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1 **Deregulation of the endometrial stromal cell secretome precedes embryo**
2 **implantation failure**

3

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27

28 **Running title:** Endometrial secretome and implantation failure

29

30

31 **Abstract**

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

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

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

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

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

55 **Main results and the role of chance:** Clonogenicity and induction of decidual marker genes
56 were comparable between EnSC cultures from pregnant and non-pregnant group groups ($P >$
57 0.05). Analysis of 23 secreted factors revealed that successful implantation was associated
58 with co-ordinated secretome changes in decidualizing EnSCs, which were most pronounced
59 on day 2 of differentiation: 17 differentially secreted proteins on day 2 of decidualization
60 relative to undifferentiated (day 0) EnSCs ($P < 0.05$); 11 differentially secreted proteins on
61 day 8 relative to day 2 ($P < 0.05$); and 8 differentially secreted proteins on day 8 relative to
62 day 0 ($P < 0.05$). By contrast, failed implantation was associated with a disordered secretome
63 response. Blastocyst development was compromised when cultured for 24 hours in medium
64 conditioned by undifferentiated EnSCs when compared to decidualizing EnSCs. Analysis of
65 the embryo microdroplets revealed that human blastocysts mount a secretory cytokine
66 response to soluble decidual factors produced during the early (day 2) but not late phase (day
67 8) of differentiation. The embryo responses to secreted factors from decidualizing EnSCs
68 were comparable between the pregnant and non-pregnant group ($P > 0.05$).

69 **Large scale data:** not applicable.

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

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

75 **Study funding and competing interest(s):** This work was supported by funds from the
76 Biomedical Research Unit in Reproductive Health, a joint initiative of the University
77 Hospitals Coventry & Warwickshire NHS Trust and Warwick Medical School, the University
78 of Nottingham and Nurture Fertility, and the National Medical Research Council, Singapore
79 (NMRC/BNIG14NOV023). The authors have declared that no conflict of interest exists.

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

81

82 **Introduction**

83 Implantation is the rate-limiting step in ART. Clinically, failed implantation is defined by the
84 lack of detectable hCG levels in either serum or urine 14 days after intrauterine transfer of
85 one or more embryos. By default, failed implantation is a retrospective diagnosis, rendering it
86 difficult, if not impossible, to discern if caused by a hostile endometrium, impaired embryo
87 quality, or an iatrogenic event. Nevertheless, genome-wide expression studies have identified
88 endometrial signature genes that are predictive of recurrent implantation failure (RIF),
89 defined as the absence of pregnancy following serial transfers of high quality embryos (Koler
90 *et al.*, 2009, Koot *et al.*, 2016). Interestingly, these studies have also challenged the prevailing
91 concept that implantation failure merely reflects a lack of expression of receptivity genes
92 during the window of implantation (Diaz-Gimeno *et al.*, 2011). Instead, the gene signatures
93 associated with RIF point towards more fundamental defects pertaining to cell cycle
94 regulation, cell motility, epithelial-mesenchymal transition (EMT), and signalling pathways
95 involved in stem cell maintenance (e.g. WNT and Notch) (Koler *et al.*, 2009, Koot *et al.*,
96 2016).

97

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

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

114

115 Decidualization of the human endometrium is not dependent on embryo implantation but is
116 initiated during the mid-luteal phase of each cycle in response to the postovulatory rise in
117 progesterone and increasing endometrial cAMP levels. Consequently, decidualization is a
118 reiterative process, linked to cyclic activation of mesenchymal stem-like cells (MSCs) and
119 subsequent differentiation into mature stromal cells in regenerating endometrium (Gellersen
120 and Brosens, 2014, Lucas *et al.*, 2016). The endometrium harbors abundant MSCs that are
121 multipotent, immuno-privileged, and highly regenerative (Cervello *et al.*, 2011, Gargett *et al.*,
122 2012, Gargett *et al.*, 2016). Functional repair of the endometrium requires activation of
123 poised progenitor cells, induction of transit-amplifying cells and differentiation of mature
124 progeny, which collectively determine the tissue response to decidualogenic and embryonic
125 cues. The mechanisms that control this regenerative pathway and ensure homeostatic
126 balancing of the different stromal cell populations are poorly understood. Arguably,
127 disruption of this process will give rise to dysfunctional EnSCs. Studies on primary EnSC
128 cultures have provided ample evidence that programming defects are linked to reproductive
129 failure. For example, endometriosis is associated with progesterone resistance, defined by the
130 refractoriness of cultured EnSCs to decidualogenic cues (Aghajanova *et al.*, 2010a, Aghajanova
131 *et al.*, 2010b, Al-Sabbagh *et al.*, 2012, Klemmt *et al.*, 2006, Sherwin *et al.*, 2010, Velarde *et*

