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Uterine Selection of Human Embryos at Implantation

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Human embryos frequently harbor large-scale complex chromosomal errors that impede normal development. Affected embryos may fail to implant although many first breach the endometrial epithelium and embed in the decidualizing stroma before being rejected via mechanisms that are poorly understood. Here we show that developmentally impaired human embryos elicit an endoplasmic stress response in human decidual cells. A stress response was also evident upon *in vivo* exposure of mouse uteri to culture medium conditioned by low-quality human embryos. By contrast, signals emanating from developmentally competent embryos activated a focused gene network enriched in metabolic enzymes and implantation factors. We further show that trypsin, a serine protease released by pre-implantation embryos, elicits Ca²⁺ signaling in endometrial epithelial cells. Competent human embryos triggered short-lived oscillatory Ca²⁺ fluxes whereas low-quality embryos caused a heightened and prolonged Ca²⁺ response. Thus, distinct positive and negative mechanisms contribute to active selection of human embryos at implantation.

Reproduction in humans is marred by early pregnancy failure. Approximately 15% of clinically recognized pregnancies miscarry. When combined with pre-clinical losses, the true incidence is closer to 50%, rendering miscarriage by far the most common complication of pregnancy^{1,2}. This exceptional attrition rate is attributed to the intrinsic invasiveness of human embryos and the high prevalence of chromosomal errors. Based on genome-wide screening of individual blastomeres, in excess of 70% of high-quality cleavage-stage IVF embryos reportedly harbor cells with complex large-scale structural chromosomal imbalances, some caused by meiotic aneuploidies but most by mitotic non-disjunction^{3–5}. The incidence of aneuploidy in human embryos is estimated to be an order of magnitude higher than in other mammalian species. Further, a vast array of chromosomal errors has been detected in human embryos throughout all stages of pre-implantation development. Many of the chromosomal abnormalities observed in blastocysts have never been recorded in clinical miscarriage samples³, suggesting that these embryos either fail to implant or are rejected soon after breaching the endometrial luminal epithelium^{2,6–8}.

Evidence from several mammalian species indicates that the endometrium is intrinsically capable of mounting an implantation response that is tailored to individual embryos. For example, microarray analysis of bovine endometrium has identified gene signatures that are dependent on the origins (e.g. somatic cell nuclear transfer, IVF, or artificial insemination) and developmental potential of the attaching embryo⁹. Using a co-culture system, we reported previously that human endometrial stromal cells (HESCs) become sensitive to embryonic signals upon differentiation into decidual cells and respond selectively to low-quality human embryos by inhibiting the secretion of key implantation factors, including interleukin-1 beta, heparin-binding EGF-like growth factor, and leukemia inhibitory factor¹⁰. Furthermore, aberrant decidualization of HESCs and lack of embryo sensing are strongly associated with recurrent pregnancy loss^{11–14}. These observations led to the hypothesis that active embryo selection



at implantation is essential for reproductive success^{11–14}, although the underlying mechanisms remain as yet poorly characterized.

A major obstacle is that human implantation events cannot be studied directly. In culture, the developmental potential of human embryos can only be assessed indirectly, foremost on morphological criteria, and over a legally restricted period. To overcome these hurdles, we prospectively collected conditioned medium of individually cultured human pre-implantation embryos and then characterized the maternal response, *in vitro* as well as in a heterologous *in vivo* model, to soluble factors produced by low-quality human embryos and embryos of proven developmental competence. We report that human embryos presage their developmental competence even prior to implantation. The spectrum of endometrial responses to cues from different embryos ranges widely, from enhanced expression of key implantation factors to overt endoplasmic reticulum (ER) stress. We also provide evidence that embryo-derived serine proteases are involved in eliciting a maternal response tailored to the developmental potential of the conceptus.

