations suggest that decidualizing ESCs actively encapsulate the early human conceptus. If so, the phenomenal response of decidual cells to a developmentally impaired embryo could represent a mechanism for controlled embryo disposal, mediated by induction of menstruation-like tissue breakdown and shedding. Intriguingly, women with early pregnancy losses bled less than their typical menses following pregnancies of very short duration and more than usual for the pregnancies lasting approximately six weeks. This indicates that menses might reflect endometrial factors associated with early pregnancy loss[31,32].

Our observations raise a number of important but as yet unanswered questions. First, the nature of the embryonic signals capable of modulating the decidual secretome remains elusive, although it is tempting to speculate that they are generated in response to the much higher metabolite or nutrient turnover rates that characterize arresting embryos[33]. Secondly, while we show that ESCs become biological sensors of embryo quality upon decidualization, the underpinning mechanism and that of the subsequent secretory response are unclear. Decidualized but not undifferentiated ESCs form dense intercellular connections, such as adherens and tight junctions[34], which may be essential to propagate embryo-derived factors, such as metabolites, throughout the culture in a receptor-independent manner. Thirdly, whether or not the developmentally impaired embryos in our co-culture system retain an invasive phenotype remains to be determined. Conversely, it is also important to ascertain if the nature and magnitude of the decidual response correlates somehow with the frequency and complexity of gross chromosomal errors in the co-cultured blastocyst. A finely tuned, tailored decidual response is not beyond the realm of possibilities as a recent study in the bovine demonstrated that endometrial gene expression varies dramatically in response to implantation of an embryo conceived *in vivo*, after IVF or following somatic cell nuclear transfer[35].

Finally, and most importantly, clinical studies are required to determine the extent to which impaired decidualization and embryo recognition underpins miscarriage and other pregnancy complications. The accompanying paper by Salker *et al*. in this journal describes our attempt at addressing this important question. In summary, rather than being biologically silent or inert, our data suggest that developmentally impaired human embryos can trigger a formidable maternal response, which requires decidual transformation of the endometrium. From an evolutionary perspective, our

findings are in agreement with the genetic conflict theory of pregnancy[36,37], which predicts that changes in the embryonic genome are opposed by maternal countermeasures. Within this context, we propose that the emergence of cyclic decidualization of the human endometrium, which is inextricably linked to menstruation, is an adaptive response to the high incidence of chromosomal instability in human embryos. If correct, our findings predict that failure of the endometrium to express an adequate decidual phenotype disables natural embryo selection upon implantation and causes subsequent pregnancy failure.

## Materials & Methods

### Ethics statement and patient selection

This study was approved by the Medical Review Ethics Committee University Medical Center Utrecht and the Central Committee on Research inv. Human Subjects in The Netherlands (NL 12481.000.06). Written informed consent was obtained from all participating subjects, either for the use of supernumerary cryopreserved embryos or endometrial samples. All patients were investigated according to the standard clinic protocols, but the outcome of these routine investigations was not taken in account in either the recruitment into this study or in the analysis of the data.



### Embryo collection and co-culture

Ovarian stimulation, ovulation induction, oocyte retrieval and IVF/ICSI were performed according to standard clinical protocols. Supernumerary, good quality embryos were subsequently cryopreserved as previously described[38]. For this study, 140 day 4 embryos from 39 patients were thawed, taken through consecutive washes of 1.25, 1.00, 0.75 and 0.375 mol/l DMSO for 5 minutes each, then transferred to Human Tubal Fluid (HTF) culture medium supplemented with 10% GPO (human plasma solution; CLB, The Netherlands), overlaid with 1 ml of light paraffin oil (Irvine Scientific, Santa Ana, USA), and cultured until day 5. Sixty-two embryos from 27 patients survived the thawing procedure and extended culture period. These embryos were subjected to 0.1% Pronase / 10% GPO treatment to remove the zona pellucida. For the co-culture experiments, primary ESCs were purified, as previously described[22], from a single proliferative phase biopsy sample obtained from a patient with no uterine pathology or a history of RPL. The culture was expanded and the cells frozen at -80°C in aliquots, which were subsequently thawed consecutively to ensure identical conditions in all co-culture experiments. Thawed ESCs were seeded into 16 mm wells ($0.5 \times 10^5$ cells per well) in DMEM/F12 complete medium and grown until confluence. Decidualization was induced by the addition of 0.5 mM of 8-bromoadenosine 3′, 5′-cyclic monophosphate (8-Br-cAMP; Sigma, UK) and 1 µM medroxyprogesterone acetate (MPA; Sigma, UK). This medium was changed every 48 hours. Individual blastocysts were then seeded onto a confluent layer of either undifferentiated ESCs or cells first decidualized for 5 days. Co-cultures were maintained in 250 µl of DMEM/F-12 complete medium for 72 hours. At the end of the co-culture period, the embryos were assessed for the quality of development. Culture medium was collected and stored at -80°C. ESC viability, assessed by trypan blue exclusion, was >90% for all cultures.

**Multiplex immunoassay and hCG measurements**

The supernatant samples were analysed using a multiplex immunoassay capable of detecting interleukin (IL)-1β, IL-5, IL-6, IL-10, IL-12, IL-15, IL-17, IL-18, tumour necrosis factor a (TNF-α), interferon g (IFN-γ), monocyte chemotactic protein 1 (MCP-1), chemokine (C-X-C motif) ligand 10 (IP-10), eotaxin [also known as chemokine (C-C motif) ligand 11 or CCL11] and heparin-binding EGF-like growth factor (HB-EGF)[25]. Antibodies were covalently coupled to the microspheres and the assay was carried out as described previously[25,39]. Co-culture supernatants were analysed for hCG (human chorionic gonadotropin) production by the embryo using the Immulite 1000® immunoanalyser (Siemens). The functional sensitivity of the assay was 0.06 mU/mL with 20% coefficient of variation[25].

**GATA6 immunostaining and confocal imaging**

Co-cultured day 8 human blastocysts were washed in PBS and fixed for 10 minutes at room temperature in 4% formaldehyde. After fixation, embryos were washed in 0.1% triton x-100 in PBS (PBST) and subsequently permeabilized in 0.5% triton x-100 in PBS for 15-30 minutes at room temperature (RT). Blocking was performed by incubating the embryos in 10% fetal calf serum in PBST (blocking solution) for 1 hour at RT followed by overnight incubation at 4°C in primary antibody (rabbit anti-GATA6; SC9055, Santa Cruz Biotechnology, Santa Cruz, US), diluted in blocking solution. The next day, the embryos were washed in PBST and transferred to blocking solution containing Alexa fluor conjugated secondary antibodies (Molecular probes, Invitrogen, Venlo, the Netherlands). After 1 hour embryos were washed, counterstained with TOPRO-3 (Molecular Probes, Invitrogen) and mounted in Vectashield mounting medium (Brunschwig Chemie, Amsterdam, the Netherlands). Fluorescent signals were visualized using a confocal laser scanning microscope.

**Statistical analyses**

For multiple comparisons, ANOVA test with Bonferroni correction was used. $P < 0.05$ was considered significant.

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# Chapter 5

## MATERNAL RECOGNITION AND REJECTION OF COMPROMISED HUMAN EMBRYOS

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The high incidence of developmentally compromised human preimplantation embryos represents a major reproductive obstacle and accounts for the limited efficacy of assistant reproductive technologies, such as *in vitro* fertilization (IVF) treatment. Whether or not human embryos are capable of signaling their competence to the mother upon implantation is not known. We performed genome-wide microarray analysis on decidualizing human endometrial stromal cells (HESCs) incubated for 12 hours with pooled culture supernatants from poor quality IVF embryos or from embryos that resulted in pregnancies upon transfer. Unexpectedly, the transcriptional response of HESCs to secreted signals from competent embryos was extremely limited, confined to merely 47 genes. In contrast, 1909 maternal genes were dysregulated upon incubation of HESCs with culture supernatant of compromised embryos, with a preponderance implicating in cellular transport, transcription or cell adhesion. *HSPA8*, encoding the multifaceted heat-shock protein HSC70, was amongst the most sensitive genes selectively repressed in response to signals from compromised embryos. HSC70 knockdown in differentiating HESCs triggered an endoplasmic reticulum stress response and impaired the secretion of prototypical decidual markers. Thus, decidualizing endometrial cells are exquisitely adapted to sense poor quality embryos, thus enabling active maternal rejection of unwanted pregnancies.

Pregnancy in humans is marred by early failure. Approximately 15% of clinically recognized pregnancies miscarry but, when combined with pre-clinical losses, the true incidence is closer to 50%[1]. Consequently, 25-50% of women experience one or more pregnancy failures during the reproductive years, rendering miscarriage by far the most common complication of pregnancy. This extraordinary burden of early pregnancy wastage has been attributed to the high prevalence of chromosomal errors in human preimplantation embryos and the requirement of the conceptus to establish a deeply invading hemochorial placenta for survival[2-4]. Based on genome-wide screening of individual blastomeres, 90% of good-quality cleavage-stage IVF embryos reportedly harbor cells with complex large-scale structural chromosomal imbalances, some caused by meiotic aneuploidies but mostly by mitotic non-disjunction[4]. Thus, while not all chromosomal errors in preimplantation embryos will impede further development, many affected embryos will either fail to implant or lead to early pregnancy failure. In agreement, 50-60% of all miscarriages exhibit gross chromosomal anomalies on conventional karyotyping[1,5].

The luminal endometrial epithelium is the primary barrier in the implantation process[6]. As is the case for most mammalian species, the human endometrium transiently expresses a receptive phenotype in response to postovulatory surge in progesterone levels, which first allows apposition and attachment of the blastocyst to the apical membrane of the luminal epithelium, followed by invasion and then encapsulation of the conceptus by decidualizing (differentiating) stromal cells[7-9]. Arguably, this limited 'window of implantation', confined to 2 to 4 days during the mid-secretory phase of the cycle, ensures coordinated embryonic and endometrial development, thus minimizing the risk of late implantation of compromised embryos. While there is evidence in support for this concept[10-12], many developmentally compromised human embryos are capable of breaching the

luminal epithelial barrier and embed within the decidualizing stroma before being rejected as a clinical or pre-clinical miscarriage.

The mechanisms underlying maternal rejection of a compromised embryo are not well understood. It is widely assumed that miscarriage is the inevitable endpoint of the aberrant developmental trajectory of such embryos, which precludes the establishment of a functional decidua-placental interface. An equally plausible scenario involves the implanting embryo signalling its competence to the surrounding decidual cells, which in turn elicit a response that enables active maternal rejection of unwanted pregnancies.

It has proven difficult to discriminate between these models of pregnancy failure as early human implantation events cannot be studied *in vivo* and developmentally compromised embryos are rare in many species, including the mouse. Human co-culture systems, consisting of hatched blastocysts cultured on decidualizing endometrial stromal cells, potentially address many of these intrinsic problems[13,14]. A major drawback, however, is that the developmental potential of human embryos can only be assessed indirectly, commonly on morphological criteria, and over a legally restricted co-culture period. To overcome this hurdle we collected the medium of day-4 human embryos cultured for 3 days in single microdroplets (30 µl) overlaid with mineral oil as well as the unconditioned medium from microdroplets that did not contain a human embryo. Next we incubated primary HESCs, first decidualized with 8-bromo-cAMP and medroxyprogesterone acetate for 5 days[15,16], with pooled culture supernatants from poorly developing embryos (n=30; Table 1) and from embryos that resulted in ongoing pregnancies after single embryo transfer (n=30).

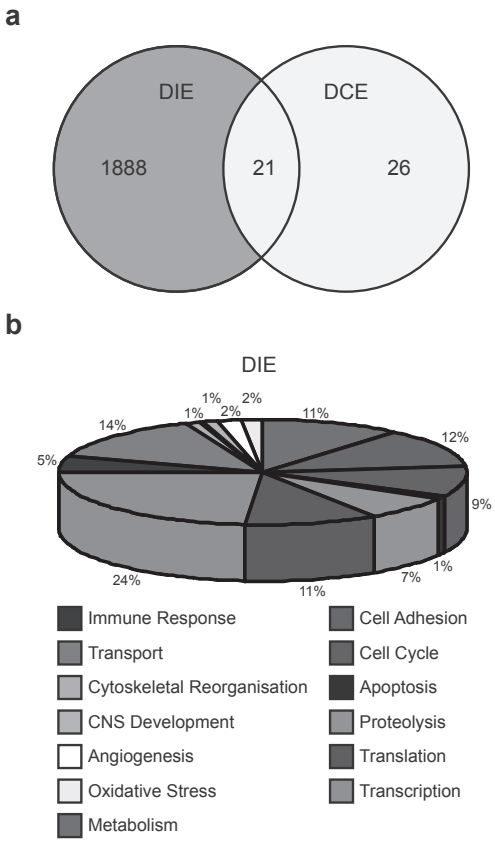


**Table 1.** Patient demographics and embryo characteristics.

| | DIE (n=30) | DCE (n=30) |
|---|---|---|
| Age (years): | 33.5 ± 4.2 | 35.2 ± 4.3 |
| Duration of infertility (years): | 2.6 ± 1.9 | 3.3 ± 2.4 |
| Primary infertility (%): | 64.3% | 50% |
| No. of oocytes collected: | 10.3 ± 6.0 | 9.5 ± 4.2 |
| No. of developing embryos: | 4.6 ± 2.7 | 4.4 ± 2.4 |
| Top quality embryos (%): | 0% | 53.6%* |

DIE, developmentally incompetent embryos; DCE, developmentally competent embryos. The data are expressed as mean ± standard deviation. * $P < 0.001$. Embryos were scored on standard morphological criteria[30], top quality embryos were defined as morula stage embryos with less than 10% of fragmentation.

**Figure 1.** Endometrial decidual cells are biosensors of embryo quality. (**a**) Venn diagram visualizing the number of maternal genes responsive to signals from developmentally competent embryos (DCE) and developmentally incompetent embryos (DIE). (**b**) Gene Ontology (GO) classification of decidual genes regulated in response to soluble factors secreted by DIE.




Control cultures consisted of decidualizing HESCs incubated with unconditioned embryo culture medium. Total RNA was harvested after 12 hours of incubation from biologically triplicate experiments and subjected to genome-wide expression profiling. Genes with a $P < 0.01$ after false discovery rate (FDR) multiple testing comparison were considered significant. Surprisingly, only 47 decidual genes were found to be responsive to signals emanating from developmentally competent embryos. In contrast, 40-times more maternal genes were perturbed in response to conditioned medium from developmentally incompetent embryos (Fig. 1 and Tables 2 & 3). Gene ontology annotation categorized half of these maternal genes into three broad biological processes: transcription (24%), transport (14%), and cell adhesion (12%). Real-time PCR analysis was used to validate the differential expression of four decidual genes (*HSPA8*, *THBS1*, *PDGFC* and *MEG3*) in response to soluble factors secreted by developmentally competent and incompetent human embryos (Fig. 2).

**Figure 2.** Real-time quantitative PCR validation of the microarray analysis.
Primary HESCs decidualized with 8-bromo-cAMP and MPA for 5 days were incubated for 12 h with unconditioned embryo culture medium (control. C) or culture medium from developmentally incompetent embryos (DIE) or developmentally competent human embryos (DCE). The results confirm that maternal expression of *HSPA8*. *THBS1*. and *PDGFC* are sensitive to signals from DIE whereas *MEG3* is responsive to signals from DCE. The results are mean ± standard deviation of triplicate experiments.

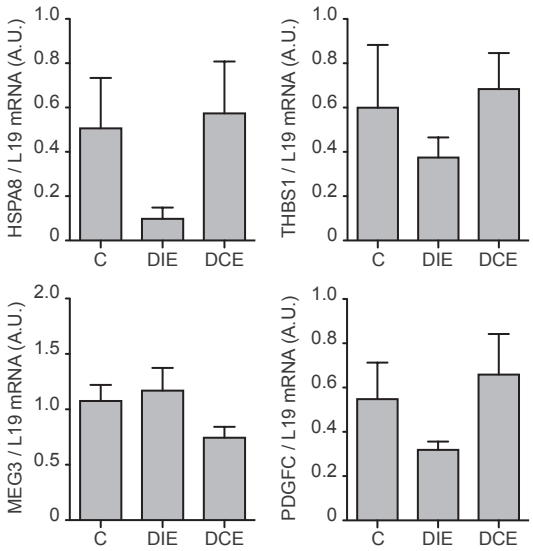


**Table 2.** Genes regulated in decidualizing HESCs upon incubation of culture medium from developmentally incompetent human embryos (DIE).