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

136

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

143

144 **Materials and Methods**

145 **Study design and participants**

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

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

162

163 **Endometrial biopsy and sample preparation**

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

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

182

183 **ART protocols**

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

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

213 A home urinary pregnancy test was performed 14 days after the embryo transfer. If positive,
214 a transvaginal scan was performed at 6-8 weeks gestation to confirm viability. The main
215 clinical outcome of the study was a positive biochemical pregnancy per woman, defined as a
216 positive urinary pregnancy test 14 days after embryo transfer. Secondary outcomes included a
217 clinical pregnancy defined as evidence of a viable fetus on a transvaginal scan performed at
218 6-8 weeks gestation, and live birth (delivery of a viable fetus after the 24th completed week of
219 gestation).

220

221 **Primary EnSC culture**

222 Once the outcome of the IVF treatment was known, frozen endometrial cell suspensions from
223 20 patients were thawed and expanded in DMEM/F12 containing 10% DCC-FBS, 1% L-
224 glutamine (Thermo Fisher Scientific) , 1% antibiotic-antimycotic solution, insulin (2 μ g/mL;
225 Sigma-Aldrich) and estradiol (1nM; Sigma-Aldrich). At passage 2, cells were plated in two
226 6-well plates. When confluent, the cultures were either maintained in phenol red-free
227 DMEM/F12 containing 2% DCC-FBS and 1% antibiotic-antimycotic solution (Thermo
228 Fisher Scientific) or decidualized in the same medium supplemented with 0.5 mM 8-
229 bromoadenosine cAMP (8-bromo-cAMP; Sigma-Aldrich) and 1 μ M medroxyprogesterone
230 acetate (MPA; Sigma-Aldrich). The media was changed every 48 h. EnSCs remained either
231 untreated for 8 days or decidualized in a reversed time-course, meaning that the medium on

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

237

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

239 Colony-forming assays were performed at passage 1 as previously described (Murakami *et*
240 *al.* , 2013, Murakami *et al.* , 2014). Briefly, EnSCs were seeded at a clonal density of 30
241 cells/cm² on 60 mm fibronectin-coated culture dishes and cultured in DMEM/F12
242 supplemented with basic fibroblast growth factor (10 ng / mL; Merck Millipore, Watford,
243 UK). A partial medium change was performed on day 7, 10, and 12 of culture. Microscopic
244 examination of colonies was performed periodically to confirm that the colonies arose from
245 single cells. The culture was continued for 15 days and stained with haematoxylin. Colonies
246 consisting of more than 50 cells were counted and cloning efficiency was calculated using the
247 formula: CE (%) = (number of colonies / number of seeded cells) × 100.

248

249 **Real-time Quantitative (RTq)-PCR**

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

257 *RPL19*, coding for ribosomal protein L19, to nullify the variances in input cDNA. The
258 measurements were made in triplicate. Amplification specificity of the primers was
259 confirmed by melting curve analysis and agarose gel electrophoresis. Gene-specific primers
260 were designed using Primer 3 software: prolactin (*PRL*) sense 5'-AAG CTG TAG AGA TTG
261 AGG AGC AAA C-3', *PRL* antisense 5'-TCA GGA TGA ACC TGG CTG ACT A-3';
262 insulin-like growth factor binding protein 1 (*IGFBP1*) sense 5'-CGA AGG CTC TCC ATG
263 TCA CCA-3', *IGFBP1* antisense 5'-TGT CTC CTG TGC CTT GGC TAA AC-3'; 11 β -
264 hydroxysteroid dehydrogenase 1 (*HSD11B1*) sense 5'-AGC AAG TTT GCT TTG GAT GG-
265 3', *HSD11B1* antisense 5'-AGA GCT CCC CCT TTG ATG AT-3'; L19 sense 5'-GCG GAA
266 GGG TAC AGC CAT-3', L19 antisense 5'-GCA GCC GGC GCA AA-3'.