Results

Developmentally impaired human embryos induce ER stress response in decidualizing HESCs. We systematically collected the conditioned medium of day 4 human IVF embryos, which had been cultured individually for 72 h in microdroplets overlaid with mineral oil. Next, we incubated primary decidualizing HESCs with pooled culture supernatants from developmentally impaired embryos (DIEs), which were deemed unsuitable for transfer¹⁵, and from embryos that resulted in ongoing pregnancies after single embryo transfer (developmentally competent embryos, DCEs; Table S1). Control cultures consisted of decidualizing HESCs incubated with unconditioned embryo culture medium (ECM). Incubation of primary cultures was repeated three times with separate pools of conditioned media from DCEs and DIEs. RNA was isolated from decidualizing HESCs after 12 h of incubation and analyzed by

genome-wide expression profiling using DNA microarrays. Surprisingly, only 15 decidual genes were found to respond significantly ($P < 0.01$) to signals emanating from DCEs. In contrast, 449 maternal genes were perturbed in response to medium conditioned by DIEs (Fig. 1A; Table S1). Gene ontology annotation categorized half of these maternal genes into three broad biological processes: transport (20%), translation (17%), and cell cycle (13%) (Fig. 1B).

To investigate further the response of decidual cells to compromised embryos, we focused on *HSPA8*, the most downregulated gene in the array analysis (Table S2). This gene encodes HSC70, a ubiquitously and constitutively expressed member of the heat shock protein 70 family of molecular chaperones involved in the assembly of multi-protein complexes, transport of nascent polypeptides and regulation of protein folding^{16,17}. HSC70 levels increased in primary HESCs decidualized for 4 or more days (Fig. 1C). Small interfering (si)RNA-mediated knockdown of this molecular chaperone reduced the secretion of prolactin (PRL) and insulin-like growth factor-binding protein 1 (IGFBP1) (Fig. 1D and E), two highly sensitive differentiation markers¹⁸. Cell viability was not affected significantly (Fig. 1F).

Because of its role in protein homeostasis^{19,20}, we postulated that loss of HSC70 causes ER stress in decidual cells. In fact, decidualizing HESCs mount a physiological unfolded protein response (UPR) associated with ER expansion and acquisition of a secretory phenotype²¹. This UPR was characterized by synchronous induction of various chaperones, including protein disulfide isomerase (PDI), BIP (GRP78), endoplasmic oxidoreductin-1 α (ERO1 α), and calnexin (Fig. 2A). In addition, differentiating HESCs upregulate the expression of the three key ER signaling proteins, the serine/threonine kinase inositol-requiring enzyme 1 α (IRE1 α), PKR-like ER-localized eIF2 α kinase (PERK), and the protease-activated transcription factor ATF6, which collectively determine the cellular response to ER stress signals²¹. Interestingly, HSC70 knockdown had little or no effect on the expression of other ER chaperones or ATF6

