Deregulation of the endometrial stromal cell secretome precedes embryo implantation failure

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Running title: Endometrial secretome and implantation failure

Abstract

Study question: Is implantation failure following ART associated with a perturbed decidual response in endometrial stromal cells (EnSCs)?

Summary answer: Dynamic changes in the secretome of decidualizing EnSCs underpin the transition of a hostile to a supportive endometrial microenvironment for embryo implantation; perturbation in this transitional pathway prior to ART is associated with implantation failure.

What is known already: Implantation is the rate-limiting step in ART, although the contribution of an aberrant endometrial microenvironment in IVF failure remains ill defined.

Study design, size, duration: *In vitro* characterisation of the temporal changes in the decidual response of primary EnSCs isolated prior to a successful or failed ART cycle. An analysis of embryo responses to secreted cues from undifferentiated and decidualizing EnSCs was performed. The primary clinical outcome of the study was a positive urinary pregnancy test 14 days after embryo transfer.

Participants/materials, setting, methods: Primary EnSCs were isolated from endometrial biopsies obtained prior to IVF treatment and cryopreserved. EnSCs from 10 pregnant and 10 non-pregnant patients were then thawed, expanded in culture, subjected to clonogenic assays, and decidualized for either 2 or 8 days. Transcript levels of decidual marker gene [prolactin (*PRL*), insulin-like growth factor binding protein 1 (*IGFBP1*) and 11β-hydroxysteroid dehydrogenase (*HSD11B1*)] were analysed using real-time quantitative PCR and temporal secretome changes of 45 cytokines, chemokines and growth factors were measured by multiplex suspension bead immunoassay. The impact of the EnSC secretome on human blastocyst development was scored morphologically; and embryo secretions in response to EnSC cues analyzed by multiplex suspension bead immunoassay.

Main results and the role of chance: Clonogenicity and induction of decidual marker genes were comparable between EnSC cultures from pregnant and non-pregnant group groups ($P > 0.05$). Analysis of 23 secreted factors revealed that successful implantation was associated with co-ordinated secretome changes in decidualizing EnSCs, which were most pronounced on day 2 of differentiation: 17 differentially secreted proteins on day 2 of decidualization relative to undifferentiated (day 0) EnSCs ($P < 0.05$); 11 differentially secreted proteins on day 8 relative to day 2 ($P < 0.05$); and 8 differentially secreted proteins on day 8 relative to day 0 ($P < 0.05$). By contrast, failed implantation was associated with a disordered secretome response. Blastocyst development was compromised when cultured for 24 hours in medium conditioned by undifferentiated EnSCs when compared to decidualizing EnSCs. Analysis of the embryo microdroplets revealed that human blastocysts mount a secretory cytokine response to soluble decidual factors produced during the early (day 2) but not late phase (day 8) of differentiation. The embryo responses to secreted factors from decidualizing EnSCs were comparable between the pregnant and non-pregnant group ($P > 0.05$).

Large scale data: not applicable.

Limitations, reasons for caution: Although this study uses primary EnSCs and human embryos, caution is warranted when extrapolating the results to the *in vivo* situation because of the correlative nature of the study and limited sample size.

Wider implications of the findings: Our finding raises the prospect that endometrial analysis prior to ART could minimise the risk of treatment failure.

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Key words: implantation, embryo, endometrium, decidualization, stem cells, secretome

Introduction

Implantation is the rate-limiting step in ART. Clinically, failed implantation is defined by the lack of detectable hCG levels in either serum or urine 14 days after intrauterine transfer of one or more embryos. By default, failed implantation is a retrospective diagnosis, rendering it difficult, if not impossible, to discern if caused by a hostile endometrium, impaired embryo quality, or an iatrogenic event. Nevertheless, genome-wide expression studies have identified endometrial signature genes that are predictive of recurrent implantation failure (RIF), defined as the absence of pregnancy following serial transfers of high quality embryos (Koler *et al.*, 2009, Koot *et al.*, 2016). Interestingly, these studies have also challenged the prevailing concept that implantation failure merely reflects a lack of expression of receptivity genes during the window of implantation (Diaz-Gimeno *et al.*, 2011). Instead, the gene signatures associated with RIF point towards more fundamental defects pertaining to cell cycle regulation, cell motility, epithelial-mesenchymal transition (EMT), and signalling pathways involved in stem cell maintenance (e.g. WNT and Notch) (Koler *et al.*, 2009, Koot *et al.*, 2016).

Endometrial stromal cells (EnSCs) play a critical role in the implantation process, not only by relaying hormonal signals to the overlying surface epithelium (Chen *et al.*, 2013, Cooke *et al.*, 1997, Li *et al.*, 2011), but also by controlling the influx and function of various immune cells, including uterine natural killer cells (Collins *et al.*, 2009, Gellersen and Brosens, 2014, Nancy *et al.*, 2012). Upon invasion, the implanting embryo is rapidly surrounded and encapsulated by migrating decidualizing stromal cells. As the differentiation process unfolds, gap and tight junctions form between decidualizing cells. Consequently, the conceptus becomes anchored in the endometrium and forms a matrix that enables co-ordinated trophoblast invasion (Gellersen and Brosens, 2014, Wang *et al.*, 2004, Weimar *et al.*, 2013).

Growing evidence indicates that decidual cells are exquisitely responsive to embryonic signals and play a critical role in embryo biosensoring and selection (Brosens *et al.*, 2014a, Macklon and Brosens, 2014, Teklenburg *et al.*, 2010). On the other hand, decidualization transforms EnSCs into secretory cells that determine the embryonic microenvironment upon breaching of the luminal epithelium. Whether or not failed implantation can be attributed to an altered endometrial microenvironment that impacts directly on embryo development and survival is not known.

Decidualization of the human endometrium is not dependent on embryo implantation but is initiated during the mid-luteal phase of each cycle in response to the postovulatory rise in progesterone and increasing endometrial cAMP levels. Consequently, decidualization is a reiterative process, linked to cyclic activation of mesenchymal stem-like cells (MSCs) and subsequent differentiation into mature stromal cells in regenerating endometrium (Gellersen and Brosens, 2014, Lucas *et al.*, 2016). The endometrium harbors abundant MSCs that are multipotent, immuno-privileged, and highly regenerative (Cervello *et al.*, 2011, Gargett *et al.*, 2012, Gargett *et al.*, 2016). Functional repair of the endometrium requires activation of poised progenitor cells, induction of transit-amplifying cells and differentiation of mature progeny, which collectively determine the tissue response to deciduogenic and embryonic cues. The mechanisms that control this regenerative pathway and ensure homeostatic balancing of the different stromal cell populations are poorly understood. Arguably, disruption of this process will give rise to dysfunctional EnSCs. Studies on primary EnSC cultures have provided ample evidence that programming defects are linked to reproductive failure. For example, endometriosis is associated with progesterone resistance, defined by the refractoriness of cultured EnSCs to deciduogenic cues (Aghajanova *et al.*, 2010a, Aghajanova *et al.*, 2010b, Al-Sabbagh *et al.*, 2012, Klemmt *et al.*, 2006, Sherwin *et al.*, 2010, Velarde *et*

al., 2009). By contrast, purified EnSCs from women with a history of recurrent miscarriage mount a prolonged and highly disordered pro-inflammatory response upon treatment with deciduogenic stimuli (Lucas *et al.*, 2016, Macklon and Brosens, 2014, Salker *et al.*, 2011, Salker *et al.*, 2012).

