Novel decidual determinants of recurrent miscarriage

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Novel decidual determinants

of recurrent miscarriage

Nieuwe deciduale cel kenmerken van vrouwen met herhaalde miskraam
(met een samenvatting in het Nederlands)

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Prof. dr. N.S. Macklon
Prof. dr. A. Kavelaars

Voor mijn ouders

Contents

Chapter 1	General introduction	9
Chapter 2	Endometrial stromal cells of women with recurrent miscarriages fail to discriminate between high- and low-quality human embryos *PLOS One 2012; 7(7): e41424*	19
Chapter 3	Impaired migration of endometrial stromal cells of women with recurrent miscarriage in response to trophoblast-derived factors *Submitted*	39
Chapter 4	Dexamethasone modulates human endometrial stromal cell migration *in vitro* *Submitted*	57
Chapter 5	The active human decidual phenotype: opening the black box of early pregnancy loss? *Invited review by Human Reproduction Update*	73
Chapter 6	Cell lineage specific distribution of H3K27 trimethylation accumulation in an in vitro model for human implantation *PLOS One 2012; 7(3):e32701*	103
Chapter 7	Summary and general discussion	121
Chapter 8	Nederlandse samenvatting	139
Chapter 9	Authors and affiliations	149
	List of publications	153
	Dankwoord/ Acknowledgements	157
	About the author	163

Chapter 1

General introduction

Human reproduction

With 134.754.000 annual births worldwide, the rate of human reproduction may be considered high (UN data, 2010). However, the efficiency of human reproduction is very low (Norwitz *et al.*, 2001). The monthly fecundity rate (MFR), the probability of achieving a pregnancy within one menstrual cycle, is only around 20-30% in humans and decreases to <10% above the female age of 35 (Chard, 1991; Evers, 2002; Taylor, 2003; Stevens, 1997). As a comparison, in baboons the MFR is 80% and in rabbits it is 90% (Foote and Carney, 1988; Stevens, 1997).

The fact that the majority of human conceptions have chromosomal abnormalities may explain a great deal of the conception wastage and the low MFR observed in humans. Following fertilization around 60% of the conceptions fail preclinically; 30% are wasted prior implantation (pre-implantation loss) and an additional 30% is lost within 6 weeks after fertilization (early-pregnancy loss) (*Figure 1*) (Farquharson *et al.*, 2005; Macklon *et al.*, 2002). On top of that, around 10% of the conceptions will miscarry with accompanying clinical symptoms (Chard, 1991; Macklon *et al.*, 2002). It was estimated that last year 10 million couples sought medical assistance for infertility or recurrent miscarriage (RM) (Patel and Lessey, 2011).

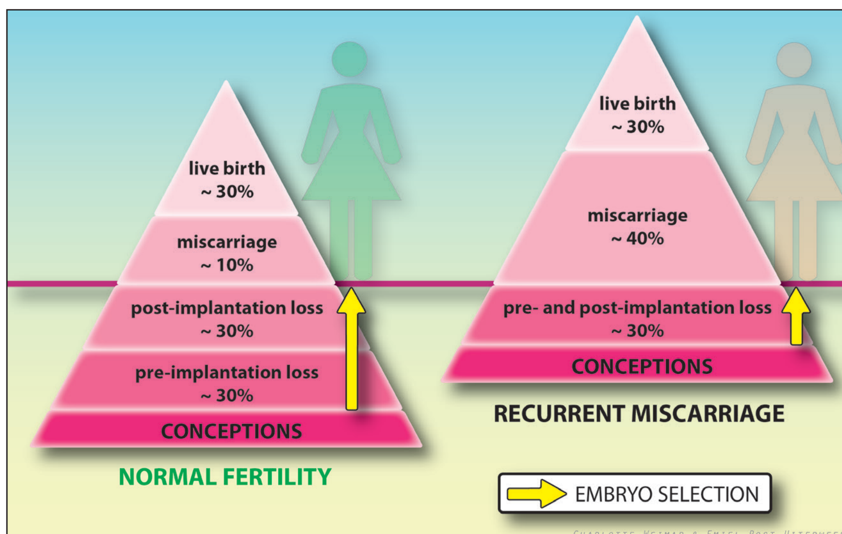

Figure 1. The icebergs of pregnancy loss in normal fertile women (left) and super- fertile women suffering from recurrent miscarriage (right). Embryo selection (represented by the yellow arrows) enabling maternal recognition and elimination of compromised embryos by decidualizing endometrial stromal cells may be restricted in women with recurrent miscarriage.

Recurrent miscarriage

Miscarriage is the commonest complication of pregnancy, occurring in around 10-15% of clinically recognized gestations, and is defined as the loss of a conception before a gestational age of 20 weeks (Rai and Regan, 2006). RM, defined as two or three or more (subsequent) miscarriages, affects 1-2% of couples who try to conceive (Jauniaux *et al.*, 2006; Rai and Regan, 2006; Stirrat, 1990). In many cases, RM is accompanied by psychological morbidities such as depression and anxiety, making RM a very distressing and costly condition (Rai and Regan, 2006). Possible causative factors for RM include maternal primary anti-phospholipid syndrome, structural uterine abnormalities, endocrine factors such as hyperprolactinaemia, thrombophilia, parental balanced translocations, and most commonly chromosomal abnormalities of the fetus (Rai and Regan, 2006). However, in only half of the cases a clear cause for RM is identified (Li, 1998; Rai and Regan, 2006). Limited understanding of the mechanisms underlying RM means that options for effective interventions are lacking; and clinical management is either empirical or primarily focused on providing supportive care.

Thus far, several therapies have been tested in women with unexplained RM, such as immunotherapy (Coulam and Acacio, 2012; Scott, 2003), human chorionic gonadotropin (hCG) (Scott and Pattison, 2000), progesterone (Oates-Whitehead *et al.*, 2003) and heparin (Kaandorp *et al.*, 2010). Unfortunately, these therapies either lacked efficacy (immunotherapy) in increasing live birth rate or the studies generated insufficient evidence to justify the treatment (progesterone and hCG) in randomized studies. Whether prednisolone improves pregnancy outcome in women with unexplained RM is currently under investigation in a randomised, double-blind, placebo controlled trial (Tang *et al.*, 2009). Glucocorticoid administration aims at improving the intrauterine environment by immunomodulating natural killer (NK) cell activity (NK cells are thought to be important in the process of implantation) (Pountain *et al.*, 1993). Indeed, the high numbers of uterine NK in pre-implantation endometrium of women with RM were reduced after daily administration of prednisolone for twenty days (Quenby *et al.*, 2005). However, the effect of glucocorticoids on pregnancy outcome in women with RM still remains to be investigated. In a recent meta-analysis of glucocorticoid supplementation in IVF, the evidence supporting their use was found to be weak, and the need for a properly powered RCT remains (Boomsma *et al.*, 2012).

In 2002, a novel possible cause for RM, 'Selection Failure' , was suggested (Quenby *et al.*, 2002). This hypothesis was first described in 1996 and states that the endometrium of women with RM may be less selective for embryo quality than the endometrium of normal fertile women (Aplin *et al.*, 1996; Quenby *et al.*, 2005) (schematically depicted in *Figure 1*). As a result, compromised embryos may be allowed to implant and as the embryo fails to develop further, a miscarriage will follow. A remarkable clinical feature of the women with RM is that many of these women are characterized by remarkably high pregnancy rates; around 40% of the 560 studied RM women can be categorized in the 'super fertile' group (defined as a mean time-to-pregnancy (TTP) interval of three months or less) (Salker *et al.*, 2010). In contrast, in a normal population only 3% of the women are estimated to belong to the super fertile group (Tietze *et al.*, 1950). As super fertile couples are characterized by a MFR of 60% or higher, it can be estimated that 94 and 100% of these couples would achieve a pregnancy within three and six months of timed intercourse (Evers, 2002). As a comparison, of the normal fertile couples with a MFR of 20%, only 74% achieve a pregnancy within six months (Evers, 2002).
The concept of super-fertility or super-receptivity in RM is supported by several other recent observations. Women with RM demonstrated lower levels of endometrial mucin-1, an anti-adhesion molecule that contributes to the barrier function of the epithelium (Aplin *et al.*, 1996). Moreover, human endometrial stromal cells (H-ESCs) of women with RM demonstrate abnormal decidualization *in vitro* and this phenotype may result in the extension of the window of implantation (Salker *et al.*, 2010) . In addition, implantation occurring later in the luteal phase has been shown to be associated with pre-clinical pregnancy loss (Wilcox *et al.*, 1999).

Embryo implantation and endometrial stromal cells migration

Following menstruation, both H-ESC proliferation and H-ESC migration support regeneration of the functional layer of the uterine endometrium (Mansouri-Attia *et al.*, 2009; Salamonsen, 2003). Recently, it was found that H-ESC migration is an important factor for human embryo implantation as it was found to actively promote embryo implantation (Grewal *et al.*, 2008). H-ESC motility has been found to be more pronounced in decidualizing (receptive) H-ESCs compared to undifferentiated (non-receptive) H-ESCs (Gellersen *et al.*, 2010). In addition, H-ESC migration was significantly enhanced when the decidualizing H-ESCs were placed in contact with either extra-villous trophoblast cells or extra-villous trophoblast cell-derived supernatant (Gellersen *et al.*, 2010).

How decidualizing H-ESC from normal fertile women migrate in response to (high- or low-quality) embryos is not yet known. In addition, H-ESC migration of women with RM in response to trophoblast-derived factors or to high-or low-quality embryos has also not been investigated.

Aims and outline of the thesis

The main aim of the work presented in this thesis was to test the hypothesis that decidualizing H-ESCs, the main component of the luteal phase endometrium, are capable of embryo selection, i.e. recognizing a signal from compromised embryos which results in altered migratory activity, and that this selective response may be altered in women with RM. Using *in vitro* models, in which the implantation environment is mimicked by exposing an endometrial monolayer to an embryonic stimulus, H-ESC migration from normally fertile controls and women with RM was studied in response to high- or low-quality (chromosomal abnormal) human embryos or trophoblast-derived signals. Moreover, we investigated putative trophoblast-derived signals that may modulate H-ESC migration in the ORIS migration assay. In addition, an *in vitro* co-culture model of H-ESCs and human embryos enabled us to investigate the regulation of early human embryo development in our model of implantation. For example, to support optimal embryo development, X linked gene expression should be similar in male and female cells. To achieve this, one X chromosome should be inactivated in female cells to obtain similar X-linked gene expression levels as male cells, which have just one X-chromosome. How this is regulated in human embryos is yet unclear. Our *in vitro* model allowed us to study the mechanisms and dynamics behind X chromosome inactivation in human embryo development.

In **chapter 2** migration of decidualizing H-ESCs is studied in order to provide evidence for the "Selection Failure" hypothesis for RM. The migratory response of decidualizing H-ESCs of normal fertile women and women with RM was tested and compared in the presence of a chromosomally abnormal (low-quality)- and chromosomally normal (high-quality) embryo. In addition, H-ESC migration of RM and control women was investigated in the presence and absence of trophoblast spheroids. The migratory behaviour of H-ESCs was further studied in **chapter 3**. Using the ORIS migration assay, H-ESCs of both women with RM and normal fertile women were tested in response to trophoblast-derived factors. In search of candidate factors that regulate H-ESC migration, we additionally determined the cytokine and chemokine profile of the trophoblast cell line AC-1M88.

As glucocorticoid treatment has been suggested as an immunomodulatory therapy for women with RM in order to optimize the implantation environment, we studied in **chapter 4** the influence of dexamethasone on the migratory activity of H-ESCs. Possible mechanisms behind the increased H-ESC migratory response to dexamethasone of women with RM are investigated on the gene expression level and through determination of the number of glucocorticoid receptors define by their dexamethasone binding capacity.

Chapter 5 reviews the literature of the classically known and novel concepts of H-ESC function, such as ESC migration at the implantation site and high-quality embryo selection by H-ESCs. It further discusses whether dysfunctional H-ESCs may underlie several reproductive disorders such as RM and implantation failure.

Chapter 6 shows data of our *in vitro* co-culture model that was performed to give insight in the mechanisms of X-chromosome inactivation in human day 5 embryos that are cultured until day 8. In this chapter the embryonic lineage development and the spatial accumulation of H3K27me3 (the epigenetic mark that leads to the silencing of the X-chromosome) on the X-chromosomes was examined using immunohistochemistry on day 5 and day 8 human embryos.

Finally, in **chapter 7** the thesis will be concluded with a summary of the main findings and with a general discussion in which also the future directions for RM research are discussed.

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Chapter 2

Endometrial stromal cells of women with recurrent miscarriage fail to discriminate between high- and low-quality human embryos

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Abstract

Background: The aetiology of recurrent miscarriage (RM) remains largely unexplained. Women with RM have a shorter time to pregnancy interval than normally fertile women, which may be due to more frequent implantation of non-viable embryos. We hypothesized that human endometrial stromal cells (H-ESCs) of women with RM discriminate less effectively between high-and low-quality human embryos and migrate more readily towards trophoblast spheroids than H-ESCs of normally fertile women.

Methodology/Principal Findings: Monolayers of decidualized H-ESCs were generated from endometrial biopsies of six women with RM and six fertile controls. Cell-free migration zones were created and the effect of the presence of a high-quality (day five blastocyst, n=13), a low-quality (day five blastocyst with three pronuclei or underdeveloped embryo, n=12) or AC-1M88 trophoblast cell line spheroid on H-ESC migratory activity was analyzed after eighteen hours. In the absence of a spheroid or embryo, migration of H-ESCs from fertile or RM women was similar. In the presence of a low-quality embryo in the zone, the migration of H-ESCs of control women was inhibited compared to the basal migration in the absence of an embryo ($P < 0.05$) and compared to the migration in the presence of high-quality embryo ($P < 0.01$). Interestingly, the migratory response H-ESCs of women with RM did not differ between high- and low-quality embryos. Furthermore, in the presence of a spheroid their migration was enhanced compared to the H-ESCs of controls ($P < 0.001$).

Conclusions: H-ESCs of fertile women discriminate between high- and low-quality embryos whereas H-ESCs of women with RM fail to do so. H-ESCs of RM women have a higher migratory response to trophoblast spheroids. Future studies will focus on the mechanisms by which low-quality embryos inhibit the migration of H-ESCs and how this is deregulated in women with RM.

Introduction

The relative inefficiency of human reproduction is reflected in a high prevalence of pre-implantation embryo losses, pre-clinical pregnancy losses and clinical miscarriages (Evers, 2002; Macklon *et al.*, 2002). This high rate of early loss is considered to represent a strategy for dealing with the high prevalence of chromosomal abnormalities in human embryos (Teklenburg *et al.*, 2010b; Vanneste *et al.*, 2009). More than ten percent of clinical pregnancies end in miscarriage (Rai and Regan, 2006). Recurrent miscarriages (RM), defined as three or more consecutive miscarriages, is experienced by 1-2 % of couples that try to conceive (Rai and Regan, 2006; Stirrat, 1990). Since the prevalence of RM is higher than what would be expected by probability alone, it is likely to indicate specific aetiologies in affected women. Known causes include fetal genetic abnormalities, uterine abnormalities, antiphospholipid syndrome and thrombophilic disorders. However, in more than 50% of cases, no cause is identified (Rai and Regan, 2006).

Increasing evidence suggests that some women may experience RM when 'super-receptive' endometrium allows embryos of low viability to implant, presenting as a clinical pregnancy before miscarrying (Aplin *et al.*, 1996; Quenby *et al.*, 2002; Teklenburg *et al.*, 2010b). The concept of super-receptivity is supported by the recent observation of a reduced interval between pregnancies in women with RM compared to that reported by normally fertile women (Salker *et al.*, 2010). Further evidence comes from studies demonstrating lower levels of endometrial mucin-1, an anti-adhesion molecule that contributes to the barrier function of the epithelium in women with RM (Aplin *et al.*, 1996). Moreover, endometrial stromal cells (H-ESCs) of women with RM demonstrate abnormal decidualization *in vitro* (Salker *et al.*, 2010). This phenotype may result in the window of implantation being extended (Salker *et al.*, 2010), while reducing the ability of the decidualized endometrium to be 'selective' in response to embryo quality (Teklenburg *et al.*, 2010a). This concept is consistent with the previously reported association between implantation occurring later in the luteal phase and pre-clinical pregnancy loss (Wilcox *et al.*, 1999).

Decidualized H-ESCs appear to play an active role in the process of embryo implantation, demonstrating motility at the site of embryo implantation (Grewal *et al.*, 2008). This process is regulated by Rho GTPases in the H-ESCs (Grewal *et al.*, 2010; Grewal *et al.*, 2008). Moreover, decidualized H-ESCs demonstrate invasion into Matrigel coated

inserts, and the invasion of decidualized H-ESCs increases significantly in co-culture with extra-villous trophoblast cells (AC1M88 cell line) (Gellersen *et al.*, 2010).

Our aim was to investigate the migratory activity of H-ESCs from women with RM to extravillous trophoblast cells or high- or low- quality embryos. We hypothesized that H-ESCs from women with RM discriminate less efficiently between high- or low-quality embryos than H-ESCs from normal fertile women. In addition, we hypothesized that H-ESCs from women with RM migrate more readily to extravillous trophoblast cells than H-ESCs from normal fertile. To test these hypotheses we first determined the migratory response to extravillous trophoblast cells by comparing the *in vitro* migration of H-ESCs obtained from women with RM and fertile controls in the presence of trophoblast spheroids. Having successfully demonstrated this, we were able to examine the migratory response of H-ESCs from both groups in the presence of high- or low-quality human embryos.

Materials and Methods

Human endometrial stromal cells and surplus embryos

This study was approved by the Medical Review Ethics Committee University Medical Center Utrecht and the Central Committee on Research involving Human Subjects in The Netherlands (NL30143.000.09). Written informed consent was obtained from all participating subjects, either for the use of surplus cryopreserved embryos or endometrial biopsies.

Day four embryos were thawed by taking them through consecutive washes of 1.25, 1.00, 0.75 and 0.375 mol/l DMSO for five minutes each, after which they were transferred to Human Tubal Fluid culture medium supplemented with 10% GPO (human plasma solution; CLB, The Netherlands) and were overlaid with 1 ml of light paraffin oil (Irvine Scientific, Santa Ana, USA), and cultured until day five. At day five the embryos were scored according to previously published morphological criteria (Gardner *et al.*, 2000). Embryos with two pronuclei (PN) on day one that reached the blastocyst stage with clear cavitation on day five were considered high-quality embryos. Day five blastocysts with 3PN on day one or those which had failed to progress beyond the morula stage were considered low-quality embryos.

H-ESCs were isolated from endometrial biopsies obtained from six women with a history of unexplained RM (defined as three consecutive miscarriages before a gestational age of twenty weeks, with no identifiable cause) and from hysterectomy specimens

of six premenopausal control women (operated for benign indications) with no history of RM. Endometrial biopsies were performed in the mid-proliferative phase of the cycle of women with RM and the biopsies of normal control women were taken randomly in the cycle. All H-ESCs cultures were expanded and the cells frozen at -150ºC in aliquots and thawed consecutively for the co-culture experiments ensuring identical co-culture conditions in all migration experiments.

Culture conditions and decidualization

All twelve individual H-ESC cultures were isolated from proliferative phase endometrium. The endometrial tissue was finely minced and enzymatically digested in 10 ml 417 U/ml collagenase type IA (Sigma) in digest medium (phenolred-free Dulbecco's modified Eagle medium (DMEM)/F12 medium supplemented with 1% L-glutamine (Gibco), 1% amphotericin-B (Sigma, UK) and 1% penicillin/streptomycin solution (Gibco)) for one hour in the incubator (37°C under atmospheric oxygen levels and 5% CO_2). To stop collagenase action, the digested tissue was placed in DMEM/F12 medium supplemented with 10% heat-inactivated fetal bovine serum (FCS) and pelleted by centrifugation at 670 g for eight minutes. The cells were cultured in standard medium (digest medium supplemented with 10% FCS (Gibco) in 75cm^2 tissue culture flasks in the incubator. After three hours medium was replaced to out select the glands.
In 48-well plates 50.000 pre-decidualized H-ESCs were seeded. In 4-well plates, 25.000 undifferentiated H-ESCs were plated and subsequently decidualized for five days. 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 (Sigma, UK) for five days. For this medium and for the experimental medium the FCS was replaced by 10% charcoal stripped FCS. The medium was changed every 48 hours. All primary H-ESCs were used before passage six.

Trophoblast spheroids

The AC-1M88 cell line, derived from human extravillous trophoblast cells, was cultured until 70-80% confluence, trypsinized and counted. Spheroids were formed as previously described (Gonzalez *et al.*, 2011). In short, in each well 100 µl medium containing twelve, 40, 120 or 3000 cells and methylcellulose was plated in a round bottom non-adherent plate. Overnight in an incubator (37ºC, 5% CO_2) the cells formed spheroids. One hour prior to the start of the experiment the spheroids were transferred to experimental medium.

Migration assay

A cell-free strip, the migration zone, was created by scratching a select area of confluent H-ESC monolayers in the 4- and 48-well plates using a 1000 μl pipette-tip. A trophoblast spheroid, a high-quality embryo (n=13), a low-quality embryo (n=12), or no (controls) human embryo was placed in the migration zone. All but one H-ESC primary cell lines were tested with two high- and two low-quality embryos. One control H-ESC primary cell line was tested with two high-quality and three low-quality embryos. The culture medium was overlayed with mineral oil to prevent evaporation. Phase contrast pictures of the migration zone were taken using a Zeiss Axio Observer inverted microscope and the AxioVision imaging system (Zeiss, Germany) directly after creating the migration zone and eighteen hours later. Using Photoshop software, the migratory response was quantified over the eighteen hours.

Staining

H-ESC monolayers were washed in PBS and successively fixed in 4% paraformaldehyde at room temperature for ten minutes, and permeabilized with 1% Saponin. Tetramethyl rhodamine isothiocyanate- conjugated phalloidin was used to detect filamentous actin and DAPI was used to stain DNA. Fluorescent images were obtained using an EVOS® fl digital inverted fluorescence microscope (Advanced Microscopy Group, USA).

Statistical analysis

Statistical testing was performed by Student's t-tests and two-way ANOVA with Bonferroni correction. A P-value below 0.05 was considered statistically significant.

Results

Participant characteristics

Table 1 summarizes characteristics of the women with RM and normally fertile controls included in this study. No statistically significant differences in age, BMI or menstrual cycle length were observed. All women described themselves as non-smokers. As anticipated, the women in the recurrent miscarriage group reported significantly more pregnancies and spontaneous abortions than the control group. The benign conditions for which the hysterectomies were performed were uterus myomatosis (2x), vaginal bleeding disorder e.c.i. (2x), metrorrhagia (1x) and abdominal pain e.c.i. (1x).

Table 1. Characteristics of the women enrolled in the study

Characteristic	RM (n=6)	Controls (n=6)	P-value
Age (y) [a]	33 (2)	36 (5)	0.19
Weight (kg) [a]	67(9)	69 (7)	0.72
BMI [a]	24 (3)	24 (2)	0.81
Cycle (days) [a]	28 (3)	29 (2)	0.57
Gravidity[b]	6 (4-7)	2 (1-3)	0.0001
Parity[b]	0 (0-1)	2 (1-3)	0.006
Spontaneous abortions[b]	6 (4-7)	0 (0-1)	<0.0001

[a] Data represent mean (SD).
[b] Data presented as median (range).

Migratory response towards trophoblast spheroids

To determine whether H-ESCs of RM women and of control fertile women differ in their migratory response to extravillous trophoblast cells and to refine our understanding of the role of different cell types in embryo-endometrial signalling, we analyzed the migratory activity of decidualized H-ESCs of RM women and controls in response to a trophoblast spheroid based on a previously described migration assay (Gonzalez *et al.*, 2011).

In the presence of a trophoblast spheroid, the migration of decidualized H-ESCs from women with RM was significantly more extensive compared to the decidualized H-ESCs of normally fertile women (P < 0.001) (*Figure 1*).

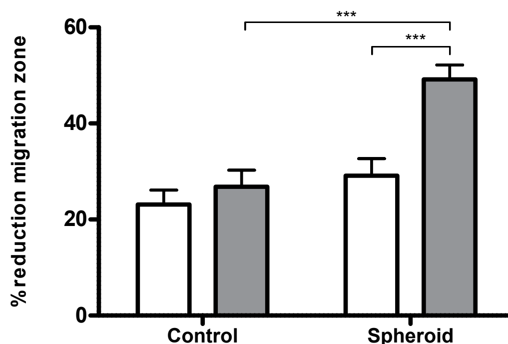

Figure 1. Migration of H-ESCs of fertile control and RM women in response to a trophoblast spheroid. In a confluent well of a 48-well plate a migration zone was created. H-ESCs were left to migrate in the presence or absence of a trophoblast spheroid in the migration zone. Data is shown as a reduction of the migration zone after eighteen hours. Experiments were performed in triplicates. Data represent means ± SEM of six women with RM (grey bars) and six normally fertile women (white bars) and was analysed by 2-way ANOVA and Bonferroni post hoc tests, *** P < 0.001.

The difference in migratory response seen between the control and RM group was not due to differences in spontaneous migration, as the migration in the absence of a trophoblast spheroid was comparable between the two groups (*Figure 1*). The pronounced migratory activity of H-ESCs from women with RM is illustrated in the Supporting information, Video S1. This video shows an eighteen hour timelapse imaging of decidualized H-ESCs from a RM woman in the presence of a spheroid consisting of 3000 extravillous trophoblast cells. Trophoblast spheroid attachment to the culture dish, spheroid expansion and trophoblast shedding are clearly visible. Interestingly, H-ESC migration seems to increase once the shedded trophoblast cells reach the H-ESCs.
An example of cytoskeletal changes of decidualized H-ESCs of a RM woman in the leading edge of the migration zone is depicted in *Figure* 2. In the presence of a spheroid in the migration zone, their actin filaments in decidualizing cells orientate to the direction of the trophoblast spheroid (*Figure 2A*). This directed actin filament positioning is not observed in the absence of trophoblast spheroid in the migration zone (*Figure 2B*).
To investigate whether H-ESC migration is dependent on the distance from the trophoblast spheroid, we split up the migration zone in five parts and compared H-ESC migration in the central zone, adjacent to the trophoblast spheroid, to the average migratory activity of cells in the two most peripheral parts. Migration proximal and distal to the trophoblast spheroid was comparable for both decidualized H-ESCs of women with RM and normal fertile women ($P > 0.05$). As day five human embryos consist of fewer trophoblast cells than contained in the spheroids (Bielanska *et al.*, 2002),

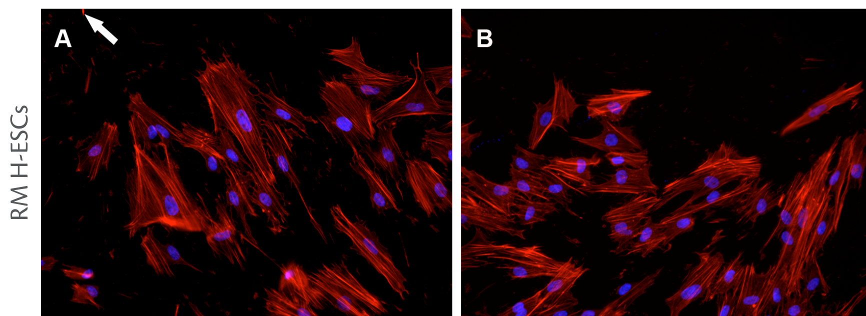

Figure 2. F-Actin architecture in H-ESCs in the presence and absence of a trophoblast spheroid.
In a confluent well of a 48-well plate a migration zone was created. Decidualized H-ESCs of a RM patient were left to migrate in the presence (A) or absence (B) of a trophoblast spheroid consisting of 3000 cells. The white arrow in panel A indicates the position of the trophoblast spheroid. Both micrographs were obtained from the same location in the well. Cells were fixed and stained for F-actin (red) and DNA was stained with DAPI (blue). Magnification: 20.

we determined whether the migration of H-ESCs of RM women is also enhanced in the presence of small trophoblast spheroids. *Figure 3* shows that the migratory activity of decidualized H-ESCs from RM patients in the presence of the small trophoblast spheroids was significantly greater than of decidualized H-ESCs from control women ($P < 0.0001$). Even in the presence of a 40-cell and 120-cell sized trophoblast spheroid (fewer than normally present in a day five human embryo), migration of H-ESCs of women with RM was significantly enhanced compared to the basal migration ($P < 0.001$, not shown) and compared to the migration in the presence of a 12-cell spheroid ($P < 0.001$) (*Figure 3*.). In contrast, migration of H-ESCs of normal fertile women was no longer affected by the presence of the 12-, 40- and 120-cell sized spheroids. The basal migration of decidualized H-ESCs from women with RM did not differ significantly in comparison to the decidualized H-ESCs from control women ($P > 0.05$).
This data highlights the more active migratory responsiveness of decidualized H-ESCs of RM women towards extravillous trophoblast cell signals.