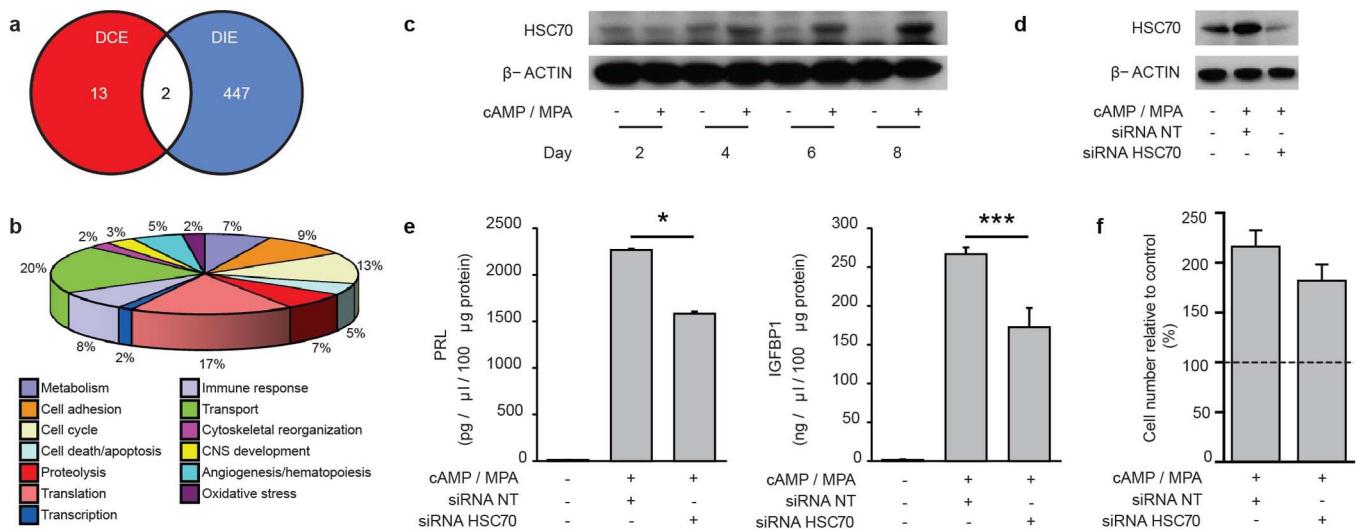


Figure 1 | Decidualizing endometrial cells are biosensors of embryo quality. (a) Venn diagram presenting the number of transcripts regulated in decidualizing HESCs significantly ($P < 0.01$) regulated in response to signals from developmentally competent embryos (DCE) and developmentally impaired embryos (DIE). (b) Gene Ontology classification of decidual genes regulated in response to soluble factors secreted by DIE. (c) Western blot analysis demonstrating the kinetics of HSC70 induction in primary HESC cultures decidualized with cAMP and MPA in a time-course lasting 8 d. β -ACTIN served as a loading control. A representative result from three different primary cultures is shown. Full length images are presented as Supplementary Information. (d) Primary HESCs were transfected with non-targeting (NT) siRNA or siRNA targeting HSC70, decidualized for 5 d, and then immunoblotted for HSC70. β -ACTIN served as a loading control. Full length images are presented as Supplementary Information. (e) HSC70 knockdown inhibits the secretion of decidual markers, PRL and IGFBP1, in primary HESC cultures differentiated *in vitro* for 5 d. The data represent mean (\pm SD) of triplicate experiments. * indicates $P < 0.05$, and *** $P < 0.001$. (f) The percentage of viable HESCs, transfected first with non-targeting (NT) siRNA or HSC70 targeting siRNA and then decidualized for 5 d, is presented relative to the number of viable cells in mock-transfected, undifferentiated cells (dotted line). The data represent the mean (\pm SD) of three biological repeat experiments.