**A.** Decidual genes down-regulated ≥ 1.2-fold.

| Gene symbol | Gene name | Fold down |
|---|---|---|
| HSPA8 | Heat shock cognate 71 kDa protein | 1.88 |
| THBS1 | Thrombospondin-1 Precursor | 1.76 |
| PDGFC | Platelet-derived growth factor C Precursor | 1.66 |
| SBDS | Ribosome maturation protein SBDS | 1.59 |
| CTGF | Connective tissue growth factor Precursor | 1.56 |
| ST3GAL5 | Lactosylceramide alpha-2.3-sialyltransferase | 1.51 |
| CALD1 | Caldesmon | 1.49 |
| ERICH1 | Glutamate-rich protein 1 | 1.42 |
| KIAA1913 | Transmembrane protein 200A | 1.42 |
| EIF4G2 | Eukaryotic translation initiation factor 4 gamma 2 | 1.41 |
| UBE2D3 | Ubiquitin-conjugating enzyme E2 D3 | 1.40 |
| LAMB1 | Laminin subunit beta-1 Precursor | 1.38 |
| ACTR2 | Actin-related protein 2 | 1.38 |
| FXR1 | Fragile X mental retardation syndrome-related protein 1 | 1.37 |
| BPGM | Bisphosphoglycerate mutase | 1.37 |
| IGFBP7 | Insulin-like growth factor-binding protein 7 Precursor | 1.37 |
| SSB | Lupus La protein | 1.35 |
| SUB1 | Activated RNA polymerase II transcriptional coactivator p15 | 1.34 |
| ZFAND5 | AN1-type zinc finger protein 5 | 1.34 |
| PTP4A1 | Protein tyrosine phosphatase type IVA 1 | 1.33 |
| HNRNPM | Heterogeneous nuclear ribonucleoprotein M | 1.33 |
| ZNF588 | Zinc finger protein 107 | 1.32 |
| EPS8 | Epidermal growth factor receptor kinase substrate 8 | 1.32 |
| TMEM212 | Transmembrane protein 212 | 1.32 |
| ZNF549 | Zinc finger protein 549 | 1.31 |
| MUC16 | Mucin-16 | 1.31 |
| PHLDA1 | Pleckstrin homology-like domain family A member 1 | 1.31 |
| UACA | Uveal autoantigen with coiled-coil domains and ankyrin repeats | 1.30 |
| KLF5 | Krueppel-like factor 5 | 1.30 |
| UBE2E3 | Ubiquitin-conjugating enzyme E2 E3 | 1.29 |
| hsa-mir-568 | hsa-mir-568 | 1.29 |
| ZNF565 | Zinc finger protein 565 | 1.29 |
| NDFIP1 | NEDD4 family-interacting protein 1 | 1.29 |
| LIMS1 | LIM and senescent cell antigen-like-containing domain protein 1 | 1.29 |
| ECHDC1 | Enoyl-CoA hydratase domain-containing protein 1 | 1.28 |
| DOCK4 | Dedicator of cytokinesis protein 4 | 1.28 |

| | | |
|---|---|---|
| SERINC1 | Serine incorporator 1 | 1.27 |
| ADAMTS5 | ADAM with thrombospondin motifs 5 Precursor | 1.27 |
| SCOC | Short coiled-coil protein | 1.27 |
| RHBDD1 | Rhomboid domain-containing protein 1 | 1.27 |
| IGSF9 | Protein turtle homolog A Precursor | 1.26 |
| LIMS1 | LIM and senescent cell antigen-like-containing domain protein 1 | 1.26 |
| SLC38A2 | Sodium-coupled neutral amino acid transporter 2 | 1.26 |
| CALD1 | Caldesmon | 1.26 |
| PNN | Pinin | 1.26 |
| FBXL3 | F-box/LRR-repeat protein 3 | 1.26 |
| PPM1B | Protein phosphatase 1B | 1.26 |
| JMJD1C | Probable JmjC domain-containing histone demethylation protein 2C | 1.26 |
| DNAJA1 | DnaJ homolog subfamily A member 1 | 1.26 |
| THOC7 | THO complex subunit 7 homolog | 1.25 |
| EIF2A | Eukaryotic translation initiation factor 2A | 1.25 |
| CHML | Rab proteins geranylgeranyltransferase component A 2 | 1.25 |
| FAM20C | Dentin matrix protein 4 Precursor | 1.24 |
| MRFAP1L1 | MORF4 family-associated protein 1-like 1 | 1.24 |
| LEPROT | Leptin receptor gene-related protein | 1.24 |
| MAPK13 | Mitogen-activated protein kinase 13 | 1.24 |
| RAB9A | Ras-related protein Rab-9A (Rab-9A) | 1.24 |
| NMD3 | 60S ribosomal export protein NMD3 | 1.23 |
| CBX3 | Chromobox protein homolog 3 | 1.23 |
| NEK7 | Serine/threonine-protein kinase Nek7 | 1.23 |
| OGFRL1 | Opioid growth factor receptor-like protein 1 | 1.23 |
| TANK | TRAF family member-associated NF-kappa-B activator | 1.22 |
| BCAR3 | Breast cancer anti-estrogen resistance protein 3 | 1.22 |
| CCNG1 | Cyclin-G1 | 1.22 |
| TNFSF15 | Tumor necrosis factor ligand superfamily member 15 | 1.22 |
| CCT6A | T-complex protein 1 subunit zeta | 1.22 |
| CCNG2 | Cyclin-G2 | 1.22 |
| HCG11 | HLA complex group 11 | 1.21 |
| PRDX4 | Peroxiredoxin-4 | 1.21 |
| MED10 | Mediator of RNA polymerase II transcription subunit 10 | 1.21 |
| NDUFB6 | NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 6 | 1.21 |
| THBS1 | Thrombospondin-1 Precursor | 1.20 |
| AC092416.3 | Putative uncharacterized protein ENSP00000381576 | 1.20 |
| FSIP2 | Fibrous sheath-interacting protein 2 | 1.20 |
| ROCK1 | Rho-associated protein kinase 1 | 1.20 |
| RP2 | Protein XRP2 | 1.20 |

**B.** Decidual genes up-regulated ≥ 1.2-fold.

| Gene symbol | Gene name | Fold up |
|---|---|---|
| HMOX1 | Heme oxygenase 1 | 1.54 |
| ZNRF3 | Zinc/RING finger protein 3 Precursor | 1.46 |
| TMEM87B | Transmembrane protein 87B Precursor | 1.44 |
| IGSF1 | Immunoglobulin superfamily member 1 Precursor | 1.39 |
| KHSRP | Far upstream element-binding protein 2 | 1.37 |
| GNA11 | Guanine nucleotide-binding protein subunit alpha-11 | 1.34 |
| FCHO1 | FCH domain only protein 1 | 1.32 |
| ARHGDIA | Rho GDP-dissociation inhibitor 1 | 1.31 |
| BMP5 | Bone morphogenetic protein 5 Precursor | 1.28 |
| LIMK1 | LIM domain kinase 1 | 1.26 |
| MAP1LC3A | Microtubule-associated proteins 1A/1B light chain 3A Precursor | 1.26 |
| SRC | Proto-oncogene tyrosine-protein kinase Src | 1.26 |
| WASH1 | WAS protein family homolog 1 | 1.24 |
| RHOC | Rho-related GTP-binding protein RhoC Precursor | 1.24 |
| LRRN1 | Leucine-rich repeat neuronal protein 1 Precursor | 1.24 |
| FOSL2 | Fos-related antigen 2 | 1.23 |
| SKIP | Inositol polyphosphate 5-phosphatase K | 1.23 |
| NAB1 | NGFI-A-binding protein 1 | 1.23 |
| MICAL2 | Protein MICAL-2 | 1.22 |
| SUPT5H | Transcription elongation factor SPT5 | 1.22 |
| S100A11 | Protein S100-A11 | 1.22 |
| CDK4 | Cell division protein kinase 4 | 1.22 |
| LDB2 | LIM domain-binding protein 2 | 1.22 |
| TRIM17 | Tripartite motif-containing protein 17 | 1.22 |
| BSG | Basigin Precursor | 1.22 |
| VPS37B | Vacuolar protein sorting-associated protein 37B | 1.21 |
| COLEC12 | Collectin-12 | 1.21 |
| H2AFX | Histone H2A.x | 1.20 |
| CANT1 | Soluble calcium-activated nucleotidase 1 | 1.20 |
| LPCAT4 | Lysophosphatidylcholine acyltransferase | 1.20 |

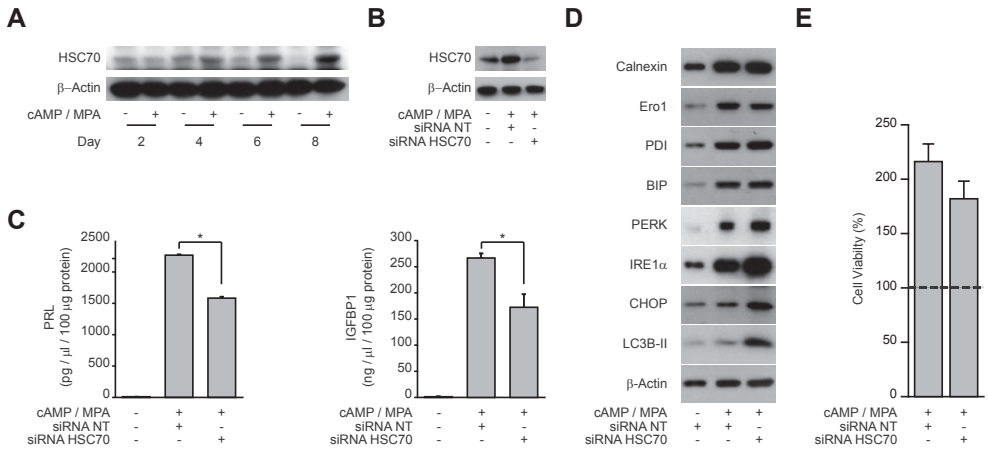
**Table 3.** Genes regulated (≥ 1.2-fold) in decidualizing HESCs upon incubation of culture medium from developmentally competent human embryos (DCE).

| Gene symbol | Gene name | Fold down |
|---|---|---|
| MEG3 | maternally expressed 3 | 1.49 |
| MAGT1 | Magnesium transporter protein 1 Precursor | 1.26 |
| COL7A1 | Collagen alpha-1(VII) chain Precursor | 1.24 |
| IGSF9 | Protein turtle homolog A Precursor | 1.23 |
| SULT1A4 | Sulfotransferase 1A3/1A4 | 1.23 |

To provide proof of concept that signals from poor quality embryos disrupt decidual cell homeostasis and function, we focused on *HSPA8*, which encodes heat shock cognate 70 (HSC70) protein. HSC70 is a cytosolic protein and member of the heat shock protein 70 (HSP70) family of molecular chaperones involved in the assembly of multiprotein complexes, transport of nascent polypeptides and regulation of protein folding[17,18]. HSC70 is reportedly constitutively and ubiquitously expressed, although expression in decidualizing HESCs was found to be biphasic, characterized by a transient reduction followed by a marked increase upon 4 or more days of differentiation (Fig. 3A). This induction is important for the decidual phenotype as small interfering RNA-mediated HSC70 knockdown reduced the secretion of prolactin (PRL) and insulin-like growth factor-binding protein 1 (IGFBP1), two highly sensitive differentiation markers (Fig. 3B & C)[15,19,20]. The HSP70 family plays a critical role in preventing accumulation of unfolded proteins in the endoplasmic reticulum (ER), especially in response to environmental stress signals. Accumulation of misfolded proteins in the cell triggers the evolutionarily conserved unfolded protein response (UPR) and, if prolonged or excessive, ER stress and cell death[21,22]. A physiological UPR is also observed in cells that dramatically expand their ER upon differentiation[23]. Decidualization of HESCs is associated with strong UPR (Fig. 3D), characterized by the marked and synchronous induction of ER chaperones, including calnexin, BiP, PDI (protein disulfide isomerase), and Ero1 (endoplasmic oxidoreductin-1), and resident kinases, such as the serine/threonine kinase IRE1$\alpha$ (inositol-requiring enzyme 1$\alpha$) and PERK (PKR-like ER-localized eIF2α kinase). In contrast, the levels of CHOP, a bZip transcription factor that couples ER stress to cell cycle arrest and death[21,24], increased only marginally upon decidualization. Knockdown of HSC70 converted the physiological UPR associated with decidualization into an overt ER stress response, exemplified foremost by the pronounced upregulation of CHOP (Fig. 3D). LC3B-II, a marker of autophagic activity, also increased dramatically upon HSC70 knockdown in decidualizing cells. The autophagy pathway, which degrades and recycles cellular components, has been linked to non-apoptotic cell death although, depending on the nature of the stress signal and cellular context, it is more commonly associated with a pro-survival response[25]. HSC70 knockdown adversely impacted on the viability of primary endometrial cultures (Fig. 3E), albeit modestly ($P = 0.05$), suggesting that ER stress-associated autophagy in decidualizing HESCs is primarily cytoprotective.

In summary, we compared the transcriptional responses of decidualizing endometrial cells to secreted signals from human embryos that differ widely in their developmental potential. To eliminate possible confounders associated with the transfer procedure, and the uterine environment *in vivo*, we focused on poor quality embryos and on embryos that resulted in successful pregnancies. We demonstrate that maternal decidual cells mount an extraordinarily graded transcriptional response to embryos of different quality, ranging from very pronounced to exceptionally discrete. This means that human embryos, even at the preimplantation stage, signal their developmental competence, although the nature of this signal remains to be defined. Compromised human embryos are more likely to harbor gross and chaotic chromosomal abnormalities[26], comparable to those found in cancer cells[27].

**Figure 3.** HSC70 expression and function in decidualizing HESCs. **(a)** Western blot analysis demonstrating the kinetics of HSC70 induction in primary HESCs decidualized with 8-bromo-cAMP and medroxyprogesterone acetate (cAMP/MPA) in a time-course lasting 8 days. β-actin served as a loading control. A representative result from three different primary cultures is shown. (**b**) primary HESCs were transfected with non-targeting (NT) siRNA or siRNA targeting HSC70, treated with cAMP and MPA for 5 days, and immunoblotted for HSC70. (**c**) HSC70 knockdown inhibits the secretion of decidual markers, PRL and IGFBP1, in primary HESC cultures differentiated with cAMP and MPA for 5 days. The data are the mean ± standard error of biological triplicate experiments. * indicates $P < 0.05$. (**d**) HSC70 knockdown induces ER stress in decidual cells. Total cell lysates from primary HESC cultures, transfected first with non-targeting (NT) siRNA or siRNA targeting HSC70 and then cAMP and MPA for 5 days, were immunoprobed for various proteins involved in UPR and ER stress, as indicated. LC3B-II is a marker of autophagic activity β-actin served as a loading control. A representative result from three different primary cultures is shown. (**e**) The number of viable cells in HESCs, transfected first with non-targeting (NT) siRNA or siRNA targeting HSC70 and then decidualized with cAMP and MPA for 5 days, was measured by the ApoTox-Glo™ Triplex Assay, and expressed relatively (%) to the number of viable cells in mock-transfected undifferentiated cells (dotted line). The data are the mean ± standard error of biologically triplicate experiments. Although HSC70 knockdown affected decidual cell viability, this was not statistically significant ($P = 0.05$).




Like cancer cells, poor quality embryos also display heightened metabolism[26,28], the byproducts of which could possibly serve as a gradient signal for maternal recognition. Conversely, preimplantation competent human embryos are known to be metabolically quiet, which may render them virtually invisible to decidual cells, at least until the onset of secretion of major peptide hormones, such as human chorionic gonadotropin. Based on our findings, we extrapolate that poor quality embryos that breach the luminal endometrial epithelium trigger a complex maternal response, leading to ER stress in the surrounding decidual cells, disruption of the local microenvironment and ultimately, menstruation-like shedding. Perturbations in ER homeostasis could also account for the previous observation that decidualizing cells respond to the presence of an arresting blastocyst in co-culture by inhibiting secretion of key implantation mediators and immunomodulators[14]. Undifferentiated HESCs do not mount such a response[14], which emphasizes the importance of the decidual process

in enabling maternal quality control of implanting embryos. Thus, strategies aimed at optimizing endometrial differentiation prior to conception may be key to prevent inappropriate maternal investment in failing pregnancies, thus reducing the formidable burden of clinical miscarriages.

## METHODS

**Experimental ethics policy**. This study was approved by the Medical Review Ethics Committee of the University Medical Center Utrecht, the Central Committee on Research inv. Human Subjects in The Netherlands (NL 12481.000.06), and the Hammersmith and Queen Charlotte's & Chelsea Research Ethics Committee (1997/5065). Written informed consent was obtained from all participating subjects.

**Primary cultures.** HESC cultures were established as previously described[29] and decidualized with 0.5 mM 8-Bromo-cAMP (Sigma, UK) and 1 µM medroxyprogesterone acetate (MPA; Sigma, UK).

**Embryo conditioned media.** Human embryos were cultured in microdroplets (30 µl) of Human Tubal Fluid media, supplemented with 5% GPO (40 gr/l pasteurized plasma protein, containing 95% albumin) under mineral oil from the second day after oocyte retrieval until day 4. The supernatants were collected from individually cultured embryo that resulted in pregnancy after single embryo transfer (n=30) and from embryos deemed of poor quality (n=30), based on standard morphological criteria[30]. Unconditioned medium cultured under the same conditions without the presence of an embryo was collected for control cultures. HESCs from a single biopsy were plated in 48-well tissue culture-grade plates, decidualized first for 5 days, and then incubated for 12 hours with 100 µl of separately pooled supernatants, each derived from 10 individually cultured embryos. Three pools of conditioned media were used from developmentally competent embryos and 3 from poor quality embryos. Total RNA from individual wells was subjected to microarray analysis.

**Microarray analysis and gene ontology.** Human 70-mer oligos (Operon, Human V2 AROS) spotted onto Codelink Activated slides (Surmodics USA) were used for genome-wide expression profiling. RNA amplifications, labeling and hybridisations were performed as described[31]. Briefly, 500 ng of each amplified cRNA was coupled to Cy3 or Cy5 fluorophores (Amersham, UK) and subsequently hybridized on a Tecan HS4800PRO and scanned on an Agilent G2565BA microarray scanner. After data extraction using Imagene 8.0 (BioDiscovery), print-tip Loess normalization was performed[32] on mean spot-intensities without background subtraction. Data were analyzed using ANOVA (R version 2.2.1/MAANOVA version 0.98-7) (http://www.r-project.org/). Genes with $P < 0.01$ after false discovery rate (FDR) multiple testing comparison were considered significantly changed. Regulated genes were mapped to GO-slim categories according to the Gene Ontology Consortium (http://www.geneontology.org/GO_slims/goslim_generic.obo).

**Real-time quantitative PCR.** Total RNA from primary cultures was isolated using RNeasy Micro kits (Qiagen, UK) according to the manufacturer's protocol. cDNA was synthesized with SuperScript Reverse Transcriptase (Invitrogen, UK). The PCR reaction was performed with iQ5 Real-Time PCR Detection System (Biorad, UK) using gene-specific primer pairs. Melting curve analysis and agarose gel electrophoresis confirmed amplification specificity. Data were normalized to expression of L-19. The expression levels of the samples were expressed as arbitrary units defined by the $\Delta\Delta C_T$ method.

**Transfections**. HESCs were cultured until 80 % confluency and transiently transfected by the calcium phosphate co-precipitation method using the Profection mammalian transfection kit (Promega, Madison, WI), as previously described[15]. Transfections were performed with 100 nM of siCONTROL non-targeting siRNA and HSPA8 siGENOME SMARTpool (Dharmacon, USA). Experiments were performed on 3 or more primary cultures.

**Western blot analysis**. Whole cell protein extracts were prepared by lysing cells in RIPA buffer. Protein yield was quantified using the Bio-Rad DC protein assay kit (Bio-Rad, USA). Equal amounts of protein were separated by 10% SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) before wet-transfer onto PVDF membrane (Amersham Biosciences, UK). Nonspecific binding sites were blocked by overnight incubation with 5% nonfat dry milk in Tris-buffered saline with 1% Tween (TBS-T; 130 mmol/L NaCl, 20 mmol/L Tris, pH7.6 and 1% Tween). Primary antibodies used were anti-HSC70 (Abcam, UK), anti-BiP, anti-Calnexin, anti-ERo1$\alpha$, anti-CHOP, anti-PERK, anti-PDI, anti-LC3B (Cell Signaling, USA) and β-actin (Abcam, UK) which was used as a loading control. Protein complexes were visualized with a chemiluminescent detection kit (GE Healthcare UK, UK).

**PRL and IGFBP1 measurements.** PRL and IGFBP-1 levels in the HESC culture media were determined using an amplified two-step sandwich-type immunoassay (R&D Systems, USA) according to the manufacturer's protocol.