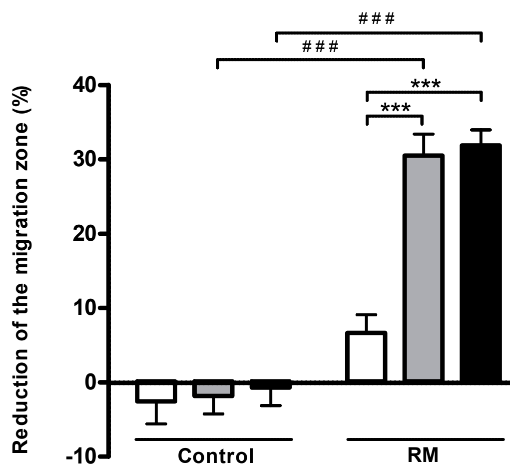

Figure 3. Migration of H-ESCs in response to three different sizes of trophoblast spheroids.
In a confluent well of a 48-well plate a migration zone was created. H-ESCs were left to migrate in the presence or absence of a three different sizes of trophoblast spheroids (consisting of either twelve, 40 or 120 cells as depicted by the white, grey or black bars respectively) in the migration zone. Data is shown as a reduction of the migration zone after eighteen hours (the percentage reduction of the migration zone in the presence of a trophoblast spheroid minus the percentage reduction in the absence of a trophoblast spheroid). Experiments were performed in triplicates. Data represent means ± SEM of two women with RM and three normally fertile women and was analysed by 2-way ANOVA and Bonferroni post hoc tests, *** and $^{###}$ $P < 0.001$.

Migration in the presence of human day five embryos

To test whether H-ESCs from women with RM also demonstrate increased migratory activity in the presence of a human embryo compared to H-ESCs from fertile controls, we measured the migratory response of decidualized H-ESCs in the presence or absence of day five human high-quality embryos.

Before the start of the experiment, the migration zones of H-ESCs of women with RM and control women were comparable in size ($P > 0.05$, data not shown). Of the 26 day-five embryos used, three were in the morula stage M3 (embryo with signs of compaction). Of the 23 blastocysts used, two were graded as B1 (clearly expanded blastocyst, at least twice as big as the egg, with signs of hatching), fourteen were graded as B2 (expanded blastocyst without signs of hatching) and seven were graded as B3 (blastocyst with little or no expansion). All embryos used had less than 10% fragmentation and all blastocysts had an adequate number of cells in the inner cell mass and trophectoderm.

The presence of high-quality embryos did not significantly alter the migration characteristics of decidualized H-ESCs of RM women as compared to migration in the absence of an embryo (*Figure 4 and* 5). Moreover, no difference in migration between decidualized H-ESCs from fertile control women and from RM women in the presence of a high-quality embryo was observed (*Figure 4 and* 5).

Next, we compared the migratory response of the decidualized H-ESCs of both groups in the presence of a low-quality embryo (*Figure 4 and* 5). Interestingly, in response to low-quality embryos there was a clear difference between H-ESCs from RM and control women ($P < 0.01$); the presence of a low-quality embryo markedly reduced migration of control H-ESCs compared to migration in the absence of an embryo ($P < 0.05$). In contrast, however, migration of H-ESCs from women with RM was not reduced by the presence of a low-quality embryo compared to the migration in the absence of an embryo. The migration of H-ESCs from women with RM was comparable in the presence of a high- or a low-quality embryo and comparable to the migration of H-ESCs from control women in the presence of a high-quality embryo (*Figure 4 and* 5). These findings indicate that H-ESCs from RM women fail to discriminate between high- and low-quality embryos.

Video S2 of the supporting information shows timelapse recording of decidualized H-ESCs of a woman with RM migrating towards a human 3PN blastocyst. In a period of eighteen hours both H-ESCs migration, and simultaneous growing and rolling of the 3PN embryo towards the H-ESCs monolayer is seen. Most embryos (both 3PN

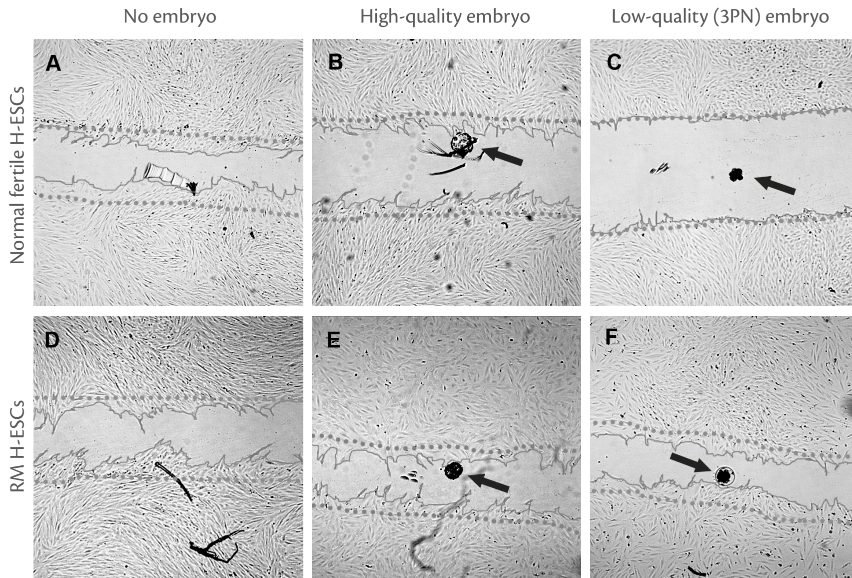

Figure 4. The migration zone after adding a high-quality, low-quality or no embryo. The migratory response of decidualized H-ESCs from normally fertile (A-C) and RM women (D-F) was analyzed in absence of a human embryo (A and D), in presence of a high-quality embryo (B and E) or a low-quality embryo (C and F). Phase contrast pictures were taken eighteen hours after creating the migration zone. The dotted line represents the front of the migration zone directly after its creation. As a reference for the position of the embryo, the bottom of the plate was marked. The arrows indicate the position of the embryo. All pictures were taken with 25x magnification.

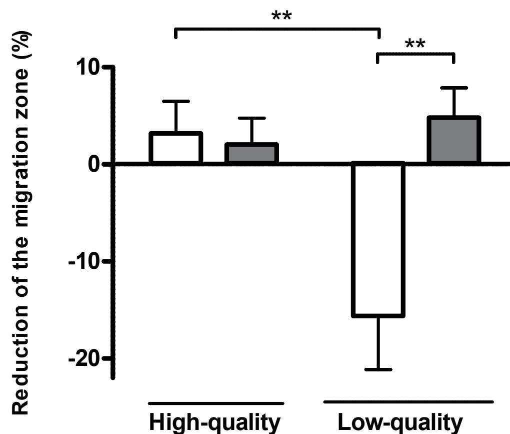

Figure 5. Migration of decidualized H-ESCs in response to a high-quality or a low-quality human embryo. In a confluent well of a 4-well plate a migration zone was created. Decidualized H-ESCs from control women (white bars) and RM women (grey bars) were left to migrate for eighteen hours in the presence or absence of a high- or low-quality embryo. Data is shown as percentage reduction of the migration zone (the percentage reduction of the migration zone in the presence of an embryo minus the percentage reduction in the absence of an embryo). Data represent means ± SEM of three women with RM and three controls in the presence of a high-quality embryo (n=13) or a low-quality embryo (n=12) and were analysed by 2-way ANOVA and Bonferroni post hoc tests ** $P < 0.01$.

and 2PN) observed, however, do not roll but remain in the migration zone. Embryo adherence to the migrating H-ESCs is therefore rarely observed. Finally, as also became apparent from Video S2, in our experiments hatched blastocysts did not appear to adhere to the plastic dishes, which is in line with previous observations (Gonzalez *et al.*, 2011; Grewal *et al.*, 2008; Teklenburg *et al.*, 2010a).

Discussion

Here we show for the first time that decidualized H-ESCs from fertile women are able to adjust their migratory activity in response to the quality of an embryo. In addition, decidualized H-ESCs from RM women fail to discriminate between high- and low-quality embryos as the cells do not regulate their migratory response in response to the quality of the embryo. This inability to discriminate with respect to migration between low- and high-quality embryos may be regarded as a failure of nature's quality control and is consistent with the 'Selection Failure' hypothesis for women with RM (Aplin *et al.*, 1996; Quenby *et al.*, 2002). This hypothesis proposes that super-receptive endometrium may not be able to select high- from low-quality embryos leading to the implantation of low-quality embryos and -as their development fails- may cause a subsequent miscarriage.

The ability to distinguish between high- and low embryo qualities may have evolved as a response to the pressure of the high percentage of chromosomally abnormal (poor-quality) embryos present in fertile women (Vanneste *et al.*, 2009). A preference for the implantation of high-quality embryos may offer a reproductive advantage; higher monthly fecundity rate and larger offspring size thereby ensuring a more effective gene pool spread. A situation where the endometrial bio-sensor does not function properly may then result in the implantation of low-quality embryos which are rejected later, presenting as a clinical miscarriage. Consistent with this concept, we have recently shown that decidualized human endometrium responds to the presence of a low-quality embryo by downregulating the production of key pro-implantation cytokines (Teklenburg *et al.*, 2010b; Teklenburg *et al.*, 2010a). Moreover, bovine endometrium gene profiles have been shown to alter in response to embryos of low versus high development potential (Mansouri-Attia *et al.*, 2009).

Several studies have investigated the migratory role of H-ESCs in regulating implantation. Decidualized H-ESCs from fertile women display profound motility around a high-quality human blastocyst, as was illustrated by 24-hour timelapse pictures of a blastocyst

co-culture with CellTracker stained H-ESCs (Grewal *et al.*, 2008). However, this study did not provide information about net migratory activity because the embryo was placed on top of the endometrial monolayer. Furthermore it has been shown that the migratory activity of H-ESCs is increased in the secretory phase, since decidualized H-ESCs of premenopausal women migrate significantly more than undifferentiated H-ESCs in the presence of extra-villous trophoblast secretory products (Gellersen *et al.*, 2010).

In this paper we confirm the migratory nature of decidualized H-ESCs. In addition, a series of key novel findings shed light on the mechanisms which underlie the 'selective' phenotype of H-ESCs. For decidualized H-ESCs originating from both control and RM women, migration in the presence and absence (basal migration) of a high-quality embryo is comparable. Since the presence of a high-quality embryo did not appear to result in a difference in migration compared to the basal migration observed when no embryo was present, we further hypothesized that high-quality embryos do not elicit a migratory response. The comparable basal migration and migration in the presence of a high-quality embryo is also not due to a ceiling effect as on average only 20-30% of the migration zone of the cultures with high-quality embryos was closed after eighteen hours. Moreover, we observed an inhibition of the migration of normal fertile H-ESCs in the presence of a low-quality embryo. This implies that the distance between the embryo and the H-ESC monolayer is not a limiting factor in regulating H-ESC migration. It also suggests that normal fertile H-ESCs are able to sense and respond differently to low-quality embryos.

The proposed mechanism that may underlie the 'super-receptive' phenotype seen in women with RM is supported by their inability to discriminate high- from low-quality embryos. This makes these RM women more receptive for low-quality embryos than normally fertile women. The 'super-receptive' phenotype of women with RM is also supported by the observation that these cells are more sensitive to extravillous trophoblast cell stimuli, as the migration of H-ESCs from RM women is also enhanced by exposure to trophoblast spheroids containing fewer cells than normally present in the day five human embryo.

The difference in response pattern of H-ESCs from women with RM to trophoblast spheroids and embryos is likely to reflect major differences in signalling from trophoblast spheroids and from embryos, with the former producing a stronger pro-migration signal unmodulated by signals from the other cell types represented in an em-

bryo. It may be that H-ESCs from women with RM are more sensitive to a stimulatory signal coming from the trophoblast spheroid or it may be that the H-ESCs from women with RM are not able to sense an inhibitory signal coming from the trophoblast cells. In the cultures with human day five embryos, the failure of H-ESCs from women with RM to respond to inhibitory signals coming from the 3PN embryo may underlie the difference between RM and fertile control women.

An interesting observation from the Supporting information, Video S1 and S2 is the intense interaction between the H-ESCs and the trophoblast spheroid or day five 3PN human embryo. In Video S1 it is seen that H-ESC migration is enhanced from the moment spheroid derived satellites have reached the H-ESC monolayer. In this case extensive directed migration towards the trophoblast spheroid is also observed, which however is not observed consistently in all spheroid migration experiments. Video S2 provides some novel insights into the earliest human embryo implantation events. It shows how a 3PN blastocyst travels to one side of the H-ESC monolayer and rolls alongside it. It also shows the fast H-ESC response to the 3PN blastocyst by migrating towards and away from the blastocyst, retraction and repulsion. In our experience, hatched human blastocysts do not adhere to the plastic cultures dishes, which may reflect the disposable material used. Also other groups noted a similar lack of adhesion or outgrowth when a trophoblast spheroid or embryo was placed on or adjacent to decidualized H-ESCs (Gonzalez *et al.*, 2011; Grewal *et al.*, 2008).

We have chosen to use 3PN embryos as we consider they are a suitable model for the 'low-quality embryo'. The rate of aneuploid concepti in mid-late first trimester RM has been reported to be 30%, versus around 2% detected by chorion villus sampling of intact first trimester pregnancies (Carp *et al.*, 2001). Moreover, triploidy is one of the more commonly found aneuploidies in concepti examined after RM (Carp *et al.*, 2001). Since triploid fetuses very rarely reach term (Lakovschek *et al.*, 2011) there is clearly a selection mechanism in place, and this is likely to occur prior to any pregnancy becoming clinically evident (Macklon *et al.*, 2002).

Although we should acknowledge that morphology is only part of what determines embryo quality, karyotyping of some blastomeres may not reflect the rate of mosaicism. For this, comparative genomic hybridization (CGH) would have to be done in all blastomeres, which was not feasible.

The mechanisms by which healthy H-ESCs sense the difference between high- and low-quality embryos remain to be fully elicited. Compromised embryos are metabolically

very active, producing increased ATP and reactive oxygen species and demonstrating increased amino acid turnover when compared to viable embryos (Leese *et al.*, 2007; Leese *et al.*, 2008; Stokes *et al.*, 2007). Conversely, increased metabolism (as illustrated by amino acid turnover) has been shown to be associated with increased DNA damage and lower cytogenic health in the embryo (Picton *et al.*, 2010; Leese *et al.*, 2007; Leese *et al.*, 2008; Stokes *et al.*, 2007). These metabolites might be good candidate signals of embryo quality to be sensed by H-ESCs. Secondly, it is known that syncytiotrophoblast shed microparticles that can subsequently reach the maternal circulation (Redman and Sargent, 2008). Early trophoblast cells might also shed microparticles. Decidualized H-ESCs may then adjust their migration in response to these particles. Thirdly, human chorionic gonadotropin (hCG), a hormone secreted by the embryonic trophoblast, may modulate the response of H-ESCs. It has been shown that decidualized H-ESCs of RM women display a dysregulated response to hCG with regard to prolactin and prokineticin1 mRNA expression (Salker *et al.*, 2010). In line with this, it is possible that decidualized H-ESCs from women with RM also have a dysregulated migratory response to hCG. Decidualized H-ESCs from women with RM may be less discriminatory in response to one of the products released by low-quality embryos (e.g. byproducts of increased metabolism, trophoblast microparticles or hormones such as bHCG) and as a result show no inhibition of migration.
An interesting follow-up experiment would be to stimulate the decidualized H-ESCs with culture medium of the human embryo or trophoblast spheroid. So far, we have not been able to identify factors that are secreted by the blastocyst. Extensive crosstalk between the H-ESCs and the trophoblast has been reported to occur at the gene expression level in a co-culture system (Popovici *et al.*, 2006).

The 'selection failure' hypothesis would lead us to expect a somewhat higher live-birth rate in RM women that concieve following preimplantation genetic screening (PGS) than in RM patients that conceive naturally. However, we would not expect this increase to be dramatic because PGS only screens for a small number of chromosomal abnormalities, while there is a high incidence of mosaicism in young couples that undergo IVF (Baart *et al.*, 2006) and the fecundity rate of RM is not low (with a live birth rate of around 35%). Furthermore, although the best available evidence suggests a similar live-birth rate in women with unexplained RM after PGS vs. natural conception (42 vs. 35 % respectively), this is still under debate as no RCT or non-randomized comparative studies (directly comparing PGS vs. natural conception) have been performed in this study group (Musters *et al.*, 2011).

Thus far no treatment options exist for women with unexplained RM. There is insufficient evidence to support the use of immunotherapy, hCG or progesterone in women with unexplained RM to increase live birth rate.

In conclusion, we report several new findings that describe the mechanisms behind the 'selective phenotype' seen in fertile women and behind the 'super-receptive' phenotype seen in women with RM. Decidualized H-ESCs of fertile women may actively select a high-quality embryo for implantation by inhibiting their migratory response in the presence of low-quality embryos. In contrast, the migration of H-ESCs of RM women that is already elicited by trophoblast spheroids and by low-quality embryos highlight the super-receptive state of the endometrium of women suffering from RM.

This enhanced migratory response of H-ESCs of RM women towards a low-quality embryo or towards small spheroids may become a biomarker for identifying 'selection failure' as the aetiology in those patients diagnosed with otherwise unexplained RM.
Future studies will focus on the mechanisms by which low-quality embryos inhibit the migration of decidualized H-ESCs of fertile women and how this is deregulated in the H-ESCs of RM women. Clarification of the mechanism of non-discriminative migration of to high- and low-quality embryos of H-ESCs of women with RM may have important clinical implications both for understanding the aetiology of this distressing condition, and for the future development of a therapeutic target for the prevention of further miscarriages.

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Supporting information

Video S1 Timelapse of H-ESCs of a woman with RM migrating towards a 3000 cell trophoblast spheroid. *See online at PLoS One. 2012; 7(7): e41424*

Video S2 Timelapse of H-ESCs of a woman with RM migrating towards a human 3PN blastocyst. *See online at PLoS One. 2012; 7(7): e41424*

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Chapter 3

Impaired migration of endometrial stromal cells of women with recurrent miscarriage in response to trophoblast-derived factors

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Submitted

Abstract

Background: Abnormal migratory behaviour of human endometrial stromal cells (H-ESCs) may compromise feto-maternal interactions and contribute to the aetiology of early pregnancy loss. We investigated whether the migratory response of decidualizing primary H-ESCs to trophoblast signals differs between recurrent miscarriage (RM) and control subjects.

Methods: H-ESCs were isolated from endometrial biopsies obtained from six women with RM and from five control subjects. Multiplex immunoassay was performed to determine the cytokine and chemokine profile of the trophoblast cell line AC-1M88. The ORIS migration assay was used to determine the responsiveness of decidualizing H-ESCs from RM and control subjects to either trophoblast conditioned medium (TCM) or specific factors, including platelet-derived growth factor-BB (PDGF-BB), vascular endothelial growth factor A (VEGF-A) and interleukin-8 (IL-8).

Results: Baseline migration of H-ESCs was comparable between both groups. Further, the potent chemoattractant PDGF-BB stimulated migration of RM and control H-ESCs, although this response was impaired in the RM group when exposed to a low PDGF-BB concentration. Migration of control H-ESCs was enhanced upon exposure to TCM in a concentration-dependent manner. In contrast, no significant effect of TCM on H-ESC migration was observed in the RM group. H-ESC migration was enhanced by IL-8, but not by VEGF-A, and this response was comparable between both groups.

Conclusions: Decidualizing H-ESCs of women with RM show a blunted migratory response to a low dose of PDGF-BB and to TCM. This is consistent with the concept of an altered response of the decidual compartment to the implanting embryo in women with RM.

Introduction

More than ten percent of all clinical pregnancies fail to continue beyond the first-trimester, rendering miscarriage the most common complication of pregnancy (Rai and Regan, 2006). Recurrent miscarriage (RM), defined as three or more consecutive miscarriages, is experienced by one to two percent of couples trying to conceive (Stirrat, 1990). Associated factors for RM include primary antiphospholipid syndrome, parental balanced translocations, structural uterine abnormalities, endocrine factors, and thrombophilia. However, in approximately 50% of cases, no risk factors are identified (Kutteh, 1999). Limited understanding of the mechanisms underlying RM means that effective interventions are lacking; and clinical management is primarily focused on providing supportive care for this distressing condition.

Recently, it has been hypothesized that one of the factors contributing to RM may originate from impaired peri-implantation embryo-maternal interactions (Brosens and Gellersen, 2010; Teklenburg *et al.*, 2010a). In humans, the endometrium is decidualized cyclically during the luteal phase prior to apposition and implantation of the blastocyst (Gellersen *et al.*, 2007). The decidual process in the stromal compartment is initiated in response to local factors, such as prostaglandin E2, that elevate intracellular cAMP levels and sustained by continuous progesterone signalling. Decidualization of human endometrial stromal cells (H-ESCs) represents a process of profound differentiation, characterized by the phenotypic transformation of estrogen-primed fibroblasts into epithelioid cells that secrete decidual markers, such as prolactin (PRL) and Insulin-like growth factor-binding protein 1 (IGFBP-1) (Gellersen *et al.*, 2007; Gellersen *et al.*, 2007; Tang *et al.*, 1993).

Conventional models of implantation infer that the stromal compartment of the decidua primarily represents a passive matrix for the invading embryo. However, recent *in vitro* studies indicate that decidualizing H-ESCs have a more active role in implantation. For example, decidualizing H-ESCs are biosensors of embryo quality, enabling maternal recognition and rejection of compromised embryos (Teklenburg *et al.*, 2010b; Weimar *et al.*, 2012). Further, rather than being passively invaded, emerging evidence suggests that decidual cells play an active role in encapsulating the early conceptus (Brosens and Gellersen, 2010; Grewal *et al.*, 2008; Grewal *et al.*, 2010; Teklenburg *et al.*, 2010b; Weimar *et al.*, 2012). In addition, decidualizing H-ESCs have been shown

to migrate in response to trophoblast cell secretions (Gellersen *et al.*, 2010), suggesting an active role in the spatial organization of the early placenta.

Recently, we have described that H-ESCs of women with RM display abnormal decidualization *in vitro* (Salker *et al.*, 2010). We therefore wished to investigate whether the migratory response of decidualizing H-ESCs to trophoblast-derived signals may be dysregulated in women with RM and to identify putative key signalling factors present in the trophoblast conditioned medium (TCM).

Methods

Primary H-ESC cultures

H-ESCs were isolated from endometrial biopsies obtained from six women presenting at the University Medical Center Utrecht, The Netherlands with a history of unexplained RM (defined as three consecutive miscarriages before a gestational age of twenty weeks with no identifiable cause) and from hysterectomy specimens of six premenopausal control women (operated for benign indications) with no history of RM.

H-ESCs were isolated and cultured as previously described (Weimar *et al.*, 2012). In short, endometrial biopsies were enzymatically digested and the H-ESCs were cultured in 75cm^2 tissue culture flasks in the incubator (37°C under atmospheric oxygen levels and 5% CO_2). After three hours medium was replaced, thereby removing the less adherent epithelial cell fraction. All H-ESC cultures were expanded in phenolred-free Dulbecco's modified Eagle medium (DMEM)/F-12 supplemented with 1% L-glutamine (Gibco), 1% amphotericin-B (Sigma, UK), 1% penicillin/streptomycin (Gibco) and 10% heat-inactivated fetal bovine serum (FCS) and then frozen in aliquots at -150ºC. These aliquots were thawed before the start of every experiment to ensure identical conditions (culture time) in all experiments.

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 (Sigma, UK) for five days. In the medium used for *in vitro* decidualization and in the medium used for the migration experiments 10% charcoal stripped (steroid-depleted, preventing steroid-induced migration) FCS was used. The medium was changed every 48 hours. All primary H-ESCs were used before passage six.

ORIS migration assay

The migration of H-ESCs in six RM and five control cultures, respectively, was examined using the ORIS™ cell migration assay (Platypus Technologies, USA). Forty-thousand decidualizing H-ESCs were seeded into the wells of the ORIS plate and allowed to attach. After six hours the central circular stoppers were removed from the wells, creating the 'migration zone'. Decidualizing cells were left to migrate under the following conditions: unstimulated, stimulated with 10%, 20%, or 50% conditioned medium from the trophoblast cell line AC-1M88, 0.1, 1, 10 or 100 ng/ml IL-8 (Sanquin, Amsterdam, The Netherlands), and 1 or 10 ng/ml PDGF-BB (Biomol, Hamburg, Germany). After eighteen hours, cells were stained with 1 μM Calcein AM and fluorescence in the migration zone was quantified using ImageJ software and expressed in relative fluorescence units (RFUs; *Figure 1*).

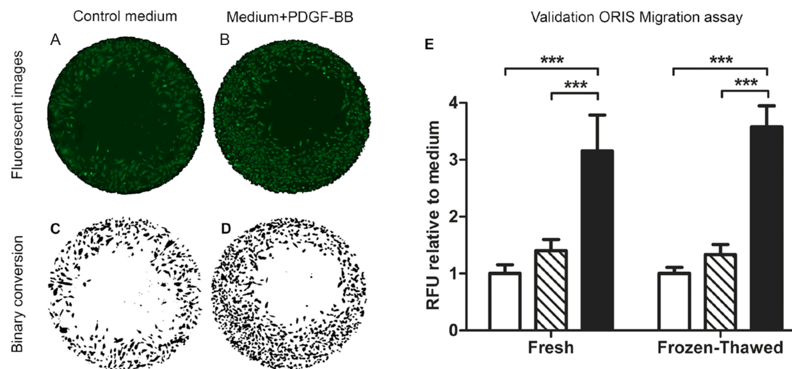

Figure 1. The ORIS migration assay. A and B. Fluorescent pictures of two wells, with the mask attached to the bottom of the plate, visualizing only the fluorescent ESCs of normally fertile women in the migration zone. C and D. Binary images of the two fluorescent pictures A and B respectively. In A and C the decidualizing ESCs were left to migrate in the absence of a stimulus. In B and D the decidualizing ESCs were left to migrate in the presence of 10 ng/ml PDGF-BB. All pictures were taken with 4x magnification. E. Migration of fresh and frozen-thawed decidualizing H-ESCs. H-ESCs from two women with RM and two fertile control women were left unstimulated or stimulated with PDGF-BB 1 ng/ml or PDGF-BB 10 ng/ml (white, striped and black bar respectively per group). After eighteen hours cells were stained with Calcein-AM after which the fluorescence in the migration zone was measured and depicted as RFU (relative fluorescence units). Data represent means from quadruplos ± SEM and were analysed by 2-way ANOVA and Bonferroni post hoc tests (*** $P < 0.001$).

In order to validate the use of frozen primary cultures, the migratory of thawed H-ESCs capacity was compared with that of matched fresh cultures from the same patients in the presence of two doses of PDGF-BB. The migratory activity was similar in both groups ($P > 0.05$) (*Figure 1*).

In the absence of published data, we examined the reproducibility of ORIS migration assay when using primary H-ESCs. To determine the inter-assay variability, we carried out two experiments a week apart using H-ESCs from three RM patients. In each experiment, the difference in migration rate between untreated cells (medium only), and cells stimulated with 10 ng/ml PDGF-BB was measured. After eighteen hours, the difference in rate appeared to be consistent at both time points with an interassay variability of 18.5% (Supplementary Material, Figure S1).

Proliferation assay

Forty-thousand cells per well were plated in flat-bottom 96 well plates (NUNC). After six hours of culture (37ºC; 5% CO_2), a small area within each well was made free of cells by scraping the bottom of the well using a pipette-tip (mimicking the migration zone in the ORIS migration plates) before adding ^{3}H-labelled thymidine (1.0 mCi) per well. Eighteen hours later, cells were harvested on a glass fiber filter and ^{3}H-thymidine incorporation was measured as counts per minute (CPM) using liquid scintillation counting.