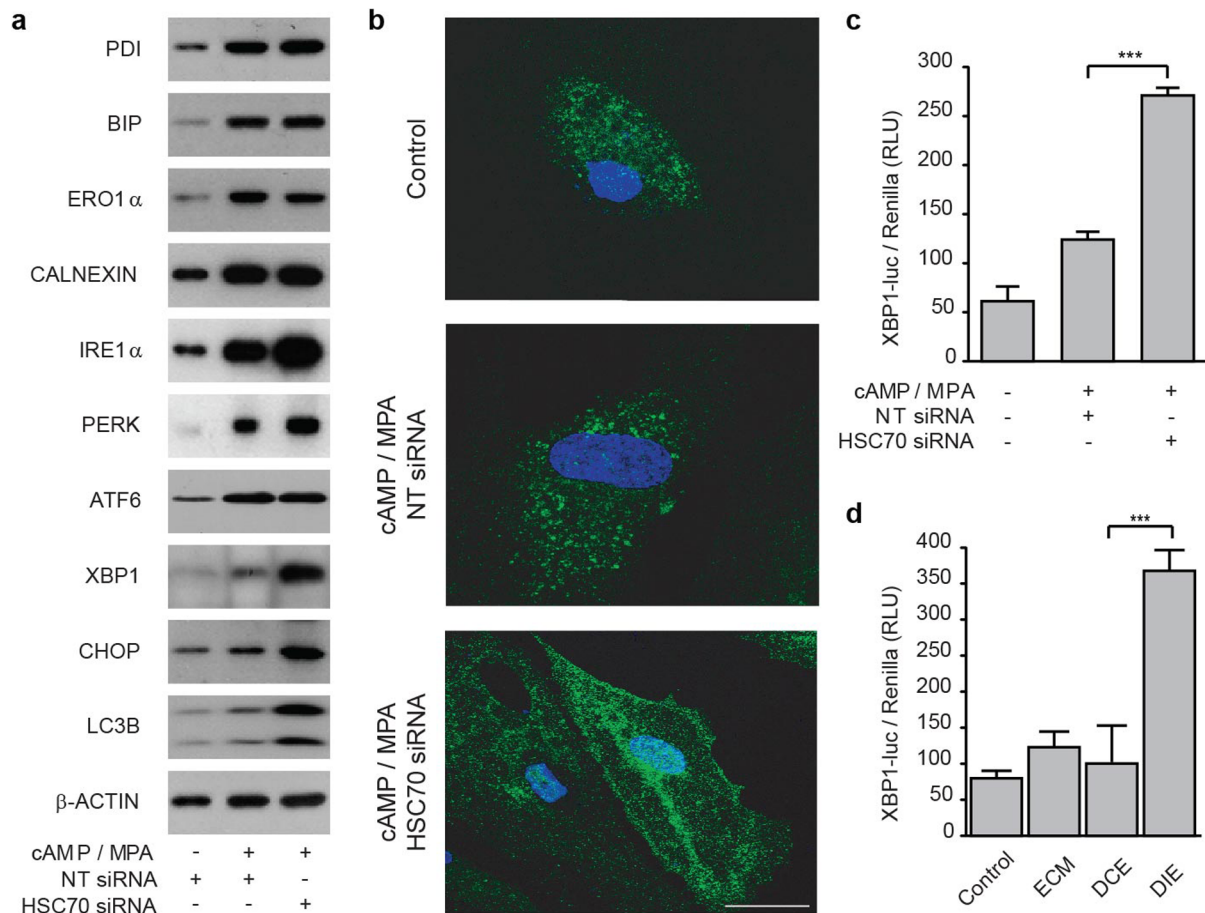


Figure 2 | HSC70 knockdown induces ER stress in decidualizing stromal cells. (a) Total cell lysates from primary HESC cultures, transfected first with non-targeting (NT) siRNA or siRNA targeting HSC70 and then decidualized with cAMP and MPA for 5 d, were immunoprobed for various proteins involved in UPR, ER stress, and autophagy as indicated. β -ACTIN served as a loading control. Full length images are presented as Supplementary Information. (b) HSC70 knockdown in HESCs promotes autophagosome formation. Primary cells cultured on chamber slides were transfected with either non-targeting (NT) or HSC70 targeting siRNA, decidualized for 5 d, stained for LC3B expression (green) and subjected to confocal microscopy. The nuclei were visualized with DAPI (blue). (c) Primary cultures were co-transfected with pcDNA3/XBP1-luc, pRL-sv40 and either siRNA targeting HSC70 or NT siRNA. The cells were left untreated or differentiated for 5 d before measuring luciferase activity. The results show the normalized mean firefly luciferase activity (\pm SD), expressed in relative light units (RLU), of four biological repeat experiments. *** indicates $P < 0.001$. (d) Confluent cultures were transfected as described in (c), left untreated (control) or decidualized for 5 d, and then exposed to 30 μ l of unconditioned embryo culture medium (ECM) or media conditioned by DCEs or DIEs for 12 h. The results show normalized mean luciferase activity (\pm SD), expressed in relative light units (RLU), of three biological repeat experiments. *** indicates $P < 0.001$.

but further enhanced the induction of IRE1 α and PERK. In addition, silencing of this molecular chaperone protein in decidualizing cells markedly upregulated the levels of X-box binding protein 1 (XBP1) and C/EBP-homologous protein (CHOP), downstream transcription factors that couple ER stress to translational inhibition, induction of ER chaperones, cell cycle arrest and death²¹. We speculated that lack of overt cell death upon HSC70 knockdown could be accounted for by augmented autophagy²¹. In keeping with this notion, the abundance of the microtubule-associated protein light chain 3B (LC3B), a marker of autophagic activity²², increased markedly upon HSC70 knockdown in decidualizing cells (Fig. 2A). Confocal microscopy showed that the pattern of LC3B staining changed from being punctate and finely granular in undifferentiated and decidualizing cells to immunoreactive aggregates upon HSC70 silencing (Fig. 2B).