267

268 **Human embryo culture**

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

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

293

294 **Secretome analysis**

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

307 30 min at 25°C protected from light. Subsequently, the plate was washed three times with
308 wash buffer, and 10 µL of streptavidin-phycoerythrin added and rotated at 450 rpm for 30
309 min at 25°C protected from light. The plate was again washed three times with wash buffer;
310 60 µL of reading buffer was then added and the samples read using the Bio-Plex Luminex
311 200 (BioRad). The beads are classified by the red classification laser (635 nm) into its distinct
312 sets, while a green reporter laser (532 nm) excites the phycoerythrin, a fluorescent reporter
313 tag bound to the detection antibody. Quantitation of the 45 analytes in each sample was then
314 be determined by extrapolation to a 6-point standard curve, as the amount of fluorescence
315 detected by the reporter laser is proportional to the amount of target present in the sample.
316 Data analysis of experimental data was carried out using five-parameter logistic regression
317 modeling (Gottschalk and Dunn, 2005). Calibrations and validations were performed prior to
318 runs and on a monthly basis, respectively. Twenty-two factors with measurements missing
319 from >50% of samples were excluded (Supplementary Table S3).

320

321

322

323 **Statistical analysis**

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

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

339

340 **Results**

341

342 **Successful versus failed implantation: demographic and treatment variables**

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

354

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

356 To assess the CFU-F activity of EnSCs, frozen endometrial cell suspensions were thawed,
357 expanded in T25 flasks following differential plating, and subjected to colony-forming assays
358 at passage 1 (Figure 1). The average endometrial CFU-F activity (mean \pm SEM) was $8.1 \pm$
359 1.3% and $6.3 \pm 1.4\%$ in the pregnant and non-pregnant group, respectively ($P > 0.05$).
360 Notably, endometrial CFU-F activity was $\leq 1\%$ in 3 women who suffered failed implantation,
361 indicating relative MSC deficiency (Figure 1).

362

363 **Responsiveness of EnSCs to decidual cues**

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

376

377 **An aberrant EnSC secretome is associated with subsequent failed implantation**

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

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

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

399 Next, we used PLSR analysis to evaluate the temporal multivariate differences in the
400 secretome profiles associated with successful or failed implantation. As shown in Figure 4,
401 the secretome profiles were less divergent in cultures from the pregnant compared to the non-
402 pregnant group, at least in undifferentiated cells and cells decidualized for 8 days. The PLSR
403 model indicated that the inherent secretome differences associated with ART failure were
404 most pronounced in undifferentiated EnSCs. Decidual transformation of the cultures led to a
405 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 1 α (MIP1 α), 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 μ l 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).

431 Analysis of the embryo growth rates demonstrated that the secretome of undifferentiated
432 EnSCs compromised blastocyst development, exemplified by a growth score of 0 in 13 out of
433 23 (57%) embryos. By contrast, only 3 out of 34 (9%) embryos had a growth score of 0 when
434 cultured in supernatant from decidualizing EnSCs (Chi-square test: $P < 0.005$; Figure 5A).
435 There was no statistical difference in the growth rates of human blastocysts cultured in the
436 supernatant with the secretome of EnSC decidualized for either 2 or 8 days. In addition, no
437 significant differences were found in the growth rates between embryos cultured in medium
438 conditioned by EnSC cultures from pregnant versus non-pregnant patients, whether
439 decidualized or not (data not shown). Finally, the embryonic response to secreted decidual
440 factors was examined more closely by comparing the cytokine content of human blastocysts
441 cultured in supernatant of EnSCs decidualized for either 2 or 8 days. Multiplex suspension
442 bead immunoassay allowed quantification of 20 analytes in microdroplets of 18 embryos
443 cultured in medium conditioned by early decidualizing cells (day 2) and 19 embryos cultured
444 in supernatant of late decidualizing cells (day 8). Following hierarchical clustering, the
445 dendrogram showed that the cytokine / chemokine profiles of embryos cultured in
446 supernatant of EnSCs decidualized for 2 days were unrelated to either growth rate or clinical
447 group, suggesting an embryo-specific response (Figure 5B). By contrast, the secretome
448 profiles of embryos cultured in day 8 supernatant clustered, with the exception of one sample,
449 with the clinical groups, indicating the lack of a significant embryonic secretory response
450 (Figure 5C).