Multiplex immunoassay of trophoblast conditioned medium (TCM)

The trophoblast cell line AC-1M88 (Hannan *et al*., 2010) was cultured near confluency in DMEM/F-12 supplemented with 10% charcoal stripped FCS for 24 hours. The supernantant (TCM) was analyzed using multiplex immunoassay (Bio-Rad Laboratories) (Boomsma *et al*., 2009; de Jager *et al*., 2003). Eleven key soluble regulators of implantation were analyzed: interleukin (IL)-1b, IL-5, IL-6, IL-10, IL-12, IL-15, IL-17, interferon-gamma (IFN-γ), vascular endothelial growth factor (VEGF), heparin-binding epidermal growth factor (HB-EGF) and tumor necrosis factor (TNF)-alpha. In addition, a panel of eighteen chemokines was assessed: C-C motif ligand 1 (CCL1), CCL2 (monocyte chemo-attractant protein-1; MCP-1), CCL3 (macrophage inflammatory protein-1α; MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL7 (monocyte-specific chemokine 3; MCP3), CCL11 (Eotaxin), CCL17, CCL18, CCL19 (MIP-3b), CCL22 (macrophage-derived chemokine; MDC), CCL27, C-X-C motif ligand 1 (CXCL1), CXCL5, CXCL8 (IL-8), CXCL9 (monokine induced by IFN-g; MIG), CXCL10 (IFN-g-inducible 10 kDa protein; IP-10) and CXCL13. Antibodies were covalently coupled to the microspheres and the assay was carried out as previously described (Boomsma *et al*., 2009; de Jager *et al*., 2003).

Statistical analysis

Statistical testing was performed by ANOVA followed by post-tests with Bonferroni correction and Student's *t*-tests. A *P*-value below 0.05 was considered significant.

Results

Participant characteristics

The demographic characteristics of participating women are summarised in *Table 1*. No statistically significant differences in body weight, body mass index or menstrual cycle length were observed between RM and fertile control subjects. All participants were non-smokers. RM subjects were on average five years younger compared to the control group. As anticipated, the RM group had significantly more pregnancies and spontaneous abortions than the control group ($P < 0.0003$ and $P < 0.0001$ respectively).

Table 1. Characteristics of the women enrolled in the study

Characteristic	RM (n=6)	Controls (n=5)	P-value
Age (y) [a]	33 (2)	38 (3)	0.03
Weight (kg) [a]	67(9)	71 (7)	0.98
BMI [a]	24 (3)	24 (2)	0.96
Cycle (days) [a]	28 (3)	29 (2)	0.55
Gravidity [b]	6 (4-7)	1 (1-3)	0.0003
Parity [b]	0 (0-1)	1 (1-3)	0.01
Spontaneous abortions [b]	6 (4-7)	0 (0-1)	<0.0001

[a] Data represent mean (SD).
[b] Data presented as median (range).

Migration of H-ESCs in response to PDGF-BB

PDGF-BB has previously been shown to stimulate migration of endometrial stromal cells through the rapid activation of both ERK1/2 and PI3K/Akt signalling pathways (Gentilini *et al.*, 2010). We used this potent chemoattractant to compare the migratory capacity of H-ESCs from control and RM patients. In both groups, PDGF-BB elicited a dose-dependent increase in the migratory response of decidualizing H-ESCs (*Figure 2*) However, while the increase in the migratory activity in response to 10 ng/ml PDGF-BB was comparable, the response to 1 ng/ml PDGF was significantly reduced in the RM compared to the control group ($P < 0.05$).

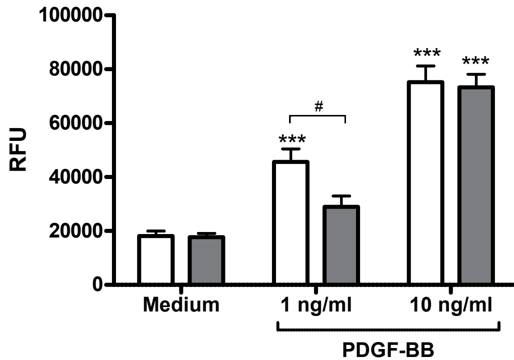

Figure 2. Migration of H-ESCs in response to platelet derived growth factor-BB (PDGF-BB). Decidualizing H-ESCs of six women with RM (grey bars) and five control women (white bars) were exposed to two different concentrations of PDGF-BB. After eighteen hours cells were stained with Calcein-AM after which the fluorescence in the migration zone was measured and depicted as RFU (relative fluorescence units). Data represent means ± SEM . # $P < 0.05$; *** $P < 0.001$ compared to the migration in the absence of PDGF-BB.

Migration of H-ESCs in response to trophoblast conditioned medium

Next, we examined the migratory response of H-ESCs to trophoblast signals. Decidualizing primary H-ESCs from RM patients migrated significantly less when exposed to TCM in comparison to decidualizing H-ESCs from control subjects ($P < 0.0001$) (*Figure 3*). Migration of control decidualizing H-ESCs showed a dose-dependent increase reaching a 2.5-fold increase when stimulated with 50% TCM ($P < 0.0001$). Decidualizing H-ESCs from RM women showed a non-significant trend towards enhanced migration in response to increasing TCM concentrations. Notably, basal migration of decidualizing H-ESCs did not differ between both groups ($P > 0.05$).

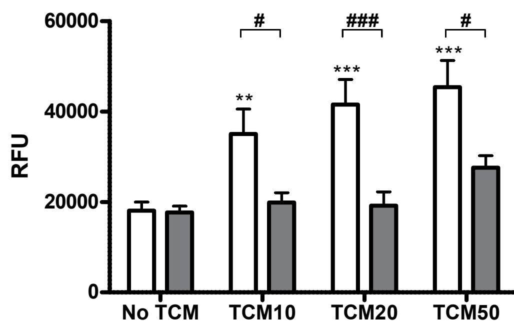

Figure 3. Migration of H-ESCs in response to trophoblast conditioned medium (TCM). Decidualizing H-ESCs of six women with RM (grey bars) and five control women (white bars) were exposed to three different concentrations of TCM or unconditioned control medium for eighteen hours. The cells were then stained with Calcein-AM after which the fluorescence in the migration zone was measured and depicted as RFU (relative fluorescence unit). Data represent means ± SEM and were analysed by 2-way ANOVA and Bonferroni post hoc tests. # $P < 0.05$; ** $P < 0.01$ (compared to the basal migration); ### and ***$P < 0.001$ (compared to the basal migration).

Cytokine and chemokine profile of trophoblast conditioned medium

As H-ESCs from women with RM and normal fertile women migrated differently in the presence of TCM, we characterized the cytokine and chemokine profile of the TCM by Multiplex immunoassay analysis. Of the eleven cytokines tested, VEGF-A was most abundant in the TCM (3324 pg/ml) (*Table 2*). Eight additional cytokines were secreted by the trophoblast cells but at much lower concentrations (*Table 2*). IFN-g and HB-EGF were not detectable in the TCM (*Table 2*). The most abundant chemokines produced by the trophoblast cells were CCL22 and CXCL8 (IL-8) with levels being 54- and 18-fold higher, respectively, in TCM compared to control medium. The concentrations of CCL2, CCL11, CXCL1, CXCL 9 and CXCL10 were 2.5- to 5-fold higher in TCM compared to unconditioned medium and the levels of CCL1, CCL19, and CCL27 between 5-10 times.

Migratory response of H-ESCs to trophoblast conditioned medium factors

Testing the effect of all identified TCM factors on H-ESC migration was not feasible. Instead, we focussed on VEGF-A and IL-8, both highly secreted by AC-1M88 trophoblast cells. VEGF-A, however, did not significantly stimulate migration of H-ESCs from RM or control women (data not shown). In contrast, IL-8 stimulated the migration of H-ESCs in a dose-dependent manner ($P < 0.05$), although this response was comparable between both groups (*Figure 4*).

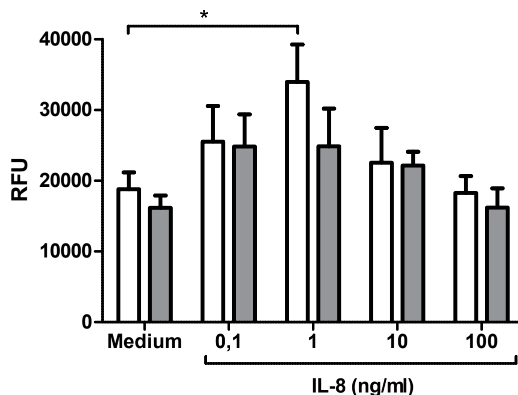

Figure 4. Migration of H-ESCs in response to interleukin-8 (IL-8). Decidualizing H-ESCs of three women with RM (grey bars) and three control women (white bars) were cultured in medium only or medium supplemented with four different concentrations of IL-8 for eighteen hours. Then the cells were stained with Calcein-AM after which the fluorescence in the migration zone was measured and depicted as RFU (relative fluorescence unit). Data represent means ± SEM and were analysed by 2-way ANOVA and Bonferroni post hoc tests.

Table 2. Cytokines and chemokines present in the TCM

Times upregulated (TCM: control medium)	N.D[a]	1.0-2.5	2.5-5.0	5.0-10	10-50	50-100	>1000
CCL chemokines							
CCL1			**x**[b]				
CCL2		x					
CCL3				x			
CCL4	x						
CCL5	x						
CCL7	x						
CCL11		x					
CCL17	x						
CCL18	x						
CCL19			x				
CCL22						x	
CCL27			x				
CXCL chemokines							
CXCL1		x					
CXCL5	x						
CXCL8					x		
CXCL9		x					
CXCL10		x					
CXCL13	x						
Cytokines							
IL-1b		x					
IL-5		x					
IL-6		x					
IL-10		x					
IL-12		x					
IL-15		x					
IL-17		x					
IFN-g	x						
HB-EGF	x						
TNF-a		x					
VEGF-A							x

[a] N.D. is not detectable (below detection limit)
[b] Values are measured in pg/ml and set in relation to the concentration present in control medium.

Taken together, the data show responsiveness of H-ESCs from RM patients to selected chemoattractants is maintained. Alternatively, it appeared possible that the differential H-ESC response between the clinical groups to TCM stimulation reflect altered proliferation rather than bona-fide differences in migratory capacity. To test this hypothesis, we measured proliferation of decidualizing H-ESCs, incubated with and without 50% TCM, in two primary cultures from RM and control subjects. Proliferation of H-ESCs was comparable between the two groups in both the presence and absence of TCM ($P > 0.05$; data not shown). Therefore, the blunted migratory response of decidualizing H-ESCs from RM patients to trophoblast signals could not be accounted for an inhibitory effect on cell proliferation.

Discussion

In this study we show that the trophoblast secretome significantly increase the *in vitro* migration of decidualizing H-ESCs obtained from normally fertile women. In contrast, the migration of decidualizing H-ESCs from women with RM was not significantly affected by trophoblast cell signals. In addition, H-ESCs from women with RM showed a lower migratory response to a low dose of PDGF-BB compared to H-ESCs from normal fertile women. Taken together, these data suggest that H-ESCs from women with RM are less responsive to trophoblast signals in comparison to H-ESCs from control women in terms of modulating their migratory activity.

Previous studies have shown that TCM increases the chemotactic migration of H-ESCs. In a transwell migration assay, the directed migration of both undifferentiated and decidualizing H-ESCs was significantly enhanced in response to factors secreted by AC-1M88 trophoblast cells compared to the migration seen in response to control medium (Gellersen *et al.*, 2010). The cell line AC-1M88 was also used in the present study and shares similarities with primary extravillous trophoblast cells with regard to cell surface molecules and secretion products (Hannan *et al.*, 2010). AC-1M88 cells are positive for cytokeratin-7, HLA-G, CD9 and several chemokine receptors such as CCR1, CCR3 and CCR5. In addition, the cells express human chorionic gonadotropin, matrix metalloproteases and various integrins (Hannan *et al.*, 2010; Samalecos *et al.*, 2009).

A whole array of implantation mediators (cytokines, chemokines) is produced at the fetal-maternal interface (Boomsma *et al.*, 2009; Hannan and Salamonsen, 2008; Moussa *et al.*, 2001; Naruse *et al.*, 2010; Red-Horse *et al.*, 2001; Salamonsen *et al.*, 2007; van

Mourik *et al.*, 2009). Analyzing the AC-1M88 proteome profile for several chemokines, cytokines and growth factors revealed the presence of eleven chemokines and nine growth factors and cytokines. From this set, the chemokines CCL11, CCL19, CCL22, CCL27 and the cytokine IL-5 have not yet been reported to be produced by human trophoblast cells. It was beyond the scope of this study to examine in-detail the effect of all TCM factors on H-ESC migration.

The most abundantly secreted product measured in the TCM was VEGF-A. Interestingly, it has been shown that decidual VEGFR1 and VEGFR2 expression is lower in a group of women suffering from RM compared to control women (Vuorela et al., 2000). In addition, polymorphisms of the -1154G/A VEGF gene have been associated with RM and VEGF−1154 A⁄A has now been identified as a risk factor for RM (Coulam and Jeyendran, 2008; Papazoglou et al., 2005). Recently VEGF, abundantly present in receptive phase human uterine fluid, was identified as an important regulator of embryo implantation (Hannan et al., 2011). Because of these observations, and our findings of the blunted migration of H-ESCs from women with RM in response to TCM, we hypothesized that the migratory response of RM H-ESCs to VEGF-A would be abnormal. Although the VEGF-A doses tested were within the range of the VEGF-A concentration found in the TCM, VEGF-A alone did not affect the migration of H-ESCs in both groups of women in the ORIS migration assay.

We further tested the migration in response to CXCL8 (IL-8), as CXCL8 was the second most abundant chemokine found in the TCM (with an eighteen times higher IL-8 concentration in the TCM compared to the control medium). IL-8 is the ligand of CXCR1 and CXCR2 and both these receptors are expressed on H-ESCs with CXCR1 peaking in the secretory phase of the cycle (Li *et al.*, 2012). Moreover, IL-8 is known to have both angiogenic and chemotactic properties (Koch *et al.*, 1992; Matsushima *et al.*, 1988). IL-8 has been found among the most highly upregulated genes in H-ESCs after 12 hour stimulation with cytotrophoblast (isolated from first and second trimester placental tissue) supernatant (Hess *et al.*, 2007). Although we showed for the first time that IL-8 was able to elicit a migratory response in H-ESCs, we did not detect differences in H-ESC migration between the normal fertile group and the RM group.

PDGF-BB, the well-described chemoattractant for H-ESCs and known to be present in first trimester placenta and receptive phase uterine fluid (Gentilini *et al.*, 2007; Hannan

et al., 2011; Matsumoto *et al.*, 2005), stimulated migration of H-ESCs of both fertile control women and women with RM. However, the migratory response of H-ESCs from RM women was impaired at the lower PDGF-BB dose compared to the migration of H-ESCs from normal fertile women. The H-ESCs from RM women are thus less sensitive to PDGF-BB alone than H-ESCs of the control women.

The mechanism underlying blunted H-ESCs migration in response to trophoblast signals in RM patients requires further investigation. Decidualization is a dynamic process, characterized by the transient expression of some, often pro-inflammatory genes such as *PROK1*, and continuous induction of other many other genes (e.g. *PRL*, *SGK1*, *LEFTY-A*). In RM, this temporal pattern of decidual gene appears disordered (Salker *et al.*, 2010; Salker *et al.*, 2011), which likely accounts for the reduced responsiveness of H-ESCs to pro-migratory trophoblast signals. The fact that we were able to use primary H-ESCs for our study contributes to the strength of this study. Ideally we would also want to test their migratory response to TCM derived from human primary trophoblast cells to more closely mimic the *in vivo* situation. Unfortunately this was not feasible in this study. Instead, we choose to use trophoblast cell line derived TCM as a migratory stimulus which has the benefit that it constitutes a highly reproducible source.

Recently, we compared the migration of decidualizing H-ESCs from women with RM and normal fertile women in the absence and presence of high- or low-quality (chromosomally abnormal) embryos using scratch assays (Weimar *et al.*, 2012). We found that migration of decidualizing H-ESCs from normal fertile women was inhibited in the presence of a low-quality embryo while this phenomenon was not observed in H-ESCs from women with RM. The H-ESCs from women with RM may thus not only be unresponsive to embryonic 'low-quality' signals, in addition H-ESCs from RM women may show impaired migratory responsiveness to stimulatory trophoblast factors.

In conclusion, we hypothesized that the migration of decidualizing H-ESCs towards trophoblast-derived factors may be altered in endometrium from women with a history of RM. In this paper we demonstrate that H-ESCs from women with RM indeed show a reduced migratory response, both to factors secreted by trophoblast cells and to low concentrations of the early pregnancy growth factor PDGF-BB. More research is needed to fully elucidate identify the responsible trophoblast secretory product(s) inducing H-ESCs migration. Possibly, an abnormal response to these

factors may underlie the impaired migratory response seen in the decidualizing H-ESCs from women with RM. As migration may be an important mediator of embryo implantation, impaired migration may compromise implantation, and in that way possibly contribute to the aetiology of miscarriages.

Acknowledgements

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Supplementary material

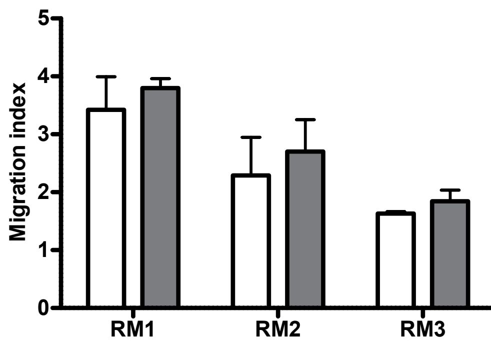

Figure S1 Migration of H-ESCs of RM women in response to platelet derived growth factor-BB (PDGF-BB). The difference in migration rate between unstimulated and PDGF-BB stimulated decidualizing H-ESCs from the same three RM patients is given at time point one (white bars) and one week later (grey bars). Data represent means ± SEM and were analysed by Student's *t*-tests.

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Chapter 4

Dexamethasone modulates human endometrial stromal cell migration *in vitro*

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Submitted

Abstract

Background: Glucocorticoid administration could develop into a therapy to support implantation in IVF and recurrent miscarriage (RM). However, the efficacy and mode of action remains unclear. Recently, human endometrial stromal cell (H-ESC) migration has been shown to contribute to embryo implantation and RM has been associated with an increased migratory H-ESC response to trophoblast spheroids and to low quality embryos. We now investigated whether dexamethasone inhibits spontaneous migration of decidualizing H-ESCs from women with recurrent miscarriage (RM) and H-ESCs from normal fertile women.

Methods: H-ESCs were isolated from endometrial biopsies obtained from six women with RM and from six control hysterectomy specimens. *In vitro* scratch assays were used to examine the influence of dexamethasone on spontaneous migration of decidualizing H-ESCs as well as migration of H-ESCs in response to trophoblast spheroids. The number of glucocorticoid receptor (GR) binding sites was determined using radiolabelled dexamethasone. Moreover, GRα, GRβ, FKBP5, GILZ and hSGK1 mRNA expression were determined in decidualizing H-ESC before and after dexamethasone stimulation using Q-PCR.

Results: Baseline migration (in the absence of dexamethasone) of H-ESCs was comparable between both groups. Dexamethasone enhanced the spontaneous migratory activity of H-ESCs from RM women in comparison to control H-ESCs ($P < 0.0001$). At higher dexamethasone doses (10^{-6}M), spontaneous migration of control H-ESC was inhibited by dexamethasone. The number of dexamethasone binding sites in H-ESCs was comparable between groups. Dexamethasone treatment decreased FKBP5 mRNA expression ($P < 0.01$) and increased GRα mRNA ($P < 0.001$) expression in RM-ESCs but not in control-H-ESCs. Dexamethasone did not alter the chemoattractive properties of trophoblast spheroids.

Conclusions: Dexamethasone enhances the spontaneous migratory response of decidualizing H-ESCs from women with RM whereas the migratory response of H-ESCs from control women is inhibited at higher dexamethasone doses. RM is associated with diminished FKBP5 mRNA expression and an elevated GR-α expression in decidualizing H-ESCs. The enhanced migratory response is, however, not due to a difference in the number of GR. Dexamethasone had no effect on the mRNA expression of the GR signaling molecules GILZ and hSGK1. These findings do not support a role for GC therapy in reducing H-ESC migratory activity in women with RM.

Introduction

The administration of glucocorticoids (GC) in the peri-implantation period has been suggested to be capable of improving endometrial receptivity and implantation rates in IVF. However, a recent meta-analysis on the efficacy of GC was inconclusive (Boomsma *et al.*, 2012). GC are sometimes prescribed for women with unexplained recurrent miscarriage (RM) in order to reduce the risk of a subsequent miscarriage (Quenby *et al.*, 2003). Again, however, data from an appropriately powered randomized controlled trial are still needed to define the therapeutic benefit in this clinical condition (Tang *et al.*, 2009).
The rationale for a therapeutic role of GC in IVF and RM is subject to debate. It has been postulated that GC may modulate immune cell activity in the endometrium (Quenby *et al.*, 2005). However, glucocorticoid receptors (GRs) have also been shown to be highly expressed by human endometrial stromal cells (H-ESCs), indicating a possible role in decidualization of H-ECS (Bamberger *et al.*, 2001).

Our group and others have shown that H-ESC migration, which can be demonstrated in *in vitro* models of implantation promoting blastocyst implantation, is part of the normal decidual phenotype (Grewal *et al.*, 2008; Weimar *et al.*, 2012). We have also reported that decidualizing H-ESCs from RM women have an increased migratory potential in the presence of trophoblast spheroids and in the presence of chromosomally abnormal embryos compared to H-ESCs of normally fertile women (Weimar *et al.*, 2012). This enhanced migration of H-ESC may render the endometrium more receptive and less 'selective' with respect to low-quality embryo implantation leading to the implantation of compromised embryos that will miscarry at a later stage (Teklenburg *et al.*, 2010; Weimar *et al.*, 2012).

The steroid hormones GC, progesterone and estrogen are crucial for H-ESC homeostasis and play an important role in human reproductive function. GRs reside in the cytosol and, upon ligand binding, translocate to the nucleus to regulate gene transcription (Munck, 2005; Pratt *et al.*, 1992; Rhen and Cidlowski, 2005). The sensitivity of GRs is regulated by heat-shock proteins (HSP70/HSP90) chaperoning GRs, which influence proper folding and maturation of the GR and subsequent translocation and gene transcription (Pratt *et al.*, 1992). FKBP5, both target gene and co-chaperone of the GR-HSP70/90 heterocomplex, lowers GR affinity and thereby alters glucocorticoid binding to the receptor (Binder, 2009; Denny *et al.*, 2000; Librach *et al.*, 1994).

Previous *in vitro* studies have shown that trophoblast cell migration and invasion is inhibited by GC treatment (Librach *et al.*, 1994). We hypothesized that GC treatment of decidualized H-ESC may also reduce migratory activity of H-ESCs *in vitro*. An inhibitory effect of GC on H-ESC migration may serve to prevent poor quality embryos from implanting and therefore decrease the risk of clinical miscarriage in those prone to this distressing condition. We therefore studied the effects of the GC dexamethasone on the spontaneous migration of decidualizing H-ESCs of normal fertile women and women with RM. In addition, we investigated whether dexamethasone influenced the chemotactic properties of trophoblast spheroids. In order to ascertain whether possible differences in migratory response between dexamethasone-treated H-ESCs from RM and control women was caused by a difference in the number of GR binding sites, and/or a differential expression of subtypes of GR, we measured the number of intracellular GRs and mRNA expression of GR-α and GR-β receptor subtypes in decidualizing H-ESCs from normal fertile and RM women. Moreover, we tested whether group differences existed in mRNA expression of the downstream signaling molecules of the GR pathway such as glucocorticoid-induced leucine zipper (GILZ) and serum and glucocorticoid-inducible kinase-1 (SGK-1), and the GR target gene and co-chaperone FKBP5.

Methods

Primary H-ESC cultures

This study was approved by the Medical Review Ethics Committee University Medical Center Utrecht and the Central Committee on Research involving Human Subjects in The Netherlands (NL30143.000.09). Written informed consent was obtained from all participating subjects. H-ESCs were isolated from endometrial biopsies obtained from six women with a history of unexplained RM (defined as three consecutive miscarriages before a gestational age of twenty weeks, with no identifiable cause) and from hysterectomy specimens of six premenopausal control women (operated for benign indications) with no history of RM.

All twelve individual H-ESCs cultures were isolated from proliferative phase endometrium. H-ESCs were isolated and cultured as previously described (Weimar *et al.*, 2012). In short, the endometrial tissue was finely minced and enzymatically digested in 10 ml 417 U/ml collagenase type IA (Sigma) in digest medium (phenolred-free Dulbecco's modified Eagle medium (DMEM)/F12 medium supplemented with 1% L-glutamine (Gibco), 1% amphotericin-B (Sigma, UK) and 1% penicillin/streptomycin

solution (Gibco)) for one hour in the incubator (37°C under atmospheric oxygen levels and 5% CO_2). To stop collagenase action, the digested tissue was placed in DMEM/F12 medium supplemented with 10% heat-inactivated fetal bovine serum (FCS) and pelleted by centrifugation at 670 g for eight minutes. The cells were cultured in standard medium (digest medium supplemented with 10% FCS (Gibco) in 75cm^2 tissue culture flasks in the incubator. After three hours medium was replaced to out select the glands. All H-ESC cultures were expanded and the cells frozen at -150°C in aliquots and thawed consecutively for the co-culture experiments ensuring identical culture conditions in all migration experiments.

In 48-well plates 25.000 undifferentiated H-ESCs were plated in standard medium for two days. Subsequently the H-ESCs were decidualized for five days. 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 (Sigma, UK) for five days. For this medium and for the experimental medium the FCS was replaced by 10% charcoal stripped FCS. The medium was changed every 48 hours. All primary H-ESCs were used before passage six.

Migration assay

A cell-free strip, the migration zone, was created by scratching a select area of confluent decidualized H-ESCs monolayers using a 1000 μl pipette-tip. All H-ESCs were subsequently cultured in the absence and presence of 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12} M dexamethasone or a trophoblast spheroid in triplo for eighteen hours. Pictures of the migration zone were taken directly after creating the migration zone and eighteen hours later. Using Photoshop software, the migratory response was quantified over eighteen hours.

Trophoblast spheroids

The AC-1M88 cell line, derived from human extravillous trophoblast cells, was cultured until 70-80% confluence, trypsinized and counted. Spheroids were formed as previously described (Gonzalez *et al.*, 2011). In short, in each well 100 μl medium containing 3000 cells and methylcellulose was plated in a round bottom non-adherent plate. Overnight in an incubator (37°C, 5% CO_2) the cells formed spheroids. One hour prior to the start of the experiment the spheroids were transferred to experimental medium.

mRNA expression

Total RNA was purified from H-ESC monolayers using RNeasy columns (Qiagen, Hilden, Germany). One µg of total RNA was used to synthesize cDNA with Super-Script Reverse Transcriptase (Invitrogen, Carlsbad, California, USA). Real-time polymerase chain reactions were performed with an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA) (see *Table 1* for primer sequences). Data were normalized to the average expression levels glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin.

Table 1. Primer Sequences Used in Quantitative Real-Time Polymerase Chain Reaction

Target	Sequence
GR-α	Forward: 5'-TCAACTgACAAAACTCTTgg-3'
	Reverse: 5'-TgATTggTgATgATTTCAgC-3'
GR-β	Forward: 5'-AgCggTTTTATCAACTgAC-3'
	Reverse: 5'-TgAgTTCTATTTTTTgAgCg-3'
GILZ	Forward: 5'-ACCgAAATgTATCAgACCCCCA-3'
	Reverse: 5'-CgATCTTgTTgTCTATggCCACC-3'
SGK-1	Forward: 5'-gAgATTgTgTTAgCTCCAAAGC-3'
	Reverse: 5'-CTgTgATCAgGCATAgCACACT-3'
FKBP5	Forward: 5'-AAATCCAAACgAAggAgCAA-3'
	Reverse: 5'-gCCACATCTCTgCAgTCAAA-3'
GAPDH	Forward: 5'-gAAggTgAAggTCggAgTC-3'
	Reverse: 5'-gAAgATggTgATgggATTTC-3'
β-actin	Forward: 5'-CCTggCACCCAgCACAAT-3'
	Reverse: 5'-gggCCggACTCgTCATACT-3'

Dexamethasone binding

For determination of the capacity of decidualizing H-ESCs to bind GRs, a validated whole cell single-point binding assay was used, as described previously (Yehuda,1995; VanZuiden, 2009). This method provides a reliable estimate of Bmax, as determined using a classical binding assay with 3–200 nM 3H-dexamethasone (r^2=0.92). Briefly, cultured decidualizing H-ESCs were trypsinized, washed and counted. 0.7 x10^6 decidualizing H-ESCs and 3x10^6 PBMCs (control) were resuspended in assay buffer (experimental medium with 5% fluorescence correlation spectroscopy), and incubated in duplicate with 100 nM ^{3}H-dexamethasone (Amersham, Buckinghamshire, United Kingdom) in the presence or absence of excess unlabeled dexamethasone (Sigma-Aldrich, Steinheim, Germany) for one hour at 37°C. Cell-bound radioactivity was quantified by liquid scintillation analysis.