**Cell viability assays.** Cultured HESCs were seeded in 96-well black plates with clear bases and maintained in 10 % DCC/DMEM until they become confluent. Cells where transfected with or without siRNA targeting HSPA8 and then subsequently decidualized or left untreated for a total of 6 days. Cell viability was evaluated using the ApoTox-Glo™ Triplex Assay (Promega, USA) according to the manufacturer's instructions.

**Statistical analysis.** Statistical testing was performed by ANOVA with Bonferroni correction was used. $P < 0.05$ was considered significant.

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# Chapter 6

## NATURAL SELECTION OF HUMAN EMBRYOS: IMPAIRED DECIDUALIZATION OF ENDOMETRIUM DISABLES EMBRYO-MATERNAL INTERACTIONS AND CAUSES RECURRENT PREGNANCY LOSS


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## Abstract

Background: Recurrent pregnancy loss (RPL), defined as 3 or more consecutive miscarriages, is widely attributed either to repeated chromosomal instability in the conceptus or to uterine factors that are poorly defined. We tested the hypothesis that abnormal cyclic differentiation of endometrial stromal cells (ESCs) into specialized decidual cells predisposes to RPL, based on the observation that this process may not only be indispensable for placenta formation in pregnancy but also for embryo recognition and selection at time of implantation.

Methodology/Principal Findings: Analysis of mid-secretory endometrial biopsies demonstrated that RPL is associated with decreased expression of the decidual marker prolactin (PRL) but increased levels of prokineticin-1 (PROK1), a cytokine that promotes implantation. These *in vivo* findings were entirely recapitulated when ESCs were purified from patients with and without a history of RPL and decidualized in culture. In addition to attenuated PRL production and prolonged and enhanced PROK1 expression, RPL was further associated with a complete dysregulation of both markers upon treatment of ESC cultures with human chorionic gonadotropin, a glycoprotein hormone abundantly expressed by the implanting embryo. We postulated that impaired embryo recognition and selection would clinically be associated with increased fecundity, defined by short time-to-pregnancy (TTP) intervals. Woman-based analysis of the mean and mode TTP in a cohort of 560 RPL patients showed that 40% can be considered 'superfertile', defined by a mean TTP of 3 months or less.

Conclusions: Impaired cyclic decidualization of the endometrium facilitates implantation yet predisposes to subsequent pregnancy failure by disabling natural embryo selection and by disrupting the maternal responses to embryonic signals. These findings suggest a novel pathological pathway that unifies maternal and embryonic causes of RPL.

## Introduction

Miscarriage is the most common complication of pregnancy. It is estimated that 30% of embryos are lost prior to implantation (preimplantation loss) and a further 30% before 6 weeks gestation (pre-clinical/biochemical pregnancy loss)[1]. In addition, in excess of 10% of clinical pregnancies result in miscarriage, mostly prior to 12 weeks gestation, and 1-2% of couples experience recurrent pregnancy loss (RPL), defined as failure of 3 or more consecutive pregnancies[2]. Beside the physical trauma, miscarriage, and especially RPL, is associated with considerable psychological morbidity with a third of patients attending specialist clinics suffering from clinical depression[2]. Moreover, a history of RPL increases the risk of a variety of adverse obstetric outcomes in a subsequent ongoing pregnancy, including preterm delivery, premature preterm rupture of membranes, placenta praevia, low birth weight and congenital malformation[3].

Early pregnancy loss is widely viewed as a dichotomous disorder, attributed either to maternal factors or chromosomal errors in the conceptus. On the maternal side, numerous anatomical, endocrine, immunological, thrombophilic and genetic perturbations have been invoked to explain RPL, yet none are specific or prevalent[2,3]. Moreover, for most of these conditions, the pathological mechanisms that account for persistent pregnancy wastage are entirely conjectural. Conversely, between 30 to 60% of miscarriages are attributed to fetal chromosomal anomalies[4]. These estimates are based on conventional karyotyping of fetal tissues, suggesting that the true incidence may be higher. However, the prevalence of gross mitotic chromosomal errors in preimplantation human embryos is also very high, affecting a staggering 90% of all embryos, even in young fertile women[5]. In other words, if chromosomal instability in the preimplantation embryo is the norm rather than the exception, then RPL could primarily reflect inadequate embryo selection, accounting for the high prevalence of aneuploidic miscarriages.

For most of the menstrual cycle, the endometrium is not primed for implantation. It only transiently acquires a receptive phenotype, starting approximately 6 days after the postovulatory progesterone surge and is estimated to last between 2 to 4 days[6,7]. Arguably, a limited 'implantation window' synchronizes implantation with embryo development, which may serve as an important mechanism to select against developmentally impaired but potentially invasive embryos. Consistent with this concept, the population study of Wilcox *et al*[7] elegantly demonstrated that implantation beyond the normal period of endometrial receptivity is strongly associated with early pregnancy loss.

Although the luminal endometrial epithelium is the primary barrier in the implantation process, the progesterone responses in this cellular compartment that underpin the receptive phenotype are mediated by signals derived from the underlying stromal cells[8]. A striking feature of the human endometrium is that the acquisition of a receptive phenotype in the mid-secretory phase of the cycle coincides with decidualization of the stromal compartment, irrespective of pregnancy. Decidualization is characterized by transient local oedema, influx of macrophages and specialized uterine natural killer cells, angiogenesis, and the extraordinary transformation of resident endometrial stromal fibroblasts into secretory, epitheloid-like decidual cells[9-11]. From a functional perspective, the

decidual process is indispensable for pregnancy in all species with invasive placentae as it establishes maternal immunologic tolerance to fetal antigens, protects the conceptus against environmental insults, and ensures tissue integrity and haemostasis during the process of trophoblast invasion and placenta formation[9,10]. Further, decidualization of human endometrial stromal cells also bestows on the endometrium the ability to selectively recognize and respond to developmentally impaired embryos, as outlined in the accompanying paper by Teklenburg *et al*. in this journal. Based on these observations, we hypothesized that impaired decidualization of the endometrium prior to conception predisposes for subsequent pregnancy failure, either by prolonging the implantation window, thereby disabling natural embryo selection, or by disrupting the maternal responses to embryonic signals. If correct, impaired decidualization and lack of natural embryo selection should not only lead to RPL but also be associated with paradoxical superfecundity, defined by persistent very short time-to-pregnancy (TTP) intervals.

## Results

### Endometrial Decidualization Is Impaired in RPL

We speculated that impaired decidualization of the stromal compartment may facilitate delayed implantation of compromised embryos by prolonging the window of endometrial receptivity, as suggested by the population study of Wilcox *et al*[7]. To test this hypothesis, we determined the expression levels of a recently identified key regulator of endometrial receptivity, prokineticin-1 (PROK1)[12], as well as prolactin (PRL), a classic decidual marker[11,13]. Transcript levels were determined in endometrial biopsies, timed to span the implantation window, from RPL patients and controls, consisting of either fertile or infertile women without a history of recurrent pregnancy failure (Table 1). Compared with controls, RPL was associated with significantly higher endometrial PROK1 mRNA levels and approximately 100-fold lower PRL levels (Fig. 1A & B). Analysis of an independent sample set (Table 2) demonstrated that elevated PROK1 transcript levels are primarily associated with biochemical rather than fetal RPL, whereas no such association was found with PRL (Fig. 1C & D).

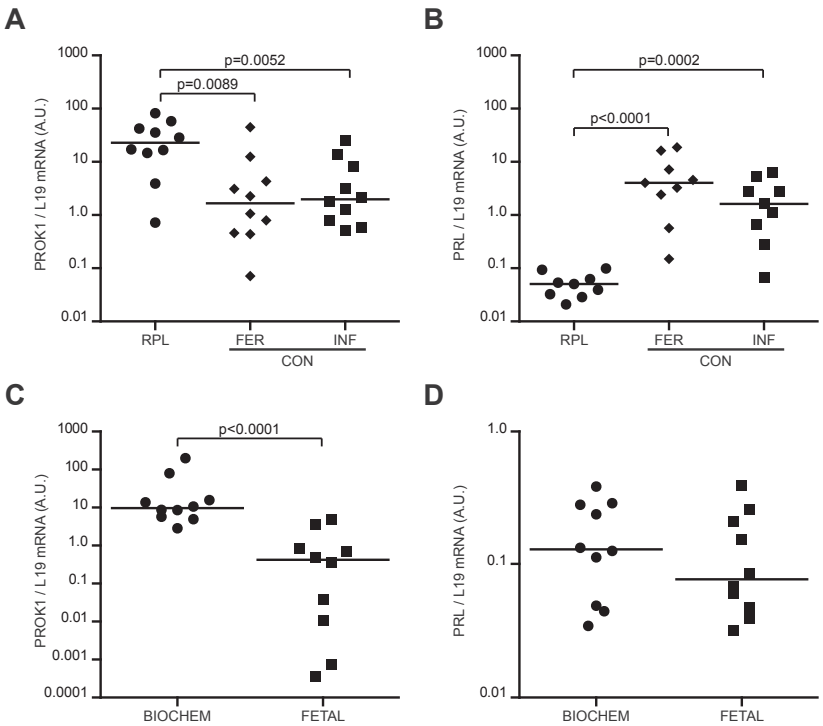
**Table 1.** Analysis of timed endometrial biopsies – patient characteristics

| | **Control** | | **RPL** (n=10) |
|---|---|---|---|
| | **Fertile** (n=10) | **Infertile** (n=10) | |
| Age (years): | 34 ± 2.9 | 32.9 ± 4.1 | 35.1 ± 2.9 |
| Live births: | 2.5 ± 1.4* | 0.2 ± 0.4 | 0.6 ± 0.7 |
| Miscarriages: | 0.3 ± 0.5 | 0 | 7.4 ± 6.1* |

**Table 2.** Analysis of fetal versus biochemical RPL - patient characteristics

| | **Fetal RPL** (n=10) | **Biochemical RPL** (n=10) |
|---|---|---|
| Age (years): | 36.6 ± 3.4 | 30.4 ± 7.4* |
| Live births: | 0 | 0 |
| Miscarriages: | 4.4 ± 1.2 | 4.0 ± 1.0 |

**Fig. 1.** PROK1 and PRL transcript levels in timed endometrial samples of 10 RPL patients and 20 control (CON) subjects, consisting of 10 fertile (FERT) volunteers and 10 infertile (INF) patients without a history of RPL. PROK1 (*A*) and PRL (*B*) mRNA levels, normalized to L19 transcript levels, are expressed in arbitrary units (a.u.). Horizontal bars indicate the median expression in each group. PROK1 and PRL mRNA levels, respectively (*C & D*), in RPL patients with recurrent biochemical (biochem; n=10) or fetal (n=10) pregnancy failure. A 'biochemical' loss was defined as a miscarriage at 4-6 weeks gestation with ultrasound evidence of either an intrauterine pregnancy sac with no fetus or retained products of conception. A 'fetal' loss was defined as a pregnancy failure between 6-13 weeks gestation with prior ultrasound evidence of fetal development. Note the logarithmic y-axes.



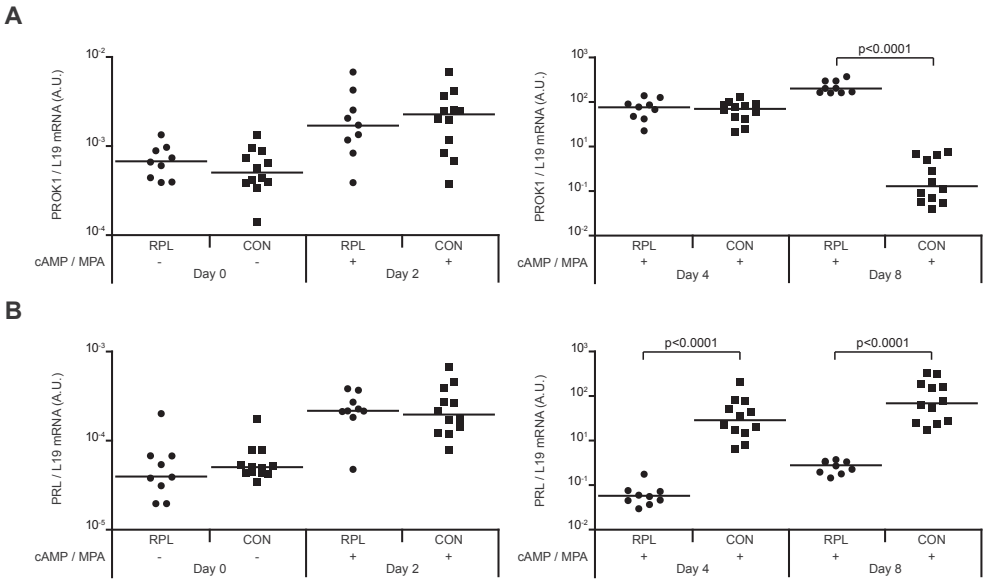

To validate these *in vivo* observations, we established primary cultures from 9 RPL patients and 12 controls (Table 3). ESCs, purified from samples taken randomly in the cycle, were passaged once, allowed to grow to confluency, and then decidualized over a time-course lasting 8 days. PROK1

and PRL mRNA levels did not differ between the two groups in undifferentiated ESCs or in cells decidualized for 48 hours. However, after 4 days of differentiation, the rise in PRL transcript levels was several magnitudes higher in the control group when compared to RPL samples (Fig. 2). PROK1 levels continued to rise with comparable kinetics in all decidualizing cultures until day 8 when expression in the control but not RPL group declined markedly.

**Table 3.** Time-course analysis - patient and culture characteristics

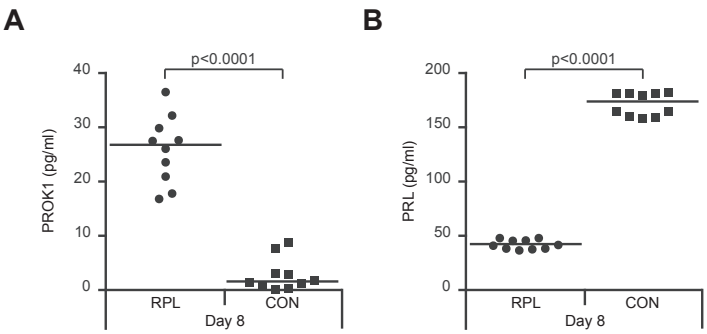
| | **Control** (n=12) | **RPL** (n=9) |
|---|---|---|
| Age (years): | 33 ± 4.9 | 34.1 ± 3.6 |
| Live births: | 0.4 ± 1 | 0.4 ± 0.7 |
| Miscarriages: | 0.1 ± 0.3* | 3.9 ± 1.2 |
| Day of biopsy from LMP: | 17 ± 5.6 | 16.2 ± 7.9 |
| Days in culture | 16 ± 5.1 | 16.3 ± 4.1 |

**Fig. 2.** RPL is associated with aberrant expression of PROK1 and PRL in decidualizing primary ESC cultures. PROK1 (*A*) and PRL (*B*) mRNA levels, normalized to L19 transcript levels and expressed in arbitrary units (a.u.), were determined in undifferentiated ESCs (day 0) or cultures decidualized with 8-Br-cAMP and MPA (cAMP/MPA) for 2, 4 or 8 days. A total of 9 primary cultures were established from RPL patients and 12 from control (CON) subjects. Horizontal bars indicate the median expression in each group. Note the logarithmic y-axes.



This expression profile in cultured cells supported the notion that an impaired decidual response, leading to prolonged endometrial receptivity and impaired embryo selection, is the primary uterine defect in RPL. Two additional observations are worth emphasizing. First, the magnitude of difference in PROK1 and PRL expression between the RPL and control group was not only high, at mRNA as well as at the secreted protein level (Fig. 3), but the pattern of expression in all samples corresponded to the clinical phenotype. Secondly, ESCs were purified from endometrial samples taken randomly in the cycle and maintained in prolonged culture, suggesting that the ability to mount a decidual response, perhaps more so than the signals responsible for differentiation, is perturbed in RPL.

**Fig. 3.** RPL is associated with impaired PROK1 and PRL secretion by decidualizing ESCs. Secreted PROK1 (A) and PRL (B) levels accumulated over 48 hours in the supernatants of confluent primary ESC cultures decidualization with 8-Br-cAMP and MPA for 8 days. Primary cultures were established from 10 RPL patients and 10 control (CON) subjects. Horizontal bars indicate the median expression in each group.





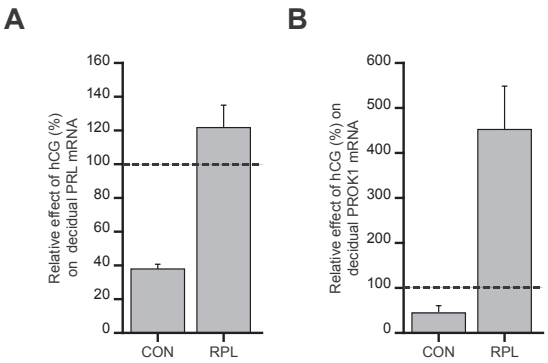

**hCG Responses in Decidual Cells Are Disrupted in RPL.**

We speculated that aberrant differentiation of ESCs observed in RPL patients would interfere directly with the maternal response to embryonic signals. To test this hypothesis, we examined the effects of human chorionic gonadotropin (hCG), one of the earliest and most abundant glycoproteins secreted by embryonic trophoblast, on *PRL* and *PROK1* expresssion in 20 additional cultures (Table 4). ESCs were decidualized with 8-Br-cAMP and MPA for 72 hours in the presence or absence of hCG. As reported previously[14], hCG strongly inhibited PRL mRNA levels in all control cultures (Fig. 4A). In contrast, this response was without exception reversed in RPL samples and characterized by a modest but significant increase in PRL transcripts upon hCG treatment. PROK1 mRNA levels were also inhibited by approximately 50% upon hCG treatment of decidualizing control cultures whereas the opposite response, a 4-fold increase, was observed in RPL cultures (Fig. 4B). The data suggest that perturbed endometrial preparation prior to conception will have profound consequences on embryo-maternal interactions in pregnancy.

**Table 4.** hCG analysis - patient characteristics

| | **Control** (n=10) | **RPL** (n=10) |
|---|---|---|
| Age (years): | 34.1 ± 3.1 | 32.3 ± 6.6 |
| Live births: | 0.4 ± 0.9 | 0.2 ± 0.4 |
| Miscarriages: | 0 | 7 ± 3.7* |
| Day of biopsy from LMP: | 17.6 ± 7.1 | 20.8 ± 5.3 |

**Fig. 4.** RPL is characterised by aberrant hCG responses in decidualizing ESCs. Primary cultures from RPL patients (n=10) and control women (n=10) were decidualized with 8-Br-cAMP and MPA for 72 hours in the presence or hCG or vehicle. The data show the percentage change (± SEM) in PRL (*A*) and PROK1 (*B*) mRNA levels upon hCG treatment of control and RPL cultures, relative to the expression levels in cultures treated with vehicle (dotted lines); $P < 0.005$.