451

452

453

454 **Discussion**

455 Aberrant decidual responses in purified primary EnSC cultures have been linked to a variety
456 of reproductive disorders, including endometriosis (Aghajanova *et al.*, 2010a, Aghajanova *et*
457 *al.*, 2010b, Klemmt *et al.*, 2006, Velarde *et al.*, 2009), polycystic ovary syndrome (Piltonen *et*
458 *al.*, 2015), and recurrent miscarriage (Salker *et al.*, 2010, Salker *et al.*, 2011, Salker *et al.*,
459 2012). These pioneering studies demonstrated that the responsiveness of EnSCs to steroid
460 signals and other cues is not predetermined but occurs in a disease-specific manner. In this
461 study, we examined if EnSCs contribute to failed implantation. Study subjects were recruited
462 solely because of the need for ART, and irrespective of the cause of infertility or previous
463 treatment cycles.

464 We reasoned that a deficiency of endometrial MSCs could lead to aberrant EnSC function
465 and implantation failure. While the overall CFU-F activity at passage 1 was comparable
466 between the pregnant and non-pregnant group, three cultures associated with failed
467 implantation were deficient in clonogenic MSCs. The secretome of these cultures was
468 markedly divergent from other cultures. This observation supports our hypothesis that the
469 abundance of endometrial stem cell populations is an important determinant of reproductive
470 outcome, although further studies are needed. Overall, the induction of ‘classical’ decidual
471 marker genes was not informative in terms of predicting reproductive success or failure,
472 which was disappointing as the same markers have been widely used to determine the impact
473 of reproductive disorders on the differentiation potential of EnSCs (Klemmt *et al.*, 2006,
474 Piltonen *et al.*, 2015, Salker *et al.*, 2010). However, the level of induction was highly variable
475 between cultures, which probably reflects the clinical heterogeneity of the study population.
476 For implantation to take place, differentiating EnSCs must transit through distinct functional
477 phenotypes in response to elevated circulating progesterone levels and rising cellular cAMP
478 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

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

508 Analysis of human blastocysts indicates that the stromal microenvironment is a major
509 determinant of embryo survival. Our findings demonstrate that the secretome changes
510 associated with undifferentiated EnSCs transitioning to fully decidualized cells induce a
511 graded embryo response, ranging from embryonic growth arrest to a transient embryo-
512 specific secretory response and finally quiescence. Although the EnSC secretome was
513 divergent between the pregnant and non-pregnant groups, no discernible difference in embryo
514 response was observed. This may reflect that EnSC conditioned medium was pooled for the
515 embryo experiments, that only a limited number of blastocysts were available for these
516 experiments, or that the duration of incubation was too short. Another potential pitfall is that
517 these experiments were performed ‘out-of-phase’ as there is no evidence that day 6 human
518 embryos are already embedded in the decidualizing stroma. However, there is histological
519 evidence of a Carnegie stage 5a human embryo (post-fertilization age of 7 to 8 days)
520 surrounded by EnSCs. Thus, while our embryo experiments may indeed be ‘out-of-phase’, it
521 is probably only by 1 or perhaps 2 days (Gardner and Schoolcraft, 1999, Gardner DK, 1999).
522 In summary, cyclic menstruation and renewal renders the endometrium intrinsically dynamic
523 and arguably capable of adapting to ensure reproductive success (Gellersen and Brosens,
524 2014, Lucas *et al.*, 2013). It is evident that a community of resident cells, ranging from
525 immature progenitor cells to mature and senescent cells, makes up the stromal compartment
526 of the endometrium. It is conceivable that the constituents of this community fluctuate from
527 cycle to cycle, with transient imbalances contributing to sporadic IVF failure. The impact of
528 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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537 Nurture Fertility and Anne Skinner for assistance in processing the endometrial biopsies.

538

539 **Authors' roles**

540 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.,
541 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
542 and processed the endometrial biopsies. A.A. and H.V.D.V. performed the human embryo
543 experiments. Y.H.L performed the secretome analysis. J.J.B. designed the study and wrote
544 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
545 the study and all authors edited and approved the manuscript.