In this study, we examined the clonogenicity, decidual response and secretome changes in cultured EnSCs purified from mid-luteal biopsies obtained in the cycle prior to ART. We demonstrate that successful implantation is associated with coordinated temporal changes in the secretome of differentiating EnSCs that transform a hostile maternal microenvironment into an optimal milieu for implantation. We further show an association between a disordered EnSC secretome response and sporadic failed implantation following ART.

Materials and Methods

Study design and participants

The endometrial samples used in this study were collected as part of an ongoing trial aimed at assessing the effect of endometrial biopsy prior to ART, including IVF, ICSI, and frozen embryo replacement (FER). This trial was conducted at the Nottingham University Research and Treatment Unit in Reproduction (Nurture), Nottingham, UK (trial registration number NCT01882842). Subsequent sample analysis was carried out at the Clinical Science Research Laboratories, University of Warwick, Coventry, UK, and the Department of Reproductive Medicine, KK Women's and Children's Hospital, Singapore. The NHS (National Health Service) National Research Ethics Service approved this study (13/EM/0277). None of the participating subjects were on hormonal treatment within 3 months prior to the treatment cycle. Major uterine anomalies and uterine instrumentation within 3 months prior to endometrial biopsy precluded inclusion in the study. Samples were selected based on the

outcome of ART: positive or negative urine hCG 14 days after embryo transfer. No other factors were taken into consideration. Written informed consent was obtained from all participants in accordance with the guidelines in The Declaration of Helsinki 2000 and all experiments were performed in accordance with relevant guidelines and regulations. Patient demographics and treatment details are summarized in Supplementary Table S1.

Endometrial biopsy and sample preparation

A home urinary ovulation kit was given to each participant with instructions to test morning urine from day 8 of their menstrual cycle immediately prior to the treatment cycle. Endometrial biopsy was performed 7 to 9 days after the LH surge. All participants were advised to use barrier methods of contraception or abstain from intercourse during the biopsy cycle.

Prior to biopsy, a urinary pregnancy test was carried out. An endometrial biopsy was then obtained using a Pipelle endometrial sampler (CCD, Paris). Briefly, the device was introduced into the uterus until resistance from the fundus was felt. The piston was withdrawn to generate negative pressure and the device rotated through 360^{o} as it was gradually withdrawn. Biopsies were transferred to the laboratory in DMEM/F12 medium supplemented with 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) (Thermo Fisher Scientific, Loughborough, UK) and 1% antibiotic-antimycotic solution (Invitrogen, Paisley, UK). The tissues were washed twice in DMEM/F12, finely minced, and enzymatically digested with collagenase type 1A (134 U/ml; Sigma-Aldrich, Gillingham, UK) and deoxyribonuclease type 1 (156 U/ml; Roche, Burgess Hill, UK) for 1 h at 37 ^{o}C. After centrifugation at 400 × g for 4 min, the pellet, consisting of dispersed endometrial cells, was re-suspended in FBS containing 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Gillingham, UK), frozen at an approximate rate of -1°C / min, and stored in liquid nitrogen.

ART protocols

In the cycle after the endometrial biopsy, participants underwent IVF/ICSI treatment using standard stimulation protocols. In brief, during a long agonist protocol, pituitary down-regulation with the GnRH agonist, buserelin (Sanofi, Guildford, UK) or nafarelin (Pfeizer, Puurs, Belgium), was commenced 7 days prior to the date of the next expected menstrual bleed. Following a withdrawal bleed, a transvaginal ultrasound scan was performed to confirm downregulation using the following criteria: quiescent ovaries with follicles < 10 mm diameter, endometrium ≤ 5mm in thickness and serum estradiol level < 200 pmol/L. Ovarian stimulation was achieved with HMG (Menopur; Ferring, West Drayton, UK) or recombinant FSH- follitropin α (Gonal-F, Merck Serono, Feltham UK) administered by s.c. injection at a daily dose tailored to the individual according to markers of ovarian reserve. From day 6 of stimulation, subjects were monitored for their ovarian response using ultrasound and serum estradiol levels. In case of a short protocol, women started GnRH agonist (Sanofi) on day 2 of the menstrual period and stimulation was on day 3 of her period. When a short antagonist protocol was used, stimulating medications (Menpur or Gonal-F) were commenced on day 2 of her period. GnRH antagonist, cetrorelix (Cetrotide, Merck Serono) was commenced when a lead follicle began to develop. Once women met the criteria for oocyte retrieval (≥3 leading follicles of ≥17 mm in diameter), 250 μg of choriogonadotrophin α, (Ovitrelle, Merck Serono), was administered subcutaneously to trigger final follicular maturation. Transvaginal ultrasound-guided oocyte retrieval was performed 36 h later. In all our patients, one or two embryos were transferred on day 5. An elective single embryo policy applied to women under the age of 37 years undergoing a first treatment cycle. Women not meeting these criteria were eligible to have double embryo transfer. Micronized progesterone (Cyclogest, 400 mg twice daily; Actavis, Barnstaple, UK)

administered vaginally was used as luteal support from the day of transfer until the time of the pregnancy test and continued to the 10th week of pregnancy in those with positive tests. In the natural FER cycle, ultrasound examinations were carried out every 2 to 3 days from day 6 of the cycle onwards. When a dominant follicle (≥ 18 mm) was identified, the LH surge was monitored using a home urinary LH detection kits. Embryo transfer was carried out 5 days after the LH surge. Luteal support was not administered.

A home urinary pregnancy test was performed 14 days after the embryo transfer. If positive, a transvaginal scan was performed at 6-8 weeks gestation to confirm viability. The main clinical outcome of the study was a positive biochemical pregnancy per woman, defined as a positive urinary pregnancy test 14 days after embryo transfer. Secondary outcomes included a clinical pregnancy defined as evidence of a viable fetus on a transvaginal scan performed at 6-8 weeks gestation, and live birth (delivery of a viable fetus after the 24th completed week of gestation).

Primary EnSC culture

Once the outcome of the IVF treatment was known, frozen endometrial cell suspensions from 20 patients were thawed and expanded in DMEM/F12 containing 10% DCC-FBS, 1% L-glutamine (Thermo Fisher Scientific) , 1% antibiotic-antimycotic solution, insulin (2 µg/mL; Sigma-Aldrich) and estradiol (1nM; Sigma-Aldrich). At passage 2, cells were plated in two 6-well plates. When confluent, the cultures were either maintained in phenol red-free DMEM/F12 containing 2% DCC-FBS and 1% antibiotic-antimycotic solution (Thermo Fisher Scientific) or decidualized in the same medium supplemented with 0.5 mM 8-bromoadenosine cAMP (8-bromo-cAMP; Sigma-Aldrich) and 1 µM medroxyprogesterone acetate (MPA; Sigma-Aldrich). The media was changed every 48 h. EnSCs remained either untreated for 8 days or decidualized in a reversed time-course, meaning that the medium on

all cultures was switched from high to low serum (10% to 2% DCC-FBS) on day 0, decidualization was then triggered at different time-points, and all cultures were harvested on day 8. This design negates potential artefacts induced by the switch of high to low serum culture conditions. Total RNA was extracted and culture supernatants snap-frozen and stored at -80 ^{o}C until further analysis.

***In vitro* colony-forming assay**

Colony-forming assays were performed at passage 1 as previously described (Murakami *et al.* , 2013, Murakami *et al.* , 2014). Briefly, EnSCs were seeded at a clonal density of 30 cells/cm^2 on 60 mm fibronectin-coated culture dishes and cultured in DMEM/F12 supplemented with basic fibroblast growth factor (10 ng / mL; Merck Millipore, Watford, UK). A partial medium change was performed on day 7, 10, and 12 of culture. Microscopic examination of colonies was performed periodically to confirm that the colonies arose from single cells. The culture was continued for 15 days and stained with haematoxylin. Colonies consisting of more than 50 cells were counted and cloning efficiency was calculated using the formula: CE (%) = (number of colonies / number of seeded cells) × 100.