Statistical analysis

Statistical testing was performed by Student's t-tests and two-way ANOVA with Bonferroni correction. A P-value below 0.05 was considered statistically significant.

Results

Spontaneous human endometrial stromal cell migration in the presence of dexamethasone

In the presence of dexamethasone H-ESCs of women with RM migrated more than H-ESCs from normally fertile women ($P < 0.0001$; *Figure 1*). Moreover, the migration of RM H-ESCs was increased in the presence of dexamethasone (10^{-10} M and higher) compared to the migration in the absence of dexamethasone. In contrast, the migration of H-ESCs from normally fertile women was inhibited in the presence of dexamethasone (10^{-10}, 10^{-9}, 10^{-8}, 10^{-6}M dexamethasone). The difference in migration was not due to a difference in dexamethasone-induced proliferation, as dexamethasone had no effect on H-ESC proliferation in both groups (data not shown).

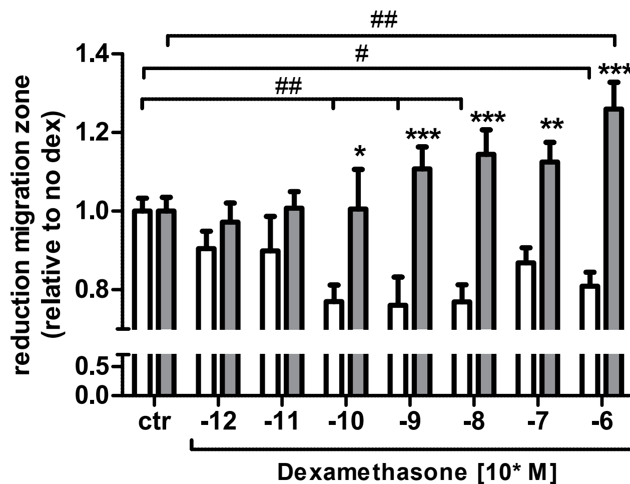

Figure 1. Migration of H-ESCs in the presence of various concentrations of dexamethasone. Decidualized H-ESCs of six women with RM (grey) and six control women (white) were cultured in seven different concentrations of dexamethasone. In a confluent well of a 48-well plate a migration zone was created. H-ESCs were left to migrate in the presence or absence of various dexamethasone concentrations. Data is shown as a reduction of the migration zone after eighteen hours and is set relative to the control migration in the absence of dexamethasone. Experiments were performed in triplicates. Data represent means ± SEM and was analyzed by 2-way ANOVA and Bonferroni post hoc tests, * and # $P < 0.05$, *** $P < 0.001$.

Human endometrial stromal cell migration in the presence of dexamethasone incubated trophoblast spheroids

In addition to a direct effect on the migratory effects of H-ESC, dexamethasone may also exert an indirect migratory effect on H-ESCs through altering the (chemoattractant) behaviour of embryonic trophoblast cells. To investigate whether dexamethasone alters the chemoattractive properties of the trophoblast spheroids (TBS), we analyzed the migration of H-ESC in the absence and presence of dexamethasone-incubated TBS. In accordance to our earlier findings we showed that TBS increase the H-ESC migration of women with RM while TBS do not alter H-ESC migration in the control women (Weimar *et al.*, 2012) (*Figure* 2). Pre-culturing the TBS with various concentrations of dexamethasone had no effect on the migratory response of both groups of H-ESCs compared to the H-ESC migration in the presence of TBS not incubated with dexamethasone.

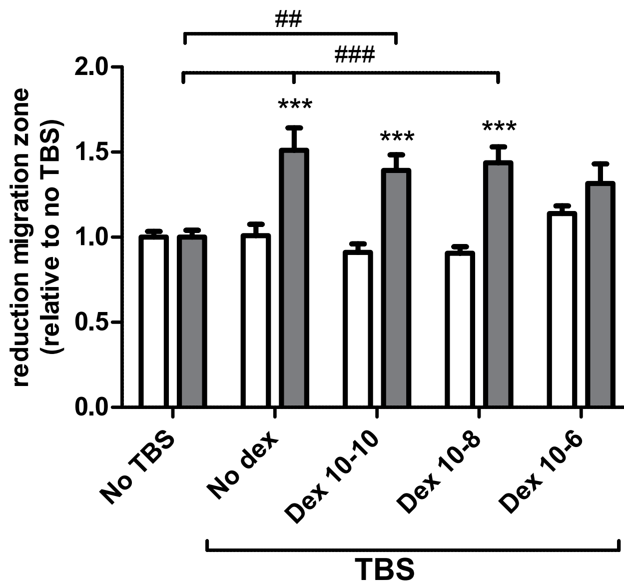

Figure 2. Migration of H-ESCs of fertile control and RM women in the presence of a dexamethasone-incubated trophoblast spheroid. In a confluent well of a 48-well plate a migration zone was created. H-ESCs were left to migrate in the presence or absence of a (dexamethasone-incubated) trophoblast spheroid in the migration zone. Data is shown as a reduction of the migration zone after eighteen hours and is set relative to the migration in the absence of a trophoblast spheroid. Experiments were performed in triplicates. Data represent means ± SEM of six women with RM (grey) and six normally fertile women (white) and was analysed by 2-way ANOVA and Bonferroni post hoc tests, *** $P < 0.001$.

Dexamethasone binding

To investigate whether the increased migratory response of RM H-ESCs to dexamethasone was associated with an increased number of GRs present in the RM H-ESCs, the number of GR binding sites was compared between the H-ESCs from three women with RM and three normally fertile women.

The number of binding sites did not differ between H-ESCs from women with RM and normal fertile women ($P < 0.05$) (*Figure* 3). H-ESCs from normally fertile women had on average 4705 GR binding sites (range 893-7859); H-ESCs from women with RM had on average 5681 GR binding sites (range 3795-7663).

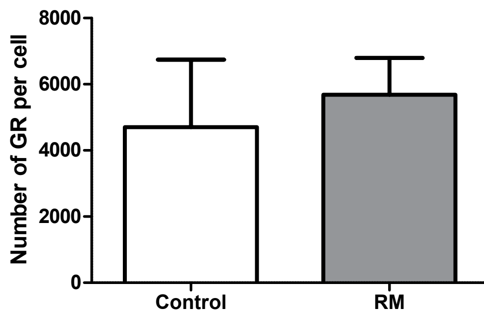

Figure 3. The number of GR binding sites. Similar numbers of GR binding sites were measured in decidualizing H-ESCs from normal fertile women (white) and women with recurrent miscarriage (grey).

Subtypes of glucocorticoid receptors

Although the total number of GR was similar between groups, we next investigated whether the increased migratory response of dexamethasone-treated decidualizing H-ESC from RM women was caused by a differential expression of subtypes of GR. To that end we determined GR-α, and GR-β mRNA expression in decidualizing H-ESC from RM and control women under unstimulated conditions. In these conditions, decidualizing H-ESCs, *GR-α* expression did not differ between the RM and the control group. The effect of dexamethasone on GR-α mRNA expression was significantly different between decidualizing H-ESCs of the RM and the control group ($P < 0.001$, *Figure 4A*). In both groups the *GR-β* gene expression was not detectable in H-ESCs cultured in the presence and absence of dexamethasone (data not shown).

Glucocorticoid receptor signaling molecules

To investigate whether the effect of dexamethasone on GR-mediated migration in the RM group was caused by differences in GR signaling, we also determined mRNA of two downstream GR signaling pathway molecules GILZ, and human serum- and glucocorticoid-inducible kinase 1 (hSGK1) in the presence of various doses of dexamethasone. However, the transcription of the genes encoding hSGK1, GILZ were unaffected by dexamethasone administration in both groups (*Figure 4B and 4C*).
mRNA expression of FKBP5, a third downstream GR target molecule was also measured in both H-ESC groups. FKBP5 is a molecule that is capable of decreasing the binding affinity of the GR. No effect of dexamethasone was observed on the expression of FKBP5. However, FKBP5 expression was significantly higher in the control group compared to the RM group (*Figure 4D*).

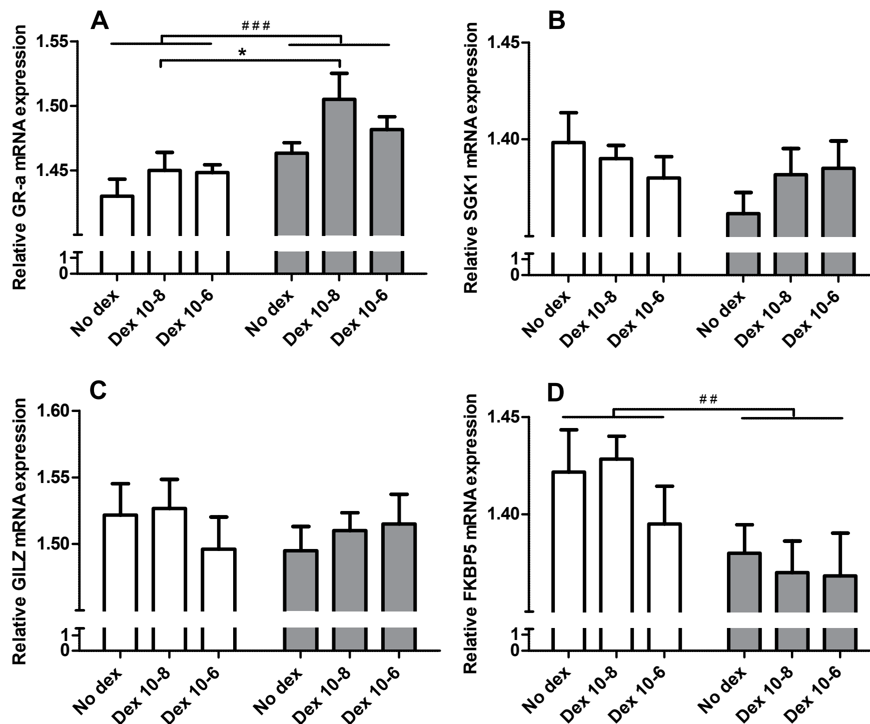

Figure 4. The relative mRNA expression of GR target genes (GR-α, SGK1, GILZ and FKBP5) in decidualizing H-ESC of six controls (white) and six women with RM (grey). Gene expression of decidualizing H-ESCs was measured in the absence and presence of two concentration of dexamethasone (10^{-8} and 10^{-6}M), and were normalized to GAPDH and b-actin levels. Data represent means ± SEM and was analyzed by 2-way ANOVA and Bonferroni post hoc tests, * $P < 0.05$, ** and ## $P < 0.01$, ### $P < 0.001$.

Discussion

In this study, dexamethasone was shown to stimulate the migration of decidualizing H-ESCs from women with RM and to inhibit the migration of decidualizing H-ESCs from normally fertile women.

H-ESC migration actively takes place during embryo implantation and may promote the implantation process. RM has been associated with an increased migratory (super-receptive) H-ESC response in the presence of chromosomally abnormal embryos and TBS (Weimar *et al.*, 2012). As RM women are occasionally treated with glucocorticoids, we wished to investigate whether the synthetic glucocorticoid dexamethasone inhibited the migratory response of H-ESCs of RM women. However, the opposite effect was observed in decidualizing H-ESCs from women with RM.

The increased migratory response of decidualizing H-ESCs from RM women in the presence of dexamethasone could not be explained by a difference in the number of GR binding sites as both were comparable between the two groups. However, FKBP5 mRNA, a co-chaperone molecule of the GR/HSP90 complex that is known to lower the GC binding affinity (Binder, 2009; Denny *et al.*, 2000), was observed to differ between the decidualizing H-ESCs of RM and control women. A lower FKBP5 expression would indicate greater affinity for GR and thus a more pronounced effect of dexamethasone, i.e. more inhibition of migration, would be expected. However, we observed an increased migratory effect of dexamethasone in H-ESC from women with RM. The latter data may indicate that the level of FKBP5 does not influence GR signaling with respect to migration and that other unknown substrates direct the functional changes in migration induced by dexamethasone.

The migratory differences observed between the two H-ESC groups may also be due to a difference in GR signaling further downstream of the GR. However, mRNA expression of two downstream GR signaling pathway molecules GILZ and hSGK1 were comparable between the RM and the control group.

Thus far, three subtypes of the GR have been identified. GR-α is the most abundant variant and is transcriptionally active (Hollenberg *et al.*, 1985), while GR-β is less ubiquitously expressed, does not bind GC and has limited transcriptional capability (Kino *et al.*, 2009). The third GR is GR-P, a broadly expressed receptor that is known to increase GR-α activity (Hagendorf *et al.*, 2005). In this study, we observed that GR-α was upregulated by dexamethasone only in the RM group. However under basal conditions there was no difference in GR-α mRNA expression and the number of GR

binding sites was comparable between decidualizing H-ESCs from normal fertile and RM women.

If the increased migratory potential of decidualizing H-ESCs from women with RM indeed contributes to a failure in embryo selection and thus the implantation of compromised embryos, then inhibition of migration may be beneficial for RM women. However, in this study dexamethasone is shown not to inhibit but to enhance H-ESC migration in women with RM. On the basis of this experiment we conclude that dexamethasone would not be the first choice to treat RM. However, in the IVF setting where many women may suffer from implantation failure, enhancing H-ESC migration may have a beneficial effect on implantation rates. How H-ESC migration of women with repeated implantation failure is affected by dexamethasone treatment is the subject of ongoing study by our group.

Future investigations should determine the effect of dexamethasone on chemokine and cytokine expression in decidualizing H-ESCs of RM and control women. It may well be that the difference in migratory effects of dexamethasone could be explained by a differential expression of chemokines or chemokine receptors.

In conclusion, dexamethasone enhances H-ESC migration in women with RM, while it inhibits H-ESC in normally fertile women. RM is further associated with diminished FKBP5 mRNA expression and an elevated GR-α expression in the presence of dexamethasone in decidualizing H-ESCs from RM women compared to the H-ESCs of control women. Deciphering how dexamethasone enhances migration of H-ESCs from women with RM, requires further investigation.

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Chapter 5

The active human decidual phenotype: opening the black box of early pregnancy loss?

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Annemieke Kavelaars, Cobi J. Heijnen

Invited review by Human Reproduction Update

Abstract

Background: In contrast to other mammals, the majority of human embryos does not result in an ongoing pregnancy. This may be explained by the high rate of chromosomal abnormalities observed in human embryos. Recently, human decidualizing endometrial stromal cells (H-ESCs) have been shown to have a role in embryo selection. Current concepts of decidual function therefore need to be reassessed.

Methods: A search of MEDLINE was performed focussing on decidualization and implantation in order to review the classically known and current concepts of H-ESC function. Articles discussing decidualizing H-ESC function of fertile women and women with recurrent miscarriage (RM), implantation failure and endometriosis were considered.

Results: H-ESCs provide a substrate for trophoblast invasion and aid implantation by protecting the embryo from oxidative stress and immune rejection. Recently, a novel feature in embryo selection has been described as a dynamic, migratory H-ESC response to the implanting embryo. Recent data indicate that endometriosis and RM may be associated with abnormal migration of H-ESCs, while implantation failure and RM may be in part explained by disruption of the decidual embryo selection phenotype.

Conclusions: Novel concepts are extending our understanding of the role of decidualizing H-ESCs in human implantation. Decidual embryo selection serves to prevent implantation by the majority of compromised embryos. Implantation involves an active migratory response by H-ESCs which is actively modulated by embryo quality. Further understanding of these emerging concepts will lead to new strategies for preventing and treating reproductive disorders such as RM.

Introduction

Despite the high number of people on our planet, human reproduction is highly inefficient (Evers, 2002). The human monthly fecundity rates (MFR), defined as the chance of a successful conception within one menstrual cycle that will lead to a live birth, is low compared to other mammals. In humans the MFR is 30% between 20 and 30 years of age and decreases to <10% above the female age of 35 (Chard, 1991). As a comparison, in baboons the MFR is 80% (Stevens, 1997) and in rabbits it is 90% (Foote and Carney, 1988). Currently, in Western countries approximately one in six couples suffers from subfertility, which is defined as the failure of conceiving after one year of unprotected regular intercourse (Evers, 2002).

Another remarkable difference between humans and other mammals is the high rate of chromosomal abnormalities in human zygotes. Chromosomal abnormalities have been found in non-human mammals with a prevalence of around 27% in bovine species, 18% in primate species, and 5 or 0% in *in vitro* or *in vivo* generated equine embryos (Dupont, Segars *et al.*, 2010; Rambags, Krijtenburg *et al.*, 2005; Viuff, Greve *et al.*, 2000). When the rate of mosaic embryos is taken into account, this percentage increases to 50% in non-human primate species and to 40% and 18% in *in vitro* or *in vivo* generated equine embryos respectively. The rate of chromosomal abnormalities in human pre-implantation embryos ranges from more than 50% as demonstrated by studies using fluorescent in situ hybridization (FISH) to 91% in studies that used comparative genomic hybridization (CGH) (Delhanty, 2005; Vanneste, Voet *et al.*, 2009; Voullaire, Slater *et al.*, 2000; Wells and Delhanty, 2000). Using CGH all chromosomes are screened and therefore this method detects the true rate of mosaicism. Interestingly, in spite of a high frequency of aneuploidy found in pre-implantation embryos, only a low percentage (0.3%) of aneuploidy is found at term birth (Hassold, Abruzzo *et al.*, 1996; Plachot, de *et al.*, 1987), which suggests that there is a negative selection of abnormal embryos.

Decidualization of the endometrium only occurs in species in which implantation is accompanied by breaching of the luminal epithelium by a blastocyst and subsequent trophoblast cell invasion in the decidua (Brosens, Pijnenborg *et al.*, 2002; Ramsey, Houston *et al.*, 1976). The third unique feature of the human reproductive system is the cyclic decidualization of the endometrium that occurs irrespective of coitus and irrespective of the presence of a conception (Brosens, Pijnenborg *et al.*, 2002). In primate species, the extent of decidualization is related to the degree of trophoblast invasion (Ramsey, Houston *et al.*, 1976). Human trophoblast invasion is very exten-

sive and even includes invasion into the myometrium. Human decidualization is also more pronounced compared to other species that undergo endometrial decidualization and affects all uterine compartments; spiral arteries, immune cells, epithelial and endometrial stromal cells (H-ESCs) (Brosens, Pijnenborg *et al.*, 2002).
H-ESCs are increasingly recognized as key players in the implantation process. During the process of implantation, the embryo becomes completely surrounded by the H-ESC compartment. H-ESCs seem not only to function passively as fertile soil for implantation but there is emerging evidence H-ESCs actively contribute to the achievement of embryo implantation and maintenance of the pregnancy. In this review, both the established and more recently reported active functions, such as H-ESC migration and embryo selection by H-ESCs in the implantation process, will be described. Finally, we will discuss the possibility that dysfunctional H-ESCs contribute to the pathofysiology of conditions such as implantation failure, recurrent miscarriage (RM) and endometriosis.

Methods

Electronic searches were performed in the MEDLINE database with keywords focused on decidualization, decidual function and implantation in health and in reproductive disorders. Search criteria included: endometrial stromal cells, uterine stromal cells, endometrium, decidualization, decidua, implantation, migration, embryo selection, trophoblast invasion, endometriosis, implantation failure, recurrent miscarriages, recurrent pregnancy loss.
The searches were restricted to articles published in the English language and covered the period from 2000 to August 2012. Earlier relevant publications were accessible from previously conducted searches and cross-references were picked up during the review process.

Decidualization and functions of H-ESCs in implantation

Endocrine regulation of decidualization

Throughout a woman's reproductive life, the functional layer of the human endometrium regularly breaks down, proliferates and decidualizes in preparation for the implantation of an embryo, should conception occur. The term decidualization encompasses a complex range of changes to the endometrium and key to these changes is the differentiation of endometrial fibroblast-like H-ESCs into secretory, epithelioid decidual H-ESCs (Gellersen, Brosens *et al.*, 2007; Tang, Guller *et al.*, 1993). The most notable secretory products of decidual H-ESCs are summarised in *Table 1*.

Table 1. Short overview of products produced by decidualizing ESCs

GROUP	PRODUCT	FUNCTION AND REFERENCE
Hormones/ neuropeptides	Prolactin	Characteristic marker of ESC decidualization (Bell, Jackson et al., 1991)
	Relaxin	Promotes decidualization of ESCs. (Hsu, Nakabayashi et al., 2003; Bartsch, Bartlick et al., 2001)
	Somatostatin	Somatostatin may control ESC migration. (Annunziata, Luque et al., 2012)
	CRH	Paracrine inducer of decidualization and may participate in the early maternal tolerance to semiallograft embryo. (Ferrari, Petraglia et al., 1995; Zoumakis, Margioris et al., 2000)
Cytokines	IL-15	Promotes ESC decidualization and stimulates lymphocyte (NK cell) proliferation (Okamoto, Uchida et al., 1991; Teklenburg, Salker et al., 2010)
	TNF-α	Proinflammatory cytokine. Inhibits *in vitro* decidualization of ESCs. (Inoue, Kanzaki et al., 1994; Teklenburg, Salker et al., 2010)
	Glycodelin-A	Glycoprotein with potent immunosuppressive function. (Bolton, Pockley et al., 1987; Seppala, Taylor et al., 2002)
Extracellular matrix proteins	Collagen IV, laminin, fibronextin, decorin	Extracellular matrix proteins provide structural support to the endometrial cells. Regulatory roles in adhesion-dependent cellular activities. (Tanaka, Wang et al., 2008; Bolton, Pockley et al., 1987; Popovici, Betzler et al., 2006)
Enzymes	Matrix metalloproteinase	Degrades the extra cellular matrix proteins. (Bolton, Pockley et al., 1987; Gellersen, Reimann et al., 2010)
	Protease inhibitors (TIMPs)	Tissue inhibitors of metalloproteinases (TIMPs) (Bolton, Pockley et al., 1987; Dimitriadis, White et al., 2005)
	Antioxidants	Enzymes with antioxidant activity, such as superoxide dismutase 2 (SOD2), thioredoxin, monoamine oxidases A and B and gluthatione peroxidase 3 (GPx3). Counteract oxidative cell death. (Maruyama, Yoshimura et al., 1999; Bolton, Pockley et al., 1987; Gellersen, Reimann et al., 2010)
	Indoleamine 2,3-dioxygenase (IDO)	Enzyme with an immunosuppressive function. (Bolton, Pockley et al., 1987; Kudo, Boyd et al., 2004; von, Krusche et al., 2007)
Growth factors	Heparin-binding EGF-like growth factor (HB-EGF)	It facilitates embryo development and mediates implantation. (Bolton, Pockley et al., 1987; Chobotova, Karpovich et al., 2005; Kudo, Boyd et al., 2004; Teklenburg, Salker et al., 2010)
	Vascular endothelial growth factor (VEGF)	Increases permeability of uterine epithelium, promotes angiogenesis and the recruitment of peripheral NK cells. (Olofsson, Jeltsch et al., 1999; Ancelin, Buteau-Lozano et al., 2002; Engert, Rieger et al., 2007; Teklenburg, Salker et al., 2010)
	Fibroblast growth factor (FGF)	May induce ESC migration. (Ancelin, Buteau-Lozano et al., 2002; Tsai, Wu et al., 2002)
Various	Insulin-like growth factor binding protein-1 (IGFBP-1)	The second characteristic marker of ESC decidualization. (Bell, Jackson et al., 1991)
	Glycogen and lipids	Causes the enlargement of the ESC during decidualization. Provide nutrition for the implanting embryo. (Cornillie, Lauweryns et al., 1985)

The initiation of decidualization seems to be a biphasic process (Telgmann, Maronde *et al.*, 1997). The first, relatively weak induction of decidualization, with only a modest upregulation of the typical marker genes prolactin (*PRL*) and IGF-binding protein 1 (*IGFBP-1*), is stimulated by the post-ovulatory rise in intracellular cyclic adenosine monophosphate (cAMP) and subsequent activation of protein kinase A, a family of enzymes whose activity is dependent on cAMP (Bell, Jackson *et al.*, 1991; Gellersen and Brosens, 2003; Telgmann, Maronde *et al.*, 1997).

The postovulatory rise in cAMP level of H-ESCs may be realized through various pathways (*Figure 1*). Prostaglandins (Frank, Brar *et al.*, 1994), human chorionic gonadotropin (hCG) (Tang and Gurpide, 1993), corticotropin-releasing hormone (CRH) (Ferrari, Petraglia *et al.*, 1995), gonadotrophins (Tang and Gurpide, 1993; Zoumakis, Margioris *et al.*, 2000) and relaxin (Hsu, Nakabayashi *et al.*, 2003) have been reported to bind G-protein coupled receptors (GPCR). After binding of the ligands to their specific receptors, adenylate cyclases are activated, leading to elevation of intracellular cAMP levels. The hormone relaxin, mainly produced by the corpus luteum and the endometrium, may also function as a phosphodiesterase inhibitor thereby preventing the breakdown of cAMP (Bartsch, Bartlick *et al.*, 2001). Decidualization is further enhanced and maintained by progesterone and androgen signalling (Brosens, Hayashi *et al.*, 1999; Cloke, Huhtinen *et al.*, 2008).

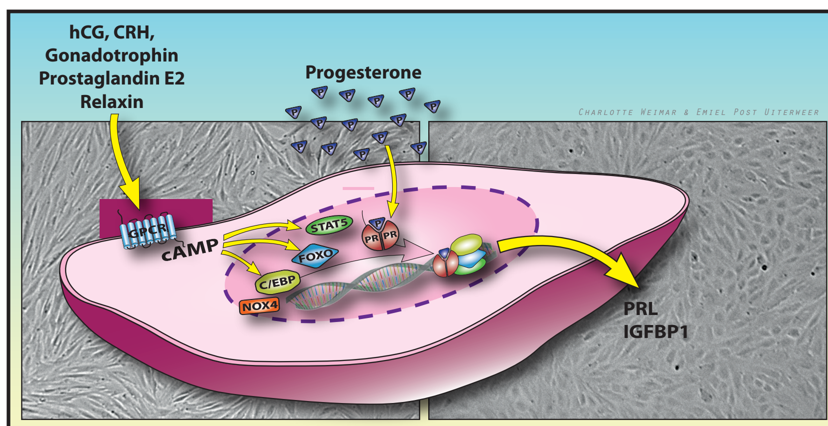

Figure 1. cAMP, progesterone and NOX-4 induced decidualization of H-ESCs. The left phase contrast picture shows primary undifferentiated human ESCs in culture. The H-ESCs transform into decidualizing secretory ESCs (the right phase contrast picture) under influence of cAMP, progesterone and NOX-4 signalling. The decidual marker genes *PRL* and *IGFBP-1* are transcribed following the formation of a transcriptome complex of the progesterone receptor (PR), CCAAT enhancer binding protein (C-EBP-β), forkhead box protein O1a (FOXO1a) and signal transducers and activators of transcription (STAT5) (see text for details).