546

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552

553 **Conflict of interest**

554 The authors have declared that no conflict of interest exists.

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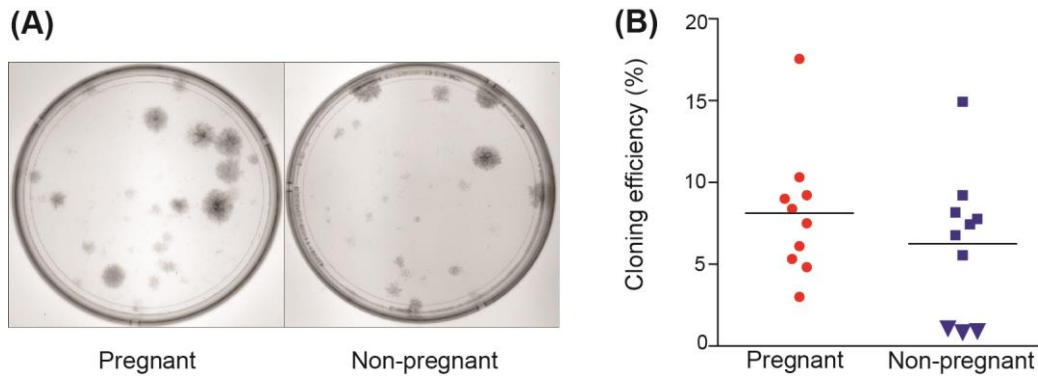
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693

694 **Figure Legends**



695

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 $\leq 1\%$.

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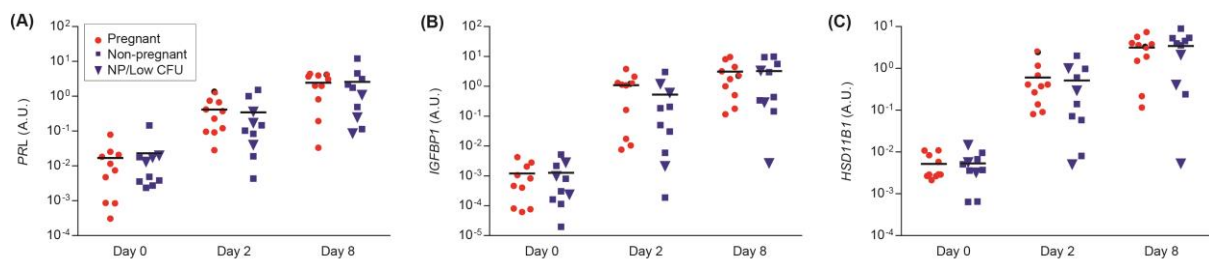
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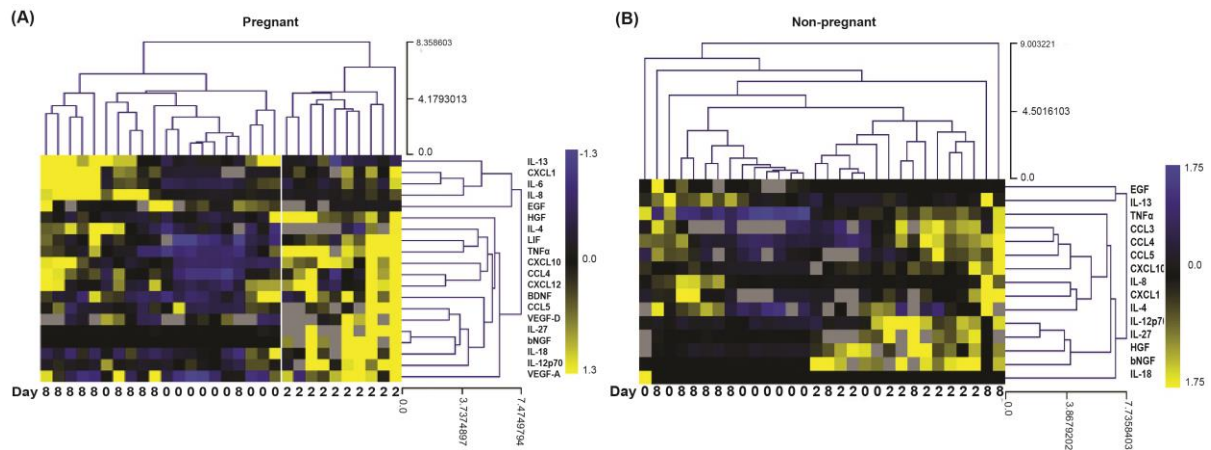
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Figure 2. Induction of decidual marker genes.

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* (11 β -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.