**RPL Is Associated with Short Time-To-Pregnancy**

RPL patients often report short periods between pregnancies, which is the predictable clinical correlate of impaired embryo recognition and selection. To substantiate this observation, we analysed the time-to-pregnancy (TTP), expressed in months, in 2076 pregnancies reported by 560 women with a history of 3 or more consecutive miscarriages. Woman-based analysis of the mean and mode TTP showed that many RPL patients are highly fecund (Fig. 5 and Table 5). Multivariate logistic regression analysis demonstrated that fecundity is further enhanced in the subgroup of patients with 5 or more recurrent miscarriages (odds ratio: 2.0; 95% confidence interval 1.1-3.4; $P = 0.01$ when adjusted for maternal age). In the absence of a suitable control group, we compared the observed incidence of achieving 3 or more pregnancies within 1, 3, or 6 months with the predicted likelihoods, based on an average MFR of 20%[15]. As shown in Table 6, the observed incidence of persistent short TTP intervals in RPL patients was much higher than predicted. In fact, 40% of RPL patients could be considered 'superfertile', defined by a mean TTP of less than 3 months[15]. Interestingly, maternal age was not an important confounding factor for TTP in RPL patients, which further suggests that implantation, rather than fertilization, is the major rate-limiting step in the fecundity of fertile couples.

**Table 5.** Analysis of time-to-pregnancy (TTP) in women with RPL

| | **≥3 miscarriages** (n=560) | **≥5 miscarriages** (n=132) | *P* |
|---|---|---|---|
| Number of pregnancies: mean ± SD | 5.0 ± 2.1 | 7.4 ± 2.7 | <0.0001 |
| Mean age group (years) | 30-34.9 | 30-34.9 | NS |
| TTP: mean ± SD (months) | 5.4 ± 4.9 | 4.5 ± 4.2 | <0.05 |
| TTP : mode ± SD (months) | 3.0 ± 3.4 | 2.2 ± 2.3 | =0.01 |
| TTP: median ± SD (months) | 4.6 ± 4.6 | 3.8 ± 3.9 | NS |
| Women with mean TTP ≤ 1 month (%) | 70 (13%) | 23 (17%) | NS |
| Women with mean TTP ≤ 1.5 months (%) | 104 (19%) | 30 (23%) | NS |
| Women with mean TTP ≤ 3 months (%) | 229 (41%) | 66 (50%) | NS |
| Women with mean TTP ≤ 6 months (%) | 381 (68%) | 97 (73%) | NS |

**Fig. 5.** Woman-based analysis of time-to-pregnancy (TTP) in 560 women with a history of ≥3 consecutive first trimester miscarriages. Histogram representing woman-based analysis of the mean (*A*) and mode (*B*) TTP in patients with RPL.

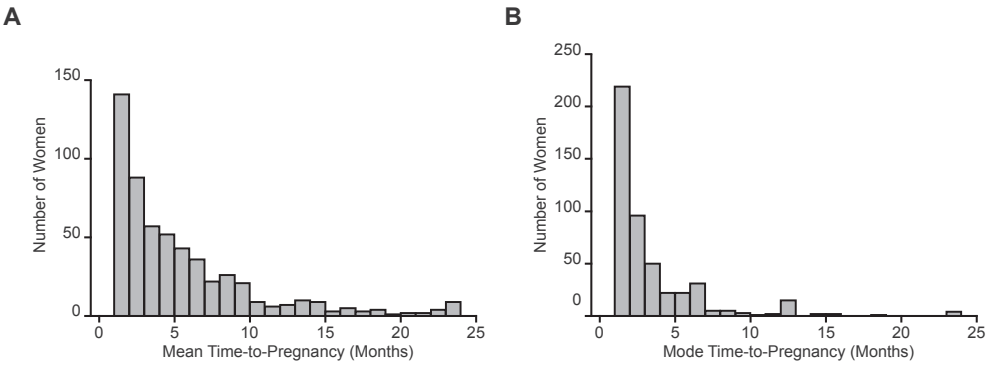



**Table 6**. Proportion of women achieving ≥3 consecutive pregnancies within 1, 3, or 6 months.

| | **1 month** | **3 months** | **6 months** |
|---|---|---|---|
| Predicted: | 0.8% | 8% | 41% |
| RPL patients: | 13% | 41% | 68% |

Predicted likelihoods are based on a MFR of 20%; $P < 0.0001$.

## Discussion

Our study provides compelling evidence that impaired decidual programming of the endometrium is an important mechanism underlying RPL. This is based primarily on the observation that expression of *PRL*, a highly sensitive and specific decidual marker gene in the endometrium[13,16], is grossly impaired in RPL patients, both *in vivo* as well in primary cultures subjected to a decidualizing stimulus. However, we also obtained evidence that uterine receptivity is enhanced and prolonged in RPL patients, which in turn may facilitate delayed implantation of compromised embryos. Differentiating ESCs abundantly express PROK1, a cytokine that promotes embryo-uterine interaction via induction of leukaemia inhibitory factor (LIF) in endometrial epithelial cells[12]. The levels of this pro-implantation cytokine were significantly higher in timed endometrial samples from RPL patients, especially in women with a history of predominantly very early (biochemical) pregnancy losses. More strikingly, the induction of PROK1 in decidualizing endometrial cultures from control women was transient, with levels declining after 4 days of differentiation, whereas the levels continued to rise in cultures established from RPL patients. The concept of enhanced endometrial receptivity in RPL is supported by a previous study demonstrating that affected women express lower levels of mucin 1, an anti-adhesion molecule that contributes to the barrier function of luminal epithelium[4,17].

We also found that RPL is further associated with paradoxical endometrial responses to embryonic hCG signalling, characterized by induction rather than inhibition of PRL and PROK1 expression in decidualizing ESCs. The ability of hCG to inhibit decidual PRL expression is in agreement with a previous study[14]. Interestingly, hCG has been shown to transiently induce PROK1 in Ishikawa cells, a cancer cell line widely used as model for uterine epithelial cells, which was followed by increased LIF expression[12]. Combined with our data, these observations indicate that under normal circumstances, hCG signalling may first transiently enhance uterine receptivity upon contact of the embryo with the luminal epithelium but upon implantation antagonize continuous receptivity by inhibiting decidual PROK1. The mechanism of normal hCG signaling in decidual cells remains to be defined[18], which is a prerequisite for understanding the disrupted transcriptional responses in RPL. Irrespective of the underlying pathway, a dysregulated response in pregnancy is likely to jeopardize the integrity of the feto-maternal interface as hCG, besides its luteotrophic role in early pregnancy, directly stimulates angiogenesis and limits cell death responses in the maternal decidua[19-21].

Although our expression analysis was confined to *PRL* and *PROK1*, the data demonstrate that endometrial programming in preparation of pregnancy is both quantitatively and qualitatively different in RPL, characterized by impaired decidualization of resident stromal cells, prolonged endometrial receptivity and a dysregulated maternal response to embryonic signals. This pathological pathway explains several established clinical features of RPL. For example, in view of the excess of chromosomally abnormal preimplantation human embryos[5], the likelihood of euploidic pregnancy failure can be predicted to increase with the number of miscarriages, which is indeed the case[22]. As mentioned, failure to limit the window of endometrial receptivity also accounts for delayed

implantation of severely compromised embryos and subsequent very early pregnancy loss[7]. A primary defect in the decidual response may further explain why the incidence of miscarriage drops dramatically after 12 weeks gestation[2]. The last few weeks of the first trimester of pregnancy are particularly perilous for the conceptus as it coincides with intense vascular remodelling, the onset of placental perfusion, and a dramatic increase in oxygen tension at the feto-maternal interface[23]. Reactive oxygen species trigger a pro-apoptotic pathway in undifferentiated ESCs, which is selectively silenced upon differentiation into decidual cells[24]. Thus an adequate decidual response is critical to prevent cell death, necrosis, and bleeding at the feto-maternal interface, especially when challenged by oxidative stress signals towards the end of the first trimester. Finally, the concept of defective embryo selection explains why some RPL patients appear to be exceptionally fertile.

Biologic fertility is measured using TTP[25]. Based on an average MFR of 20%, a simple mathematical model predicts that 74%, 93%, and 100% of normally fertile couples will conceive in 6, 12, and 24 months, respectively[15]. Along the same lines, moderate and severe subfertility are defined by MFR of 5% and 1%, respectively. On the other side of the spectrum is superfertility, characterized by a MFR of 60% or more. In this context, the term 'superfertility' refers to enhanced efficacy in achieving pregnancy but not increased life-births. Superfertile couples achieve 94% and 100% of pregnancies within 3 and 6 months, respectively[15]. It has been estimated that 79% of the population is fertile, 18% subfertile or infertile, and 3% superfertile[15,26]. Our retrospective analysis of TTP revealed that 40% of RPL patients report very short time to conception for each pregnancy. Thus, the prevalence of 'superfertile' couples in this population appears to be considerably higher than expected. Arguably, a majority of patients did not, or at least not consistently, report very short TTPs but this is not unexpected as the likelihood of conception is dependent upon many additional variables, including timing and frequency of coitus and the presence of coexisting disorders, such as suboptimal sperm quality or ovulatory, tubal and uterine defects. Hence, additional well-controlled, prospective studies are warranted to test our assumption that the superfertile end of the fecundity spectrum is as much a pathological condition as subfertility.

A striking observation is that the aberrant expression of *PRL* and *PROK1* in timed endometrial biopsies from RPL patients is recapitulated upon differentiation of purified ESCs maintained in prolonged cultures. Such cellular 'memory' suggests that the endometrial decidual response is subject to epigenetic programming in humans[9]. Interestingly, inflammatory signals are important epigenetic modifiers[27], which raises the possibility that the tissue trauma associated with pregnancy loss, or even menstrual events between pregnancies, may provide cues that dynamically modulate subsequent decidual responses in the endometrium[9]. If the epigenetic basis of RPL is correct than profiling of DNA or histone modifications in endometrial biopsies could be used to identify women at risk of adverse pregnancy outcome prior to conception and to monitor the effectiveness of medical interventions.

In summary, decidualization of uterine tissues is indispensable for placenta formation in pregnancy. We now provide evidence that failure to express an adequate decidual phenotype disables embryo

recognition and selection upon implantation, which may lead to shorter TTP intervals but also predisposes to persistent pregnancy failure. Established risk factors for RPL, such as antiphospholipid antibodies and endocrine perturbations, may directly impact on uterine function, although our data suggest that the primary defect lies in the ability of ESCs to mount a decidual response. Together, these findings establish a novel pathological pathway that unifies maternal and embryonic causes of RPL.

## Materials & Methods

### Ethics statement and patient selection

The Research Ethics Committee of each participating centre approved the study: Hammersmith and Queen Charlotte's & Chelsea Research Ethics Committee (1997/5065) and the Liverpool (Adult) Research Ethics Committee (REC reference number: 05/Q1505/147). Written informed consent was obtained from all participating subjects. All patients were investigated according to the standard clinic protocols[28], but the outcome of these routine investigations was not taken in account in either the recruitment into this study or in the analysis of the data.

### Timed endometrial biopsies

Women were asked to use commercially available urine LH kits and to contact the research team at the time of their LH surge. All samples were obtained 6-10 days after the LH surge with a pipelle sampling device. Each biopsy was divided and one portion snap-frozen in liquid nitrogen for RNA analysis. The other portion was fixed in formalin for histological dating using standard criteria. The demographic details of the RPL and control groups are summarize in Tables 1 & 2.

### Analysis of the decidual response *in vitro*

Primary ESC cultures were established from endometrial biopsies, taken randomly in the cycle, as previous described[29]. The demographic details of RPL and control patients are summarized in Table 3**.** Primary cultures were passaged once, allowed to grow to confluency, and then decidualized with 8-Br-cAMP (0.5 mM) and MPA ($10^{-6}$ M) for 2, 4 or 8 days. At each time-point, supernatants were collected and frozen and cells harvested for mRNA analysis. Additional cultures were established from control and RPL patients (Table 4) and decidualized with 8-Br-cAMP and MPA for 72 hours in the presence or absence of 10 nM hCG (Sigma, UK).

### PROK1 and PRL measurements

Total RNA was extracted from frozen tissue or cell cultures using Trizol Reagent (Invitrogen, Paisley, UK), treated with DNAsel (Ambion, Inc., Austin, TX), reversed transcribed, and the resulting cDNA subjected to real-time quantitative PCR analysis using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) with the following gene-specific primer pairs: L19-sense (5'-

GCG GAA GGG TAC AGC AAT-3′) and L19-antisense (5′-GCA GCC GGC GCA AA-3′); PRL-sense (5′-AAG CTG TAG AGA TTG AGG AGC AAA C-3′) and PRL-antisense (5′-TCA GGA TGA ACC TGG CTG ACT A-3′); PROK1-sense(5′-GTG CCA CCC CGG CAG-3′) and PROK1-antisense (5′-AGC AAG GAC AGG TGT GGT GC-3′). Secreted PROK1 and PRL levels were determined using ELISAs (R&D systems, Abingdon, UK).

**Time-to-pregnancy (TTP) analysis**

Woman-based analysis of TTP was performed on a cohort of patients attending a tertiary service level Recurrent Miscarriage Clinic at Imperial College Healthcare NHS Trust. TTP for each pregnancy was recorded by medical staff on a standardised pro forma questionnaire upon referral to the clinic. TTP data of 856 women, encompassing 4018 pregnancies, were extracted from the clinical notes and subjected to analysis. After excluding incomplete data sets, the final analysis was based on 560 fertile women with a history of 3 or more miscarriages, which included 132 patients with 5 or more miscarriages. All analyses, carried out using STATA statistical software (Version 10), were adjusted for maternal age at time of pregnancy. Sensitivity analyses excluding women with other pregnancy complications (e.g. ectopic pregnancy, terminations and multiple pregnancies) showed similar effects. The unit of analysis was a pregnancy.

**Statistical analyses**

Student's *t*-test and Mann-Whitney U test were used to determine statistical significance between two groups. For multiple comparisons, ANOVA test with Bonferroni correction was used. $P < 0.05$ was considered significant. The association between the relevant pregnancy outcome and TTP was explored using logistic regression analysis, with effects on risk being estimated by odds ratios with 95% confidence intervals. Since women could have more than one pregnancy outcome in the analysis, a robust method based on the "sandwich estimate"[30] was used to compute standard errors, with Wald tests to ascertain statistical significance of parameters[31].

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# Chapter 7

## The molecular basis of recurrent pregnancy loss: impaired natural embryo selection

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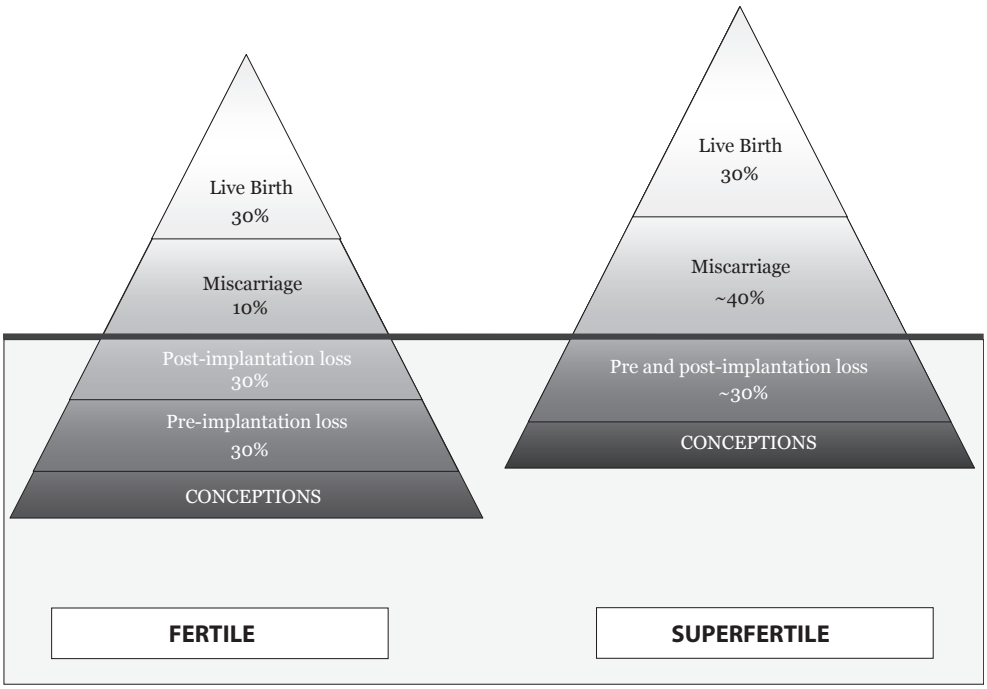
## Abstract

Recurrent pregnancy loss (RPL) is a common and distressing disorder. Chromosomal errors in the embryo are the single most common cause whereas uterine factors are invariably invoked to explain non-chromosomal miscarriages. These uterine factors are, however, poorly defined. The ability of a conceptus to implant in the endometrium is normally restricted to a few days in the menstrual cycle. A limited 'window of implantation' ensures coordinated embryonic and endometrial development, thereby minimizing the risk of late implantation of compromised embryos. In this paper we review emerging evidence indicating that RPL is associated with impaired differentiation of endometrial stromal cells into specialized decidual cells. From a functional perspective, this differentiation process, termed decidualization, is not only critical for placental development but also signals the end of the implantation window and bestows on the endometrium the ability to recognize, respond to and eliminate implanting compromised embryos. Thus, we propose that spontaneous decidualization of the human endometrium, which inevitably causes menstrual shedding in the absence of a viable conceptus, serves as functional "window for natural embryo selection". Conversely, impaired decidualization predisposes to late implantation, negates embryo quality control, and causes early placental failure, regardless of the embryonic karyotype. This pathological pathway also explains the common observation that many RPL patients seem exceptionally fertile, often conceiving within one or two cycles. Thus, as the clinical correlate of inappropriate uterine receptivity, 'superfertility' should be considered as a genuine reproductive disorder that requires targeted intervention.