After twelve hours of cAMP signalling, a second much stronger decidual response is generated which is likely to be due to nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX)-4 activation and NOX-4 -dependent reactive oxygen species (ROS) signalling (Al-Sabbagh, Fusi *et al.*, 2011). The importance of NOX-4 in the decidualization process was shown by NOX-4 knockdown in H-ESCs which strongly inhibited the expression of *PRL* and *IGFBP-1* (Al-Sabbagh, Fusi *et al.*, 2011). NOX-4 and ROS signalling lead to an increased DNA binding activity of CCAAT enhancer binding protein (C/EBP-β), an important transcription factor for the transcription of PRL and IGFBP-1 (Al-Sabbagh, Fusi *et al.*, 2011; Christian, Zhang *et al.*, 2002; Pohnke, Kempf *et al.*, 1999).
In addition to C/EBP-β, the intracellular rise in cAMP induces the expression of several other transcription factors, such as signal transducers and activators of transcription (STAT5) (Mak, Brosens *et al.*, 2002), specificity protein 1 (SP1) (Lockwood, Krikun *et al.*, 2001), forkhead box protein O1a (FOXO1a) (Christian, Zhang *et al.*, 2002; Kajihara, Jones *et al.*, 2006; Labied, Kajihara *et al.*, 2006; Takano, Lu *et al.*, 2007) and SRC kinase (Nagashima, Maruyama *et al.*, 2008), all of which are capable of interacting directly with progesterone-receptors. It is hypothesized that progesterone receptor-A in H-ESCs serves as a platform for the formation of a multimeric DNA binding complex that regulates the transcription of the decidua-specific genes *PRL* and *IGFBP-1* (*Figure 1*) (Gellersen and Brosens, 2003).

Current concepts of the role of decidualizing H-ESCs in implantation

Numerous biological substances are produced by H-ESCs during decidualization including growth factors, cytokines, neuropeptides, free radical scavengers and extracellular matrix components (Oliver, Cowdrey *et al.*, 1999; Popovici, Kao *et al.*, 2000; Tang, Guller *et al.*, 1993) enabling the H-ESCs to display a variety of characteristics essential for ensuring a pregnancy favourable environment both for embryo implantation and pregnancy maintenance (*Table* 1).
Firstly, H-ESCs function as a receptive substrate for embryo implantation (*Figure* 2). During decidualization the extracellular matrix gene expression patterns are changed, including the main components of the decidual extracellular matrix; type IV collagen, fibronectin, laminin and decorin (Aplin, Charlton *et al.*, 1988; Popovici, Kao *et al.*, 2000). Trophoblast cells migrate and invade the H-ESC compartment to ensure future metabolic support of the embryo for which degradation of extracellular matrix is necessary.

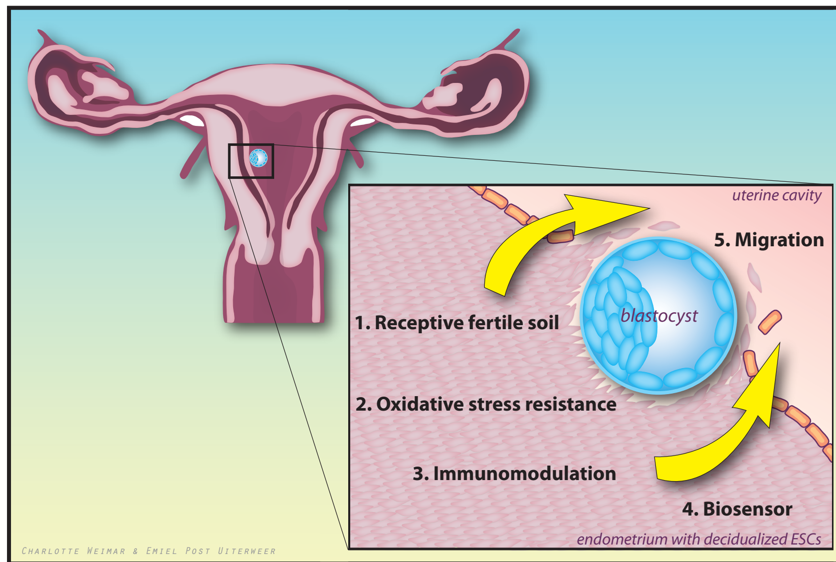

Figure 2. Schematic overview of a cross section of the female reproductive organs (upper left). In the uterine cavity a blastocyst (blue) is implanting. The box (bottom right) zooms in on the feto-maternal interface, and depicts the functions of decidualizing ESCs.

Extracellular matrix proteins can be broken down by matrix metalloproteinases (MMPs) (e.g. collagen IV is broken down by MMP-2 and MMP-9) that are produced by trophoblast cells, and to a lesser extent by decidualizing H-ESCs (Gellersen, Brosens *et al.*, 2007). In order to limit the extent of trophoblast invasion, extracellular matrix breakdown is under tight control of tissue inhibitors of MMPs (TIMPs) that are primarily produced by decidual H-ESCs (Dimitriadis, White *et al.*, 2005; Gellersen, Brosens *et al.*, 2007).

Over the course of pregnancy, some H-ESC functions may become more important. For example, the capacity of decidualizing H-ESCs to protect themselves and the embryo against environmental- and oxidative stress is most relevant at the end of the first trimester (Jauniaux, Poston *et al.*, 2006; Kajihara, Jones *et al.*, 2006; Kajihara, Tochigi *et al.*, 2012; Leitao, Jones *et al.*, 2010). In response to endometrial inflammation, remodelling and vascular changes that are present especially at the end of the first trimester, there is a profound increase in oxygen tension and oxidative stress is generated by the production of ROS (Brosens, Parker *et al.*, 2009). The integrity of the fetal-maternal interface is dependent on the resistance to oxidative stress and on the ability to maintain progesterone signalling (Leitao, Jones *et al.*, 2010). It is known that progesterone signalling in decidualizing H-ESCs ensures fetal-maternal interface

integrity by preventing proteolytic breakdown of the endometrium (menstruation) (Critchley, Kelly *et al.*, 2001). In undifferentiated H-ESCs an increase in ROS will lead to c-Jun N-terminal kinases (JNK)-induced hypersumoylation causing the transcriptional inhibition of the progesterone receptor. Repressed progesterone receptor activity may then induce the cascade of oxidative stress-induced breakdown of the endometrium (Leitao, Jones *et al.*, 2010). The activated JNK pathway in ROS-exposed undifferentiated H-ESCs also induces phosphorylation of FOXO3a, a major transcriptional regulator of pro-apoptotic genes (Kajihara, Jones *et al.*, 2006). Thus, undifferentiated H-ESCs are programmed to undergo apoptosis in response to oxidative stress signals.

Decidualizing H-ESCs, however, have developed effective strategies to counteract oxidative stress and apoptosis by producing free radical scavengers such as superoxide dismutase 2 (SOD2), thioredoxin, monoamine oxidases A and B and glutathione peroxidase (GPx3) (Gellersen, Brosens *et al.*, 2007; Cornillie, Lauweryns *et al.*, 1985; Maruyama, Yoshimura *et al.*, 1999). In addition, in decidualizing H-ESCs progesterone signalling is actively maintained by (the MAPK phosphatase-1) the downregulation of JNK signalling, preventing hypersumoylation and thus keeping the progesterone receptor active (Leitao, Jones *et al.*, 2010). Moreover, FOXO3a expression is repressed and -combined with the increased free radical scavenging potential- renders the decidualizing H-ESCs well equipped to withstand oxidative stress (Kajihara, Jones *et al.*, 2006). Protection against oxidative stress in decidualizing H-ESCs may also be regulated through androgen signalling and this was shown to be dependent on an androgen-induced increase of FOXO1 and SOD (Kajihara, Tochigi *et al.*, 2012).

In addition to functioning as 'fertile soil' and endometrial protection from oxidative stress, the ability of H-ESCs to control anti-fetal immune responses also ensures a healthy pregnancy environment. H-ESCs exert immunosuppression by the production of a plethora of soluble mediators, including glycodelin-A and indoleamine 2,3-dioxygenase (IDO) (Alok, Mukhopadhyay *et al.*, 2009; Kudo, Boyd *et al.*, 2004; Kudo, Hara *et al.*, 2004; Seppala, Taylor *et al.*, 2002; von, Krusche *et al.*, 2007). Although IDO is predominantly found to be produced by uterine epithelial cells and leukocytes, it is also expressed by H-ESCs from the mid-luteal phase onwards (von, Krusche *et al.*, 2007). IDO is able to control potentially harmful T cell responses against the semi-allogeneic fetus by depleting the essential amino-acid tryptophan that is necessary for T cell proliferation (Munn, Zhou *et al.*, 1998). Glycodelin-A has been shown to suppress lymphocyte function such as NK cell activity and cytokine production (Bolton, Pockley *et al.*, 1987; Okamoto, Uchida *et al.*, 1991). Moreover, H-ESCs may express indirect

immunomodulatory effects through the recruitment of IL-4 and IL-10 producing anti-inflammatory T cells (Hunt, Chen *et al.*, 1996; Moreau, drian-Cabestre *et al.*, 1999). In addition, decidualizing H-ESCs express HLA-G which is known to downregulate immune responses through interaction with inhibitory receptors on various immune cells: uterine NK cells, macrophages and $CD8^{+}$ T cells (Long, 1999; Ristich, Liang *et al.*, 2005).

Emerging concepts of the role of decidualizing H-ESCs in implantation

Recent evidence indicates two novel functions of decidualizing H-ESCs. The first is that of 'embryo selection'. This new concept describes the function of H-ESCs as a biosensor for embryonic derived signals and for selection of high- over low- quality embryos for implantation. In order to select high-quality embryos for implantation the H-ESCs must have the ability to respond to trophoblast- and embryonic signals (the first prerequisite for embryo selection). Indeed, decidualizing H-ESC gene expression levels, including those for multiple cytokines, have been shown to change dramatically in the presence of trophoblast cells or embryos as measured in *in vivo* and *in vitro* co-culture systems (Hess, Hamilton *et al.*, 2007; Kashiwagi, DiGirolamo *et al.*, 2007; Popovici, Betzler *et al.*, 2006; Teklenburg, Salker *et al.*, 2010). For example, in the pregnant mouse decidua, up to 1500 genes were reported to be differentially expressed compared to the non pregnant deciduas (Kashiwagi, DiGirolamo *et al.*, 2007). Many of these genes are involved in hemostasis and inflammation, interferon signaling, tissue growth and remodelling and NK cell function. Interestingly, factors secreted by NK cells (such as TNF-a, TGF-β and IFN-γ) have been found to be crucial in controlling trophoblast invasion in mouse models (Bulmer and Lash, 2005; Lash, Schiessl *et al.*, 2006). Another endometrial gene profiling study analyzed the effect of first trimester trophoblast explants on human H-ESCs in a co-culture model. Especially H-ESC genes regulating inflammatory responses, and chemotaxis were upregulated in the trophoblast explant-H-ESC co-culture model (Popovici, Betzler *et al.*, 2006). For example, pentraxin-related gene 3, CXCL2, CXCL1, CCL2, CXCL6 and CCL8 were respectively 105, 291 195 103, 83 and 30 times upregulated. IL-8, IL-1 receptor, IL-15 and its receptor were 367, 13, 10, and 4.5 times upregulated respectively.

Interestingly, prior to embryo implantation the decidualizing H-ESCs do not only sense and respond to embryonic signals but this response may be modulated by the quality of the embryo (the second prerequisite for embryo selection). Evidence for the biosensor function of embryo quality in human endometrium has been gener-

ated by our group. We have previously shown that decidualizing H-ESCs are capable of recognizing and responding differently to low-quality (arresting, developmentally compromised) or high-quality (developing) human embryos (Teklenburg, Salker *et al.*, 2010). At the level of cytokine secretion, normal H-ESCs were largely indifferent to the presence of a developing embryo. However, in the presence of an arresting embryo, the H-ESCs responded by downregulating the production of implantation cytokines and immunomodulators, such as IL-1β, HB-EGF, IL-6, IL-10, IL-17, IL-18 and eotaxin (Teklenburg, Salker *et al.*, 2010). These findings indicate that the decidualizing H-ESC has the potential to function as a biosensor for the detection of embryo quality.
The biosensor function of embryo quality is probably not confined to the human species. Also bovine endometrium gene profiles have been shown to alter in response to embryos of low (embryos generated through somatic cell nuclear transfer-SCNT- or *in vitro* fertilization) versus high developmental potential (*in vivo* fertilized embryos; control pregnancies) (Mansouri-Attia, Sandra *et al.*, 2009). According to Gene Ontology Classification, genes related to metabolism (26%) and immune function (24%) were differentially expressed in the bovine endometrium in response to embryos generated through SCNT compared to the gene expression profiles in control pregnancies. In total, 342 genes were differently expressed in SCNT pregnancies compared to 263 genes in the control pregnancies. These data suggest that in a bovine model too, lower quality embryos have more impact on the endometrial response compared to embryos with high developmental potential.

H-ESC migration

Following menstruation, both H-ESC proliferation and the migration of H-ESCs support regeneration of the functional layer of the uterine endometrium (Mansouri-Attia, Sandra *et al.*, 2009; Salamonsen, 2003). Recently it has been shown that H-ESC migration also occurs around the time of embryo implantation and may promote implantation by encapsulation of the conceptus (Brosens and Gellersen, 2010; Grewal, Carver *et al.*, 2008). In timelapse imaging studies covering a period of 48-hours, H-ESC migration was clearly depicted at the site of embryo implantation (Grewal, Carver *et al.*, 2008). Moreover, H-ESCs showed migration around the embryo suggesting an active role for H-ESCs in the implantation process (Grewal, Carver *et al.*, 2008).
H-ESC motility at the fetal-maternal interface has also been described in more detail (Gellersen, Reimann *et al.*, 2010). Using an *in vitro* cellular transwell invasion system and migration assays, it was shown that H-ESC motility was more pronounced

in decidualizing H-ESCs compared to undifferentiated H-ESCs. H-ESC invasion and migration were significantly enhanced when the decidualizing H-ESCs were placed in contact with either extra-villous trophoblast cells (AC-1M88) or AC-1M88-derived supernatant (Gellersen, Reimann *et al.*, 2010). These data strongly suggest that directed migration of decidualizing H-ESCs contributes actively to the implantation process when decidualizing H-ESCs are allowed to interact with an embryo or extra-villous trophoblast cells (Gellersen, Reimann *et al.*, 2010; Grewal, Carver *et al.*, 2008).

We recently reported that the migratory activity of decidualizing H-ESCs obtained from normal fertile women is modulated in response to the quality of the embryo (Weimar, Kavelaars *et al.*, 2012). Using so-called 'scratch' or 'wound healing' assays the migration of H-ESC towards human day five embryos was tested (Rodriguez, Wu *et al.*, 2005; Weimar, Kavelaars *et al.*, 2012). More specifically, five day decidualizing H-ESCs were left to migrate in the presence or absence of a high- or low-quality (chromosomally abnormal 3PN) embryo that was placed in a migration zone. After eighteen hours the reduction of the migration zone, i.e. the migratory activity of the H-ESCs, was assessed. The migratory activity of decidualizing H-ESCs from fertile women was completely inhibited in the presence of chromosomally abnormal (3PN low-quality) embryos, compared to the basal migration in the absence of an embryo or to migration in the presence of a high-quality embryo. Such inhibition of migration may be an effective way of H-ESCs to protect women from implanting embryos that have a low developmental potential and/or embryos that are chromosomally abnormal.
These findings are consistent with those reported by Teklenburg et al. using an H-ESC-human embryo co-culture system, in which a human embryo was placed on a confluent monolayer of decidualizing H-ESCs. In this study, down regulation of cytokine production by decidualizing H-ESCs was only observed in co-cultures with arresting embryos and not in co-cultures with high-quality (developing) embryos (Teklenburg, Salker *et al.*, 2010).

Stimuli for H-ESC migration

The novel data summarized in the previous section suggest that the decidual H-ESC compartment is capable of responding to a 'low-quality' signal released by chromosomally abnormal or arresting embryos. Thus far the exact nature of this signal is unknown. Compromised embryos have been shown to be metabolically very active, producing increased levels of ATP and ROS and demonstrating increased amino acid

turnover when compared to viable embryos (Leese, Sturmey *et al.*, 2007; Leese, Baumann *et al.*, 2008; Stokes, Hawkhead *et al.*, 2007). It is tempting to speculate therefore, that these metabolites include signals of embryo quality which are sensed by H-ESCs, inhibiting migration and subsequent embryo development.

In addition to extra-villous trophoblast cell line factors and 'low-quality' embryo markers that have been shown to enhance and inhibit H-ESC migration respectively (Gellersen, Reimann *et al.*, 2010; Weimar, Kavelaars *et al.*, 2012), molecules secreted by embryonic (trophoblast) cells might also regulate H-ESC migration. Although data on this function of embryos is scarce, CXCL12 may be a candidate embryonic derived factor to stimulate H-ESC migration. Trophoblast cell-derived CXCL12 has also been demonstrated to promote CXCR4 (receptor for CXCL12) expression and invasion of H-ESCs (Ren, Liu *et al.*, 2012). In addition, CCL2, known to be secreted by trophoblast cells, has been shown to induce H-ESC invasion in an autocrine manner through the MAPK/Erk1/2 and Akt signalling pathways, and may therefore also induce a migratory response (Li, Li *et al.*, 2012).

H-ESC migration is modulated by several other molecules as well, such as growth factors, cytokines, hormones and endocannabinoids (Gentilini, Busacca *et al.*, 2007; Gentilini, Vigano *et al.*, 2010; Matsumoto, Nasu *et al.*, 2005; Tsai, Wu *et al.*, 2002). Platelet-derived growth factor (PDGF) isoforms AA, AB and BB have been shown to stimulate H-ESC migration dose-dependently in *in vitro* scratch assays (Matsumoto, Nasu *et al.*, 2005). PDGF-BB which is produced by cyclical endometrium and early pregnancy tissue, was found to be the strongest inducer of H-ESC migration (Matsumoto, Nasu *et al.*, 2005).

Fibroblast growth factor (FGF) and epidermal growth factor (EGF) may also induce H-ESC migration (Gentilini, Busacca *et al.*, 2007; Tsai, Wu *et al.*, 2002). Using a Boyden chamber or transwell assays to study the effect of various hormones on H-ESC migration, 24 h pretreatment of H-ESC with FGF or EGF resulted in an increase in H-ESC migration compared to basal migration (Gentilini, Busacca *et al.*, 2007). In the same study progesterone had an inhibitory effect on PDGF-BB induced migration (Gentilini, Busacca *et al.*, 2007). Somatostatin, that has originally been described as a hypothalamic hormone but is now also known to be produced by the endometrium, and its natural analogue cortistatin also inhibit the PDGF-induced migration of (endometriotic) H-ESCs (Annunziata, Luque *et al.*, 2012). Somatostatin, cortistatin and progesterone may thus be important factors in controlling H-ESC migration.

Estradiol (E2) has also been shown to have a dose-dependent positive effect on H-

ESC migration in experiments using Boyden chambers (Gentilini, Busacca *et al.*, 2007; Flamini, Sanchez *et al.*, 2011). The pro-migratory effect of E2, *in vivo* primarily secreted by the ovaries and placental tissue, was confirmed in a study that used both normal and neoplastic H-ESCs (the Ishikawa human endometrial adenocarcinoma cell line) (Flamini, Sanchez *et al.*, 2011). In addition, E2 is capable of stimulating CXCL12 secretion by H-ESCs and may thus have an effect on H-ESC migration through induction of the CXCL12/CXCR4 pathway (Tsutsumi, Okada *et al.*, 2011). It could be that embryos secrete higher levels of E2, stimulating H-ESC migration and high-quality embryo implantation.

Regulation of H-ESC motility

A huge variety of intracellular signalling molecules have been implicated in cell migration (Friedl and Wolf, 2003). Studies investigating the migratory phenotype of H-ESCs from women with endometriosis showed that E2-induced H-ESC migration is regulated through phosphatidylinositol 3 kinase (PI3K) and extracellular signal-regulated protein kinase (ERK)1/2 (an important member of the mitogen-activated protein (MAP) kinase family) pathways (Gentilini, Busacca *et al.*, 2007; Gentilini, Besana *et al.*, 2010). Expression of p-Akt and p-ERK was strongly upregulated following H-ESC pretreatment with various pro-migratory growth factors, such as E2 and PDGF-BB (Gentilini, Busacca *et al.*, 2007). In addition H-ESC pretreament with Wortmannin (blocking the PI3K/Akt pathway) or PD98059 (blocking the ERK1/2 pathway) totally inhibited endocannabinoid-induced or E2- induced migration of H-ESCs (Gentilini, Busacca *et al.*, 2007; Gentilini, Besana *et al.*, 2010). Together, these data indicate the relevance of PI3K/Akt and ERK1/2 pathway in H-ESC migration.

Focal adhesion kinase (FAK) also seems to be important in regulating H-ESC migration as E2-induced migration was completely inhibited by silencing FAK. FAK activation may not only be induced by hormonal (E2) stimulation, but also by integrin engagement and, remarkably, it is upregulated during H-ESC decidualization (Ihnatovych, Hu *et al.*, 2007). FAK accumulates at cellular focal adhesions that make contact with the extracellular matrix and is a key regulator of the response to the extracellular matrix. As extracellular matrix remodelling of is required in cell migration, it may not be surprising that H-ESC migration is under the control of FAK (Flamini, Sanchez *et al.*, 2011). In a heterologous *in vitro* co-culture model (mouse blastocysts on human H-ESCs) it was shown FAK silencing disrupts focal adhesion turnover in H-ESCs and thereby inhibits embryo implantation into H-ESC monolayers (Grewal, Carver *et al.*, 2010).

One family of proteins has been found to be of particular importance for cell migration, the Rho GTPases (Jaffe and Hall, 2005). The motility of H-ESCs seemed to be dependent and regulated by the RhoGTPase family members RhoA, Rac1 and CDC42, controlling cellular dynamics through stress fiber, lamellipodia and filopodia formation (Grewal, Carver *et al.*, 2008; Grewal, Carver *et al.*, 2010). H-ESCs migration required both Rac1 expression and inhibition of RacGAP1 (a GTPase activating protein) expression and was found to be inhibited by RhoA and CDC42. Furthermore, in an *in vitro* co-culture model using human embryos and human H-ESCs, knockdown of Rac1 expression in H-ESCs inhibited human trophoblast cell invasion into H-ESC monolayers while the inhibition of RhoA activity promoted embryo invasion (Grewal, Carver *et al.*, 2008).

Clinical implication of disturbed decidual phenotype

Loss of concepti: implantation failure and recurrent miscarriage

Early pregnancy loss or implantation failure would seem an appropriate biological strategy to deal with the high incidence of aneuploidy and other abnormalities in human embryos. There is evidence that the majority of human embryos are discarded soon after conception. It has been estimated that 30% of the conceptions are lost prior to implantation (implantation failure) (Chard, 1991; Macklon, Geraedts *et al.*, 2002). Miscarriage is by far the most common complication of pregnancy, occurring in around 10-15% of clinically recognized gestations. When the incidence of preclinical pregnancy losses is also taken into account this percentage would increase even further to 40% (Macklon, Geraedts *et al.*, 2002).

Similar rates of attrition have been described in IVF concepti. In a cohort of 179 patients in an IVF setting, almost half of the embryos (49%) were lost prior to implantation (Boomsma, Kavelaars *et al.*, 2009). Around 5% of women undergoing IVF treatment will suffer from recurrent implantation failure (RIF), a distressing condition suffered by women undergoing IVF treatment, defined as absence of implantation after three or more embryo transfers with morphologically high-quality embryos (4% of the IVF/ICSI patients), or after the transfer of a total of ten or more embryos (Koot, Boomsma *et al.*, 2011; Tan, Vandekerckhove *et al.*, 2005). We would like to propose that this group may include women whose H-ESCs display an excessively selective decidual phenotype.

On the other hand, recurrent clinical miscarriage (defined as three or more consecutive miscarriages) which affects 1-2% of couples (Jauniaux, Farquharson *et al.*, 2006; Rai

and Regan, 2006; Stirrat, 1990), may represent a failure of appropriate embryo selection (Teklenburg, Salker *et al.*, 2010; Weimar, Kavelaars *et al.*, 2012). As the incidence of RM is significantly higher than may be expected by chance alone (~0.3), an underlying aetiology has been suggested in affected women although in 50% of women with RM no cause could be identified (Kutteh, 1999; Regan, 1991).

Too little or excessive selection

When the endometrial biosensor function is set to promote high-quality embryo implantation, dysfunctional bio-sensoring may result in a situation where embryos are not selected adequately (Selection Failure), leading to the implantation of both high- and low-quality embryos. It has been proposed that women who suffer from recurrent miscarriages may be less selective with respect to embryo quality than the general female population (Aplin, Hey *et al.*, 1996; Quenby, Vince *et al.*, 2002). The Selection Failure Hypothesis first proposed by Quenby et al. in 2002 suggests that the endometrium of women with RM may be unable to select only high-quality human embryos for implantation (Quenby, Vince *et al.*, 2002). The subsequent implanted low-quality embryos would then be rejected later as their development fails and is rated as a clinical miscarriage. The endometrium of these women, allowing a wider range of embryos to implant may then be considered as 'super-receptive'. On the fertility spectrum, these 'superfertile' women with RM may then be categorized at the opposite end from women suffering from implantation failure.

The hypothesis that women with RM seem to fit the 'Selection Failure' or super-receptive group is supported by several observations. It has been demonstrated that women with RM express lower levels of endometrial mucin-1, an anti-adhesion molecule that contributes to the barrier function of the epithelium (Aplin, Hey *et al.*, 1996). Moreover, H-ESCs of women with RM showed abnormal decidualization *in vitro*. Both attenuated PRL production and prolonged and enhanced prokineticin-1 (a cytokine that promotes implantation) H-ESC expression were found that may result in an extended window of implantation (Salker, Teklenburg *et al.*, 2010). Implantation occurring later in the luteal phase (beyond the window of implantation) is associated with the occurrence of preclinical pregnancy loss (Wilcox, Baird *et al.*, 1999). Clinical evidence for the concept of super-receptivity comes from a retrospective cohort study of 560 RM women demonstrating that 40% of the women fit the 'super fertile' group and had a short time-to-pregnancy-interval of three months or less (Salker, Teklenburg *et al.*, 2010).

A proposed mechanism that may underlie the 'super-receptive' phenotype seen in women with RM is supported by their H-ESC' inability to discriminate between or differentially migrate to high- and low-quality embryos (Weimar, Kavelaars *et al.*, 2012). In migration (scratch) assays the directed migration of decidualizing H-ESCs from normal fertile and RM women in the presence or absence of a high- or low- (chromosomally abnormal 3PN) quality embryo was tested. The results showed that H-ESC migration of women with RM was not capable of discriminating embryo quality: in the presence of both low-and high-quality embryo a comparable level of H-ESC migration was observed. In contrast, the migration of H-ESCs from normal fertile women was totally inhibited in the presence of a low-quality embryo. In addition, in the presence of AC-1M88 trophoblast cell line-derived spheroids, the migration of H-ESCs from women with RM was enhanced compared to the normal fertile H-ESCs. Combined, these observations suggest that H-ESCs from women with RM have an increased migratory potential in response to trophoblast signals and are more receptive (and thus less selective) for low-quality embryos than the H-ESCs from normally fertile women.
Alternatively, dysfunctional endometrial bio-sensoring may lead to a situation where embryo selection is excessively discriminatory, preventing embryos from successfully implantating irrespective of their quality. In women with RIF, around 73% of the concepti miscarry without accompanying clinical symptoms (Yakin, Ata *et al.*, 2008). It can be hypothesized that excessively discriminatory embryo selection may be a cause of RIF (*Figure 3A*).

Taken together, we would therefore propose a novel concept which considers RM and RIF to be two extremes of an abnormal decidual selection phenotype. Decidualizing H-ESCs from women with RM may fail to select sufficiently, allowing poorly viable embryos to implant, and decidualizing H-ESCs from women with RIF may be too discriminatory in embryo selection, preventing viable embryos from successfully implanting. The hypothesis is schematically visualized in *Figure 3A and 3B*.

H-ESC migration and endometriosis

Endometriosis is defined as the prevalence of endometrial tissue (glands or H-ESCs) on extra-uterine locations and is associated with clinical symptoms such as pelvic pain, dysmenorrhea and infertility (Bulun, 2009). It is estimated that around 10% of women in the fertile age suffer from endometriosis with an estimated annual treatment cost of 22 billion USD in the US (Simoens, Hummelshoj *et al.*, 2007). However, the exact prevalence is unknown as many women that have endometriosis are asymptomatic.