Real-time Quantitative (RTq)-PCR

Total RNA was extracted from cultured EnSCs using RNA STAT-60 after decidualization using cAMP and MPA. Total RNA (1 µg) was DNase (Sigma-Aldrich) treated and reverse transcribed using a QuantiTect Reverse Transcription Kit (Sigma-Aldrich). The resulting cDNA was used for RT-qPCR analysis. Power SYBR Green PCR Master Mix was used for template quantification on a 7500 Real-time PCR System (Applied Biosystems, Paisley, UK). The mRNA expression levels were calculated using the efficiency corrected ΔCT method and expressed in arbitrary units. The expression levels were normalized against the levels of

RPL19, coding for ribosomal protein L19, to nullify the variances in input cDNA. The measurements were made in triplicate. Amplification specificity of the primers was confirmed by melting curve analysis and agarose gel electrophoresis. Gene-specific primers were designed using Primer 3 software: prolactin (*PRL*) sense 5'-AAG CTG TAG AGA TTG AGG AGC AAA C-3', *PRL* antisense 5'-TCA GGA TGA ACC TGG CTG ACT A-3'; insulin-like growth factor binding protein 1 (*IGFBP1*) sense 5'-CGA AGG CTC TCC ATG TCA CCA-3', *IGFBP1* antisense 5'-TGT CTC CTG TGC CTT GGC TAA AC-3'; 11β-hydroxysteroid dehydrogenase 1 (*HSD11B1*) sense 5′-AGC AAG TTT GCT TTG GAT GG-3′, *HSD11B1* antisense 5′-AGA GCT CCC CCT TTG ATG AT-3′; L19 sense 5'-GCG GAA GGG TAC AGC CAT-3', L19 antisense 5'-GCA GCC GGC GCA AA-3'.

Human embryo culture

The ethics committee of the Institutional Review Board of the University Hospital (UZ Brussel, Vrije Universiteit Brussel) and the Belgian Federal Ethical Committee for Scientific Research on Human Embryos approved this study and reviewed and approved the informed consent form. All experiments were performed in accordance with relevant guidelines and regulations. Vitrified day 5 blastocysts, which became available for research with written informed consent and after the legally determined storage period of 5 years, were warmed using the Vitrification Thaw Kit (Vit Kit-Thaw; Irvine Scientific, Santa Ana, CA, USA) and left to recover for 3 hours before morphological scoring by an experienced independent clinical embryologist (according to Gardner and Schoolcraft criteria) (Supplementary Table S2) (Gardner and Schoolcraft, 1999, Gardner DK, 1999). For assisted hatching, the zona pellucida was removed by pronase treatment (1 mg/ml; Sigma) for 10 min at 37°C. The resulting zona-free blastocysts were washed 3 times in human tubal fluid (HTF) HEPES (IVF Basics) medium containing albumin (CAF-DCF, Brussels, Belgium). Blastocysts were left to

recover for 3 hours after pronase treatment and scored again on morphology (data not shown). Good quality, late day 5 blastocyts were allocated randomly to pooled culture supernatant from undifferentiated EnSCs and cells decidualized for 2 or 8 days. The blastocysts were incubated undisturbed for 24 hours at 37°C in 5% carbon dioxide and 6 % oxygen. Day 6 blastocysts were morphologically evaluated and growth rates (0, 1 or 2) determined, reflecting the degree of development based on expansion and morphology scores of inner cell mass (ICM) and trophectoderm (TE). A growth rate of '0' denotes that the embryo was degraded or failed to develop further; '1' indicates moderately or significantly expanded embryo with a "C"-score for the TE and/or ICM; and significantly expanded embryos with ≥ "B"-score for the TE and/or ICM were scored '2' (Supplementary Table S2). The microdroplets were stored individually at -80°C for multiplex secretome analysis.

Secretome analysis

Conditioned media collected from decidualized and undifferentiated EnSC cultures as well as embryo droplets were randomized and assayed in duplicate for 45 cytokines, chemokines and growth factors (listed in Supplementary Table S3) using a multiplex suspension bead immunoassay (Ebioscience, Singapore), according to the manufacturer's protocol and as described previously (Murakami *et al.*, 2014), but with some modifications. Briefly, 50 µL of conditioned media was mixed with 50 µL of antibody-conjugated, magnetic beads in a 96 DropArray plate (Curiox Biosystems, Singapore) and rotated at 450 rpm on a plate shaker for 120 min at 25°C while protected from light. Beads were internally dyed with different concentrations of two spectrally distinct fluorophores and covalently conjugated to antibodies against the 45 cytokines, chemokines and growth factors (Fulton *et al.* , 1997). The plate was washed three times with wash buffer (PBS, 0.05% Tween-20) on the LT210 Washing Station (Curiox, Singapore) before adding 25 µL of secondary antibody and rotating at 450 rpm for

30 min at 25°C protected from light. Subsequently, the plate was washed three times with wash buffer, and 10 μL of streptavidin-phycoerythrin added and rotated at 450 rpm for 30 min at 25°C protected from light. The plate was again washed three times with wash buffer; 60 μL of reading buffer was then added and the samples read using the Bio-Plex Luminex 200 (BioRad). The beads are classified by the red classification laser (635 nm) into its distinct sets, while a green reporter laser (532 nm) excites the phycoerythrin, a fluorescent reporter tag bound to the detection antibody. Quantitation of the 45 analytes in each sample was then be determined by extrapolation to a 6-point standard curve, as the amount of fluorescence detected by the reporter laser is proportional to the amount of target present in the sample. Data analysis of experimental data was carried out using five-parameter logistic regression modeling (Gottschalk and Dunn, 2005). Calibrations and validations were performed prior to runs and on a monthly basis, respectively. Twenty-two factors with measurements missing from >50% of samples were excluded (Supplementary Table S3).

Statistical analysis

GraphPad Prism 6 (GraphPad Software Inc. California, USA) was used for statistical analyses. Data were checked for normal distribution using the Kolmogorov-Smirnov test. Unpaired or paired Student's *t*-test was performed, as appropriate, to determine statistical significance between groups for normally distributed data. Mann-Whitney U test was used for non-normally distributed data. For comparing three or more groups, the data were analysed using one-way ANOVA, followed by the Student's *t*-test with Bonferroni adjustment for pairwise comparisons. $P < 0.05$ was considered significant. Hierarchical clustering using Euclidean distance (MeV version 4.9.0) was performed on the cytokine

profiles after the normalization of significantly differential factors by first centring the data to the median and scaling it by division with the SD. Secretome data were further analysed by partial least squares regression (PLSR) modelling (Unscrambler X version 10.1) after the normalization of data by first centering the data to the median and scaling it by division with the SD. Full cross-validation was applied in PLSR to increase model performance and for the calculation of coefficient regression values. The Chi-square test was used to determine differences in embryo growth rates.