## Fertility, subfertility and superfertility

Put simply, pregnancy requires little more than implantation of a developmentally competent embryo into the receptive endometrium. In many species, pregnancy is readily achieved and maintained. In humans, however, the prevalence of subfertility is high and increasing, affecting 1 in 6 couples in developed countries[1]. Fertility can be measured by the time taken to achieve pregnancy. Time-to-pregnancy (TTP) is expressed in monthly fecundity rates (MFR), i.e. the probability of achieving pregnancy within one menstrual cycle. The average MFR in humans is, compared to other mammalian species, relatively low at approximately 20%[1-3]. Based on this MFR, a simple mathematical model predicts that 74%, 93%, and 100% of normally fertile couples will conceive in 6, 12, and 24 months, respectively. Along the same lines, moderate and severe subfertility have been defined by MFRs of 5% and 1%, respectively[1]. At the other end of the spectrum, superfertility can be characterized with MFRs of 60% or more. According to this model, superfertile couples achieve 94% and 100% of pregnancies within 3 and 6 months, respectively. Based on the Tietze model[1,4], it has been estimated that 79% of the population is fertile, 18% subfertile or infertile and 3% superfertile. In addition to subfertility, the incidence of embryo wastage and pregnancy loss is also extraordinarily high in humans, estimated to be 30% prior to implantation (preimplantation loss), a further 30% before 6 weeks gestation (early pregnancy loss or EPL), and 10% of clinical pregnancies, mostly prior to 12 weeks gestation[5,6] (Figure 1). A recent study has shown that this pre-clinical pregnancy wastage does not present as subfertility and is therefore an additional reproductive challenge in humans[7]. Moreover, 1-2% of couples experience recurrent pregnancy loss (RPL), defined in Europe as 3 or more consecutive miscarriages[8,9]. From a clinical perspective, miscarriage, whether sporadic or recurrent, is widely viewed as a dichotomous disorder, attributed either to chromosomal or other developmental abnormalities in the embryo or to uterine factors. Although numerous anatomical, endocrine, immunological, thrombophilic and genetic perturbations have been invoked to explain non-chromosomal miscarriages, none of these are specific or prevalent[8,9]. In fact, the current paradigm of RPL and its management are firmly anchored in the conjecture that pre-existent disease, often much more relevant to subfertility, also underpins RPL.

**Figure 1. The embryo wastage iceberg in fertile and superfertile women.** The embryo wastage icebergs give an overview of the outcome of conceptions in a normal fertile population and a specific subset of a superfertile population, representing 3% of all couples. The "sea-level" in the figure distinguishes the pregnancies lost prior to the time of the missed menstrual period and clinically recognized pregnancy losses.

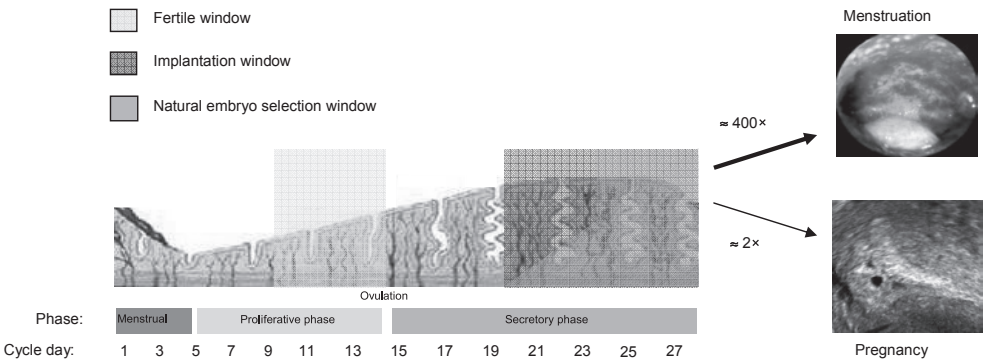



In our clinical experience, however, many women with RPL, especially those who have experienced many consecutive losses, report exceptionally high pregnancy rates. A retrospective analysis of TTP in 560 recurrent miscarriage patients revealed that 40% could be considered as 'superfertile', here defined by a mean TTP of 3 months or less[10]. If confirmed, the incidence of 'superfertile' patients suffering from RPL is remarkable, especially as the likelihood of conception is dependent upon many variables, including timing and frequency of coitus and the presence or absence of coexisting disorders, such as suboptimal sperm quality or ovulatory, tubal and uterine defects. Superfertility in humans is currently only an anecdotal concept without obvious clinical connotations. Even in a broader biological context, superfertility is a rare phenomenon, ascribed only to certain breeds of sheep. For example, the Inverdale (FecXI) sheep carries a naturally occurring X-linked mutation in the *BMP15* gene, which encodes bone morphogenetic protein 15, that causes an increased ovulation rate and twin and triplet births in heterozygotes[11]. Although often referred to as superfertile, reproduction in these animals is in fact characterized by increased prolificacy, not fertility. In absence of informative animal models, the concept of superfertility as a pathological entity must at least have a plausible biological basis. To examine if there could be a basis for superfertility in humans,

we will first discuss the two defined functional periods within the menstrual cycle, one on each side of ovulation, known to determine the rate of conception and pregnancy[12]. These functional periods are known as the 'fertile window' and 'implantation window', respectively. However, based on our recent observations that decidualizing endometrial stromal cells serve as biosensors of embryo quality[13], we will then introduce a novel functional window - *the window of natural embryo selection* – which enables maternal recognition and elimination of compromised pregnancies (Figure 2). Moreover, we will argue that perturbations in the endometrial decidual response that underpin this novel window facilitate delayed implantation of abnormal embryos, negate embryo selection, and compromise subsequent placental formation. Thus, failure to establish a functional window of natural embryo selection would result in rapid conceptions with subsequent chromosomal and non-chromosomal pregnancy failure.

**Figure 2. The functional windows of the menstrual cycle essential for reproduction.** The likelihood of conception is determined by highly coordinated changes in the cycling endometrium, which at a functional level are defined as the fertile window, the implantation window, and the functional window of natural embryo selection. Every cycle either leads to menstruation (~400 times in a woman's lifetime) or to pregnancy (~2 times in a woman's lifetime).

## The fertile window




Prospective population-based studies have shown that almost all human pregnancies result from intercourse during a six-day interval ending on the day of ovulation[14]. This interval has been termed the 'fertile window' and probably reflects the effects of increasing pre-ovulatory oestradiol levels on vaginal mucus, cervical opening (the so-called 'pupil sign')[15] and subendometrial contraction waves that permit and facilitate sperm transport through the female reproductive tract (Figure 2)[16]. The length of the fertile window varies considerably between couples and from cycle to cycle. Nevertheless, a strong inverse correlation exists between the length of the fertile window and TTP, at least in subfertile couples[17]. Thus, theoretically an abnormally long fertile window would increase fertilisation and conception rates, thus accounting for the superfertile end of the human

fecundity spectrum. However, this scenario only works if fertilisation is a rate-limiting step in normal couples, an assumption that in view of the extraordinary high rates of preimplantation loss and early pregnancy failure seems unlikely.

## The implantation window

Increased fertilisation or conception rates, however, do not necessarily equate to increased pregnancy rates. Approximately 30% of spontaneously conceived embryos are lost prior to implantation and in excess of 50% of IVF embryos fail to implant (Figure 1)[18]. Implantation is a complex biological process that dependents on two-way communication between the embryo and the endometrium[19]. This embryo-maternal dialogue requires highly orchestrated, progesterone-dependent changes in the endometrium to render it responsive to embryonic signals. Although an embryo from one species cannot implant in another[20], the factors that confer endometrial receptivity are thought to be highly evolutionarily conserved. Cardinal regulators of the implantation process include growth factors, cytokines and their receptors (e.g. heparin-binding EGF-like growth factor, leukemia inhibitory factor, prokineticin 1, interleukin-11)[21-24], cell adhesion molecules and ligands, signal intermediates and downstream transcription factors (e.g. STAT3, progesterone receptor, HOXA10, p53)[25-29]. In humans, the period of endometrial receptivity or 'implantation window' starts approximately 6 days after ovulation and lasts no longer than 4 or 5 days (Figure 2)[6,30].

From a teleological perspective, a limited implantation window ensures that the embryo achieves implantation competence in synchrony with an optimal uterine environment[31]. Indeed, different evolutionary strategies of implantation have emerged to maximise this concept for reproductive success. For instance, many mammals, including rodents, marsupials, roe deer, and the nine banded armadillo, display delayed implantation, termed embryonic diapause, which is characterized by a temporary suspension of embryo development prior to implantation[32]. Embryonic diapause can either be induced in response to environmental signals (facultative diapause) or occur in every gestation (obligate diapause) and is reversed when optimal environmental, metabolic and hormonal conditions are achieved.

There is, however, little or no evidence of such uncoupling of pre- and post-implantation development in human embryos. The prevalence of gross mitotic chromosomal errors in preimplantation human embryos is exceptionally high, even in young fertile women[33-36]. In addition, human embryos give rise to deeply invading placentae. Thus, by synchronizing implantation with embryo development, a restricted period of endometrial receptivity may protect the mother from being invaded by chaotic human embryos, which have been compared from a genetic perspective to cancer cells[37].

There is ample clinical evidence that endometrial refractoriness during the putative window of implantation is a cause of subfertility and IVF treatment failure[18,38]. In the absence of informative histological features, many investigators have adopted gene expression, and more recently proteomic profiling, to determine the 'molecular fingerprints' of receptive endometrium with a view to exploiting these for diagnostic and therapeutic purposes[30,39]. Because of differences in design

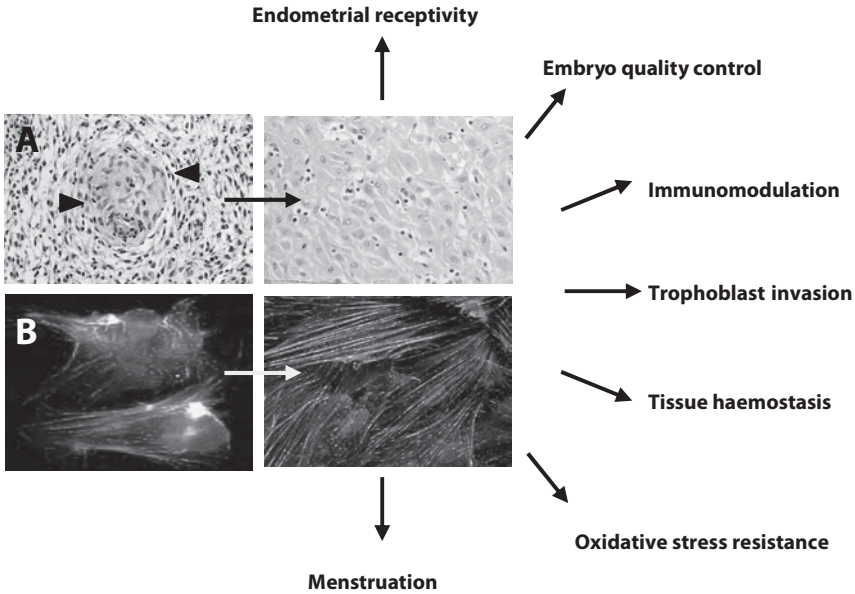
and analyses, the results of these studies have been largely disparate although a small number of common 'endometrial receptivity' genes has emerged [30]. Alternatively, uterine cavity aspirations or flushings can be used to explore the secretory factors expressed in the uterine environment. Boomsma *et al.* have shown that the expression profile of putative markers of receptivity, such as tumor necrosis factor (TNF)-alpha and interleukin (IL)-1beta, in endometrial secretions aspirated prior to embryo transfer is predictive of successful implantation[18]. While these analyses are informative, they are not yet predictive enough to be introduced in routine clinical practice.

The corollary of a restricted implantation window implies that the persistent expression of a receptive phenotype will increase implantation rates, albeit at the expense of embryo quality control and optimal embryo-maternal interactions (Figure 1). In other words, while unravelling the molecular repertoire that confers endometrial receptivity is important in the context of subfertility, understanding the signals that terminate the window of implantation may be equally important to prevent early pregnancy loss. In human beings, as well as in the handful of other menstruating species, the end of the implantation window approximately 10 days after ovulation coincides with the extraordinary transformation of endometrial fibroblast into secretory, epitheloid decidual cells[40-42]. Moreover, strong experimental evidence has emerged to show that factors secreted by decidual cells, such as LEFTY-A (also known as endometrial bleeding-associated factor or EBAF), terminate the window of implantation in fertile women and compromise endometrial receptivity in subfertile patients[43,44].

## The window of natural embryo selection

Once the luminal epithelium is breached, most implanting mammalian embryos will trigger a maternal decidual response. The situation in human beings differs in that the decidual transformation of the endometrium is primarily under maternal control and initiated in the mid- to late secretory phase of each cycle, irrespective of the presence or absence of a pregnancy. Decidualization bestows unique characteristics on the endometrium essential for placenta formation, including the ability to regulate trophoblast invasion, to modulate local vascular and immune responses, and to resist environmental and oxidative stress (Figure 3)[41,45,46]. Depending on severity, failure to express an adequate decidual phenotype inevitably results either in early pregnancy loss or predisposes to obstetrical complications associated with impaired placental function, such as pre-eclampsia, fetal growth restriction and preterm birth[47-49]. Further, once the decidual process is initiated, the integrity of the endometrium becomes entirely dependent on continuous progesterone stimulation. Consequently, falling progesterone levels in the absence pregnancy trigger a cascade of events that results in proteolytic breakdown of the decidualizing superficial endometrium, focal bleeding, cell death and, ultimately, menstruation[50-52].

**Figure 3. Decidual transformation of human endometrial stromal cells *in vivo* and *in vitro*.** (A) Decidualization of the stromal compartment (arrowheads) is initiated during the mid-secretory phase of the cycle in stromal cells that surround the terminal portion of the spiral arteries (left panel) and then spreads to encompass the entire superficial endometrial layer by the late luteal phase of the cycle (right panel). (B) Phalloidin staining of filamentous actin in undifferentiated and decidualized primary endometrial stromal cells in culture (left and right panels, respectively) highlights the phenotypic reprogramming of the cells that underpins their very diverse functions. Decidualization is characterized by a dramatic increase in filamentous actin polymerization and stress fibre formation (red staining). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue stain).




The question arises as to what the purpose of the emergence of cyclic decidualization in the absence of pregnancy could have been, especially when the only overt consequence is repeated, and often punitive, menstrual events? One possible answer to this fundamental question is provided by the 'menstrual preconditioning' hypothesis, a novel concept based on three key observations. First, it is striking that reproduction in human beings has evolved in ways that ensures that cyclic menstruation virtually always precedes pregnancy. For example, human beings do not exhibit overt oestrous behaviour, have a limited fertile window and concealed ovulation, lack embryonic diapause, and over 90% of all menstrual cycles in very young adolescent girls are anovulatory[53,54]. Second, cyclic endometrial decidualization followed by menstrual shedding is confined to species where placenta formation entails deep trophoblast invasion of maternal tissues and its vasculature[55,56]. Both menstruation and pregnancy are inflammatory conditions that cause a degree of physiological ischemia-reperfusion tissue injury, albeit much more so in pregnancy[57]. Third, repetitive brief exposures of any organ to a harmful stimulus at a dose below the threshold for tissue injury provides robust protection against, or tolerance to, the injurious effects of a subsequent more severe insult.

This paradoxical yet ubiquitous biological phenomenon is termed 'preconditioning'[58,59]. Put together, these observations indicate that cyclic decidualization of the endometrium followed by menstrual shedding preconditions, and thus protects, uterine tissues from the profound hyperinflammation and oxidative stress associated with deep trophoblast invasion during pregnancy[48].

Serendipitously, we recently obtained evidence in support of an alternative explanation for the emergence of cyclic decidualization in the absence of pregnancy, which relates to the unique human challenge of coping with the extraordinarily high prevalence of developmentally compromised but invasive embryos. We used a validated human co-culture model, consisting of hatched blastocysts cultured on decidualizing endometrial stromal cells, in an attempt to identify key factors involved in the embryo-maternal cross-talk in early pregnancy. To do this, co-culture supernatants were collected after 3 days, the levels of a host of putative implantation cytokines, growth factors and chemokines measured, and compared to the levels produced by decidualizing endometrial stromal cells in the absence of an embryo[13]. The results were disappointing in so far that developing human embryos had no detectable effects on the decidual secretions, bar for a modest decrease in interleukin (IL)-5 expression. However, a phenomenal response was noted when embryos became compromised during the co-culture period. When the blastocyst appeared morphologically to be arresting, the decidualizing cells responded by shutting down the production of key implantation mediators and immunomodulators, which included IL-1β, -6, -10, -17, -18, eotaxin, and heparin-binding EGF-like growth factor. The response of decidual cells was selective as the presence or absence of a developmentally compromised embryo had no effect on IL-12, -15, TNF-alpha, monocyte chemotactic protein-1 or chemokine (C-X-C motif) ligand 10 production[13]. We then went on to repeat the co-culture experiments, but this time with endometrial stromal cells which had not been decidualized, and found that the blastocyst no longer triggers a maternal response, irrespective of whether the embryonic development in culture was normal or not. In other words, human endometrial stromal cells are biosensors of embryo quality but only upon differentiation into decidual cells (Figure 2)[13].

These unexpected observations raise a number of important questions. For example, what is the nature of the signals emanating from compromised embryos? And what is the mechanism that allows human endometrial cells to sense and respond to this signal upon decidualization? These questions remain as yet unanswered. Nevertheless, the widely-held view that developmentally compromised embryos are biologically inert, and therefore irrelevant, is pertinently wrong. In fact, it is well established that compromised embryos are metabolically hyperactive[60] and the degree of amino acid turnover has recently been linked to the cytogenetic composition and health of human preimplantation embryos[61]. Further, the notion that maternal decidual cells mount a tailored response to individual embryos may in fact be a highly conserved reproductive feature. For example, a recent study in the bovine demonstrated that endometrial gene expression varies dramatically in response to implantation of an embryo conceived *in vivo*, after IVF or following somatic cell nuclear transfer[62].

In summary, we propose that spontaneous decidualization during the mid-secretory phase of the cycle is a unique evolutionary adaptation that, on the one hand, serves to recognize and eliminate unwanted implanting embryos and, on the other, prepares the uterus for deep placentation in pregnancy by triggering cyclic menstruations. At first glance, these proposed functions of the decidual process, natural embryo selection and uterine preconditioning, may appear disparate but both mechanisms must clearly work hand in glove to ensure reproductive success.

## Impaired natural embryo selection and recurrent pregnancy loss

As outlined, failure to establish a functional window of natural embryo selection would account for the pathological association between superfertility and persistent pregnancy loss (Figure 2). In fact, the concept of impaired embryo selection underpinning miscarriages is not new. Wilcox and colleagues, studying a cohort of 221 fertile couples, demonstrated a dramatic increase in the risk of an early loss if the pregnancy was established beyond the normal 'implantation window'[31]. This in turn fits well with the finding of another population study, demonstrating that rapid conceptions are associated with increased risk of early pregnancy loss[63]. Thus, even in unselected low-risk populations, there is evidence, albeit circumstantial, to suggest that inadequate embryo selection, reflected by short TTP, contributes to sporadic miscarriages.