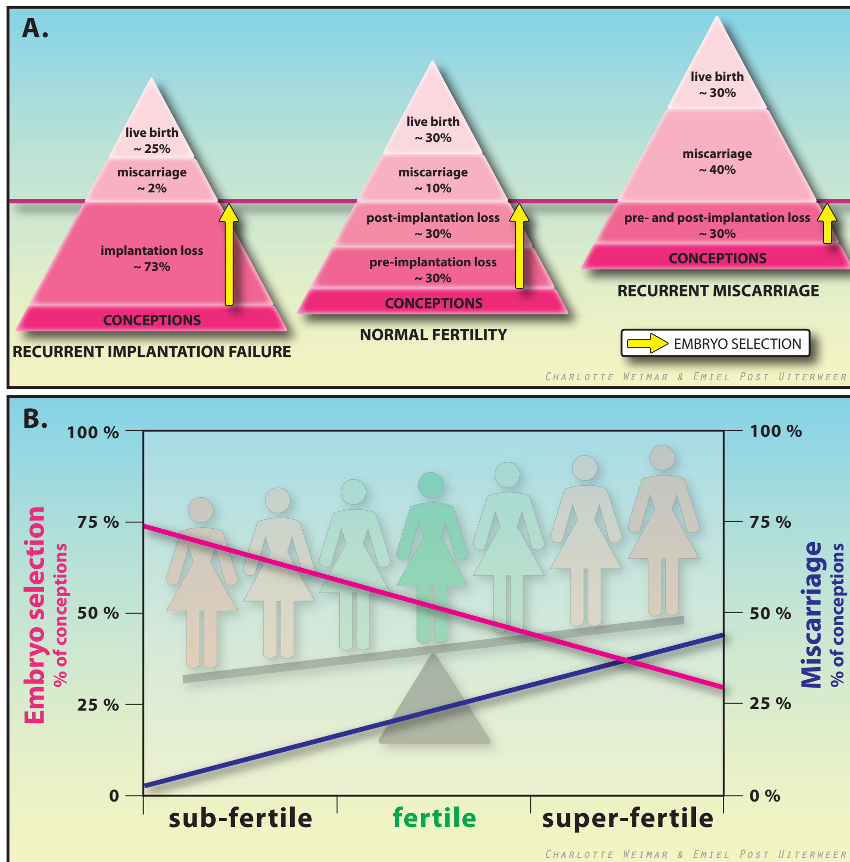

Figure 3. A. The icebergs of pregnancy loss in women with recurrent implantation failure, normal fertile women (middle) and women suffering from recurrent miscarriage (right). Too little or excessive embryo selection respectively may underlie the two reproductive disorders (represented by the yellow arrows). In **B.** the balance between embryo selection (implantation losses) and the miscarriage rate is represented for the sub-fertile, fertile and super-fertile population.

Thus far the mechanisms underlying the pathogenesis of endometriosis are not fully understood. Current hypotheses include the 'implantation theory', stating that endometrial tissue arriving at extra-uterine sites has the ability to implant and survive. The ectopic endometrial tissue reaches the pelvic area either through 'retrograde menstruation' through the fallopian tubes during menstruation or after surgery or child birth (Sampson, 1927). Another theory is that endometrial tissue reaches other sites of the body via the lymphatics or vasculature (Giudice and Kao, 2004).

The concept that the acquisition of a migratory phenotype would enhance the ability of H-ESCs to spread into ectopic sites was confirmed by several studies that tested the

migration of endometriotic H-ESCs (Banu, Lee *et al.*, 2008; Matsumoto, Nasu *et al.*, 2005; Ferreira, Witz *et al.*, 2008).

In addition, it has recently been reported that H-ESCs of patients with endometriosis display a different migratory behaviour than H-ESCs of normal fertile women (Gentilini, Vigano *et al.*, 2010). While progesterone reduced the migratory activity of normal fertile H-ESCs (with a static phenotype of the H-ESCs) in a Boyden chamber, it induced a stimulatory migratory effect in H-ESCs derived from the endometriosis group with an accompanying motile phenotype of the cytoskeleton. Moreover, the pro-migratory phenotype induced by E2 was higher in the endometriotic group compared to the controls.

Conclusions and future perspectives

Endometrial decidualization in the human is uniquely regulated among mammals, occurring in response to endocrine regulation rather than in response to conception. Another unique feature of human reproduction is the high rate of chromosomal and other abnormalities in human embryos. From an evolutionary point of view, a failure to select high- over low-quality embryos for implantation would result in a serious waste of effort. Therefore, it is likely that in addition to promoting a pregnancy favourable environment (receptive substrate for trophoblast invasion, control oxidative stress responses and immunoregulation), normal H-ESCs express an *embryo selective phenotype*. Recent data reviewed in this article support this novel concept .

In summary, data from *in vitro* studies suggest that H-ESCs from normal fertile women respond to low-quality human embryos by downregulating numerous pro-implantation cytokines which is not observed in the response of H-ESCs towards high-quality embryos. In other words, *decidualizing H-ESCs may act as a biosensor for embryo quality*. Moreover, the migration of H-ESCs of normal fertile women seems to be inhibited in response to chromosomally abnormal embryos. As migration promotes embryo implantation, we propose that *the inhibition of migration may be an effective mechanism of out-selecting low-quality embryos for implantation*, reducing the incidence of (chromosomally) abnormal embryo implantation and thus protecting women from the possible energy waste of implanting abnormal embryos. Taken together, H-ESC migration and the H-ESC biosensor function may go hand in hand and may form the basis for embryo selection; adjusting the level of migration in relation to the quality of the embryo may be a pivotal mechanism by which embryo selection is carried out. Hypothesizing that embryo selection is an evolutionary response to the high pressure

of chromosomally abnormal peri-implantation embryos, human reproduction may not be that ineffective after all.

However, when the H-ESC selection mechanism is not well regulated, reproductive problems may arise; H-ESCs from women with RM seem to fail to control the migratory activity in the presence of low-quality embryos which would suggest low-quality embryos are not out-selected for implantation (Selection Failure hypothesis). Another example of increased migratory activity is found in patients that suffer from endometriosis. While experimental evidence is still required, it can be hypothesized that recurrent implantation failure may be the result of an excessively selective decidual phenotype. More research into the nature of embryo/H-ESC signalling is required to identify the signals and their impact on the molecular pathways which govern successful implantation and the establishment of an ongoing pregnancy.

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Chapter 6

Cell lineage specific distribution of H3K27 trimethylation accumulation in an *in vitro* model for human implantation

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Abstract

Female mammals inactivate one of their two X-chromosomes to compensate for the difference in gene-dosage with males that have just one X-chromosome. X-chromosome inactivation (XCI) is initiated by the expression of the non-coding RNA *Xist*, which coats the X-chromosome in cis and triggers gene silencing. In early mouse development the paternal X-chromosome is initially inactivated in all cells of cleavage stage embryos (imprinted X-inactivation) followed by reactivation of the inactivated paternal X-chromosome exclusively in the epiblast precursors of blastocysts, resulting temporarily in the presence of two active X-chromosomes in this specific lineage. Shortly thereafter, epiblast cells randomly inactivate either the maternal or the paternal X-chromosome. XCI is accompanied by the accumulation of histone 3 lysine 27 trimethylation (H3K27me3) marks on the condensed X-chromosome. It is still poorly understood how XCI is regulated during early human development. Here we have investigated lineage development and the distribution of H3K27me3 foci in human embryos derived from an *in vitro* model for human implantation. In this system, embryos are co-cultured on decidualized endometrial stromal cells up to day eight, which allows the culture period to be extended for an additional two days. We demonstrate that after the co-culture period, the inner cell masses have relatively high cell numbers and that the GATA4-positive hypoblast lineage and OCT4-positive epiblast cell lineage in these embryos have segregated. H3K27me3 foci were observed in ~25% of the trophectoderm cells and in ~7.5% of the hypoblast cells, but not in epiblast cells. In contrast with day eight embryos derived from the co-cultures, foci of H3K27me3 were not observed in embryos at day five of development derived from regular IVF-cultures. These findings indicate that the dynamics of H3K27me3 accumulation on the X-chromosome in human development is regulated in a lineage specific fashion.

Introduction

X-chromosome inactivation (XCI) is a complex process whereby one of the two X-chromosomes in female cells is epigenetically silenced, to obtain similar X-linked gene expression levels as male cells, which have just one X-chromosome. The initiation of X-inactivation depends on RNA from the non-coding gene *Xist*, which coats the X-chromosome in cis and initiates its inactivation [1,2]. *Xist* recruits the polycomb group complex PRC2 to the X-chromosome that subsequently tri-methylates histone 3 on lysine 27 (H3K27me3), a repressive epigenetic mark that leads to further silencing of the X-chromosome [3]. The presence of H3K27me3 nuclear foci is often used to identify the inactivated X-chromosome in XX female somatic cells.

The activity status of the X-chromosomes in pre- and post-implantation development of female mouse embryos is dynamically regulated. After fertilization, the paternally inherited X-chromosome is inactivated, from the 4-cell stage onward [4]. The paternal X-chromosome remains inactive in the trophectoderm and the primitive endoderm precursors of blastocyst stage mouse embryos (imprinted XCI), but is reactivated in the epiblast precursors of the inner cell mass (ICM) between E3.5-E4.5, resulting temporarily in two active X-chromosomes (XaXa) [5-12]. Shortly thereafter in development, one of the active X-chromosomes is randomly inactivated and the resulting pattern of X-chromosome activity is epigenetically transmitted to all daughter cells [10,11].

In contrast with mouse embryos, *XIST* is initially expressed from both X-chromosomes in female human embryos, indicating that XCI in early development is not conserved between eutherian mammals [13]. According to Okamoto *et al*., H3K27me3 is not enriched at the *XIST*-coated chromosome of female human blastocysts [13]. This contrasts with the findings of van den Berg *et al*., who reported foci of H3K27me3 in ~30% of the cells of day six blastocysts [14]. This discrepancy illustrates that the full dynamics of XCI in human development are still poorly understood. Furthermore, it is unclear whether X-chromosome inactivation is differentially regulated between the different cellular lineages, as it is the case for mouse development.

X-chromosome inactivation and lineage development are difficult to study in humans because, in addition to the restricted availability of human pre-implantation embryos from *in vitro* fertilization (IVF)-cultures, there are technical and ethical obstacles to study human embryos that have initiated implantation in the endometrium.

Consequently, the processes of X-chromosome inactivation and lineage development in human embryos beyond the blastocyst stage remain largely elusive.

Culture conditions for embryos derived through IVF do not favor development beyond day six and human implantation sites are inaccessible [15]. We reasoned that using an *in vitro* model for human implantation could overcome these obstacles and open a new window on human development. A system in which human pre-implantation stage embryos are co-cultured with decidualized primary endometrial cells recapitulates a number of processes that are associated with implantation [12,15,16]. In this model stromal cells isolated from proliferative endometrium are induced to decidualize by cyclic AMP stimulation, which results in morphological changes reminiscent of the *in vivo* decidual phenotype and the induction of prolactin expression and other products of decidualization [17]. In co-culture, zona pellucida-free pre-implantation embryos attach to, penetrate and ultimately invade through the decidualized endometrial stromal cells. This is accompanied by an increased production of human chorionic gonadotrophin (hCG) by the embryo [12,16]. We therefore considered this a suitable model system to study the human peri-implantation embryo.

Here, we examined lineage formation and accumulation of H3K27me3 on the X-chromosomes in human day five embryos and in human embryos that, from day five onwards, have been cultured for an additional 72h on decidualized endometrial stromal cells. Using immunofluorescence for OCT4 and GATA4 we show that these markers are initially co-expressed in all cells of embryos at day five of development, but after 72h of co-culture expression of OCT4 is restricted to the epiblast, GATA4 expression is restricted to the hypoblast, and trophectoderm lineages are OCT4- and GATA4-negative. Female embryos that have been co-cultured on decidualized endometrial cells have distinct H3K27me3 foci, localized to the trophectoderm lineage and to a lesser extend the hypoblast lineages. Interestingly, H3K27me3 foci were not observed in the OCT4-positive pluripotent epiblast cells. We conclude that, in human development, dynamics of H3K27me3 accumulation on the X-chromosome is regulated in a lineage-specific fashion.

Materials and Methods

Ethics statement and patient selection

This study was approved by the Central Committee on Research Involving Human Subjects (CCMO) in The Netherlands (NL 12481.000.06) and by the local Medical Review Ethics Committee of the University Medical Center in Utrecht. Written informed consent was obtained from all participating subjects for confirmation that supernumerary cryopreserved embryos or endometrial samples could be used for research purposes.

Embryo collection and co-culture

Ovarian hyperstimulation, oocyte maturation triggering and *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) were performed according to standard clinical protocols as described earlier [31]. Supernumerary, good quality day four embryos were cryopreserved as previously described [32]. For this study, embryos were thawed, taken through consecutive washes of 1.25, 1.00, 0.75 and 0.375 mol/l DMSO for five minutes each, then transferred to Human Tubal Fluid 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 at 37°C until day five of development under atmospheric oxygen levels and 5% CO_2. Thirty-nine embryos from seventeen patients survived the thawing procedure and extended culture period. Fourteen embryos were subjected to 0.1% Pronase/10% GPO treatment to remove the zona pellucida, before they were transferred to decidualized endometrial cells. For the co-culture experiments, primary endometrial stromal cells were purified, as previously described [33], from a single proliferative phase biopsy sample obtained from a patient with no uterine pathology or a history of recurrent pregnancy loss. Endometrial stromal cells were seeded into 16 mm wells (0.5×10^5 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) in combination with 1 μM medroxyprogesterone acetate (MPA; Sigma). This medium was changed every 48 h. Individual blastocysts were then seeded onto a confluent layer of endometrial cells that had been decidualized for five days. Co-cultures were maintained in DMEM/F-12 complete medium for 72 h at 37°C under atmospheric oxygen levels and 5% CO_2. At the end of the co-culture period, the embryos were carefully removed from the stromal cells with a mouth pipet.

Immunofluorescence

Day five embryos were washed in PBS and fixed for ten minutes at room temperature (RT) in 4% PFA. Day eight human blastocysts were carefully detached from the decidualized endometrium cells, washed in PBS and fixed for ten minutes at room temperature RT in 4% PFA. The rest of the staining procedure was similar for day five and day eight embryos. Embryos were washed briefly in PBS with 10% Foetal Calf Serum (FCS) and 0.1% triton x-100 (PBST). Subsequently, embryos were permeabilized in PBS with 10% FCS and 0.5% triton x-100 for 15–30 minutes at RT. Embryos were incubated in PBST for one hour at RT followed by overnight incubation at 4°C in primary antibodies diluted in PBST. The primary antibodies used were: rabbit anti-H3K27me3 (Millipore, Temecula, USA), goat anti-OCT4 (Santa Cruz Biotechnology, Santa Cruz, USA), and mouse anti-GATA4 (Santa Cruz) diluted in blocking solution. The embryos were then washed in PBST and transferred to PBST containing Alexa fluor conjugated secondary antibodies (Molecular probes, Invitrogen, Venlo, the Netherlands). In the case of triple labeling, embryos were first incubated in PBST with Alexa633 donkey anti-goat, before they were transferred to PBST containing goat anti-rabbit and goat-anti mouse secondary antibodies. After one hour in secondary antibody solution, embryos were washed and mounted in Vectashield mounting medium containing DAPI (Brunschwig Chemie, Amsterdam, The Netherlands). Fluorescent signals were visualized using a Leica SPE confocal laser scanning microscope (Leica, Rijswijk, the Netherlands) and acquired images were analyzed with ImageJ [34]. We defined bright non-diffuse signals as foci of H3K27me3. A Student's two-sided t-test was used to evaluate statistical differences. A difference was determined to be significant when $P < 0.05$.

Results

Development of epiblast and hypoblast precursors in human embryos cultured on decidualized endometrial cells

Human IVF/ICSI (intracytoplasmic sperm injections) embryos that had been cryopreserved at day four were thawed and cultured until day five. At day five, embryos were fixed in 4% paraformaldehyde (PFA), or transferred to decidualized endometrial cells and cultured for an additional 72h (*Figure 1*). At 72h of co-culture, human embryos (hereafter referred to as day eight embryos) were carefully removed and fixed in 4% PFA. Day five embryos had on average 31.5 cells (n=11) and day eight embryo had on average 414.7 cells (n=10). To investigate the development of the hypoblast and epiblast lineages in human embryos, we performed immunofluorescence for the tran-

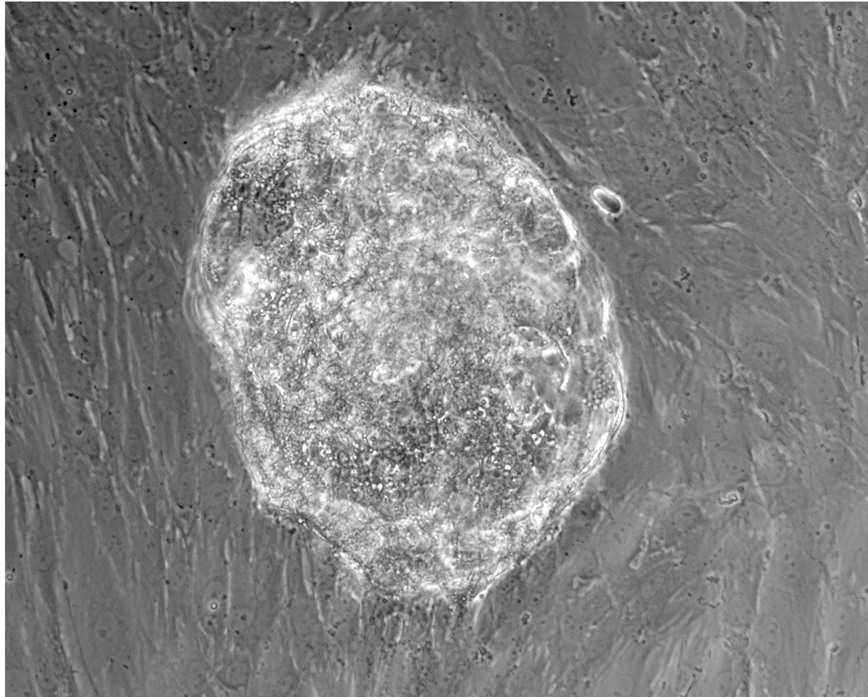

Figure 1. Embryo from co-culture. Human blastocyst attached to decidualized human endometrial stromal cells after 72h of co-culture.

scription factors OCT4 and GATA4 on fixed day five embryos and on fixed day eight embryos. In mouse post-implantation stage embryos, OCT4 is expressed in the epiblast and excluded from the primitive endoderm cells, whereas GATA4 is specifically expressed in the primitive endoderm, but not in the epiblast [18-25]. In human development, OCT4 is initially expressed in all cells at the early blastocyst stage [26,27]. In older blastocysts, OCT4 expression is downregulated in the trophectoderm lineage, while expression in the ICM remains high [27].

All nuclei of day five embryos were positive for OCT4 (in eight out of eight embryos) and GATA4 (in three out of three embryos) (*Figure* 2). However, in embryos cultured on decidualized endometrial cells for 72h, double-immunofluorescence demonstrated the presence of an OCT4-positive cell population covered by a layer of GATA4-positive hypoblast precursors. There were no cells observed that strongly co-expressed GATA4 and OCT4, although weak expression of GATA4 was observed in a few OCT4-positive cells (data not shown). In some day eight embryos, we also observed OCT4 expression in the cytoplasm of the trophectoderm cells. In day six embryos, OCT4 is

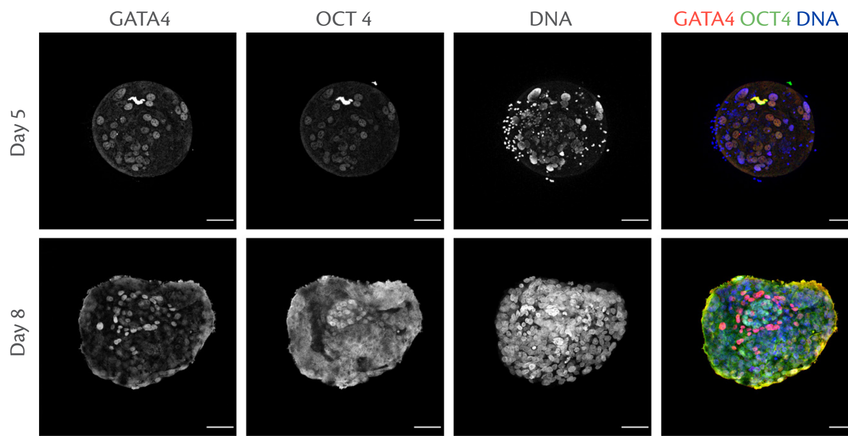

Figure 2. Lineage development in human embryos. Expression of OCT4 and GATA4 in day 5 embryos and in embryos that have been co-cultured with decidualized endometrial cells for 72h. Scale bars are 50μm.

known to be expressed in both the trophectoderm and the ICM [27]. Cytoplasmic OCT4 staining in the trophectoderm possibly reflects a stage just before its expression is fully downregulated in this lineage.

The results above indicate that the strong OCT4-positive cells and strong GATA4-postive cells represent two separate and segregated lineages at day eight of development. Quantification of the OCT4 positive cells and GATA4 positive cells demonstrated that there were significantly less OCT4 positive epiblast precursors than GATA4 positive hypoblast cells ($P < 0.01$). The majority of cells were negative for OCT4 and GATA4 and based on their outside localization we concluded these were presumptive trophectoderm cells (*Figure* 3). According to a recently published study, good quality human embryos at day six of development have on average 18.6 ICM cells [28]. This is close to our own observation of the number of ICM cells in day six human embryos (data not shown). The ICM of day eight embryos had on average 47.13±16.63 cells (n=8). Thus human embryos cultured on decidualized endometrial cells from day six until day eight of development show segregation of hypoblast precursors from epiblast precursors and an increase in the absolute number of ICM cells compared to embryos at day six of development. These data demonstrate that the co-culture system allows human embryos to progress to a further stage in development than is generally possible under regular culture conditions for IVF embryos. Importantly, our results indicate that co-staining of day eight embryos with antibodies against OCT4 and GATA4 allows identification of the epiblast, hypoblast and trophectoderm lineages.

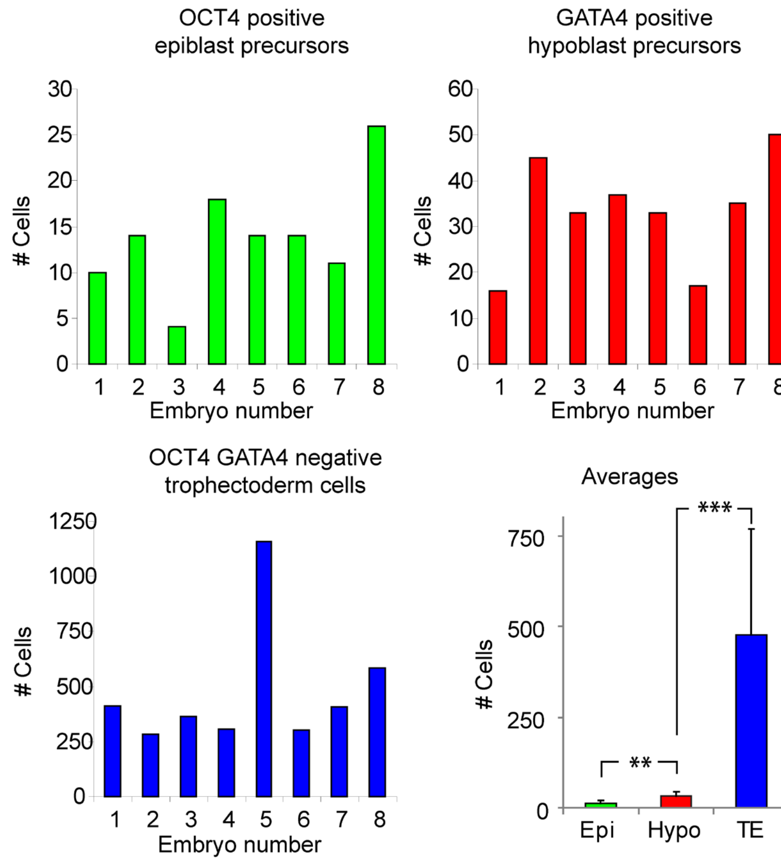

Figure 3. The number of cells per lineage in human embryos. The number of cells in each lineage in embryos that have been co-cultured with decidualized endometrial cells for 72h. Green bars are the number of OCT4 positive epiblast precursors, red bars are the number of GATA4 positive hypoblast precursors, blue bars are the number of OCT4- and GATA4- negative trophectoderm cells. Error bars denote standard deviation; asterisks denote significant differences: **: $P < 0.01$; ***: $P < 0.001$. Epi=Epiblast lineage; Hypo=Hypoblast lineage; TE=Trophectoderm lineage.

Distribution of H3K27me3 foci in early blastocysts and in post-implantation stage embryos

Female cells that carry an inactive X-chromosome can be recognized by the presence of a prominent focus of nuclear H3K27me3. We were able to detect prominent foci of H3K27me3 in >90% endometrial cells using immunofluorescence with an antibody against H3K27me3 (*Figure 4*). With the same staining we were unable to detect prominent single foci of H3K27me3 in cells of day five embryos (n=8).

By contrast, in four of the day eight embryos (n=8), prominent single H3K27me3 foci were observed, reminiscent of an inactivated X-chromosome in these cells (*Figure 5*). In the other 50% of the day eight embryos, no H3K27me3 foci were observed. H3K-27me3 foci were not observed in all cells. To determine which cell types carried H3K-27me3 foci, we examined if their distribution co-localized with OCT4 or GATA4 positive cells. Many H3K27me3 foci (87.5±21.9 per embryo) were observed in GATA4 and OCT4 negative (and therefore presumably trophectoderm) cells (*Figure 6*). Nevertheless, a large fraction (~75%) of the trophectoderm cells did not have clear H3K27me3 foci (*Figure 6*). Some H3K27me3 foci were also observed in GATA4 positive hypoblast precursors (*Figure 6*, 7), although significantly less than in the trophectoderm. In a small proportion of cells two foci were observed per cell, in both the trophectoderm and the hypoblast lineages (*Figure 7*). In the hypoblast and trophectoderm lineages, H3K27me3 domains were also observed that were too diffuse to count as bona fide foci of H3K27me3 (*Figure 7*). Nevertheless, these diffuse domains might indicate either X-chromosomes that are less compacted than fully inactivated X-chromosomes. Alternatively, these could be areas of (facultative) heterochromatin or repressive chromatin domains.

We investigated the presence of H3K27me3 foci in epiblast precursors. In the OCT4 positive cells, no H3K27me3 foci were observed (*Figure 6, 8*). These findings indicate that, in the OCT4 positive pluripotent cell population of human embryos, the accumulation of H3K27me3 on the X-chromosome does not follow the same dynamics as in the hypoblast or the trophectoderm cells.

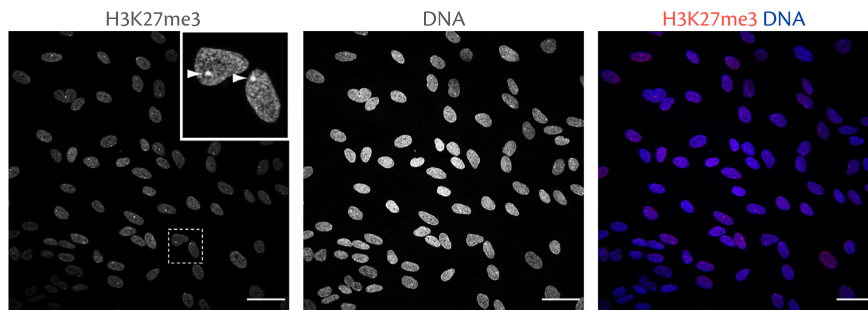

Figure 4. H3K27me3 staining of endometrial stromal cells. Accumulation of H3K27me3 on X-chromosomes was observed in >90% of the cells. Scale bars are 50μm.