Results

Successful versus failed implantation: demographic and treatment variables

Twenty women were enrolled in this study; 18 of Caucasian origin, 1 Indian and 1 mixed race. None of the women were smokers. Following IVF (n = 9), ICSI (n = 10) or FET (n = 1) in a natural cycle, 10 subjects had a positive pregnancy test, indicating that embryo implantation had occurred. Nine women, including two twin pregnancies, had live births. One woman suffered a pregnancy loss at 6 weeks gestation. The failed implantation group consisted of 10 subjects. As shown in Supplementary Table S1, there were no significant differences ($P > 0.05$) in patient characteristics, reproductive history, ovarian reserve, treatment characteristics or embryo number and quality, as assessed by standard morphological criteria, between the pregnant and non-pregnant groups. The median time between the biopsy and embryo transfer in the pregnant group was 33 days (range: 30 -36 days) and 35 days (range: 32 - 40) days in the non-pregnant group ($P > 0.05$).

Colony-forming unit-fibroblast (CFU-F) activity

To assess the CFU-F activity of EnSCs, frozen endometrial cell suspensions were thawed, expanded in T25 flasks following differential plating, and subjected to colony-forming assays at passage 1 (Figure 1). The average endometrial CFU-F activity (mean ± SEM) was 8.1 ± 1.3% and 6.3 ± 1.4% in the pregnant and non-pregnant group, respectively ($P > 0.05$). Notably, endometrial CFU-F activity was ≤ 1% in 3 women who suffered failed implantation, indicating relative MSC deficiency (Figure 1).

Responsiveness of EnSCs to deciduogenic cues

For differentiation experiments, EnSCs were plated in 6-well plates, grown to confluency, and then treated or not with 8-br-cAMP and MPA for 2 or 8 days. The median time in culture prior to the decidualization experiments was 22.5 days (range: 14 - 27 days) and 21 days (range: 12 - 40 days) in the pregnant and non-pregnant group, respectively ($P > 0.05$). The expression of three highly sensitive decidual marker genes, *PRL*, *IGFBP1* and *HSD11B1*, was determined at each time-point by RT-qPCR. As shown in Figure 2, decidualization elicited multiple-log increases in the expression of all three marker genes; but the level of induction varied greatly between cultures in both the pregnant and non-pregnant group. Furthermore, there was no significant difference ($P > 0.05$) in the expression of these decidual marker genes between the two clinical groups at any of the time-points. In addition, the induction of decidual marker genes was not distinct in the three cultures exhibiting relative MSC deficiency (Figure 2).

An aberrant EnSC secretome is associated with subsequent failed implantation

While the expression of many decidual genes, including *PRL*, *IGFBP1* and *HSD11B1*, increases exponentially as the differentiation process unfolds, a host of cytokines and immunomodulators are secreted in a bi-phasic manner, characterized by a rapid rise in

response to a deciduogenic signal followed by a gradual decline (Salker *et al.*, 2012). We used a multiplex suspension bead immunoassay to measure the secretion of 45 cytokines and immunomodulators in undifferentiated and decidualizing cultures. Out of the 23 factors detectable in a majority of samples, 19 were differentially secreted upon decidualization of cultures for either 2 or 8 days in the pregnant group. The bifurcation in the dendrogram following hierarchical clustering of regulated cytokines demonstrate that the decidual secretome profile is more divergent on day 2 of differentiation when compared to day 8 or day 0 (Figure 3A). In the pregnant group, differentially secreted factors between undifferentiated cells (day 0) and cells decidualized for 8 days (day 8) included interleukin (IL)-13, IL-6, chemokine (C-X-C motif) ligand 1 (CXCL1), and CXCL8 (IL-8).

The secretome profiles of EnSC cultures from the non-pregnant group were qualitatively different and disordered, illustrated by the lack of bifurcation in the dendrogram (Figure 3B). Hierarchical clustering of regulated cytokines showed a disordered pattern with both undifferentiated (day 0) and day 8 secretome profiles aligning with day 2 samples. When compared to cultures from the pregnant group, six factors [leukemia inhibitory factor (LIF), IL-6, vascular endothelial growth factor (VEGF)-A, VEGF-D, brain-derived neurotrophic factor (BDNF), and CXCL12] were no longer differentially secreted in cultures associated with ART failure.

Next, we used PLSR analysis to evaluate the temporal multivariate differences in the secretome profiles associated with successful or failed implantation. As shown in Figure 4, the secretome profiles were less divergent in cultures from the pregnant compared to the non-pregnant group, at least in undifferentiated cells and cells decidualized for 8 days. The PLSR model indicated that the inherent secretome differences associated with ART failure were most pronounced in undifferentiated EnSCs. Decidual transformation of the cultures led to a partial convergence of secretome profiles in the clinical groups. Notably, the three most

406 divergent cultures on both day 0 and day 8 in the failed implantation group corresponded to
407 the cultures with the lowest CFU-F activities (Figure 1).
408 Regression coefficient values were used to identify factors that are most closely associated
409 with successful implantation. C-C motif chemokine 3 (CCL3), also known as macrophage
410 inflammatory protein 1α (MIP1α), exhibited the highest regression coefficient value in
411 undifferentiated cultures, indicating a positive association with implantation (Supplementary
412 Figure S1). Congruent with the PLSR plots (Figure 4), the strength of this association
413 diminished upon decidual transformation of EnSCs (Supplementary Figure S2). A
414 combination of weakly associated factors was required to generate an association between
415 implantation and secreted factors in decidualizing cells (Supplementary Figure S2). For
416 example, a combination of a positive factor (IL-6) and several negative factors [CCL11,
417 CCL5, IL-18, epidermal growth factor (EGF) and VEGF-D], all with a regression coefficient
418 less than a magnitude of 0.2, is associated with successful implantation in cultures
419 decidualized for 8 days.
420
421 **Impact of the EnSC secretome on human blastocyst development**
422 To examine if and how temporal changes in decidualizing EnSC secretome impact on
423 embryo development, vitrified day 5 human blastocysts were warmed and scored on
424 morphology (Supplementary Table S2). Late day 5 embryos were subjected to assisted
425 hatching and then cultured for 24 hours in 40 µl microdroplets of culture supernatant of
426 undifferentiated EnSCs and cells first decidualized for either 2 or 8 days. Separate pools of
427 culture supernatants were established from the pregnant and non-pregnant groups. The
428 morphology of the embryos was rescored following 24 hours of incubation and the
429 development of the embryos was evaluated and growth rates (0, 1 or 2) determined.
430 Increasing growth rates correspond to better development (Supplementary Table S2).

Analysis of the embryo growth rates demonstrated that the secretome of undifferentiated EnSCs compromised blastocyst development, exemplified by a growth score of 0 in 13 out of 23 (57%) embryos. By contrast, only 3 out of 34 (9%) embryos had a growth score of 0 when cultured in supernatant from decidualizing EnSCs (Chi-square test: $P < 0.005$; Figure 5A). There was no statistical difference in the growth rates of human blastocysts cultured in the supernatant with the secretome of EnSC decidualized for either 2 or 8 days. In addition, no significant differences were found in the growth rates between embryos cultured in medium conditioned by EnSC cultures from pregnant versus non-pregnant patients, whether decidualized or not (data not shown). Finally, the embryonic response to secreted decidual factors was examined more closely by comparing the cytokine content of human blastocysts cultured in supernatant of EnSCs decidualized for either 2 or 8 days. Multiplex suspension bead immunoassay allowed quantification of 20 analytes in microdroplets of 18 embryos cultured in medium conditioned by early decidualizing cells (day 2) and 19 embryos cultured in supernatant of late decidualizing cells (day 8). Following hierarchical clustering, the dendrogram showed that the cytokine / chemokine profiles of embryos cultured in supernatant of EnSCs decidualized for 2 days were unrelated to either growth rate or clinical group, suggesting an embryo-specific response (Figure 5B). By contrast, the secretome profiles of embryos cultured in day 8 supernatant clustered, with the exception of one sample, with the clinical groups, indicating the lack of a significant embryonic secretory response (Figure 5C).