Biological evidence in support of the lack of embryo selection has come from endometrial studies in recurrent miscarriage patients. Comparative analysis of timed mid-secretory endometrial biopsies showed that RPL is associated with reduced epithelial secretory function, altered uterine fluid composition, and lower expression levels of mucin 1, an anti-adhesion molecule that contributes to the barrier function of luminal epithelium[64-66]. These initial observations, which focussed on epithelial cell function and markers of uterine receptivity, led to the hypothesis that RPL is primarily caused by failure of natural embryo quality control[67]. In other words, embryos that are destined to fail are allowed to implant.

Although the endometrial luminal epithelium is the primary barrier in the implantation process, the progesterone responses in this cellular compartment that underpin the receptive phenotype are mediated by signals derived from the underlying stromal cells[68]. Moreover, while dysregulation of epithelial receptivity markers may account for the excess of genetically abnormal pregnancies in RPL patients, it does not explain why many affected women will experience a mixture of chromosomal and non-chromosomal losses[69]. Our observation that endometrial stromal cells function as biosensors of embryo quality upon decidualization provides the possible 'missing link', as it associates lack of embryo selection with subsequent impaired placentation, irrespective of the intrinsic developmental potential of the conceptus. Notably, differentiating endometrial stromal cells produce factors, such as IL-11 and IL-15, responsible for the recruitment and programming of specialized uterine natural killer (uNK) cells[70-72]. Ever since Sir Peter Medawar first posed the problem of the fetus-as-allograft in 1953[73] there have been numerous attempts to establish an immunological

basis for persistent pregnancy failure, with much of the work focussed on uNK cells[74-76]. A detailed discussion of uNK cells in RPL is beyond the scope of this article, although it is fair to state that no causal links have as yet been established[77]. Perhaps this is not unexpected as immunological adaptation of the uterus to the feto-placental semi-allograft is, unlike cyclic decidualization and menstruation, an evolutionary tested phenomenon that occurs in all mammalian species with an invading conceptus.

Evidence that the ability of endometrial stromal cells to express a decidual phenotype is impaired in RPL came from a series of deceptively simple experiments that measured the expression of two marker genes[10]. The first marker gene was *PROK1*, which encodes prokineticin 1 (PROK1), a recently discovered cytokine that promotes endometrial receptivity and embryo-uterine interaction via induction of leukaemia inhibitory factor in endometrial epithelial cells[21]. The second marker was prolactin (PRL), which is the prototypic marker of decidualizing endometrial stromal cells[40,78-80]. PRL in differentiating endometrial cells is transcribed from an alternative promoter upstream of a non-coding exon, located approximately 6 kb upstream of the pituitary-specific transcriptional start site[81,82]. Interestingly, the key regulatory region of the decidua-specific *PRL* promoter is a transposon, termed MER20, which emerged in evolution upon divergence of eutherian (placental) from non-placental mammals[80,83]. Transcript levels of both marker genes were first determined in endometrial biopsies, timed to span the implantation window, from RPL patients and controls, consisting of either fertile or infertile women without a history of recurrent pregnancy failure. Compared with controls, RPL was associated with significantly higher endometrial PROK1 mRNA levels and, strikingly, with approximately 100-fold lower PRL transcript levels[10].

Increased endometrial PROK1 and decreased PRL levels fit well with the concept that enhanced uterine receptivity but decreased embryo recognition and selection underpin RPL. Intuitively, the results seem to suggest that the regulatory signals that control differentiation of endometrial stromal cells into specialized decidual cells are perturbed in RPL. Decidualization is first apparent approximately 10 days after ovulation, indicating that progesterone is not the primary trigger of this differentiation process. Instead, there is overwhelming evidence that initiation of the decidual process is dependent on elevated levels of the second messenger cAMP[40,84,85], which is accounted for by increased expression of local factors that activate adenylate cyclase in stromal cells (e.g. relaxin, corticotropin releasing hormone and prostaglandin E2) and the simultaneous downregulation of PDE4, a phosphodiesterase family member that converts cAMP back to AMP[86]. Upon continuous cAMP stimulation and protein kinase A activation, endometrial stromal cells become first responsive and then dependent on steroid hormones, foremost on progesterone but also on androgens[40,78]. Thus, theoretically, perturbation in one or more of the endocrine or paracrine signals involved in regulating the decidual phenotype could be causal to RPL. Alternatively, subtle differences in the timing of the endometrial biopsies could conceivably also account for the differential expression of *PROK1* and decidual *PRL* in RPL and non-RPL patients.

However, a second set of experiments demonstrated that the dysregulation of the decidual

phenotype in RPL was neither the consequence of sampling errors nor the results of an altered endocrine environment. These experiments were inspired by recent studies on endometriosis, demonstrating that purified eutopic endometrial cells from affected patients continue to respond differently to hormonal stimuli when compared to primary cultures from disease-free controls[87-89]. Briefly, we obtained endometrial samples randomly in the cycle from RPL and non-RPL patients, purified the stromal cells, passaged the cultures once, and then decidualized the cells *in vitro* in a time-course lasting 8 days[10]. PROK1 and PRL mRNA levels did not differ between the two groups in undifferentiated cells or in cultures decidualized for 2 days. However, after 4 days of differentiation, the rise in PRL transcript levels was several magnitudes higher in the control group when compared to RPL samples. PROK1 levels continued to rise with comparable kinetics in all decidualizing cultures until day 8 when expression in the control but not RPL group declined markedly. Thus, the perturbed PROK1 and decidual PRL expression in secretory endometrium *in vivo* was perfectly recapitulated upon decidualization in primary cultures from RPL patients, even when the cultures were established from biopsies taken randomly in the cycle. In addition to attenuated PRL production and prolonged and enhanced PROK1 expression, we also found that RPL is further associated with a complete dysregulation of both markers upon treatment of primary cultures with human chorionic gonadotropin, a glycoprotein hormone abundantly expressed by the implanting embryo[10].

Thus, rather than being caused by perturbations in maternal or embryonic signals, RPL appears to be a consequence of the intrinsic failure of endometrial stromal cells to express an appropriate decidual phenotype. However, even after 3 consecutive miscarriages, many RPL patients will ultimately achieve a successful pregnancy[9], suggesting that this intrinsic failure may relate to a modifiable programming event in endometrial cells, as suggested by the 'menstrual preconditioning' hypothesis[48]. Sustained programming of cellular responses likely involves epigenetic changes like DNA methylation or posttranslational histone tail modifications. Interestingly, inflammatory signals are important epigenetic modifiers[90], which raise the possibility that the tissue trauma associated with menstrual events between pregnancies, may provide cues that dynamically modulate subsequent decidual responses in the endometrium[91]. Perhaps the most compelling evidence that inflammatory signals modify endometrial differentiation responses has come from the baboon model of endometriosis. Kim and co-workers demonstrated that induction of endometriosis and chronic inflammation in this model resulted in a gradual decrease in endometrial HOXA10 expression, a homeobox transcription factor involved in endometrial development and differentiation[92]. Importantly, this down-regulation was only significant 6 to 12 months after the induction of endometriosis and corresponded to increased methylation of the proximal promoter of the *HOXA10* gene. In fact, the ability of local tissue injury to modify subsequent decidual responses has been long recognized and increasingly exploited for clinical purposes. In 1971, Karow et al. reported that an endometrial biopsy decreases subsequent miscarriage rates, especially in subfertile patients[93], a finding confirmed in several more recent observational studies[94-97].

## Conclusions and perspective

The efficacy of human reproduction is unequivocally constrained by the high prevalence of chromosomal errors in preimplantation embryos and the requirement of the conceptus to establish a deeply invading placenta for survival. We propose that the emergence of cyclic decidualization of the endometrium, which is inextricably linked to menstruation, is not an unfortunate evolutionary 'accident' but the adaptive maternal response to these embryonic innovations[98,99]. We have coined the term 'window of natural embryo selection' as it reflects the functional role of the decidualizing stromal cells in assessing the quality of embryos that have breached the luminal epithelium. Moreover, the decidual process plays a role in terminating the window of endometrial receptivity and enables the mother to dispose compromised embryos by inducing menstruation-like tissue breakdown and shedding upon falling progesterone levels[100]. Importantly, the ability of the human endometrium to mount an adequate decidual response for pregnancy may neither be innate nor solely dependent on the right endocrine and paracrine cues. Instead, repeated inflammatory events and tissue trauma, such as cyclic menstruation and even early pregnancy failures, may play a major role in sensitizing uterine tissues to decidualizing signals[10,48].

Lack of natural embryo selection inevitably causes biological superfertility, characterized by prolonged endometrial receptivity, inadequate decidualization of resident stromal cells and a dysregulated maternal response to embryonic signals. A substantial proportion of affected patients will report persistent short TTP, whereas others will not or may suffer from subfertility, depending on the presence or absence of concurrent reproductive disorders[10].

The concept of a window of natural embryo selection during the cycle emphasizes the need for human models to study early implantation events and reproductive failure; and cautions against uncritical extrapolations of findings in animal models, such as the mouse[101]. More importantly, lack of embryo selection, i.e. biological superfertility, provides a single pathological pathway to explain chromosomal and non-chromosomal pregnancy failure and correctly predicts the association between miscarriage and late obstetrical complications in subsequent ongoing pregnancy caused by impaired deep placentation[102]. At the same time, it explains why miscarriage in many patients tends to be a transient disorder, often resolving spontaneously, even after 3 or more consecutive losses[9]. Finally, and potentially far-reaching, the natural human embryo selection paradigm infers that early pregnancy complications may be preventable by targeting the endometrial decidual response prior to pregnancy or immediately after implantation. In fact, many drugs currently employed in the management of RPL, such as progesterone, weak androgens like dehydroepiandrosterone, glucocorticoids, and even heparin, directly modulate the decidual process[48,78,103,104]. Whether or not these drugs, alone or in combination, are truly effective may foremost lie in the timing of their administration.

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# Chapter 8

## GENERAL DISCUSSION

Implantation still is a major obstacle in human reproduction and remains the rate-limiting step in fertility treatments where morphologically good quality embryos are transferred. It remains unclear which player, the embryo or the endometrium, initiates the preimplantation dialogue. Does a viable embryo actively signal the endometrium to prepare for implantation or is the receptive endometrium by default available for implantation, unless it recognizes the presence of an abnormal embryo? The aim of the work presented in this thesis was to address this question by investigating the interactions between the receptive endometrium and the preimplantation human embryo.

In vitro models provide a means of addressing a number of challenges that have hindered progress in understanding human implantation. In this thesis, we describe a model that is not only informative for the study of human implantation, but also provides the opportunity to study the chromosomal status and developmental competence of a human peri-implantation embryo beyond day 6 of development. The possible implications of a better understanding of embryo development and human implantation extend from improving success rates in fertility treatment to the development of treatment regiments of disorders related to implantation and placentation, including recurrent pregnancy loss, pre-eclampsia and fetal growth restriction.

## Embryonic development *in vitro*

Unravelling the mechanisms that control implantation is paramount to improving reproductive outcome, especially in couples suffering from infertility or recurrent pregnancy loss. Gene deletion studies leading to reproductive failure in mice have been hugely important in defining factors essential for embryo-endometrial interactions [1-3]. However, differences between species hamper direct translation of these animal studies directly to the human situation. First, it is the embryo that triggers a decidual response in mice and most other species, yet initiation of this process is uncoupled from embryonic signals in humans [4]. Second, gross embryonic chromosomal abnormalities are rare in most mice strains, which means that little if anything is known about the fate of developmentally impaired embryos. In **chapter 2** we discuss the available models to study human embryo implantation available today. Though we believe that a model should mimic the in vivo situation as closely as possible, a model should also be easy to establish and generate reproducible results to investigate a specific event during implantation. **Chapter 3** exploits a model combining human endometrial stromal cells (HESCs) and a hatched human embryo. In the study, we performed FISH analysis of human embryos in order to assess the frequency of chromosomal mosaicism at three different stages of development (morula, preimplantation blastocyst and peri-implantation blastocyst). Consistent with previous findings [5], we found almost all embryos to be mosaic at the morula stage. This high rate of mosaicism is reflected in our results obtained after biopsy and FISH analysis of two cells from day 4 embryos. The high incidence of chromosomal abnormalities in cleavage stage embryos has been brought to light by several studies employing PGS-FISH over the past years [6]. In this study we found that the incidence of mosaic embryos decreased

over time. Also, we found a positive correlation between the total number of cells in the embryo and the proportion of chromosomally normal cells in developing day 5 and day 8 blastocysts. We observed that FISH diagnosis on 1 or 2 blastomeres of day 4 embryos was not predictive of subsequent developmental potential, which questions the added value of PGS in routine clinical practice. Apparently, the presence of chromosomally abnormal cells does not exclude blastocyst development [7-10]. However, our data also suggest that a significant proportion of mosaic embryos undergo developmental arrest before reaching the blastocyst stage. According to the model proposed by Evsikov and Verlinsky [11], if the number of aneuploid cells at the morula stage reaches a certain threshold level, there is self-elimination (arrest) of the whole embryo. Embryos with a number of aneuploid cells below the threshold level develop further and reach the blastocyst stage. So far this hypothesis of a threshold has not been directly investigated, but mouse knockout models have shown that up to 30% of aneuploid cells can be tolerated in apparently healthy animals [12]. This study is the first to provide insight in the fate of chromosomally abnormal cells in human embryos during development from the morula stage up to the peri-implantation stage of day 8. It is however important to note that the embryos studied have been cultured *in vitro* for an extended time period. Furthermore, embryos have been selected (first for cryopreservation and later for co-culture from day 5 to day 8) and therefore may not reflect the general blastocyst population. Additionally, we only analysed the copy number of 10 of the 23 pairs of chromosomes and we have no information on the incidence of structural abnormalities. These have recently been demonstrated to be highly prevalent in human IVF embryos [13]. However, the embryos used in this study represent the best currently available model accessible for research, allowing normal normal embryo developmental trajectory as discussed in **chapter 4**.

## Preimplantation embryo-endometrial interactions

The study presented in **chapter 4** addresses one of the key research questions posed at the outset of this project. We found that decidualized, but not undifferentiated HESCs selectively recognize the presence of a developmentally impaired embryo and respond by inhibiting the secretion of key implantation mediators (e.g. IL-1β and HB-EGF) and immunomodulators (e.g. IL-5, -6, -10, -11, -17, and eotaxin). Strikingly, developmentally competent embryos did not influence the panel of implantation mediators measured in these experiments. The importance of the endometrial stromal compartment in regulating early human implantation events has been demonstrated before [14,15]. Our and others work have thus shown that the endometrium is actively involved in early implantation events rather than simply playing a non-selective, passive role in allowing invasion [14-16]. Two important limitations of this study must be recognized. First, though we intend to faithfully score the morphology of the embryos in co-culture to assess developmental potential, the ultimate proof of embryo quality remains a viable pregnancy. Second, the nature of the embryonic signals capable of modulating the decidual secretome and the underlying mechanism remain elusive. Conversely, it is also important to ascertain if the nature and magnitude of the decidual response correlates

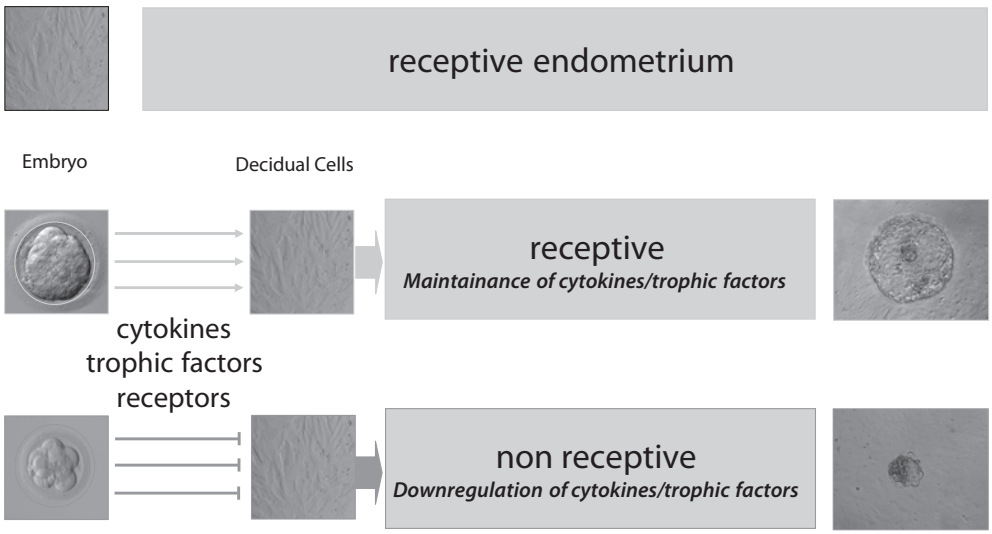
somehow with the frequency and complexity of gross chromosomal errors in the co-cultured blastocyst. A finely tuned, tailored decidual response is not beyond the realm of possibilities as a recent study in the bovine demonstrated that endometrial gene expression varies dramatically in response to implantation of an embryo conceived *in vivo*, after IVF or following somatic cell nuclear transfer [17].

**Chapter 5** aims to address some of these questions. We compared the transcriptional responses of decidualizing endometrial stromal cells to secreted signals from human poor quality embryos and from embryos that resulted in viable pregnancies. Consistent with the observations reported in **chapter 4**, we demonstrated that maternal decidual cells mount an extraordinarily graded transcriptional response to embryos of different quality. A possible mechanism behind this is described in the same chapter, in which we have shown that poor quality embryos trigger a complex maternal response, leading to endoplasmic reticulum (ER) stress in the surrounding decidual cells. This means that human embryos, even at the preimplantation stage, signal their developmental competence, although the nature of this signal remains to be defined. It is tempting to speculate that the heightened metabolism and the byproducts displayed by poor quality embryos can serve as a gradient signal for maternal recognition. Conversely, preimplantation competent human embryos are known to be metabolically quiet, which may render them virtually invisible to decidual cells, at least until the onset of secretion of major peptide hormones, such as human chorionic gonadotropin. The mechanism behind this is illustrated in Figure 1. Within this context, we propose that the expression of a decidual phenotype in the human endometrium serves as a sensor of embryo quality. Furthermore we speculate that aberrant decidualization hampers natural embryo selection and lead to the implantation of developmentally impaired embryos that will miscarry later in pregnancy.