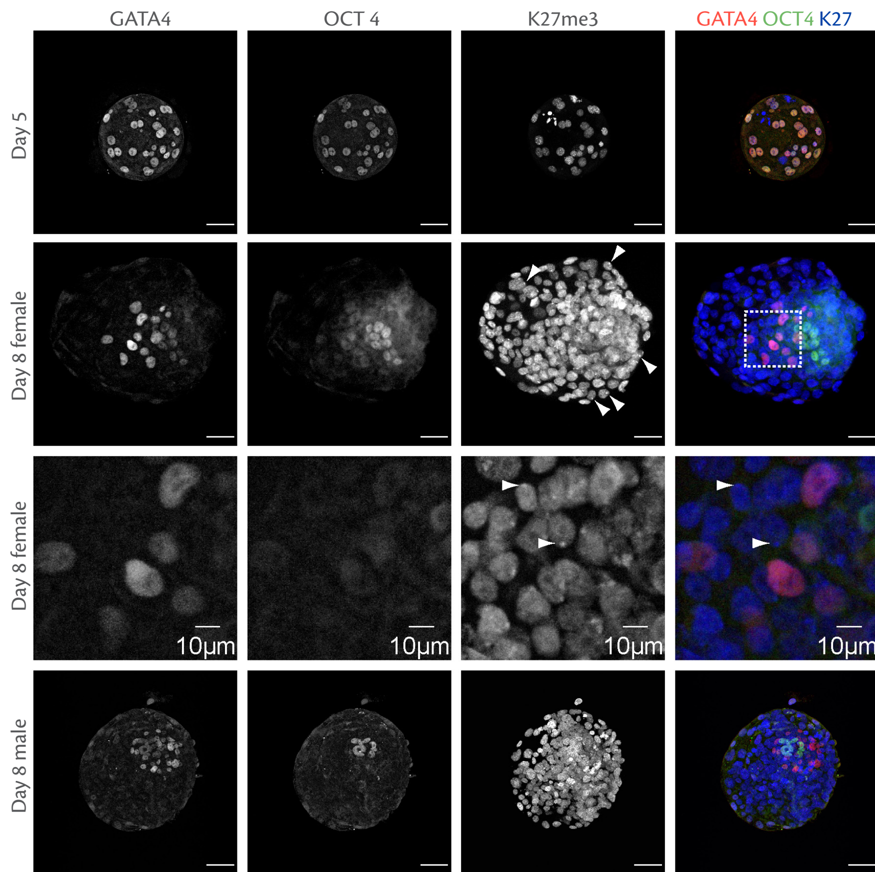

Figure 5. Lineage development and H3K27me3-accumulation on the X-chromosome in human embryos. Expression of GATA4, OCT4, and H3K27me3 in human day five pre-implantation and embryos that have been co-cultured with decidualized endometrial cells for 72h (day eight). Foci of H3K27me3 are diagnostic for an inactivated X-chromosome. Arrowheads denote H3K27me3 foci. The third row panels are enlargements of the indicated area in the merge panel in the above row. Scale bars are 50μm unless denoted otherwise.

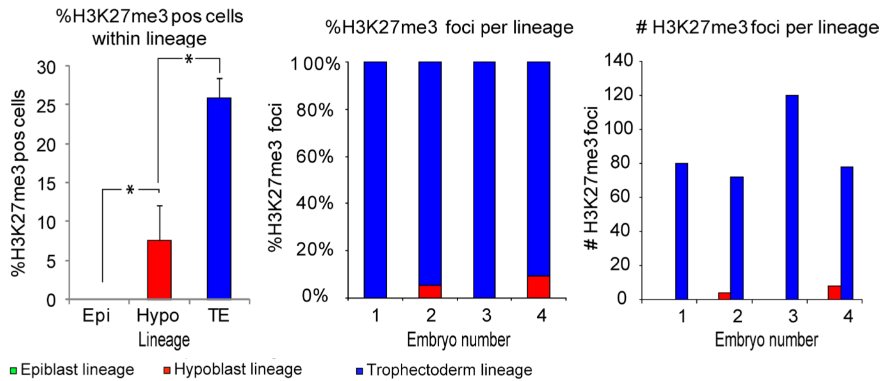

Figure 6. Degree of H3K27me3 foci per lineage. The percentages of cells in each lineage that carry a distinct H3K27me3 focus in embryos that have been co-cultured with decidualized endometrial cells for 72h. Left panel: relative number of H3K27me3 per lineage as a function of the total number of cells within that lineage; middle panel: absolute values of H3K27me3 foci counted within each lineage for each embryo; right panel: relative number of H3K27me3 foci in each lineage as a function of the total number of foci observed in that embryo. H3K27me3 foci did not reveal any preferential distribution within the embryo (see also figure S3). Green: H3K27me3 foci in OCT4 positive epiblast precursors; red: H3K27me3 foci in GATA4 positive hypoblast precursors; blue: H3K27me3 foci in OCT4- and GATA4- negative trophectoderm cells. Asterisks denote significant differences: *: $P < 0.05$. Epi=Epiblast lineage; Hypo=Hypoblast lineage; TE=Trophectoderm lineage.

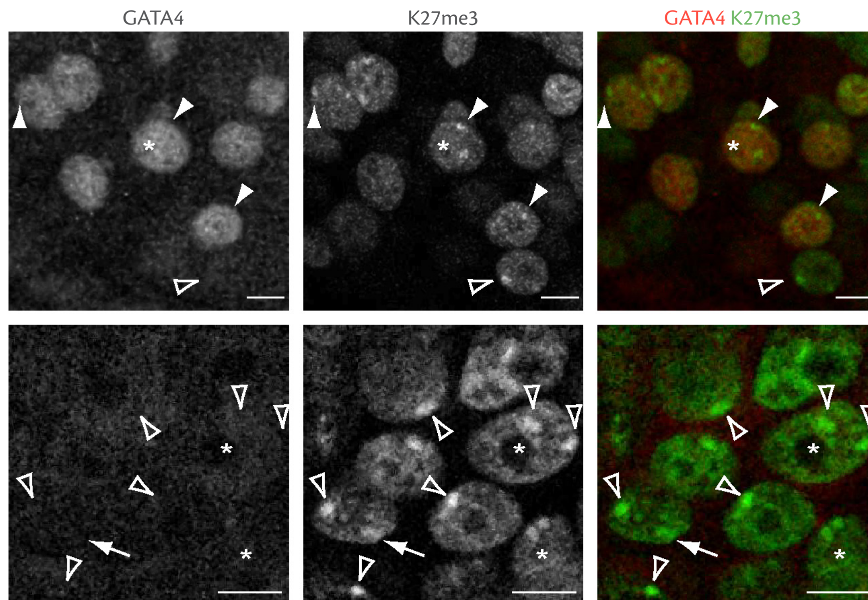

Figure 7. H3K27me3 accumulation in trophectoderm and hypoblast precursors. Expression of GATA4, and H3K27me3 in human embryos that have been co-cultured with decidualized endometrial cells for 72h. Closed arrowheads denote GATA4 positive cells with H3K27me3 foci. Open arrowheads denote trophectoderm cells negative for OCT4 and GATA4 with distinct H3K27me3 foci. The asterisks mark cells with two H3K27me3 foci. Arrows mark the location of a diffuse H3K27me3 domain. Scale bars are 10µm.

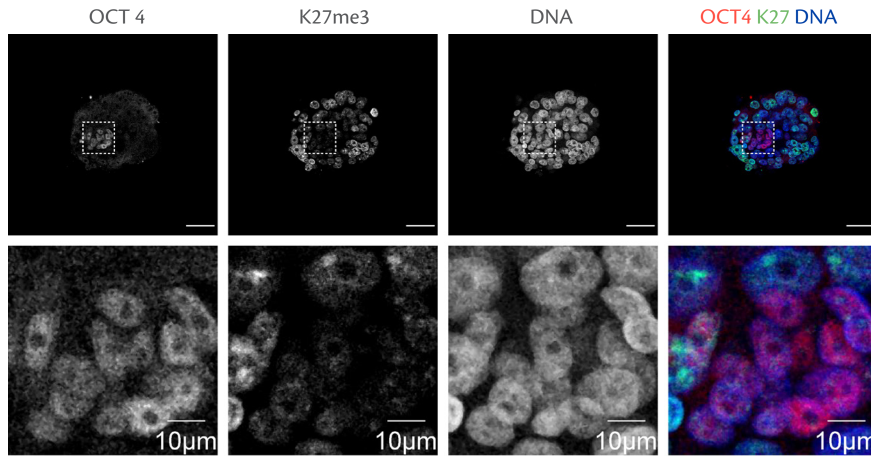

Figure 8. No accumulation of H3K27me3 on the X-chromosomes of epiblast precursors. Expression of OCT4, and H3K27me3 in human embryos that have been co-cultured with decidualized endometrial cells for 72h. The four bottom panels are an enlargement of the indicated area in the panels directly above. Scale bars are 50µm unless otherwise indicated.

Discussion

In this study, we have co-cultured of human embryos with decidualized endometrium cells. This system supports the embryo to progress development to a later stage than would be possible in conventional culture systems [15]. In the present study, we demonstrate that human embryos that have been cultured on decidualized endometrial cells have relatively large ICMs in which the epiblast precursors have segregated from the hypoblast precursors. The possibility to extend development of *in vitro*-cultured human embryos enabled us to study the accumulation of H3K27me3 (presumably on the inactive X-chromosome) in the trophectoderm, hypoblast and epiblast cell lineages of human peri-implantation stage embryos. Our findings indicate that X-chromosome-wide accumulation of H3K27me3 in human embryos is regulated in a lineage-specific manner during peri-implantation.

The accumulation of H3K27me3 on the presumably silent X-chromosome was markedly different in embryonic versus extra-embryonic lineages of implanting human day eight embryos. The trophectoderm contained the highest number of cells with clear H3K27me3 foci, followed by the hypoblast precursors. In a few cells of the hypoblast and the trophectoderm lineages, 2 foci of H3K27me3 could be identified (*Figure 7*). The presence of two H3K27me3 foci in one cell has also been described for rabbit em-

bryos [13]. H3K27me3 foci were not observed in the OCT4 positive epiblast precursors. This indicates that human embryos have lineage specific dynamics of H3K27me3 accumulation on the X-chromosome.

We observed expression of OCT4 in all blastomeres of day five embryos. Interestingly, OCT4 is initially also expressed in all cells of human blastocysts [26,27], after which it is first downregulated in the trophectoderm lineage [27], followed by exclusion from the hypoblast lineage and restricted expression in the epiblast lineage during peri-implantation (this study). The current study suggests that there is a lineage specific degree of H3K27me3 accumulation on the X-chromosome, which might be a reflection of the lineage specific loss of OCT-4 expression.

Finally, if the H3K27me3 foci we observed in the current study represent fully silenced X-chromosomes, our findings indicate that in the majority of the cells of human embryos, silencing of the X-chromosome may occur after the embryo has implanted. In the mouse, XCI is regulated by pluripotency factors [29,30]. The slow onset of H3K27me3 accumulation on the X-chromosomes in human development could be the consequence of the ubiquitous co-expression of pluripotency factors at relative late stages of pre-implantation development. In a similar line of reasoning, the lineage-specific degree of H3K27me3 accumulation in human peri-implantation stage embryos might reflect the lineage specific loss of OCT4 expression.

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Chapter 7

Summary and general discussion

Compared to other mammalian species, human reproduction is considered inefficient. The average chance of conception within one menstrual cycle is approximately 20-30% (Evers, 2002). In addition, the tendency of delaying childbirth in Western modern societies and thus an increased maternal age and lower oocyte quality at the time of the first conception, may contribute to a further decline in spontaneous conceptions. To date, almost one in six couples attend fertility clinics because of unwanted delayed conception (ESHRE, 2012). One in twelve couples eventually opt for some kind of assisted reproductive technology to help them conceiving. Based on the number of months it takes to get pregnant (time-to-pregnancy TTP interval), women may be termed to be either sub-, normal- or superfertile. Around 79% of the population is estimated to be normal fertile (Tietze *et al.*, 1950). Subfertility is the failure to conceive after one year of timed intercourse and affects almost one in five couples (18%; (Tietze *et al.*, 1950). On the other end of the fertility spectrum, around 3% of the couples are considered superfertile and experience persistent short TTP intervals (Salker *et al.*, 2010; Tietze *et al.*, 1950). Forty percent of the women that suffer from recurrent miscarriage (RM) fit the superfertile phenotype and have a TTP interval of three months or less. Despite series of clinical and scientific investigations, RM remains unexplained in approximately half of the cases (Li, 1998; Rai and Regan, 2006). The major role of the endometrium and in particular of the human endometrial stromal cells (H-ESCs) is to create a receptive environment for embryo implantation and to support a pregnancy, should fertilization occur. The primary aim of this thesis is to investigate whether H-ESCs of women with RM have a super-receptive phenotype with consequently low embryo-selective properties.

Human embryo implantation involves attachment and breakdown of the extracellular matrix, survival ability in the presence of oxidative stress, and migration of H-ESCs (Friedl and Wolf, 2003; Gellersen *et al.*, 2010; Grewal *et al.*, 2008). In the first part of the thesis we studied the migratory behaviour of decidualizing H-ESCs of both women with RM and normally fertile women in response to human embryo- or trophoblast-derived products as a possible mechanism that contributes to the pathology of RM. In addition, the migratory behaviour of H-ESCs might be used as an (indirect) marker for endometrial receptivity.

Studying the early implantation environment

Due to ethical obstacles and the relative inaccessibility of the human implantation environment, our knowledge of the early implantation events is mainly based on *in vivo* (knock out) mouse models, and *in vitro* studies of murine, baboon and human endometrial tissue. In addition, many immunological (observational) studies have been performed in the murine and human decidua. Together these studies have generated an incredible amount of knowledge on H-ESC function, the physiology of implantation as well as on factors associated with RM (Dimitriadis *et al.*, 2005; Gellersen *et al.*, 2007; Laird *et al.*, 2003; Li *et al.*, 2002).

Peri-implantation endometrium of normal fertile women has been associated with a Th2- type cytokine shift (more IL-4, IL-10 and IL-6; less IL-2 and IFNγ), which was ten times more pronounced in pregnant endometrium compared to non-pregnant endometrium from normal fertile woman (Krasnow *et al.*, 1996). Skewing the cytokine balance towards the Th2 phenotype was thus suggestive for a more receptive endometrial state. The cytokine profile of pregnant RM women was characterized by a more pronounced Th2 balance than in normal fertile women, suggesting an increased endometrial receptivity in the RM group (Bates *et al.*, 2002). Glucocorticoids (GCs) in general induce a shift in cytokine milieu from Th1 to Th2 mediator production. The rationale of GC administration for RM is therefore not very clear with respect to normalizing cytokine balance.

Another major finding in the endometrial milieu was the cyclical shift in the immune cell population in the uterine endometrium; an accumulation of NK cells from 5 to 7% and in the post-ovulatory phase of the menstrual cycle (Bulmer and Lash, 2005). The decidual NK cells (dNK cells; $CD16^-$, $CD56^{high}$) have been found to differ in gene expression, phenotype and function in comparison to the highly cytotoxic peripheral NK cells (pNK cells $CD16^+$, $CD56^{low}$) (Tabiasco *et al.*, 2006). Furthermore, the dNK cells have been found to play an important role in vascular remodelling (Croy *et al.*, 2003; Morales *et al.*, 2003), trophoblast invasion (Hanna *et al.*, 2006), and possibly immune cell modulation and decidualization of the fetal-maternal interface (Bulmer and Lash, 2005). Interestingly, an excess of $CD16^+$, $CD56^{low}$ NK cells in non-pregnant endometrium and NK cell activity has been implicated in RM (Lachapelle *et al.*, 1996; Quenby and Farquharson, 2006). Increased dNK cell counts in women with RM may lead to blood flow-induced oxidative stress damage to the developing placenta (Jauniaux *et al.*, 2000; Tang *et al.*, 2009). A second rationale for GC administration for

women with RM therefore is to lower the uterine NK cell count, that may lead to a decrease in oxidative stress induced placental damage (Jauniaux *et al.*, 2000; Tang *et al.*, 2009; Quenby *et al.*, 2005). A clear relation between the role of dNK cells in the aetiology of recurrent miscarriage is yet unclear (Quenby and Farquharson, 2006) and the GC effect on miscarriage and pregnancy rate is currently under investigation (Tang *et al.*, 2009).

Studies in abortion-prone mice have highlighted the importance of the maternal cytokine and leucocytes response to the fetal trophoblast cells (Clark *et al.*, 1999). In Leukemia inhibitory factor (LIF) knockout mice it was shown that LIF may control the amount of various immune cells in the endometrium around the time of implantation and was found to be a crucial cytokine for blastocyst implantation (Schofield and Kimber, 2005; Stewart *et al.*, 1992). Although the role of LIF on human fertility is not yet fully clarified, in one study elevated levels of LIF in human uterine flushings have been shown to be detrimental for successful implantation (Ledee-Bataille *et al.*, 2002).

Even though the - mostly murine - studies have generated an enormous amount of information, it must be noted that data derived from animal models can not directly be extrapolated to the human situation due to the apparent interspecies differences. For example, the human species distinguishes itself by the high rate of chromosomal abnormalities in human pre-implantation embryos and by cyclic endometrial decidualization that occurs irrespective of the presence of a pregnancy. Both features are (normally) not present in the mouse. Moreover, human embryos are not able to undergo diapause, i.e. temporality suspending development while awaiting a more favorable pregnancy environment, a murine strategy to optimize reproductive success.

Therefore, to better understand the needs of the human implantation environment, human *in vitro* implantation models have been designed which have been proven to be of considerable value. For instance these studies have taught us that H-ESC migration actively takes place at the site of implantation and decidualizing H-ESCs may function as a biosensor for embryo quality (Grewal *et al.*, 2008; Teklenburg *et al.*, 2010). Increasing our effort in studying the human implantation environment may eventually improve the implantation- and take home baby rate in IVF treatments that have lately been unimproved and stable (Ferraretti *et al.*, 2012).

In this thesis we used two human *in vitro* models that enabled us to investigate the migratory H-ESC response in the early implantation environment of women with RM and of normally fertile women. The first model, an adapted 'scratch' or 'wound healing' assay, makes use of a confluent monolayer of decidualizing H-ESCs in which a cell-free migration zone is created. The H-ESCs are allowed to migrate towards a human embryo or a trophoblast spheroid that is placed in the migration zone. The decrease in migration zone surface area is a measure of the level of migration (**chapter 2 and 4**). A second model we used to study the motile behaviour of decidualizing H-ESCs in the presence of various trophoblast-derived factors was the ORIS migration assay (**chapter 3**). In addition, the endometrium-human embryo co-culture model (the third model), in which a human day five embryo was placed on top of a confluent monolayer of decidualizing H-ESCs, allowed us to study the process of X-chromosome inactivation during early human embryo development (**chapter 6**).

The concept of natural embryo selection and endometrial stromal cell migration

Based on the observation that 93% of the karyotypically normal pregnancies continue and 90% of the karyotypically abnormal pregnancies miscarry in the first trimester, miscarriages may be regarded as a process of nature's quality control (Quenby *et al.*, 2002). The concept that normal fertile endometrium may not allow low-quality embryos to implant is now supported by an increasing amount of evidence (Mansouri-Attia *et al.*, 2009; Teklenburg *et al.*, 2010). Using an *in vitro* H-ESC-human day 5 blastocyst co-culture model, it has been demonstrated previously that H-ESCs of normal fertile women may function as a biosensor for embryo quality (Teklenburg *et al.*, 2010). The profile of cytokine production by decidualizing H-ESCs in co-culture with developing ('silent') embryos was comparable to the basal cytokine production in the absence of an embryo. In contrast, H-ESC co-cultures with arresting (low-quality) embryos showed a decrease in the level of pro-implantation cytokines in the supernatant. It appears that low-quality ('noisy') embryos can downregulate the mediator production of decidualizing H-ESCs.

In chapter 2, 3 and 4 two different *in vitro* models are applied to study the migration of H-ESCs. In **chapter 2** we show for the first time that decidualizing H-ESCs from normally fertile women are capable of adjusting their migratory behaviour in response to the quality of an embryo. Our data demonstrates that the migration of in the presence of a high-quality embryo is not different from the basal migration of H-ESCs

in the absence of an embryo, supporting the concept of the intact 'silent' embryo. However, migration of normal H-ESC towards a low-quality embryo is actively inhibited. We hypothesize that the low-quality embryo signals ('noisy' embryo) cause a downregulation of migratory signaling in decidualizing H-ESCs. So, migration may promote embryo implantation, but the important message is that migration may also be used as an embryo selection mechanism. In addition, decidualizing H-ESCs from RM women display an increased migratory response in the presence of trophoblast spheroids, compared to the decidualizing H-ESCs of normal fertile women. Possibly, the enhanced migratory response of RM H-ESCs towards a trophoblast spheroid (or a low-quality embryo) may be used as a biomarker to identify 'selection failure' for women with RM.

Although **chapter 2** adds evidence to the biosensor function of the H-ESCs, in order to truly confirm the selective function of the H-ESCs we would want to repeat the experiments in a larger group of normal fertile women. Interesting additional experiments would be to extend the culture period from 18 h to 24 or 48 h. This would allow us to further study the encapsulation process until the embryo is fully embedded by the H-ESCs. In case of selective migration (inhibition of migration in the presence of low-quality embryos and no inhibition of migration in the presence of high-quality embryos), the migratory difference observed between RM and control decidualizing H-ESCs may then also become more clear in the presence of low-quality embryos.

In agreement with the co-culture study by Teklenburg *et al*., we noted that H-ESCs primarily respond to low-quality embryos (Teklenburg *et al*., 2010). In addition, unpublished results derived from a microarray study showed that a much larger number of H-ESC genes were differently expressed in decidualizing H-ESCs that were co-cultured with supernatant coming from developmentally compromised (low-quality) embryos in comparison to decidualizing H-ESCs that were co-cultured with supernatant derived from developing (high-quality) embryos (Teklenburg *et al*., unpublished data).

Combined, our data suggests that normal fertile decidualizing H-ESCs are primarily responsive to 'low-quality' human embryo signal(s). As the content of this signal is still unclear, it is now of utmost importance to try to elucidate the mechanisms by which healthy H-ESCs sense the difference between high- and low-quality embryos. An ideal future experiment- in case the 'low-quality' signal has been identified - would be to

block this signal in normal fertile decidualizing H-ESCs and see how H-ESC migration would be affected.
As the last few years we and others have become increasingly aware of the extent of chromosomal abnormal embryos in humans, criteria to better grade the quality of the embryo will be needed. Additional markers in characterizing the quality of the embryo may be hCG measurements of the embryo supernatant (as was done in chapter 6).

Recurrent miscarriage and the Selection Failure Hypothesis

Based on lower uterine epithelial mucin-1 levels (an anti-adhesive for embryo attachment) observed in the endometrium of women with RM, it was hypothesized that women with RM may be more receptive to implantation including to accept low-quality embryos (Aplin *et al.*, 1996). Subsequently, in 2002, this concept was described in 'the Selection Failure Hypothesis' that was put forward as a possible explanation for the aetiology of RM (Quenby *et al.*, 2002). This hypothesis states that the endometrium of women with RM is not able to select high-quality embryos for implantation and instead will implant both high- and low-quality embryos. When low-quality embryos are allowed to implant and their development fails this will inevitably lead to a clinical miscarriage.

Chapter 2 provides strong evidence in support of the Selection Failure Hypothesis for women with RM, on the level of decidualizing H-ESCs. The most striking finding is that the migration of decidualizing H-ESCs was not inhibited in the presence of a chromosomally abnormal embryo, a phenomenon that was observed in the H-ESCs of normal fertile women. In other words, decidualizing H-ESCs from RM women were unable to discriminate high- from low-quality embryos, possibly making the RM women more receptive for low-quality embryos than normally fertile women. The 'super-receptive' phenotype of women with RM is also supported by the observation that these cells are more sensitive to extravillous trophoblast cell factors, as the migration of decidualizing H-ESCs from RM women is also enhanced by exposure to trophoblast spheroids. The increased migratory potential and the inability to control migration in the presence of chromosomally abnormal embryos may thus lead to the implantation of developmentally compromised embryos (that are so abundant in humans). The latter data tempted us to hypothesize that decidualizing H-ESC from RM women do not recognize the 'developmentally-compromised-associated' signals of the low-quality embryos and miss some kind of 'brake' in the migration process.

An interesting future experiment would be to repeat the microarray experiment of Teklenburg *et al.* now also with decidualizing H-ESCs from women with RM exposed to supernatants of high-quality or supernatant of low-quality embryos. We hypothesize that the large difference in gene expression in the presence of low-quality signals (compared to high-quality signals) that is observed in normal fertile H-ESCs may not be observed in H-ESCs from women with RM. In addition, it would be very interesting to relate the increased migratory response of RM- H-ESCs observed in response to trophoblast spheroids to the level of receptivity markers such as prokineticin-1.
The nature of the inhibiting signal expressed by the 'noisy' low-quality embryo is an as yet undiscovered field, open for further investigation. Compromised embryos have been associated with a highly increased metabolic ('noisy') state, with a joint high ATP and reactive oxygen species production and an increased amino acid turnover rate (Leese *et al.*, 2007; Leese *et al.*, 2008; Picton *et al.*, 2010). Candidate 'low-quality' signals that are sensed by decidualizing H-ESCs may therefore be byproducts of this increased metabolism. Alternatively, the 'low-quality' signal could release damage-associated molecules (damage-associated molecular patterns, DAMPs). These DAMPs such as high-mobility group box protein 1 (HMGB1) have been shown to function as active chemoattractants, possibly inducing a migratory response of RM H-ESCs towards low-quality embryos (Hirsiger *et al.*, 2012). It would be worthwhile to investigate whether these embryo-derived DAMPs play a role in the non-selective behaviour of H-ESCs from women with RM. Moreover, as trophoblast microparticles have been found in the maternal circulation, it is possible that these trophoblast-microparticles are also recognized by the H-ESCs but lead to a disturbed response in RM H-ESCs. Alternatively, human chorionic gonadotropin (hCG) may cause the dysregulated migratory response of H-ESCs from women with RM. bHCG, the key hormone secreted by the embryonic trophoblasts, has been shown to cause a dysregulated prolactin and prokineticin-1 mRNA response in decidualizing H-ESCs of women with RM (Salker *et al.*, 2010).

Chapter 3 discusses the migratory behaviour of decidualizing H-ESCs in the presence of trophoblast-derived factors and PDGF-BB measured in the ORIS model. Data of this chapter again show an altered migration pattern of decidualizing H-ESCs from women with RM compared to normal fertile woman. However, the results seem to be in contradiction to the results in chapter 2. The main finding of chapter 3 is that H-ESC migration in response to trophoblast-derived factors is much higher in the control group than in the RM group. When we compare this data to the observation

of increased migration in the presence of trophoblast spheroids (that would secrete identical factors as they are derived from the same trophoblast cell line), the results seem to be contradictive, both for the decidualizing H-ESC of women with RM and for the decidualizing H-ESCs of healthy fertile women. A possible explanation would be that the differences measured relate to the difference in method used to study the migration between **chapter 2 and 3**; the first making use of a scratch assay for directed migration (chemotaxis) and the latter of the ORIS migration assay for non-directed migration (chemokinesis). Although the ORIS migration assay is a valid method for testing the effect of various individual trophoblast-derived factors on H-ESC migration, when we would compare the methods used to the implantation environment in the *in vivo* situation, the scratch assay method may be more relevant. Another explanation might be that the migratory pattern of decidualizing H-ESCs is decided by the local paracrine secretion of the chemoattractant gradient by embryo or spheroids. We would like to propose that the molecular composition of the local chemoattractant gradient secreted in a paracrine way by the embryo or spheroid differs from the molecular composition of the supernatant, leading to a different milieu in the proximity of the H-ESCs. We advise therefore to use spheroids or embryos when testing the migratory behaviour of decidualizing H-ESC as a potential biomarker and to refrain from the use of supernatants since supernatants in this experimental set-up do not reflect the chemoattractive constitution of the fetal maternal interface.

Glucocorticoids and endometrial stromal cell migration

If the concept of the increased migratory potential in RM underlying the failed embryo selection and the aetiology of unexplained RM holds true, it is tempting to think of possible treatment options to restore the uncontrolled ESC migration in patients with RM. Agents that have been known to control or inhibit migration at least in trophoblast cells *in vitro* models are glucocorticoids (GCs) (Librach *et al.*, 1994). Interestingly, GCs are administered empirically to women with RM and women with implantation failure (Quenby *et al.*, 2003). Since dexamethasone has been shown to inhibit migration, one might think that this drug may have the potential to downregulate the enhanced migratory potential of H-ESCs from women with RM. However, in **chapter 4** we describe that the migration of normal fertile H-ESCs is inhibited in the presence of dexamethasone, but that the migration of RM-H-ESCs is not inhibited but stimulated by dexamethasone. As failed embryo selection in women with RM may be associated with an increased migratory potential, together with the

notion that GC may skew the cytokine pattern to an anti-inflammatory (Th2) state, we propose that dexamethasone treatment may not be an efficacious therapeutic strategy for women with RM.