Discussion

Aberrant decidual responses in purified primary EnSC cultures have been linked to a variety of reproductive disorders, including endometriosis (Aghajanova *et al.*, 2010a, Aghajanova *et al.*, 2010b, Klemmt *et al.*, 2006, Velarde *et al.*, 2009), polycystic ovary syndrome (Piltonen *et al.*, 2015), and recurrent miscarriage (Salker *et al.*, 2010, Salker *et al.*, 2011, Salker *et al.*, 2012). These pioneering studies demonstrated that the responsiveness of EnSCs to steroid signals and other cues is not predetermined but occurs in a disease-specific manner. In this study, we examined if EnSCs contribute to failed implantation. Study subjects were recruited solely because of the need for ART, and irrespective of the cause of infertility or previous treatment cycles.

We reasoned that a deficiency of endometrial MSCs could lead to aberrant EnSC function and implantation failure. While the overall CFU-F activity at passage 1 was comparable between the pregnant and non-pregnant group, three cultures associated with failed implantation were deficient in clonogenic MSCs. The secretome of these cultures was markedly divergent from other cultures. This observation supports our hypothesis that the abundance of endometrial stem cell populations is an important determinant of reproductive outcome, although further studies are needed. Overall, the induction of 'classical' decidual marker genes was not informative in terms of predicting reproductive success or failure, which was disappointing as the same markers have been widely used to determine the impact of reproductive disorders on the differentiation potential of EnSCs (Klemmt *et al.*, 2006, Piltonen *et al.*, 2015, Salker *et al.*, 2010). However, the level of induction was highly variable between cultures, which probably reflects the clinical heterogeneity of the study population.

For implantation to take place, differentiating EnSCs must transit through distinct functional phenotypes in response to elevated circulating progesterone levels and rising cellular cAMP levels (Gellersen and Brosens, 2014, Jones *et al.*, 2006). This decidual transitional pathway is

479 characterized first by an acute auto-inflammatory phase, which is followed by a profound
480 anti-inflammatory response. The initial auto-inflammatory response associated with decidual
481 transformation of EnSCs renders the endometrium receptive to embryo implantation (Salker
482 *et al.*, 2012), whereas acquisition of a mature secretory phenotype enables the endometrium
483 to respond to individual embryos in a manner that either supports further development or
484 facilitates early rejection(Brosens *et al.* , 2014b). Secretome analysis demonstrated that
485 EnSCs from the pregnant group closely phenocopied this transitional pathway in culture,
486 exemplified by the marked and distinct secretory response on day 2 of decidualization. By
487 contrast, this transitional pathway was clearly disordered in cultures from the failed
488 implantation group. In addition, these cultures no longer exhibited temporal changes in the
489 secretion of key factors involved in inflammation (IL-6), angiogenesis and
490 lymphangiogenesis (VEGF-A, VEGF-D), endometrial receptivity and embryo development
491 (LIF, BDNF) (Cha *et al.*, 2012, Kawamura *et al.*, 2009, Kim *et al.*, 2014, Krussel *et al* , 2003,
492 Stewart *et al.*, 1992).
493 Notably, the difference in secretome between the two groups of cultures was most
494 pronounced in undifferentiated cells. CCL3, a potent inflammatory chemokine involved in
495 innate immunity as well as wound repair (Chen *et al.*, 2013, DiPietro *et al.*, 1998), was most
496 strongly associated with successful pregnancy, which emphasizes further the importance of
497 transient endometrial inflammation for implantation. Upon decidual transformation, the
498 secretory profiles in both the pregnant and non-pregnant group tended to converge, albeit
499 with some notable exceptions. This is a potentially important observation for a number of
500 reasons. First, it suggests that endometrial defects associated with reproductive failure could
501 be more prominent in the proliferative phase of the cycle. Second, the convergence of
502 secretomes, if recapitulated *in vivo*, suggests that delaying transfer by a few days could
503 potentially be beneficial for women at risk of implantation failure. Finally, previous studies

reported that the decidual response in cultured EnSCs from recurrent miscarriage patients becomes increasingly divergent as the differentiation process unfolds (Salker *et al.*, 2010, Salker *et al.*, 2011, Salker *et al.*, 2012), which indicates that different pathways are involved in failed implantation and early pregnancy loss.

Analysis of human blastocysts indicates that the stromal microenvironment is a major determinant of embryo survival. Our findings demonstrate that the secretome changes associated with undifferentiated EnSCs transitioning to fully decidualized cells induce a graded embryo response, ranging from embryonic growth arrest to a transient embryo-specific secretory response and finally quiescence. Although the EnSC secretome was divergent between the pregnant and non-pregnant groups, no discernible difference in embryo response was observed. This may reflect that EnSC conditioned medium was pooled for the embryo experiments, that only a limited number of blastocysts were available for these experiments, or that the duration of incubation was too short. Another potential pitfall is that these experiments were performed 'out-of-phase' as there is no evidence that day 6 human embryos are already embedded in the decidualizing stroma. However, there is histological evidence of a Carnegie stage 5a human embryo (post-fertilization age of 7 to 8 days) surrounded by EnSCs. Thus, while our embryo experiments may indeed be 'out-of-phase', it is probably only by 1 or perhaps 2 days (Gardner and Schoolcraft, 1999, Gardner DK, 1999).

In summary, cyclic menstruation and renewal renders the endometrium intrinsically dynamic and arguably capable of adapting to ensure reproductive success (Gellersen and Brosens, 2014, Lucas *et al.*, 2013). It is evident that a community of resident cells, ranging from immature progenitor cells to mature and senescent cells, makes up the stromal compartment of the endometrium. It is conceivable that the constituents of this community fluctuate from cycle to cycle, with transient imbalances contributing to sporadic IVF failure. The impact of damaged or deficient progenitor populations is likely to be more severe and prolonged and

529 could account for RIF. Our finding that endometrial defects associated with implantation
530 failure are present prior to ART has potentially important clinical ramifications as it raises the
531 prospect that screening of the endometrium prior to ART could minimise the risk of
532 subsequent treatment failure.
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534

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Authors' roles

R.R.P.D., A.A., Y.H.L., Y.M., performed the experimental work. Y.H.L., J.K.Y.C., R.R.P.D., A.A., H.V.D.V, E.S.L. and J.J.B assisted in the analysis. L.P., M.N.B., and N.R-F. collected and processed the endometrial biopsies. A.A. and H.V.D.V. performed the human embryo experiments. Y.H.L performed the secretome analysis. J.J.B. designed the study and wrote the manuscript. Y.H.L., H.V.D.V, B.C., S.Q., J.K.Y.C. and N.R.-F. contributed to design of the study and all authors edited and approved the manuscript.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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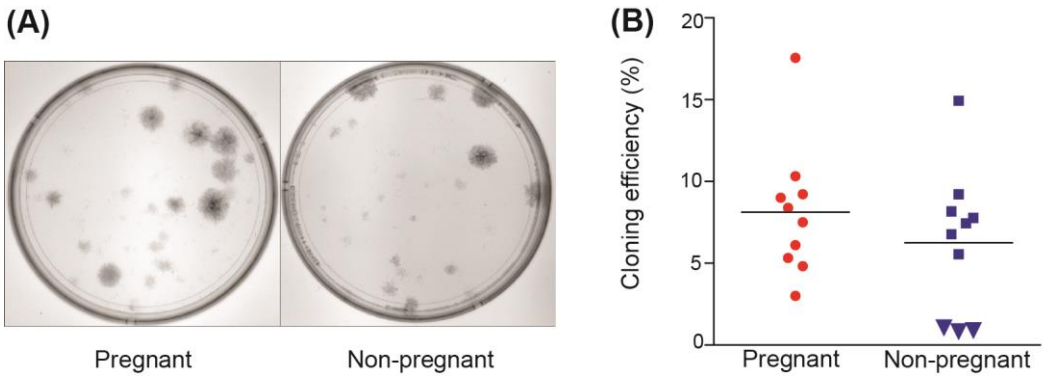
694 **Figure Legends**



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696 **Figure 1. Abundance of clonogenic cells in primary endometrial stromal cell cultures**
697 **prior to successful (pregnant) or failed embryo implantation (non-pregnant) after ART**
698 **in women.**

699 *(A)* representative images of clones obtained after subjecting primary endometrial stromal
700 cells (EnSCs) to colony-forming assays. (*B*) colonies with > 50 cells were counted and cloning
701 efficiency (CE) calculated. Triangles in the failed implantation group indicate primary EnSC
702 cultures deficient in clonogenic mesenchymal stem cells (MSCs), defined by CE of ≤ 1%.