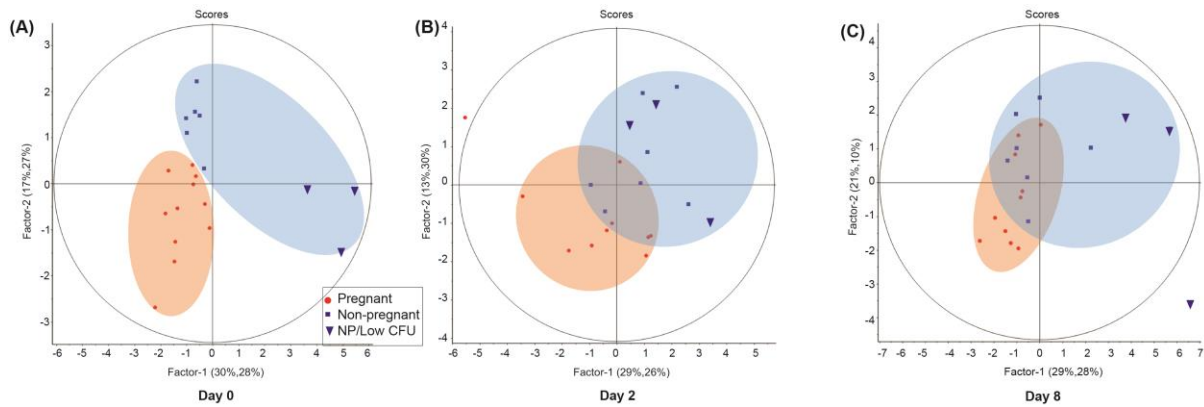
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Figure 3. Divergent human EnSC secretome prior to successful or failed implantation.

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(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)

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

759 Partial least squares (PLS) regression analysis was used to evaluate the temporal multivariate
 760 differences in cytokine and immune-modulatory secretome profile as a result of implantation.

761 The analysis showed that the secretome profiles are less divergent in implantation-positive
 762 cultures, at least in undifferentiated cells and cells decidualized for 8 days. Furthermore, the
 763 differences in secretomes were most pronounced in undifferentiated cells. Decidualization
 764 leads to convergence of secretomes, although not in MSC-deficient cultures (blue triangles).

765 The X-axis represents principal component 1, and the Y-axis principal component 2. NP/Low
 766 CFU refers to cultures from the non-pregnant group with MSC deficiency.

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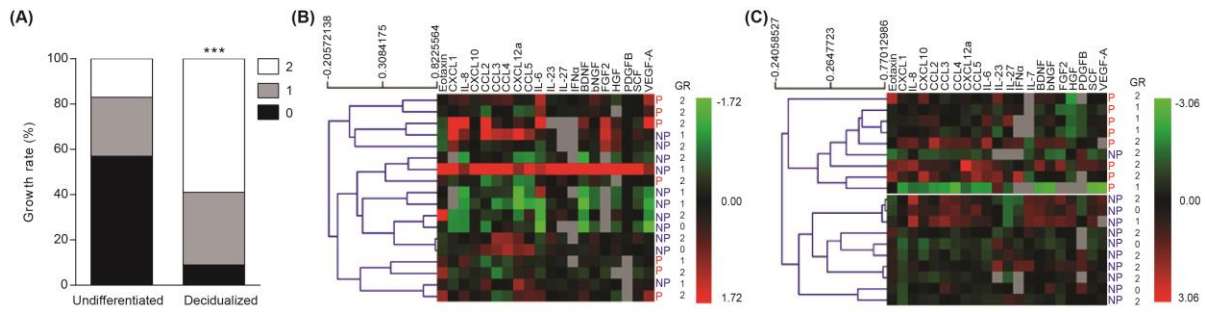
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776 **Figure 5. Impact of EnSC secretome on human blastocysts.**

777 A, growth rates (scored 0, 1, and 2) of human blastocysts cultured in conditioned medium
 778 from undifferentiated (n = 24) and decidualizing EnSCs (day 2 and day 8) (n = 33). ***
 779 indicates $P < 0.005$ (Chi-square test). B, Hierarchical clustering using Euclidean distance of
 780 20 cytokines and chemokines measured in microdroplets of human blastocysts cultured in
 781 conditioned medium of EnSC decidualized for 2 days. P: pregnant, NP: non-pregnant, GR:
 782 growth rate. C, Hierarchical clustering using Euclidean distance of 20 cytokines measured in
 783 microdroplets of human blastocysts cultured in conditioned medium of EnSC decidualized
 784 for 8 days. The colour keys are depicted on the right of the heatmaps (green: decreased
 785 expression; red: increased expression)