In trying to understand the mechanisms underlying the differential dexamethasone effect on H-ESC migration between RM and control women as described in **chapter 4**, we measured the number of dexamethasone binding receptors, and the gene expression of glucocorticoid associated genes. The mRNA expression of the downstream signaling molecules of the GR pathway included glucocorticoid-induced leucine zipper (GILZ), serum and glucocorticoid-inducible kinase-1 (SGK-1), and the GR co-chaperone FKBP5 (decreasing the glucocorticoid binding capacity). In addition, we quantified the mRNA expression of the GR subtypes (GR-α and GR-β) in H-ESCs from normal fertile and RM women.

In our study group, RM seemed associated with diminished FKBP5 mRNA expression and an elevated GR-α expression in decidualizing H-ESCs compared to the H-ESCs of control women. How this translates into a higher migratory response of H-ESCs from RM women remains unclear. Future studies should focus on the role of FKBP5 expression and function in the regulation of GR function in H-ESC from women with RM.

Therapeutic options for recurrent miscarriage

Thus far no treatment options exist for women with unexplained RM (Christiansen *et al.*, 2005). There is insufficient evidence to support the use of immunotherapy, hCG or progesterone in women with unexplained RM to increase live birth rates (Coulam and Acacio, 2012; Oates-Whitehead *et al.*, 2003; Scott and Pattison, 2000; Scott, 2003). As RM has been associated with an increased Th2 cytokine profile and an increased migratory response of the H-ESC compartment, and glucocorticoid (GC) administration seems to further promote the Th2 profile and the H-ESC migratory response, GC may therefore not be beneficial for women with RM. However, NK cells may be a good target for GC therapy and the effect of GC administration to women with RM on the uterine NK cell count, miscarriage and pregnancy rates is currently under investigation in a randomized controlled trial (Tang *et al.*, 2009).

With no available treatment options and 'embryo selection failure' as a possible cause underlying the aetiology of RM, the future may lie in the improvement of embryo selection for RM couples. Improving embryo selection may be realized through

preimplantation genetic screening (PGS) to select high-quality embryos, or screening of the blastocyst culture medium to look for high quality markers.
The idea stated in **chapter 2** is that decidualizing H-ESCs from women with RM may be less discriminatory in response to one of the products released by low-quality ('noisy') embryos and as a result show no inhibition of migration, may be an interesting alley to pursue in the search for therapeutical targets for RM. Compromised embryos have been associated with a very active metabolic ('noisy') state, with secretion of damage-associated molecular pattern molecules (DAMPs) such as ATP, reactive oxygen species, and demonstrate an increased amino acid turnover rate that is associated with a lower cytogenic health in the embryo (Leese *et al.*, 2007; Leese *et al.*, 2008; Picton *et al.*, 2010; Stokes *et al.*, 2007). We propose that increased sensitivity of RM-H-ESC to DAMPs secreted by low-quality embryos may be an attractive candidate as an underlying mechanism of the increased migration of RM-H-ESC towards a low-quality embryo, since DAMPs have been shown to be powerful chemoattractants.

Preimplantation genetic screening (PGS) on embryos from women with RM, revealed that RM women produce more aneuploid embryos than normal fertile women (Gianaroli *et al.*, 2000; Simon *et al.*, 1998). In an IVF population one study showed that when PGS was used to discard (out-select) karyotypically abnormal embryos, the miscarriage rate after IVF decreases (Munne *et al.*, 1999).
The best available evidence on the effect of PGS on increasing pregnancy rates in women with unexplained RM, comes from four observational and seven intervention studies. These studies indicate that PGS could not succeed in increasing pregnancy rates in women with RM (Musters *et al.*, 2011). However, it appeared that all studies included had limitations, such as small samples size and large heterogeneity (that did not allow for meta-analysis). In the view of the 'selection failure' hypothesis we would expect a somewhat higher live-birth rate in RM after PGS vs. RM patients that conceive naturally. However, we would not expect a dramatic increase in live birth rate following PGS for patients that suffer from RM. This is because PGS only screens for a few number of chromosomal abnormalities, there is a high incidence of mosaicism in young couples that undergo IVF (Baart *et al.*, 2006) and the baseline fecundity rate of RM is not low (with a live birth rate of around 35%). In addition, karyotyping does not detect pregnancies complicated by structural abnormalities in the presence of chromosomally normal embryos.
Although the best available evidence suggests a similar live-birth rate in women with

unexplained RM after PGS vs. natural conception (42 vs. 35 % respectively), this is still under debate as no RCT or non-randomized comparative studies (directly comparing PGS vs. natural conception) have been performed in this study group (Musters *et al.*, 2011).

As maternal age is the major risk factor for miscarriages and RM, an important recommendation is for these women, if possible, is not to delay childbirth any further. A deteriorating oocyte quality that is associated with advancing maternal age is an important contributory factor to miscarriages (Nybo Andersen *et al.*, 2000; Quenby and Farquharson, 1993).

Early human embryo development *in vitro*

Implantation and maintenance of a pregnancy is not only dependent on the behaviour of H-ESCs. A normal healthy development of the embryo also plays a crucial role. One crucial event in normal embryo development is X-chromosome inactivation in maternal cells. The *in vitro* decidualizing H-ESC-blastocyst co-culture model enabled us to study this early embryo developmental phenomenon, and has been described in **chapter 6**.

The main result in this chapter is that in day eight embryos, the hypoblast and epiblast cell lineages have segregated. In addition, we showed that H3K27me3 foci (that X-chromosome inactivation) are absent on day five embryos but are present on day 8 embryos. More specifically, H3K27me3 accumulation was regulated in a lineage specific manner, as the H3K27me3 foci were only present on trophectoderm cells (25%) and on hypoblast cells (7.5%).

In addition, the data in this chapter also shows that all three embryonic lineages are able to develop well in an *in vitro* model during these early stages of embryo development.

General conclusion

In conclusion, in this thesis we report several new findings that describe the mechanisms behind the 'selective phenotype' seen in fertile women and behind the 'super-receptive' phenotype seen in women with RM. Decidualized H-ESCs of fertile women may actively select a high-quality embryo for implantation by inhibiting their migratory response in the presence of low-quality embryos.

In contrast, the migration of H-ESCs of RM women that is elicited by trophoblast spheroids and by low-quality embryos highlights the super-receptive state of the

endometrium of women suffering from RM. This enhanced migratory response of H-ESCs of RM women towards a low-quality embryo or towards trophoblast spheroids may become a biomarker for identifying 'selection failure' as the aetiology in those patients diagnosed with otherwise unexplained RM.

More research is needed to fully elucidate the responsible trophoblast cell factors inducing H-ESCs migration and the mechanisms by which low-quality embryos inhibit the migration of decidualized H-ESCs of fertile women and how this is dysregulated in the H-ESC of RM women.

Clarification of the mechanism of non-discriminative migration of too high- and low-quality embryos of H-ESCs of women with RM may have important clinical implications for understanding the aetiology of this distressing condition, for the future development of establishing biomarkers of RM and for identifying novel therapeutic targets for the prevention of further miscarriages. When in the future better markers for embryo quality have been found, embryo selection using PGS may become an important tool to increase the live birth rate in women with RM.

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Chapter 8

Nederlandse samenvatting

Hoofdstuk 1

In de inleiding van dit proefschrift wordt duidelijk dat het niet best gesteld is met de vruchtbaarheid van de mens. De kans op een succesvolle zwangerschap na gericht seksueel contact is tussen de 20 en 30% per cyclus en daalt tot onder de 10% wanneer vrouwen de leeftijd van 35 jaar gepasseerd zijn. Ter vergelijking: bij bavianen is het succespercentage 80%. Van de 100 bevruchte eicellen blijkt er bij mensen al ongeveer 60% in een vroeg stadium van de zwangerschap verloren te gaan. Mogelijk kan het hoge percentage aan embryo's met chromosomale afwijkingen dit hoge verlies verklaren. In een later stadium zal nog eens 10% van bevruchte eicellen verloren gaan hetgeen gepaard gaat met klinische symptomen van een miskraam. Maar ongeveer 30% van de bevruchte eicellen zal uiteindelijk leiden tot een voldragen zwangerschap.

Deze vrij lage maandelijkse zwangerschapskans in combinatie met het (in toenemende mate) uitstellen van een zwangerschap tot het einde van de vruchtbare leeftijd, leidt ertoe dat tegenwoordig één op de zes koppels zich in het ziekenhuis meldt met een vruchtbaarheidsprobleem. Eén op de twaalf koppels zal uiteindelijk overgaan tot een fertiliteitsbehandeling. Vruchtbaarheidsproblematiek kan grofweg ingedeeld worden in twee categorieën: sub-(of in-)fertiliteit en super-fertiliteit. Vrouwen in deze laatste categorie worden snel en vaak zwanger, echter de zwangerschappen zullen veelal eindigen in miskramen. Gemiddeld eindigt één op de tien zwangerschappen in een miskraam en miskramen zijn daarmee de meest voorkomende complicatie van een zwangerschap. Echter sommige vrouwen krijgen te maken met drie of meer miskramen zonder dat daar een oorzaak voor gevonden kan worden. Dit wordt ook wel 'herhaalde miskraam' genoemd en komt voor bij 1-2% van de koppels. Helaas kan in meer dan de helft van deze gevallen geen oorzaak voor de herhaalde miskramen gevonden worden en zijn er geen behandelingen beschikbaar.

In een 'in vitro' kweeksysteem, waarin menselijke baarmoederslijmvliescellen (endometrium stromale cellen) worden samengebracht met vijf dagen oude embryo's, is vastgesteld dat cellen van gezonde vrouwen 'slechte' van 'goede' kwaliteit embryo's kunnen onderscheiden. De baarmoederslijmvliescellen fungeren als het ware als een biosensor voor de kwaliteit van embryo's.

Een aantal jaar geleden werd het idee geopperd dat vrouwen met herhaalde miskraam mogelijk super-fertiel (super-receptief) baarmoederslijmvlies hebben. Dit baarmoederslijmvlies zou, in tegenstelling tot dat van normaal vruchtbare

vrouwen, niet kunnen differentiëren tussen kwalitatief goede en slechte embryo's. Chromosomaal abnormale embryo's, die frequent voorkomen bij de mens, hebben daardoor een grotere kans te implanteren en wanneer vervolgens hun ontwikkeling staakt, zal dit leiden tot een klinische miskraam.

Het primaire doel van dit proefschrift is te onderzoeken of baarmoederslijmvliescellen van vrouwen met herhaalde miskraam super-receptief zijn, dat wil zeggen dat zij geen onderscheid kunnen maken tussen kwalitatief goede en slechte embryo's en dus ook slechte kwaliteit embryo's selecteren. Daartoe hebben wij middels twee laboratorium ('in vitro') modellen de eigenschappen van baarmoederslijmvliescellen onderzocht. Met deze modellen bootsen wij het werkelijke ('in vivo') implantatiemilieu in de baarmoeder na. Eén van de belangrijkste taken van baarmoederslijmvliescellen is een vruchtbare bodem te creëren waarin een embryo kan gaan nestelen. De migratie van deze baarmoederslijmvliescellen bevordert het innestelingsproces. Deze migratie hebben wij beschouwd als een indirecte marker voor receptiviteit van het baarmoederslijmvlies. In dit proefschrift hebben wij de migratie van baarmoederslijmvlies bestudeerd richting kwalitatief goede en kwalitatief slechte (chromosomaal abnormale) embryo's. Wij hebben de migratie capaciteit vergeleken tussen baarmoederslijmvliescellen van normaal vruchtbare vrouwen en vrouwen met herhaalde miskraam. Ook hebben wij gekeken naar factoren die in het innestelingsmilieu aanwezig zijn en die deze migratie kunnen beïnvloeden.

Hoofdstuk 2

In dit hoofdstuk hebben wij onderzocht of baarmoederslijmvliescellen van vrouwen met herhaalde miskraam niet alleen migreren in aanwezigheid van goede kwaliteit embryo's maar ook in aanwezigheid van slechte kwaliteit embryo's. Wij hebben dit vergeleken met de migratiecapaciteit van baarmoederslijmvliescellen van normaal vruchtbare vrouwen.

Het specifieke model waarmee de migratie gemeten is, hebben we een 'scratch' of 'wound healing' assay genoemd. In deze assay wordt gebruik gemaakt van een aaneengesloten laag baarmoederslijmvliescellen waarin één strook celvrij gemaakt wordt. In het midden van deze strook (de migratiezone) wordt één vijf dagen oud humaan embryo (kwalitatief goed of slecht) of een trophoblast spheroide (pseudo embryo's gemaakt van cellijn afkomstige embryonale cellen) geplaatst, waar de baarmoederslijmvliescellen naartoe kunnen migreren. Na achttien uur wordt de

verkleining in migratie zone bepaald, hetgeen een mate is voor de migratie van baarmoederslijmvliescellen die heeft plaatsgevonden.

Een belangrijke bevinding is dat baarmoederslijmvliescellen van normaal vruchtbare vrouwen net zo goed migreren in de aanwezigheid van goede kwaliteit embryo's als in de afwezigheid van een embryo, maar dat de migratie richting chromosomaal abnormale embryo's geremd is. Baarmoederslijmvliescellen blijken dus te kunnen differentiëren tussen goede en slechte kwaliteit embryo's en daar hun migratiepatroon op aan te passen. Als migratie de embryo-implantatie bevordert, is de migratie van baarmoederslijmvliescellen mogelijk een mechanisme achter embryo selectie. Hoe baarmoederslijmvliescellen een verschil kunnen opmerken tussen een goed of slecht embryo zal onderwerp moeten zijn van vervolg onderzoek.

Een tweede belangrijke bevinding beschreven in hoofdstuk twee is dat baarmoederslijmvliescellen van vrouwen met herhaalde miskraam géén verschil in migratie vertonen ten opzichte van goede of slechte kwaliteit embryo's. Met andere woorden deze baarmoederslijmvliescellen zijn niet in staat om onderscheid te maken tussen goede of slechte kwaliteit embryo's. Dit 'super-receptieve' fenotype werd bevestigd door de observatie dat deze baarmoederslijmvliescellen gevoeliger zijn voor trophoblast celfactoren; baarmoederslijmvlies van vrouwen met herhaalde miskraam vertoonden een verhoogde migratie respons in de aanwezigheid van pseudo embryo's. De verhoogde migratie en het gebrek aan selectief vermogen kan leiden tot de implantatie van abnormale embryo's. Deze gegevens ondersteunen de 'Selection Failure Hypothesis' voor herhaalde miskraam. Mogelijk herkennen de baarmoederslijmvliescellen van vrouwen met herhaalde miskraam de signalen die gemaakt worden door de kwalitatief slechte kwaliteit embryo's niet, waardoor zij niet geremd worden in hun migratie. In dit hoofdstuk suggereren wij dat het bepalen van de migratie ten opzichte van pseudo embryo's als biomarker voor herhaalde miskraam kan worden gebruikt.

Hoofdstuk 3

In dit hoofdstuk wordt de migratie van baarmoederslijmvliescellen verder bestudeerd waarbij we ons afvragen hoe deze cellen migreren onder invloed van verschillende vroege zwangerschapsfactoren en of er een verschil in migratie bestaat tussen baarmoederslijmvliescellen van vrouwen met herhaalde miskraam en die van normaal vruchtbare vrouwen. Ditmaal bestuderen we de migratie van de baarmoederslijmvliescellen met een ORIS migratie assay. In het midden van

ieder kweekbakje wordt een ronde migratiezone gecreëerd waarna verschillende concentraties PDGF-BB (een bekende factor die migratie van cellen kan induceren, en welke ook door vroeg placentaweefsel gemaakt wordt) of andere factoren die gemaakt worden door embryonale cellen toegevoegd worden. Achttien uur later wordt, als maat voor migratie, de verkleining van de celvrije migratiezone gemeten. De resultaten blijken anders dan verwacht. In tegenstelling tot de resultaten in hoofdstuk twee wordt in het bijzijn van embryonale (trophoblast) factoren geen toename in migratie gezien in de kweekbakjes met de baarmoederslijmvliescellen van vrouwen met herhaalde miskraam. In plaats daarvan wordt juist een veel hogere migratie van de baarmoederslijmvliescellen van normaal vruchtbare vrouwen gevonden in respons op trophoblast factoren.

Een mogelijke verklaring voor de gevonden resultaten in hoofdstuk twee en drie is het verschil in methodiek In hoofdstuk twee wordt chemotaxie (gerichte migratie) gemeten, terwijl in hoofdstuk drie gefocust werd op chemokinese (willekeurige, niet-gerichte migratie). De chemotaxie assay uit hoofdstuk twee is waarschijnlijk relevanter vanwege de chemoattractieve gradiënt die uitgaat van de embryo's. Wij veronderstellen dat deze gradiënt de in vivo situatie beter benadert dan de samenstelling van het toegevoegde supernatant aan het in vitro chemokinese systeem. Daarom adviseren wij het gebruik van embryo's of pseudo embryo's als methodiek voor dit soort onderzoek omdat dit de chemoattractie tussen foetus en moeder beter weergeeft.

Hoofdstuk 4

Tot dusver is geen behandeling effectief gebleken in het voorkomen van miskramen bij vrouwen met herhaalde miskraam. Wanneer we aannemen dat bij vrouwen met herhaalde miskraam een verhoogde (ongeremde) migratie van baarmoederslijmvliescellen een maat is voor verminderde embryo selectie, zal een behandeling die deze ongeremde migratie kan herstellen een mogelijke therapeutische optie zijn. In dit kader hebben wij het effect van glucocorticoïden (een klasse geneesmiddelen die vooralsnog empirisch gebruikt wordt in deze patiëntengroep) op de migratie van baarmoederslijmvliescellen onderzocht. Aangezien dexamethason (een glucocorticoïd) migratie van andere celsoorten kan remmen, verwachten wij dat dit middel hetzelfde effect zou hebben op baarmoederslijmvliescellen van vrouwen met herhaalde miskraam. Echter, de gegevens beschreven in hoofdstuk vier laten iets anders zien. Hoewel de migratie van baarmoederslijmvliescellen

van normaal vruchtbare vrouwen geremd is in de aanwezigheid van (hoge doses) dexamethason, blijkt dexamethason juist een stimulerend effect te hebben op de migratie van baarmoederslijmvliescellen van vrouwen met herhaalde miskraam. Wanneer verminderde embryo selectie, mogelijk door een verhoogde migratie van baarmoederslijmvliescellen, er toe leidt dat er bij vrouwen met herhaalde miskraam meer (chromosomaal) abnormale embryo's geïmplanteerd worden, zal dexamethason toediening geen effectief therapeutisch middel zijn voor vrouwen met herhaalde miskraam.

Om erachter te komen welke cellulaire mechanismen het verschil in migratie op dexamethason zouden kunnen verklaren hebben we metingen verricht naar het aantal glucocorticoïd receptoren en hebben we de genexpressie niveaus bepaald van een aantal glucocorticoïd-geassocieerde eiwitten. Wij vergeleken de effecten van dexamethason op baarmoederslijmvlies van vrouwen met herhaalde miskraam met die van normaal vruchtbare vrouwen. Bestudeerde genen waren de 'downstream signalling' moleculen in de glucocorticoïd signaal transductie zoals 'glucocorticoid-induced leucine zipper' (*GILZ*), 'serum and glucocorticoid-inducible kinase-1' (*SGK-1*), en de glucocorticoïd receptor co-chaperon *FKBP5* (dat de glucocorticoïd bindingscapaciteit verlaagt). Ook hebben we het mRNA expressieniveau bepaald van de glucocorticoïd receptor GR-α en GR-β in de baarmoederslijmvliescellen van beide groepen vrouwen.

In baarmoederslijmvliescellen van vrouwen met herhaalde miskraam blijkt de FKBP5 mRNA genexpressie verminderd en de GR-α genexpressie verhoogd te zijn ten opzichte van expressie in de cellen van normaal vruchtbare vrouwen. Aangezien in normaal vruchtbare vrouwen dexamethason zorgt voor remming van de migratie van baarmoederslijmvliescellen, zou in baarmoederslijmvlies van vrouwen met herhaalde miskraam, doordat de FKBP5 remming wegvalt, de glucocorticoid bindingscapaciteit worden verhoogd, zodat een grotere migratieremming van dexamethason kan worden verwacht. Hoe deze bevindingen zich vertalen naar de verhoogde migratie respons op dexamethason blijft vooralsnog onduidelijk.

Hoofdstuk 5

In het literatuur-overzichtsartikel van dit hoofdstuk worden verschillende functies van baarmoederslijmvliescellen ten tijde van de implantatie uiteengezet en onderverdeeld in bekende functies (zoals het fungeren als een vruchtbare bodem waarin het embryo zich kan nestelen en het beschermen van de foetus tegen oxidatieve stress en

immunologische afstoting) en de nieuw gevonden concepten in baarmoederslijmvliescel functie waarbij we de focus leggen op de migratie van deze cellen en hun rol in embryo selectie. Tevens wordt vruchtbaarheidsproblematiek besproken in het kader van van migratie van baarmoederslijmvliescellen. Zowel endometriose als herhaalde miskraam is namelijk geassocieerd met abnormale baarmoederslijmvliescel migratie. Ook stellen wij dat implantatiefalen en herhaalde miskraam verklaard kunnen worden door een abnormaal gereguleerde embryo selectie. Bij implantatiefalen kan er sprake zijn van te strenge embryoselectie waardoor embryo's niet de kans krijgen te implanteren terwijl bij herhaalde miskraam de embryoselectie juist matig of onvoldoende is met als gevolg implantatie van zowel goede als slechte kwaliteit embryo's. Wij veronderstellen dat een beter begrip van baarmoederslijmvliescel functie kan leiden tot de ontwikkeling van nieuwe therapeutische strategieën bij de voorkoming en behandeling van voortplantingsproblematiek.

Hoofdstuk 6

Het verloop van de innesteling van een embryo en behoud van de foetus is niet alleen afhankelijk van baarmoederslijmvliescellen. Ook het embryo zelf zal zich goed moeten ontwikkelen. De geslachtschromosomen zijn één van de 23 paar chromosomen waarin ons erfelijk materiaal opgeslagen ligt. Een man heeft één X-chromosoom en één Y-chromosoom en een vrouw twee X-chromosomen. Een belangrijk moment in de gezonde ontwikkeling van een vrouwelijk embryo is het inactiveren van een van de X-chromosomen, om ervoor te zorgen dat de hoeveelheid actief genetisch materiaal tussen mannen en vrouwen in elke cel gelijk blijft. Over de mechanismen van X-chromosoom inactivatie in menselijke embryo's is niet veel bekend. Ons in vitro co-cultuur systeem, waarbij een menselijk embryo tot dag acht gekweekt kon worden op een laag baarmoederslijmvliescellen, gaf de mogelijkheid om naar deze vroege embryonale ontwikkeling onderzoek te doen. In ons model blijken embryo's zich goed te kunnen ontwikkelen, zodat op dag acht de embryo's een ontwikkelingsstadium bereikt hebben met een gescheiden hypoblast en epiblast (beide afkomstig van de 'inner cell mass' van het embryo). Ook hebben we aangetoond dat de H3K27me3 foci (die zorgen voor X-chromosoom inactivatie) pas op dag acht aanwezig waren. Deze H3K27me3 foci blijken alleen aanwezig te zijn op trophectoderm en hypoblast cellen en niet op het epiblast. Het in vitro co-cultuur model blijkt niet alleen nuttig te zijn voor het bestuderen van baarmoederslijmvliescellen, maar ook voor het bestuderen van vroege embryonale ontwikkeling.

Hoofdstuk 7

In dit laatste samenvattende hoofdstuk wordt een algemene beschouwing gegeven van de studies beschreven in dit proefschrift met de daaruit voortgekomen nieuwe visie op baarmoederslijmvliescel kenmerken van vrouwen met herhaalde miskraam. Deze kenmerken zijn onder andere: verhoogde migratie in aanwezigheid van chromosomaal abnormale embryo's en pseudo embryo's, hetgeen mogelijk het 'super-receptieve' fenotype van de vrouwen kan verklaren. De verhoogde in vitro baarmoederslijmvliescel migratie na stimulatie met dexamethason is een ander niet eerder bekend kenmerk van vrouwen met herhaalde miskraam.

Meer kennis over het gedrag van baarmoederslijmvliescellen van vrouwen met herhaalde miskraam kan belangrijke klinische ontwikkelingen teweeg brengen, zowel in het inzicht in hoe herhaalde miskraam wordt veroorzaakt, als voor de toekomstige ontwikkeling van biomarkers voor herhaalde miskraam en het identificeren van therapeutische aangrijppunten ter behandeling en voorkoming van herhaalde miskraam.

Ook in dit proefschrift blijven nog veel vragen onbeantwoord. Een aantal vragen zijn hieronder uiteengezet.

- Wat is de 'kwalitatief slechte factor' gemaakt door chromosomaal abnormale embryo's die de migratie van baarmoederslijmvliescellen remt? Via welk mechanisme treedt deze remming op?
- Hoe komt het dat de migratie van baarmoederslijmvlies van vrouwen met herhaalde miskraam niet geremd is in aanwezigheid van kwalitatief slechte embryo's?
- Hoe komt het dat de migratie van baarmoederslijmvlies van vrouwen met herhaalde miskraam zo veel hoger is dan die van normaal vruchtbare vrouwen in aanwezigheid van pseudo embryo's?
- Wat is de rol en functie van FKBP5 genexpressie in het reguleren van de functie van glucocorticoid signaal transductie in baarmoederslijmvliescellen van vrouwen met herhaalde miskraam?

Chapter 9

Authors and affiliations
List of publications
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Chapter 9

Authors and affiliations

List of publications

Dankwoord/ Acknowledgements

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Chapter 9

Authors and affiliations

List of publications

Dankwoord/ Acknowledgements

About the author

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Chapter 9

Authors and affiliations

List of publications

Dankwoord/ Acknowledgements

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Lotte Weimar werd op 30 juli 1981 geboren in Rotterdam. In 1999 behaalde ze haar middelbare school diploma aan het Erasmiaans Gymnasium aldaar en startte zij haar studie Biologie aan de Universiteit Utrecht. Voor het behalen van haar master heeft zij onder andere onderzoek gedaan aan de Nuffic Department of Surgical Sciences van de Universiteit van Oxford. Hier deed zij onderzoek naar het ontstaan van memory-like T cellen door homeostatische proliferatie onder leiding van Prof dr. Kathryn Wood en dr. Nick Jones. In maart 2005 behaalde zij haar titel Master of Science in Biologie, een maand na gestart te zijn met de verkorte opleiding geneeskunde (SUMMA) van het Universitair Medisch Centrum Utrecht (UMCU). Het co-schap gynaecologie in het Gelre Ziekenhuis in Apeldoorn (opleider dr. Peter van de Weijer) wekte haar belangstelling in de gynaecologie en verloskunde. Tijdens de wetenschappelijke stage van de opleiding heeft zij onder begeleiding van Prof. dr. Nick Macklon en dr. Gijs Teklenburg onderzoek gedaan naar endometrium stromale cellen en 'natural killer' cellen aan de afdeling Voortplantingsgeneeskunde en Gynaecologie van het UMCU. In december 2008 behaalde zij haar arts-examen. Na toekenning van de beurs van het MD/PhD Programma 'Alexandre Suerman' kon zij in juli 2009 beginnen met haar promotietraject onder leiding van Prof. dr. Nick Macklon, Prof. dr. Cobi Heijnen en Prof. dr. Annemieke Kavelaars. Het project bleek een vruchtbare samenwerking tussen de afdeling voortplantingsgeneeskunde en het laboratorium 'Neuroimmunology and Developmental Origins of Disease' (NIDOD) van het UMCU en heeft geleid tot dit proefschrift. Eind 2012 heeft zij, onder leiding van Prof. dr. Nick Macklon, aan vervolgonderzoeken gewerkt op de afdeling 'Human Development and Health' van het Princess Anne Ziekenhuis van de Universiteit van Southampton. Begin 2013 zal zij starten als arts-assistent Gynaecologie in het Cluster Utrecht (opleider Prof. dr. Arie Franx).