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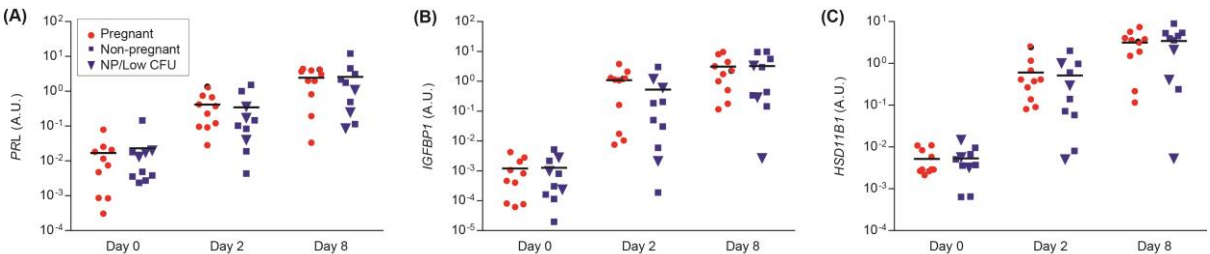


Figure 2. Induction of decidual marker genes.

EnSCs from the pregnant and non-pregnant group (n = 10 in each group) were subjected to decidualization for the indicated time-points. Total RNA was then extracted and subjected to RT-qPCR analysis. *PRL* (prolactin), *IGFBP1* (insulin-like growth factor binding protein 1) and *HSD11B1* (11β-hydroxysteroid dehydrogenase 1) transcript levels were normalized against the levels of *L19* and expressed in arbitrary units (A.U.). The induction of decidual marker genes was comparable between the two groups at each time-point ($P > 0.05$; Mann–Whitney U test). Note the logarithmic Y-axis. NP/Low CFU (non-pregnant / low colony-forming unit) denotes cultures from the non-pregnant group with MSC deficiency.

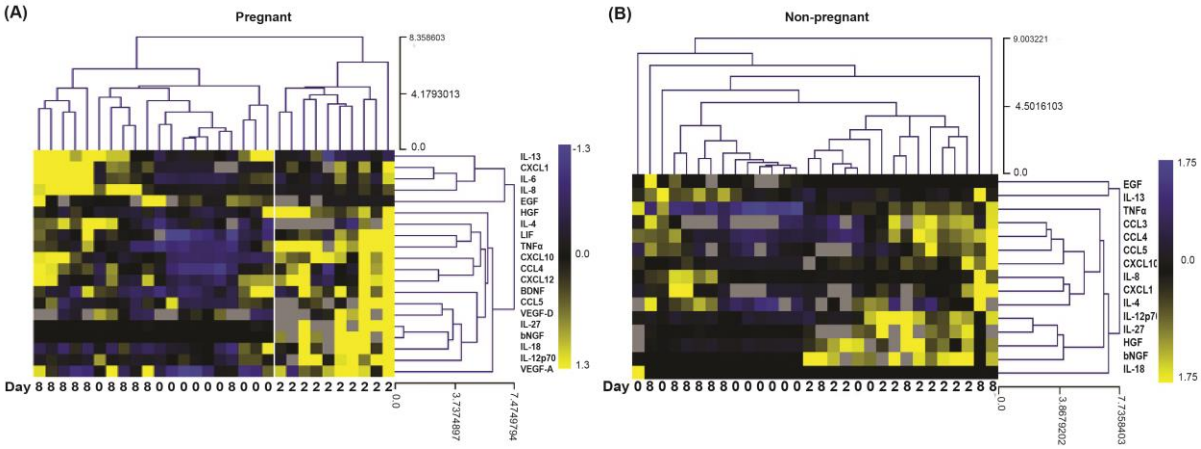


Figure 3. Divergent human EnSC secretome prior to successful or failed implantation.

(A) Hierarchical clustering using Euclidean distance of factors that were differentially secreted upon decidualization of EnSCs associated with successful implantation. (B) The same analysis was performed on decidualizing cultures from the non-pregnant group. The bifurcation in the dendrogram above the heatmap in the pregnant group indicates that the secretory response in decidualizing EnSCs is most distinct after 2 days of differentiation. By contrast, the temporal secretome changes in cultures associated with implantation failure (non-pregnant) were both qualitatively different and disordered. The colour keys are depicted on the right of the heatmaps (blue: decreased expression; yellow: increased expression)

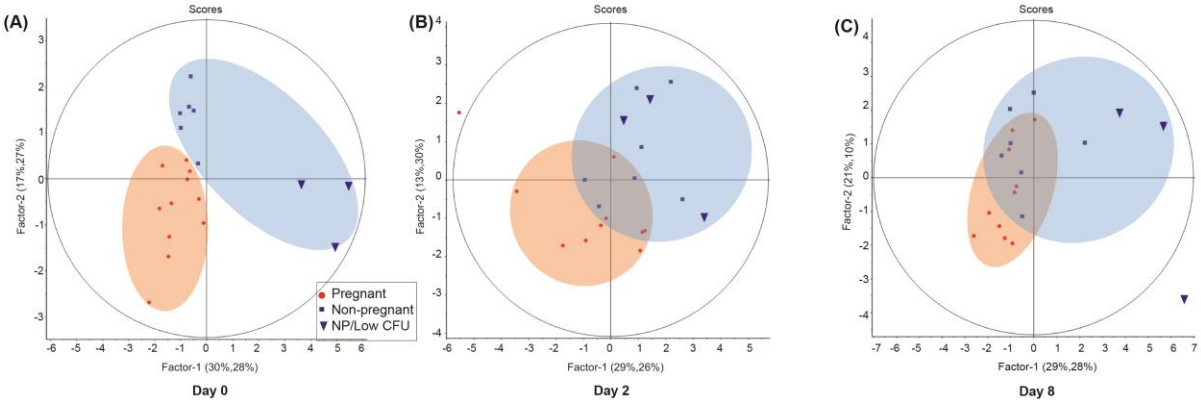


Figure 4. Two-dimensional partial least squares loading plots of undifferentiated and decidualized secretomes in primary EnSC cultures from pregnant and non-pregnant patients.

Partial least squares **(**PLS) regression analysis was used to evaluate the temporal multivariate differences in cytokine and immune-modulatory secretome profile as a result of implantation. The analysis showed that the secretome profiles are less divergent in implantation-positive cultures, at least in undifferentiated cells and cells decidualized for 8 days. Furthermore, the differences in secretomes were most pronounced in undifferentiated cells. Decidualization leads to convergence of secretomes, although not in MSC-deficient cultures (blue triangles). The X-axis represents principal component 1, and the Y-axis principal component 2. NP/Low CFU refers to cultures from the non-pregnant group with MSC deficiency.