To test further the assertion that decidualizing HESCs mount an ER stress response upon HSC70 knockdown, we transfected primary cultures with pcDNA3/XBP1-luc, a plasmid in which human XBP1 cDNA is fused upstream of luciferase cDNA²³. Under non-ER stress conditions, the presence of an in-frame stop codon prevents the

expression of the downstream luciferase gene. Under ER stress conditions, however, activated IRE1 α splices the XBP1-luc transcript, allowing translation of the luciferase cDNA. As shown in Fig. 2C, decidualization of HESCs transfected with pcDNA3/XBP1-luc only marginally enhanced luciferase levels whereas simultaneous HSC70 knockdown elicited a 5-fold induction. We then reasoned that decidualizing HESCs transfected with pcDNA3/XBP1-luc could serve as a bioassay to monitor ER stress responses induced by limited amounts of ECM. Primary cells seeded in 96-well plates were first transfected with the reporter construct, decidualized for 5 d, and then exposed for 12 h to either unconditioned ECM or pooled spent medium from DCEs or DIEs. In keeping with the array findings, soluble factors derived from DIEs strongly induced luciferase expression whereas this response was entirely absent upon incubation with unconditioned ECM or medium conditioned by DCEs (Fig. 2D).

Developmentally competent human embryos signal to promote implantation. We next explored if the developmental potential of human embryos impacts on the expression of uterine implantation genes *in vivo*. Female C57BL/6 mice were hormonally stimulated to