## Recurrent pregnancy loss and the concept of natural embryo selection

In **chapter 6** we provide evidence that impaired decidual programming of the endometrium is an important mechanism underlying recurrent pregnancy loss (RPL). This is based primarily on the observation that prolactin expression [18,19] is grossly impaired in RPL patients, both *in vivo* as well in primary cultures subjected to a decidualizing stimulus. We also show that uterine receptivity is enhanced and prolonged in recurrent pregnancy loss patients, as differentiating HESCs from RPL patients abundantly express PROK1, a cytokine that promotes embryo-uterine interaction via induction of leukaemia inhibitory factor (LIF) [20]. The concept of enhanced endometrial receptivity in RPL is supported by a previous study demonstrating that affected women express lower levels of mucin 1, an anti-adhesion molecule that contributes to the barrier function of luminal epithelium [21,22]. As the signal initiating the maternal response described in **chapter 4 and 5** remains elusive, we

**Figure 1. Preimplantation embryo-endometrial signalling**
In these three different scenarios, a possible mechanism for maternal recognition of embryo quality is proposed. The upper scenario illustrates the situation before embryo interaction, where the endometrium is supposed to be receptive by default, but act as a sensor of embryo quality. In the middle, a developmentally competent embryo is able to implant because the metabolically silent embryo doesn't alter the receptive phenotype of the endometrium. Below, the scenario of a developmentally compromised embryo is outlined. The metabolic overdrive in such a "noisy" embryo, signals the endometrium to terminate receptivity and shed the endometrium during menstruation.




set out and investigated the endometrial responses to embryonic hCG signalling. We found that the endometrial response in RPL patients to hCG is characterized by induction rather than inhibition of PRL and PROK1 expression as compared to controls. Although our expression analysis was confined to *PRL* and *PROK1*, the data demonstrate that endometrial programming in preparation of pregnancy is both quantitatively and qualitatively different in RPL, characterized by impaired decidualization of resident stromal cells, prolonged endometrial receptivity and a dysregulated maternal response to embryonic signals. The concept of defective embryo selection explains why some RPL patients appear to be exceptionally fertile. Our retrospective analysis of TTP revealed that 40% of RPL patients report very short time to conception for each pregnancy. Thus, the prevalence of 'superfertile' couples in this population appears to be considerably higher than expected.

The efficacy of human reproduction is unequivocally constrained by the high prevalence of chromosomal errors in preimplantation embryos and the requirement of the conceptus to establish a deeply invading placenta for survival. We propose that the emergence of cyclic decidualization of the endometrium, which is inextricably linked to menstruation, is not an unfortunate evolutionary 'accident' but the adaptive maternal response to these embryonic innovations [23,24]. We propose

a new 'window of natural embryo selection' as it reflects the functional role of the decidualizing stromal cells in assessing the quality of embryos that have breached the luminal epithelium. Moreover, the decidual process plays a role in terminating the window of endometrial receptivity and enables the mother to dispose compromised embryos by inducing menstruation-like tissue breakdown and shedding upon falling progesterone levels [25]. Importantly, the ability of the human endometrium to mount an adequate decidual response for pregnancy may neither be innate nor solely dependent on the right endocrine and paracrine cues. Instead, repeated inflammatory events and tissue trauma, such as cyclic menstruation and even early pregnancy failures, may play a major role in sensitizing uterine tissues to decidualizing signals [4,26]. Lack of natural embryo selection inevitably causes biological superfertility, characterized by prolonged endometrial receptivity, inadequate decidualization of resident stromal cells and a dysregulated maternal response to embryonic signals. A substantial proportion of affected patients will report persistent short TTP, whereas others will not or may suffer from subfertility, depending on the presence or absence of concurrent reproductive disorders [26].

## Preimplantation embryo-endometrial signalling

Investigating preimplantation embryo-endometrial signalling remains a challenge. The concept of a window of natural embryo selection during the cycle emphasizes the need for human models to study early implantation events and reproductive failure; and cautions against uncritical extrapolations of findings in animal models, such as the mouse [27]. More importantly, lack of embryo selection, i.e. biological superfertility, provides a single pathological pathway to explain chromosomal and non-chromosomal pregnancy failure and correctly predicts the association between miscarriage and late obstetrical complications in subsequent ongoing pregnancy caused by impaired deep placentation [28]. At the same time, it explains why miscarriage in many patients tends to be a transient disorder, often resolving spontaneously, even after 3 or more consecutive losses [29]. Finally, and potentially far-reaching, the natural human embryo selection paradigm infers that early pregnancy complications may be preventable by targeting the endometrial decidual response prior to pregnancy or immediately after implantation. In fact, many drugs currently employed in the management of RPL, such as progesterone, weak androgens like dehydroepiandrosterone and even heparin, directly modulate the decidual process [30-33]. Whether or not these drugs, alone or in combination, are truly effective may foremost lie in the timing of their administration.

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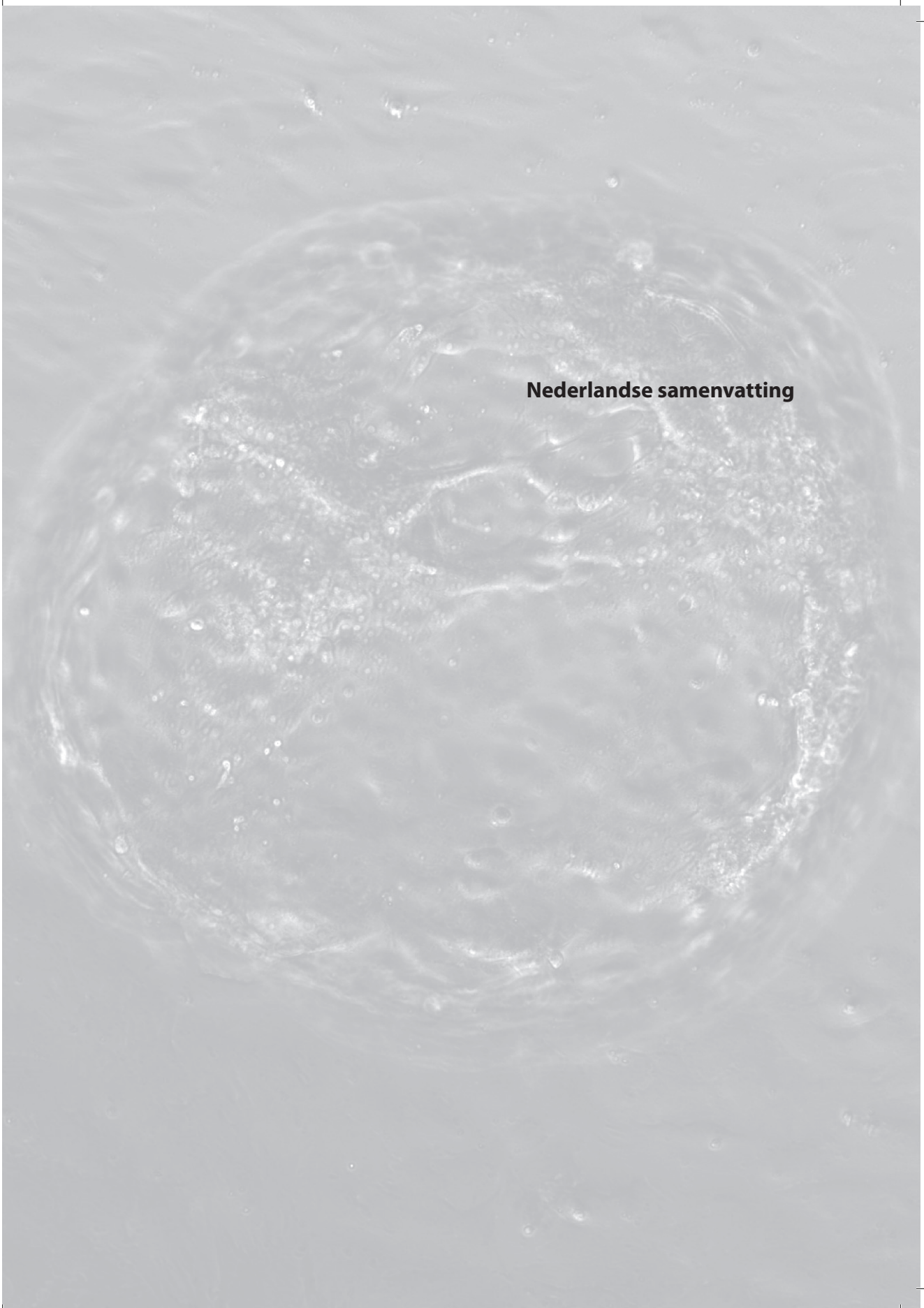


# Nederlandse samenvatting

## Hoofdstuk 1

In de introductie van dit proefschrift wordt de observatie besproken dat de menselijke voortplanting bijzonder ineffectief is. Zwanger worden is voor veel mensen een groot probleem. Zelfs als beide partners volledig gezond zijn, is de kans op een zwangerschap maandelijks slechts 20%. Een belangrijke reden hiervoor is dat een groot deel van de embryo's die op natuurlijke wijze of na In Vitro Fertilisatie (IVF) tot stand komen, van slechte kwaliteit zijn. Daarnaast nestelen embryo's van goede kwaliteit vaak in het geheel niet in en een groot gedeelte dat zich wel innestelt leidt later tot een miskraam. Het is echter niet bekend hoe en waarom dit bij mensen zo werkt.

Eerder onderzoek heeft aangetoond dat het endometrium slechts gedurende een specifieke periode in een menstruatiecyclus ontvankelijk is voor een embryo, deze periode wordt "the window of implantation" genoemd. Tijdens deze periode worden er lokaal in het endometrium cytokinen, chemokinen en groeifactoren geproduceerd welke in aanwezigheid van een competent embryo zorgen voor een gereguleerd proces van implantatie. Vanzelfsprekend speelt het embryo hierin zelf ook een belangrijke rol. De wisselwerking tussen een embryo en het endometrium bepaalt uiteindelijk of implantatie succesvol zal verlopen. Na implantatie is er echter nog een lange weg te gaan tot de geboorte van een gezond kind. In dit eerste hoofdstuk schetsen we daarom het probleem van een bijzondere groep patiënten. Sommige vrouwen ervaren namelijk geen problemen om zwanger te worden, maar wel om het te blijven: vrijwel alle zwangerschappen eindigen in een miskraam. Vrouwen die drie of meer miskramen hebben gehad, hebben volgens de medische definitie herhaalde miskramen. De oorzaak hiervan is vaak onbekend. Om het implantatieproces en de vroege complicatie van (herhaalde) miskramen beter te begrijpen, hebben wij verschillende studies verricht die in dit proefschrift worden besproken.

## Hoofdstuk 2

Het proces van humane implantatie laat zich niet gemakkelijk onderzoeken. Om voor de hand liggende redenen is het niet mogelijk om embryo-endometrium interactie *in vivo* bij de mens te bestuderen. De meeste beschikbare resultaten zijn afkomstig van dierexperimenteel onderzoek. Dankzij de techniek van gene knock-down kan de rol van individuele factoren tijdens implantatie onderzocht worden in de muis. Dit heeft geleid tot de indentificatie van enkele belangrijke cytokinen, waarvan de functie bij de mens nog verder wordt onderzocht (bijv. LIF [Leukemia Inhibitory Factor]). Doordat er grote verschillen bestaan in endometriumdifferentiatie, implantatie en uiteindelijk placentatie, is het onmogelijk om dierexperimentele resultaten altijd te extrapoleren naar de mens. Sinds de introductie van IVF is het mogelijk om niet alleen het humane embryo zelf, maar ook de interactie ervan met het endometrium te bestuderen. In dit hoofdstuk worden de huidige beschikbare *in vitro* modellen besproken, waarmee de interactie van een menselijk embryo met het endometrium kan worden onderzocht. De inventarisatie van deze modellen heeft ertoe geleid dat wij een geschikt model hebben gekozen voor het beantwoorden van enkele onderzoeksvragen. De uitgevoerde studies zullen in de navolgende hoofdstukken worden besproken.

## Hoofdstuk 3

Zoals eerder gesteld, zijn menselijke embryo's vaak van slechte kwaliteit. Een veelvoorkomende observatie in vroeg ontwikkelende embryo's zijn chromosomale afwijkingen. Pre-implantatie Genetische Screening (PGS) heeft laten zien dat slechts een klein deel van de embryo's een normaal chromosomenpatroon heeft na het screenen van één of twee blastomeren van een acht-cellig stadium van ontwikkeling. Een klinisch dilemma ontstaat wanneer één van beide gescreende blastomeren een afwijkend chromosomenpatroon heeft en de ander chromosomaal normaal is, dit wordt mozaïcisme genoemd. Het is nog onvoldoende onderzocht hoe een dergelijk embryo zich verder ontwikkelt. In dit hoofdstuk laten we zien dat het mogelijk is om met een *in vitro* model een humaan embryo te laten ontwikkelen. Dit brengt ons in de unieke positie om het lot van een embryo met mozaïcisme van dag 4 tot dag 8 van ontwikkeling te onderzoeken. Met fluorescent in situ hybridisation (FISH) hebben wij zowel met PGS op dag 4 in één of twee blastomeren, als op dag 5 en 8 in alle individuele cellen van embryo's de chromosomen 1, 7, 13, 15, 16, 18, 21, 22, X en Y geanalyseerd. De diagnose op dag 4 was in 54% van de gevallen mozaïcisme. Uit het onderzoek blijkt dat het percentage embryo's met mozaïcisme op dag 8, in vergelijking met dag 4, ongeveer gehalveerd is. Verder stellen wij vast dat er een positieve correlatie bestaat tussen het aantal cellen van een embryo op dag 5 en 8 en het percentage cellen met een normaal chromosomenpatroon. De observaties van deze studie suggereren dat een groot gedeelte van de embryo's met mozaïcisme zich reeds in een vroeg stadium niet verder ontwikkelt. In embryo's met mozaïcisme die zich wel ontwikkelen tot na dag 5, neemt het percentage normale cellen toe naarmate de ontwikkeling vordert. Hieruit blijkt dat embryo-ontwikkeling een dynamisch proces is en dat het onvoorspelbaar is hoe een embryo met een PGS diagnose mozaïcisme zich verder zal ontwikkelen.

## Hoofdstuk 4

Embryo innesteling is een complex proces, waarbij de communicatie tussen embryo en het endometrium cruciaal is. Er is toenemend bewijs dat de functie van het endometrium wordt bepaald door cytokinen, chemokinen en groeifactoren. In dit hoofdstuk maken wij gebruik van hetzelfde *in vitro* model als in hoofdstuk 3, waarbij stromale cellen van het endometrium worden gedecidualiseerd en vervolgens in contact gebracht met een humaan embryo. Het model stelt ons, in combinatie met een geavanceerde Multiplex Immunoassay, in staat om de concentraties van 14 verschillende implantatie factoren te meten en te relateren aan de ontwikkeling van een embryo tot dag 8 *in vitro*. Zoals verwacht, ontwikkelt slechts een minderheid van de embryo's (25%) zich goed tot dag 8. De bepaling van de implantatie factoren in deze groep embryo's laat een opmerkelijk resultaat zien. De aanwezigheid van een goed ontwikkelend embryo heeft nagenoeg geen effect op de gemeten factoren na 3 dagen van embryo-endometrium interactie in het *in vitro* model in vergelijking met controle condities zonder embryo. Dit in tegenstelling tot de aanwezigheid van een slecht ontwikkelend embryo in dezelfde situatie. Het blijkt dat 8 (Interleukine [IL]-1b, -5, -6, -10, -17, -18, eotaxin en heparin binding epidermal growth factor [HB-EGF]) van de 14 factoren significant lager

tot expressie komen als er een slecht embryo in contact komt met het endometrium, in vergelijking met controle condities zonder embryo. Vervolgens hebben wij laten zien dat deze respons alleen geldt voor gedifferentieerde deciduale cellen en niet voor ongedifferentieerde stromale cellen. Deze resultaten wijzen erop dat het endometrium in staat is om onderscheid te maken tussen goed en slecht ontwikkelende embryo's, door de expressie van cytokinen, chemokinen en groeifactoren hierop aan te passen. Het mechanisme achter deze observatie blijft vooralsnog onbekend. In de volgende hoofdstukken zal dieper worden ingegaan op de achtergrond en de mogelijke betekenis van deze bevindingen.

## Hoofdstuk 5

Dit hoofdstuk beantwoordt de belangrijke vraag hoe en op welk niveau de endometrium respons gereguleerd wordt. Hiertoe hebben wij de supernatanten van individueel gekweekte IVF embryo's opgeslagen en gebruikt als kweekmedium voor deciduale cellen. De supernatanten waren afkomstig van embryo's die hebben geleid tot een zwangerschap, ofwel van embryo's die niet voldeden aan de criteria voor terugplaatsing of invriezen. Kweekmedium waarin geen embryo gekweekt was, werd gebruikt als controle. Om de endometrium respons te meten, werden de deciduale cellen na een periode van kweken met het medium afkomstig van verschillende kwaliteiten embryo's en controles onderworpen aan genexpressie analyse. Geheel in lijn met onze eerdere resultaten, blijkt dat de supernatanten van embryo's van goede kwaliteit nagenoeg geen effect hadden op de genexpressie van de deciduale cellen (47 genen). De supernatanten van slecht ontwikkelende embryo's leidden tot een veranderde expressie van bijna twee duizend genen in deciduale cellen, waarvan de voornaamste functies celtransport, transcriptie en celadhesie zijn. Veruit het meest sensitief gereguleerde gen in deze studie is HSPA8 wat codeert voor Heat Shock Cognate 70 (HSC70), een moleculair chaperone eiwit. Dit eiwit speelt een belangrijke rol bij cell stress, waarbij het helpt de cel te beschermen tegen verval. Als wij de verminderde HSC70 expressie in het eerste experiment nabootsen in een knock-down experiment van deze factor in deciduale cellen, dan blijkt dat het eiwit ook een rol heeft in de differentiatie van deciduale cellen. Het heeft een belangrijke functie om de fysiologische unfolded protein response (UPR), waarbij er o.a. een verhoogde secretoire capaciteit van de cel wordt gevraagd, niet te laten uitmonden in een extreme vorm van stress (Endoplasmic Reticulum-stress) voor de cel. Omdat HSC70 verlaagd is in endometrium dat in contact is geweest met factoren geproduceerd door een kwalitatief slecht embryo, kan het zo zijn dit endometrium minder bestand is tegen stress en zal vervallen tijdens bijvoorbeeld een menstruatie.