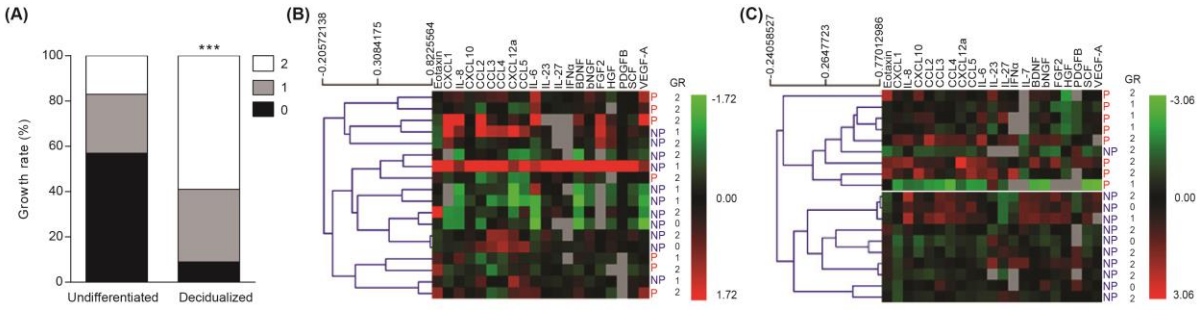


Figure 5. Impact of EnSC secretome on human blastocysts.

A, growth rates (scored 0, 1, and 2) of human blastocysts cultured in conditioned medium from undifferentiated (n = 24) and decidualizing EnSCs (day 2 and day 8) (n = 33). *** indicates $P < 0.005$ (Chi-square test). B, Hierarchical clustering using Euclidean distance of 20 cytokines and chemokines measured in microdroplets of human blastocysts cultured in conditioned medium of EnSC decidualized for 2 days. P: pregnant, NP: non-pregnant, GR: growth rate. C, Hierarchical clustering using Euclidean distance of 20 cytokines measured in microdroplets of human blastocysts cultured in conditioned medium of EnSC decidualized for 8 days. The colour keys are depicted on the right of the heatmaps (green: decreased expression; red: increased expression)

Supplementary Figure S1. Factors secreted by undifferentiated EnSCs associated with successful implantation

Regression coefficient analysis was used to identify which secreted factors were most closely associated with successful implantation in undifferentiated EnSCs. Out of 23 detectable factors, CCL3 exhibited the highest regression coefficient value in undifferentiated cultures, indicating a strong positive association with implantation.

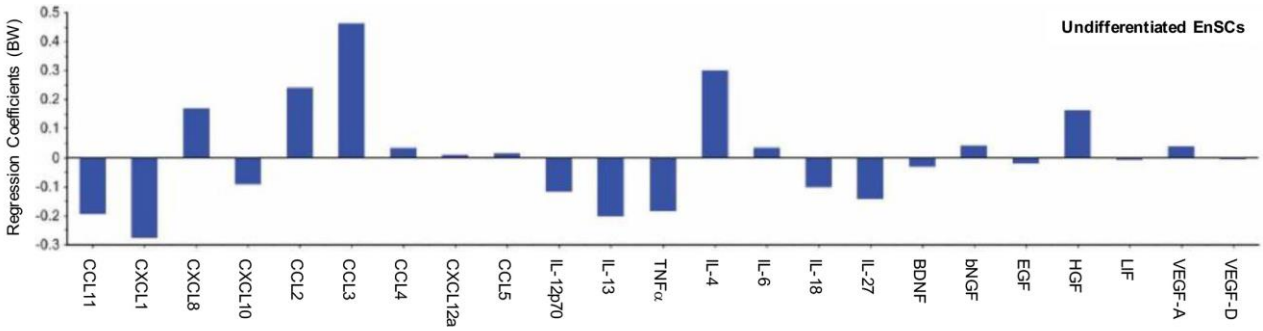
Supplementary Figure S2. **Factors secreted by decidualizing EnSCs associated with successful implantation**

Regression coefficient analysis of decidualizing cultures (day 2 or day 8) of the pregnant group demonstrated that the strength of the association of CCL3 and successful implantation diminishes upon differentiation of EnSCs. In decidualizing cells, a positive factor (IL-6) and several negative factors (CCL11, CCL5, IL-18, EGF and VEGF-D), all with a regression coefficient of < 0.2, were associated with successful implantation.

Deregulation of the endometrial stromal cell secretome precedes embryo implantation failure

Ruban Rex Peter Durairaj, Asma Aberkane, Lukasz Polanski, Yojiro Maruyama, Miriam Baumgarten, Emma S Lucas, Siobhan Quenby, Jerry K Y Chan, Nick Raine-Fenning, Jan J Brosens, Hilde Van de Velde, and Yie Hou Lee

Supplementary Figures and Data



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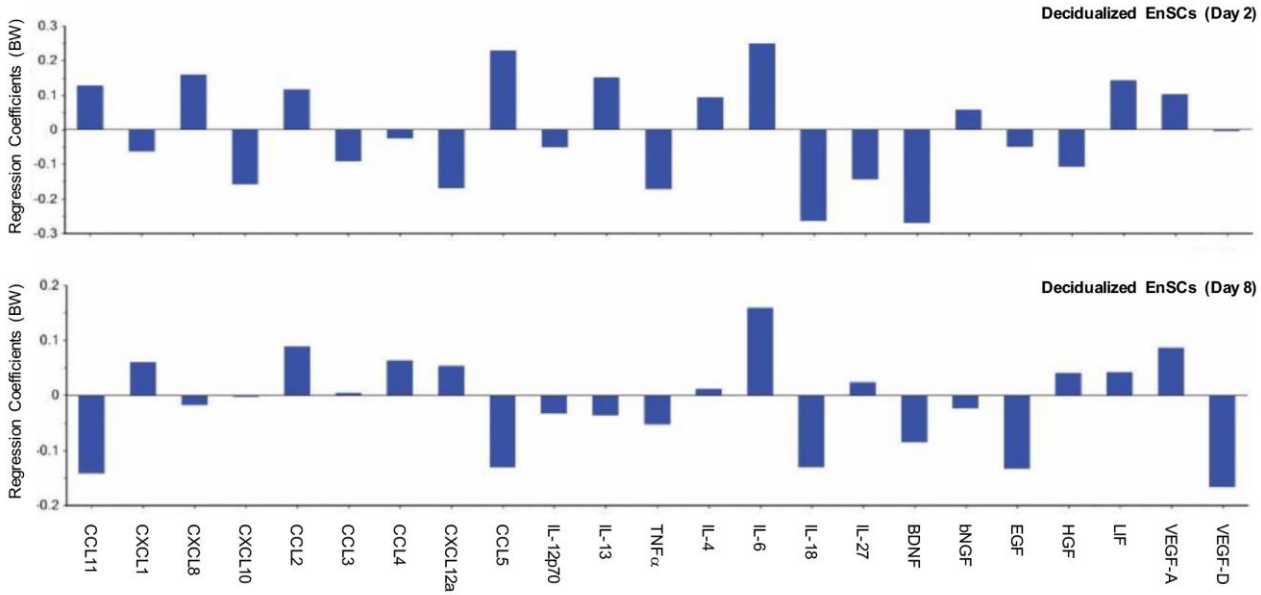