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

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

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

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

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Deregulation of the endometrial stromal cell secretome precedes embryo implantation failure

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Supplementary Figures and Data

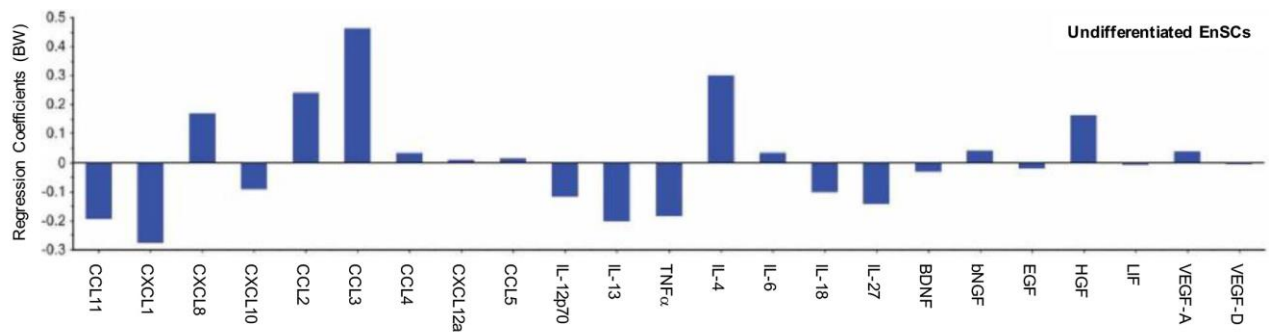


Fig. S1. Regression coefficient analysis was used to identify which secreted factors were most closely associated with successful implantation in undifferentiated EnSCs. Out of 23 detectable factors, CCL3 exhibited the highest regression coefficient value in undifferentiated cultures, indicating a strong positive association with implantation.

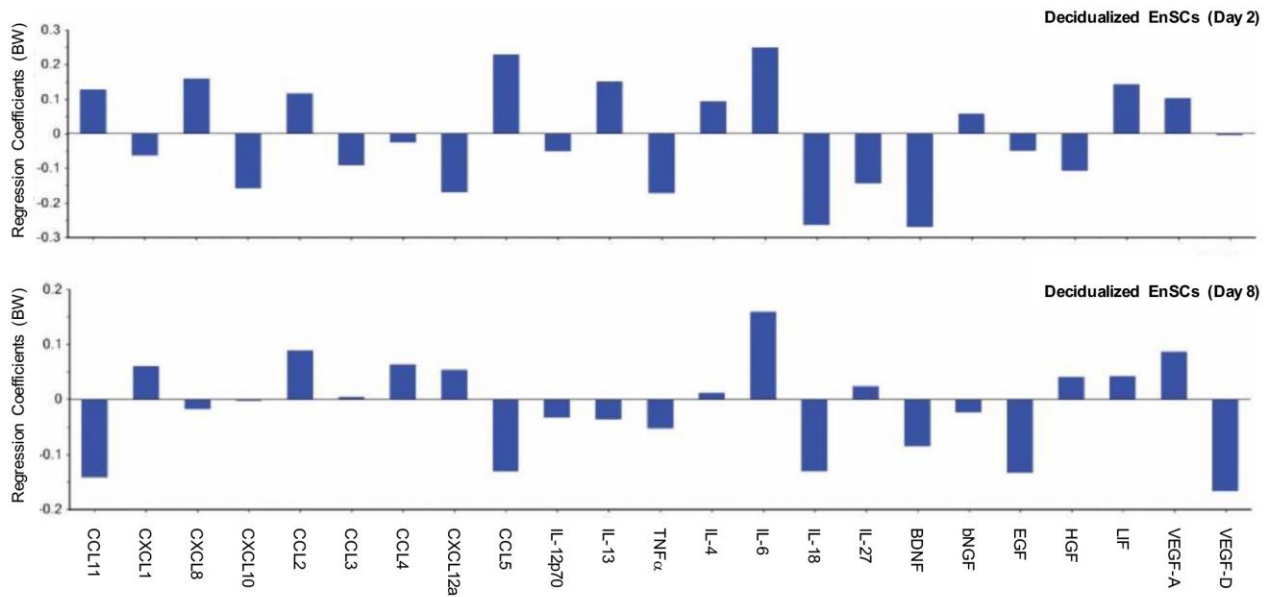


Fig. S2. Regression coefficient analysis of decidualizing cultures (day 2 or day 8) of the pregnant group demonstrated that the strength of the association of CCL3 and successful implantation diminishes upon differentiation of EnSCs. In decidualizing cells, a positive factor (IL-6) and several negative factors (CCL11, CCL5, IL-18, EGF and VEGF-D), all with a regression coefficient of < 0.2 , were associated with successful implantation.