## Hoofdstuk 6

Uit de resultaten van hoofdstuk 4 en 5 blijkt dat deciduale cellen in staat zijn om onderscheid te maken tussen goede en slechte kwaliteit embryo's door verschillende cytokinen en groeifactoren te maken. Dit heeft geleid tot de gedachte dat het baarmoederslijmvlies zich maandelijks voorbereid op twee belangrijke taken: ontvankelijkheid voor goede kwaliteit embryo's én negatieve selectie

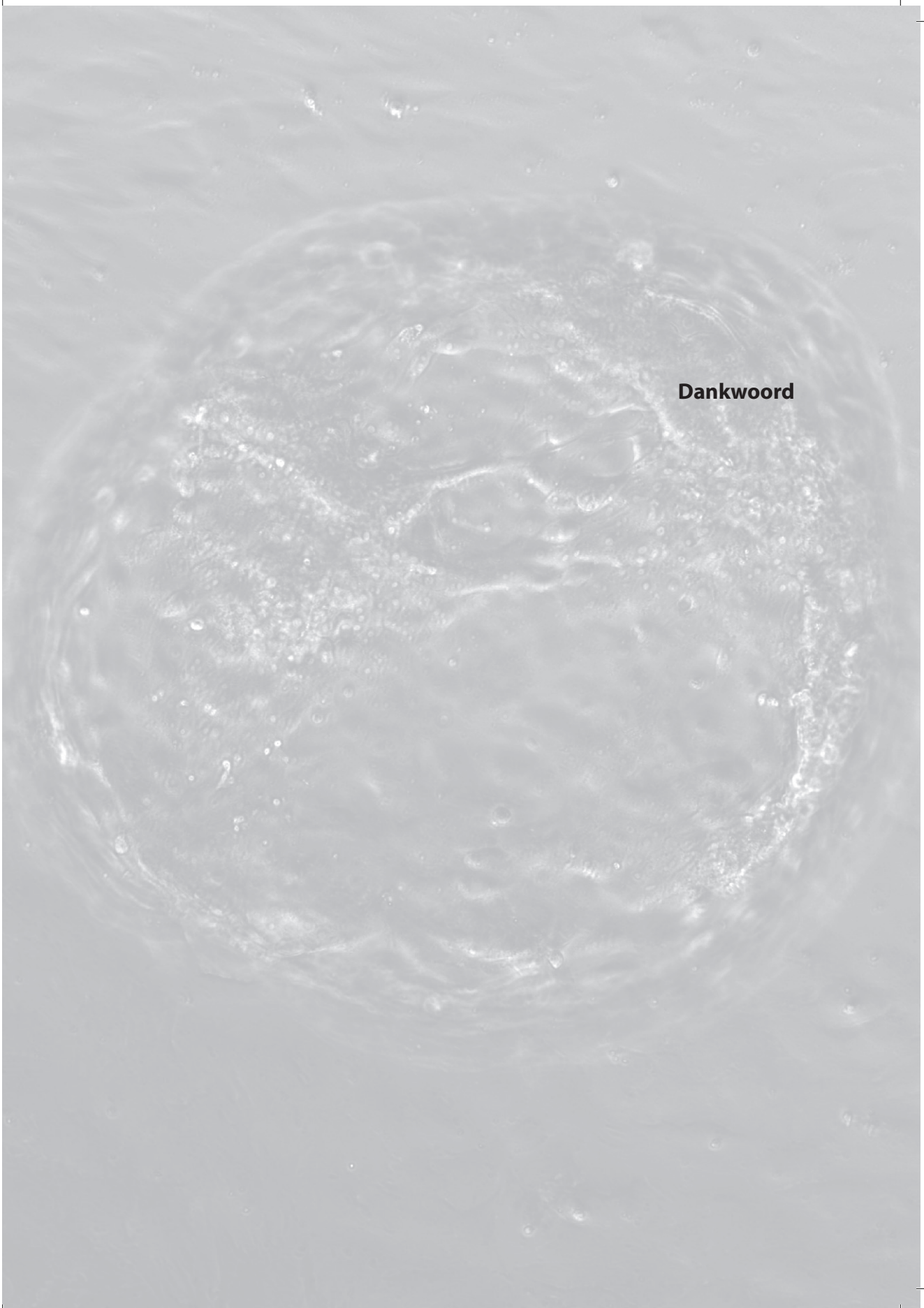
van slechte kwaliteit embryo's. Deze tweede taak is minstens zo belangrijk, omdat een slecht ontwikkelend embryo dat zich innestelt, zou kunnen leiden tot een zwangerschap die eindigt als een miskraam. Vrouwen die meer dan drie miskramen hebben gehad, hebben volgens de Nederlandse definitie herhaalde miskramen. Wij laten in dit hoofdstuk in een patiëntenstudie zien dat het endometrium van patiënten met herhaalde miskramen zich in mindere mate differentieert. Prolactine (PRL), de belangrijkste marker van endometriumdifferentiatie komt minder tot expressie bij vrouwen met herhaalde miskramen *in vivo* en *in vitro*. Daarnaast komt prokineticin-1 (PROK1), een belangrijke factor voor receptiviteit in het endometrium, juist veel meer tot expressie bij vrouwen met herhaalde miskramen. Als laatste reageert het endometrium van deze patiënten overdreven ontvankelijk op het bekende embryo signaal human Chorionic Gonadotropin (hCG). Indien het endometrium té ontvankelijk voor embryo's is, dan zou er aan de negatieve selectie van slechte embryo's voorbij kunnen worden gegaan. Door het ontbreken van natuurlijke embryo selectie worden deze patiënten snel zwanger, maar leiden zwangerschappen vaker tot een miskraam. In een cohort van vrouwen die 3 of meer miskramen hebben gehad, laten wij inderdaad zien dat de gemiddelde tijd tot elke zwangerschap veel korter is dan je op grond van normale zwangerschapskansen zou mogen verwachten.

## Hoofdstuk 7

De resultaten van het proefschrift geven meer inzicht in de rol van het endometrium tijdens het contact met goede en slecht ontwikkelende embryo's. Dit hoofdstuk bespreekt de resultaten van enkele voorgaande hoofdstukken en werpt de hypothese op dat er bij mensen sprake is van een vorm van natuurlijke embryo selectie en dat indien deze functie gestoord is, dit kan leiden tot het klinische probleem van herhaalde miskramen.

## Hoofdstuk 8

Het laatste hoofdstuk vormt de algemene beschouwing van de uitgevoerde studies en bespreekt de implicaties ervan. In de toekomst kunnen de resultaten van dit proefschrift mogelijk een eerste aanzet zijn om bijvoorbeeld vrouwen met herhaalde miskramen te behandelen met medicatie die de differentiatie van het endometrium bevordert.



# Dankwoord

Toen ik jong was, wist ik het al: uitvinder wilde ik worden! Terugkijkend op enkele jaren promotieonderzoek, ben ik een rijker mens geworden. Of de resultaten van het proefschrift zullen leiden tot werkelijke uitvindingen valt nog te bezien, maar het was mij een groot genoegen om met velen samen te werken aan dit proefschrift. De afgelopen jaren heb ik bijzondere mensen leren kennen, die nu en ook in de toekomst van blijvende invloed zullen zijn op mijn leven.

Allereerst wil ik de patiënten bedanken die hun embryo's ter beschikking hebben gesteld aan de studies voor dit proefschrift. Nadat hun vaak lang gekoesterde kinderwens in vervulling is gegaan na een IVF of ICSI behandeling, doneerden zij het begin van menselijk leven voor onderzoek. Ik heb het als een voorrecht beschouwd om tijdens dit onderzoek met humane embryo's te werken en heb getracht daar met de grootste zorgvuldigheid mee om te gaan.

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Geachte professor Heijnen, beste Cobi. Dankzij jou en je team van ervaren onderzoekers kon er invulling worden gegeven aan het basaal wetenschappelijke karakter van dit proefschrift. De breedte van het vakgebied komt maximaal tot zijn recht door de samenwerking van het NIDOD met de afdeling voortplantingsgeneeskunde.

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Geachte professor Prakken, beste Berent. Het is alweer meer dan acht jaar geleden dat we elkaar voor het eerst spraken. Wat begon als een oriëntatie in wetenschappelijk onderzoek, leidde tot een afstudeerstage Medische Biologie. In jouw lab bleef ik enthousiast voor translationeel onderzoek en dankzij jou kreeg ik de mogelijkheid om onderzoekservaring op te doen in het buitenland. Ook nu ben je nog steeds betrokken, zowel op werk als privégebied, voor Sarah en voor mij. Dank voor je mentorschap!

Dear professor Albani, dear Salvo. San Diego has changed my life in many ways. I think one of the reasons your lab used to be in the dungeon of the Stein Research Building, was to appreciate the

Californian sun better every day when I finished work! Fortunately working in your lab wasn't a punishment at all and it left plenty of time to spend in the stunning natural environment of California (with Sarah). It is great to share the most precious moments in our lives with you, Erika and Isabella!

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Mijn collega uitvinders in het AZU en in het WKZ, sinds de dag dat ik leerde dat jullie niet allemaal dagelijks overnachten in het AZU, voel ik me volledig thuis in de uitvindersfamilie. Wat is het heerlijk om dagelijks om te gaan met mensen die moeiteloos een helikopter kunnen besturen! We stonden

allen weleens bovenaan op "de lijst", maar voordat je het wist was het weer "little Friday" en een kleintje kan immers altijd… Kamertje 1, de Gutenberg, de Basket, een beachhouse in San Diego, cheapy girls, de cappuccino's, dynamite, kroketten, het broodje van de week en muziekjes op de vrijdag. Ik mis het allemaal, maar prijs mezelf gelukkig dat we nu opnieuw collega's zijn of worden als AIOS. Lotte, jouw onderzoek is het einde van een tijdperk, maak er wat moois van!

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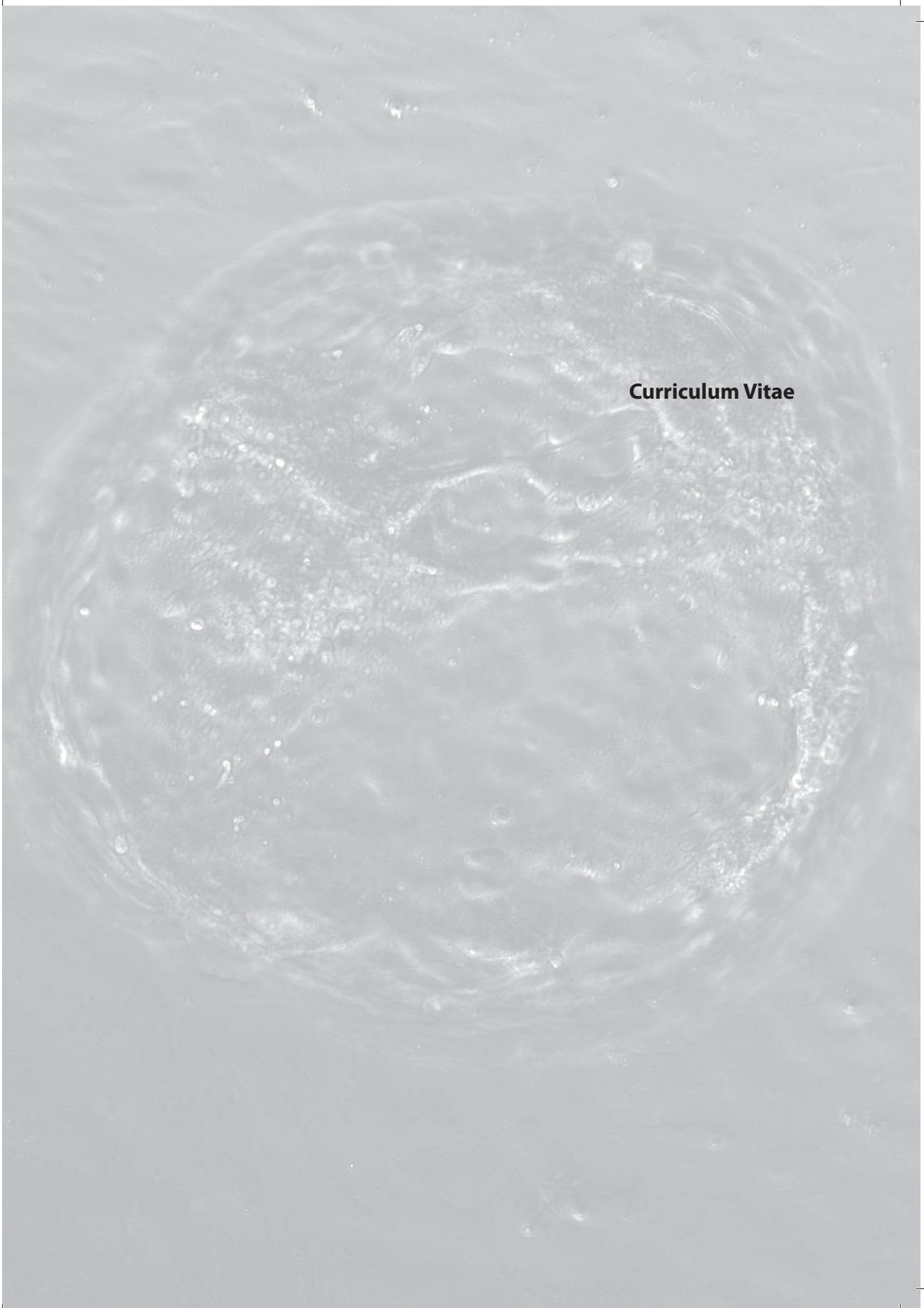
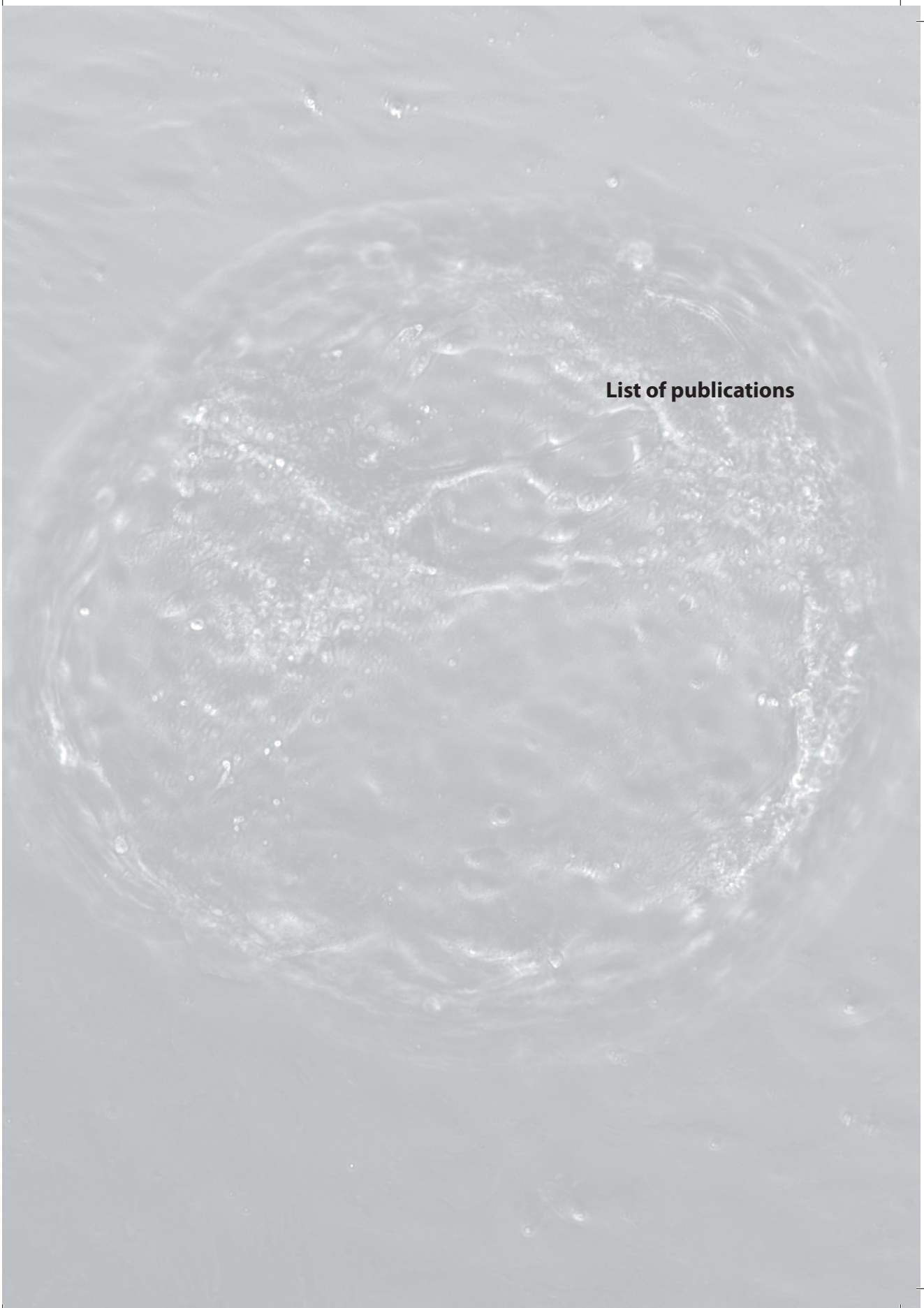


# Curriculum Vitae

Gijs Teklenburg werd geboren op 21 augustus 1978 in Amsterdam. Hij groeide samen met zijn ouders en zijn jongere zus op in Tilburg. In 1996 behaalde hij zijn eindexamen VWO aan het Theresia Lyceum in Tilburg, waarna hij naar Utrecht vertrok voor de studie medische biologie. Vanaf 1999 volgde hij de studie geneeskunde aan de Universiteit Utrecht, waarbij hij gedurende een aantal jaren twee studies combineerde. In 2002 verrichtte hij basaal wetenschappelijk onderzoek in het laboratorium van Prof. dr. B. Prakken in het Wilhelmina Kinderziekenhuis naar de herkenning en rol van evolutionair geconserveerde T cel epitopen in enkele auto-immuunziekten. Dit onderzoek werd vervolgd aan de University of California in San Diego (USA), waar hij op zoek ging naar regulatoire T cellen met als doel regulatoire T cel therapie, begeleid door Prof. dr. S. Albani. Zijn buitenland ervaring werd verder uitgebreid met een coschap sociale geneeskunde en dermatologie in het Groote Schuur Ziekenhuis in Kaapstad, Zuid Afrika. In 2005 behaalde hij zijn Master of Science Medische Biologie en in 2006 zijn artsexamen.

Tijdens zijn coschap gynaecologie in Tilburg (onder begeleiding van Dr. P. Reuwer en Dr. H. Vervest) groeide zijn interesse voor dit vakgebied. In 2006 startte hij derhalve als assistent geneeskundige in opleiding tot klinisch onderzoeker (AGIKO) aan de opleiding gynaecologie in het UMC Utrecht (opleiders Prof. dr. G. Visser en Prof. dr. B. Fauser) in combinatie met promotie onderzoek. Hij deed in 2006 in Oxford ervaring op met het ontwikkelen van een *in vitro* model voor humane embryo implantatie in het lab van Prof. dr. H. Mardon. Het eerste perifere gedeelte van zijn opleiding verrichtte hij in 2009 in het Antonius Ziekenhuis in Nieuwegein (opleiders Dr. J. Schagen van Leeuwen en Dr. P. Graziosi).

Gijs is getrouwd met Sarah Teklenburg-Roord en samen hebben zij drie prachtige zoons, Teun en Hidde (2007) en Job (2009).



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