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Supplementary Tables

Table S1: Demographic and treatment details of the study population

Demographics & treatment characteristics		Pregnant (n = 10)*	Failed implantation (n = 10)*
Age, years (± SD)		34.0 (±2.9)	35.5 (±3.3)
Duration of infertility, months (± SD)		35.4 (±15.4)	39.0 (±12.9)
Cause of infertility	Male factor	3	5
	Anovulation	1	2
	Unexplained	3	2
	Mixed	3	1
Previous live births (range)		0.3 (0-2)	0.1 (0-1)
ART cycle number (range)		1.5 (1-3)	1.4 (1-4)
Body mass index (kg/m2)		24.9 (±3.0)	24.2 (±2.0)
Anti-Müllerian hormone (pmol/L)		15.2 (±13.3)	24.9 (±22.9)
Antral follicle count		22.3 (±8.9)	27.9 (±19.6)
IVF		3 (30%)	6 (60%)
Intra-cytoplasmic sperm injection (ICSI)		6 (60%)	4 (40%)
Frozen embryo replacement		1 (10%)	0
Dose of gonadotropin (IU)		2700.0 (±1002.7)	2448.8 (±872.5)
Duration of stimulation (days)		11.7 (±1.7)	11.5 (±1.3)
Number of oocytes collected		14.6 (±6.4)	14.9 (±8.9)
Single embryo transfer (n)		6	8
Total number of embryos transferred		14	12
Number of good quality embryos transferred (%)		11 (78.6%)	9 (75%)

*There were no significant difference ($P > 0.05$) in patient or treatment characteristics.

Table S2: Morphology scores and growth rates of human embryos cultured in EnSC conditioned medium.

EnSC	Pregnant	Morphology score post warming	Growth rate after 24h of culture
Undifferentiated	Yes	BL2	0
Undifferentiated	Yes	BL3BA	0
Undifferentiated	Yes	BL3CB	0
Undifferentiated	Yes	BL3CA	1
Undifferentiated	Yes	BL2	1
Undifferentiated	Yes	BL4AA	2
Undifferentiated	Yes	BL3BA	2
Undifferentiated	Yes	BL3BB	0
Undifferentiated	Yes	BL3BC	0
Undifferentiated	Yes	BL4CB	0
Undifferentiated	Yes	Collaps	0
Undifferentiated	No	BL3BA	1
Undifferentiated	No	BL2	0
Undifferentiated	No	BL3AB	2
Undifferentiated	No	BL8	2
Undifferentiated	No	BL3BB	0
Undifferentiated	No	BL2	0
Undifferentiated	No	BL4BA	1
Undifferentiated	No	BL3BB	1
Undifferentiated	No	BL3BB	0
Undifferentiated	No	BL2	0
Undifferentiated	No	BL4CB	0
Undifferentiated	No	BL3CB	1
Decidualized (D2)	Yes	BL3BB	2
Decidualized (D2)	Yes	BL4CB	1
Decidualized (D2)	Yes	BL3CB	2

Decidualized (D2)	Yes	BL4BB	2
Decidualized (D2)	Yes	BL3CB	2
Decidualized (D2)	Yes	BLBCB	2
Decidualized (D2)	No	BL4CB	1
Decidualized (D2)	No	BL4BB	1
Decidualized (D2)	No	BL3AA	1
Decidualized (D2)	No	BL3BB	1
Decidualized (D2)	No	BL4BB	2
Decidualized (D2)	No	BL3BC	0
Decidualized (D2)	No	BL2	2
Decidualized (D2)	No	BL3AA	2
Decidualized (D2)	No	BL3AA	2
Decidualized (D2)	No	BL3BB	1
Decidualized (D8)	Yes	BL3BA	2
Decidualized (D8)	Yes	BL3CB	2
Decidualized (D8)	Yes	BL3BB	1
Decidualized (D8)	Yes	BL3BA	1
Decidualized (D8)	Yes	BL3CC	2
Decidualized (D8)	Yes	BL8	1
Decidualized (D8)	Yes	BL2	1
Decidualized (D8)	No	BL3BB	0
Decidualized (D8)	No	BL3BA	2
Decidualized (D8)	No	BL3AA	2
Decidualized (D8)	No	BL3CB	2
Decidualized (D8)	No	BL3CC	1
Decidualized (D8)	No	BL3BB	2
Decidualized (D8)	No	BL3BB	2
Decidualized (D8)	No	BL4BB	2
Decidualized (D8)	No	BL3AC	0
Decidualized (D8)	No	BL4BB	2
Decidualized (D8)	No	BL4CB	2

Embryos were scored on morphology post warming using the Gardner and Schoolcraft criteria[41,42]. After culture of 24 hours in EnSC supernatant, embryos were morphologically evaluated and scored with growth rates 0, 1 or 2 to indicate development based on expansion and morphology

scores of ICM and TE: 0: embryo was degraded or did not develop further; 1: embryo moderately expanded or significantly expanded further accompanied with a "C"-score for the TE and/or ICM; 2: embryos significantly expanded with ≥ "B"-score for the TE and/or ICM

Supplementary Table S3: Secreted cytokines, chemokines and growth factors analysed by multiplex suspension bead immunoassay

BDNF: Brain-derived neurotrophic factor EGF: Epidermal growth factor CCL11 (Eotaxin): C-C motif chemokine 11 FGF-2 (FGF basic): Basic fibroblast growth factor GM-CSF: Granulocyte macrophage colony-stimulating factor CXCL1 (GROα): Chemokine (C-X-C motif) ligand 1; Growth regulated oncogene-alpha HGF: Hepatocyte growth factor IFNγ: Interferon gamma IFNα: Interferon alpha IL-1RA: Interleukin-1 receptor alpha IL-1β: Interleukin-1 beta IL-1α: Interleukin-1 alpha IL-2: Interleukin-2 IL-4: Interleukin-4 IL-5: Interleukin-5 IL-6: Interleukin-6 IL-7: Interleukin-7 CXCL8 (IL-8): Chemokine (C-X-C motif) ligand 8; Interleukin-8 IL-9: Interleukin-9 IL-10: Interleukin-10 IL-12 p70: Interleukin-12 heterodimer IL-13: Interleukin-13 IL-15: Interleukin-15 IL-17A: Interleukin-17A IL-18: Interleukin-18 IL-21: Interleukin-21 IL-22: Interleukin-22	IL-23: Interleukin-23 IL-27: Interleukin-27 IL-31: Interleukin-31 CXCL10 (IP-10): C-X-C motif chemokine 10; Interferon gamma-induced protein 10 LIF: Leukemia inhibitory factor CCL2 (MCP-1): Chemokine (C-C motif) ligand 2; Monocyte chemotactic protein 1 CCL3 (MIP-1α): Chemokine (C-C motif) ligand 3; Macrophage inflammatory protein 1-alpha CCL4 (MIP-1β): Chemokine (C-C motif) ligand 4; Macrophage inflammatory protein 1-beta βNGF: Beta nerve growth factor PDGF-BB: Platelet-Derived Growth Factor-BB PLGF: Placental growth factor CCL5 (RANTES): Chemokine (C-C motif) ligand 5 SCF: Stem cell factor CXCL12 (SDF1α): C-X-C motif chemokine 12; stromal cell-derived factor alpha TNFα: Tumor necrosis factor alpha LTA (TNFβ): Lymphotoxin-alpha; tumor necrosis factor-beta VEGF-A: Vascular endothelial growth factor A VEGF-D: Vascular endothelial growth factor D

Factors that are underlined were undetectable in > 50% of samples and therefore excluded from subsequent analyses.