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 \geq "B"-score for the TE and/or ICM

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

<p><u>BDNF</u>: Brain-derived neurotrophic factor</p> <p><u>EGF</u>: Epidermal growth factor</p> <p><u>CCL11 (Eotaxin)</u>: C-C motif chemokine 11</p> <p><u>FGF-2 (FGF basic)</u>: Basic fibroblast growth factor</p> <p><u>GM-CSF</u>: Granulocyte macrophage colony-stimulating factor</p> <p><u>CXCL1 (GROα)</u>: Chemokine (C-X-C motif) ligand 1; Growth regulated oncogene-alpha</p> <p><u>HGF</u>: Hepatocyte growth factor</p> <p><u>IFNγ</u>: Interferon gamma</p> <p><u>IFNα</u>: Interferon alpha</p> <p><u>IL-1RA</u>: Interleukin-1 receptor alpha</p> <p><u>IL-1β</u>: Interleukin-1 beta</p> <p><u>IL-1α</u>: Interleukin-1 alpha</p> <p><u>IL-2</u>: Interleukin-2</p> <p><u>IL-4</u>: Interleukin-4</p> <p><u>IL-5</u>: Interleukin-5</p> <p><u>IL-6</u>: Interleukin-6</p> <p><u>IL-7</u>: Interleukin-7</p> <p><u>CXCL8 (IL-8)</u>: Chemokine (C-X-C motif) ligand 8; Interleukin-8</p> <p><u>IL-9</u>: Interleukin-9</p> <p><u>IL-10</u>: Interleukin-10</p> <p><u>IL-12 p70</u>: Interleukin-12 heterodimer</p> <p><u>IL-13</u>: Interleukin-13</p> <p><u>IL-15</u>: Interleukin-15</p> <p><u>IL-17A</u>: Interleukin-17A</p> <p><u>IL-18</u>: Interleukin-18</p> <p><u>IL-21</u>: Interleukin-21</p> <p><u>IL-22</u>: Interleukin-22</p>	<p><u>IL-23</u>: Interleukin-23</p> <p><u>IL-27</u>: Interleukin-27</p> <p><u>IL-31</u>: Interleukin-31</p> <p><u>CXCL10 (IP-10)</u>: C-X-C motif chemokine 10; Interferon gamma-induced protein 10</p> <p><u>LIF</u>: Leukemia inhibitory factor</p> <p><u>CCL2 (MCP-1)</u>: Chemokine (C-C motif) ligand 2; Monocyte chemotactic protein 1</p> <p><u>CCL3 (MIP-1α)</u>: Chemokine (C-C motif) ligand 3; Macrophage inflammatory protein 1-alpha</p> <p><u>CCL4 (MIP-1β)</u>: Chemokine (C-C motif) ligand 4; Macrophage inflammatory protein 1-beta</p> <p><u>βNGF</u>: Beta nerve growth factor</p> <p><u>PDGF-BB</u>: Platelet-Derived Growth Factor-BB</p> <p><u>PLGF</u>: Placental growth factor</p> <p><u>CCL5 (RANTES)</u>: Chemokine (C-C motif) ligand 5</p> <p><u>SCF</u>: Stem cell factor</p> <p><u>CXCL12 (SDF1α)</u>: C-X-C motif chemokine 12; stromal cell-derived factor alpha</p> <p><u>TNFα</u>: Tumor necrosis factor alpha</p> <p><u>LTA (TNFβ)</u>: Lymphotoxin-alpha; tumor necrosis factor-beta</p> <p><u>VEGF-A</u>: Vascular endothelial growth factor A</p> <p><u>VEGF-D</u>: Vascular endothelial growth factor D</p>
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Factors that are underlined were undetectable in > 50% of samples and therefore excluded from subsequent analyses.