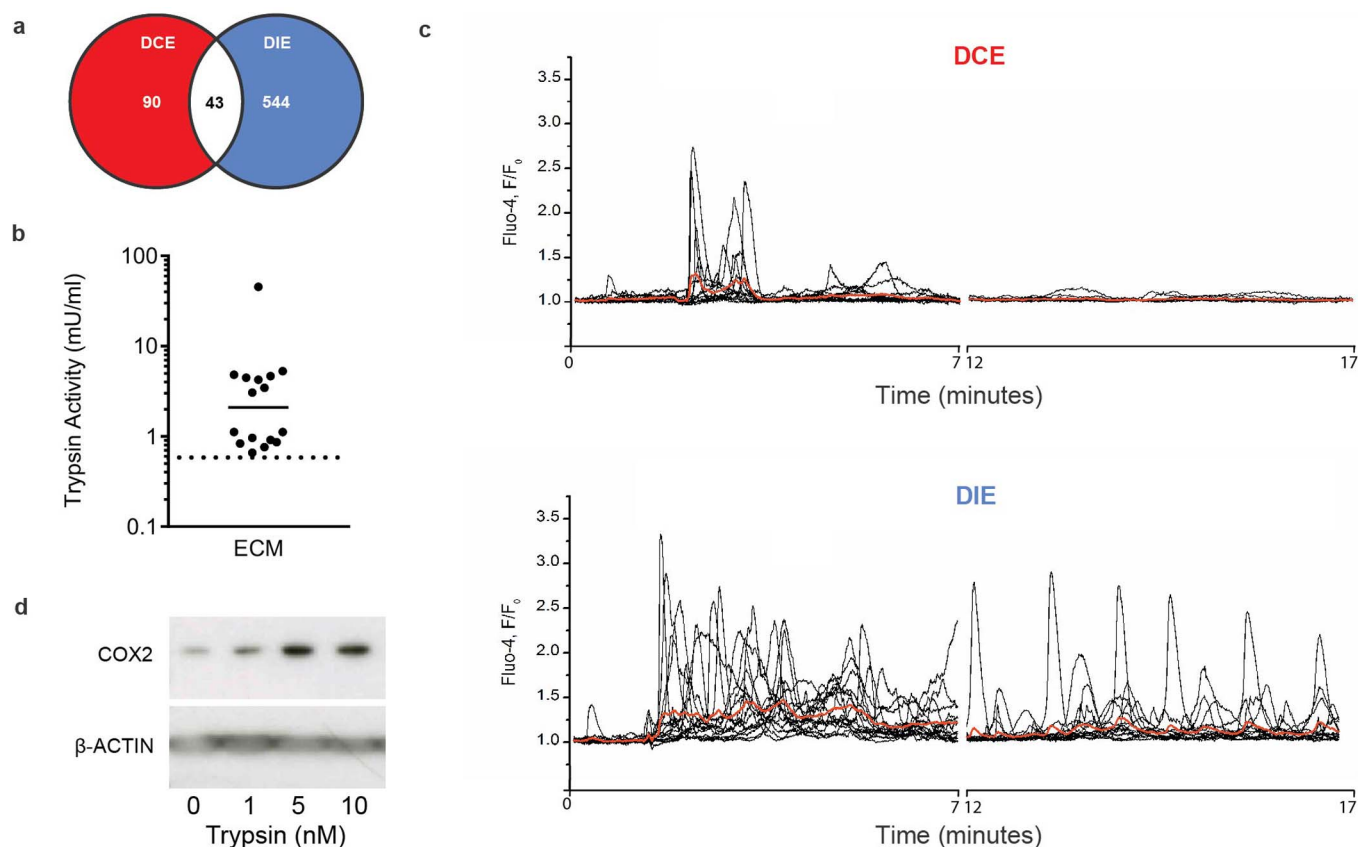


Figure 3 | Competent pre-implantation human embryos actively induce a supportive uterine environment. (a) Venn diagram presenting the number of maternal genes significantly ($P < 0.01$) altered 24 h after exposure of mouse uterus to unconditioned embryo culture medium or medium conditioned by either developmentally competent or impaired human embryos (DCE and DIE, respectively). (b) Trypsin activity was measured in 16 cultures containing a total of 163 human embryos between day 4 to 6 of development. Activity in unconditioned medium was below dotted line. (c) Application of embryo-conditioned medium induces $[Ca^{2+}]_i$ oscillations in Ishikawa cells. Black traces show $[Ca^{2+}]_i$ recordings from individual cells in response to application of 1 : 20 diluted culture medium obtained from DCE (top panel) or DIE (bottom panel). Red traces represent average of all individual traces in each panel. (d) Total protein lysates obtained from Ishikawa cells 24 h after treatment with trypsin (10 nM) for 5 min were subjected to western blot analysis and immunoprobed for COX2. β -ACTIN served as a loading control. Full length images are presented as Supplementary Information.

induce uterine receptivity and the lumen flushed with 50 μ l of unconditioned ECM or pooled conditioned culture medium from human DCEs or DIEs. The animals were sacrificed 24 h later and the uterine transcriptome sequenced. As in the experiments with primary HESCs, many more genes ($\sim 6\times$) were altered in response to exposure of the uterine lumen to DIE versus DCE conditioned medium (Fig. 3A). However, the extent and amplitude of the transcriptional response to DCE was much more pronounced *in vivo* when compared to decidualizing primary HESC cultures. Strikingly, 27 of 90 (30%) uterine genes solely responsive to DCE signals code factors that have already been implicated in the implantation process (Table S3), including COX-2 (*Ptgs2*), Cytochrome P450 26a1 (*Cyp26a1*), and osteopontin (*Spp1*)^{24–26}. In addition, exposure to DCE signals strongly upregulated a group of metabolic genes, which included *Fabp4*, *Plin1*, *Cidec*, *Adipoq*, *Retn*, and *Car3* (Fig. S1). Fatty acid binding protein 4 (*Fabp4*) is a widely used marker of differentiating adipocytes²⁷. Cell death-inducing DFF45-like effector c (*Cidec*) promotes lipid droplet expansion whereas perilipin 1 (*Plin1*) prevents lipase-dependent breakdown of lipid droplets under basal conditions²⁸. Further, adiponectin (*Adipoq*) and resistin (*Retn*) are key adipokines involved in regulating energy intake and expenditure as well as insulin sensitivity²⁹. Taken together, the data point towards the existence of an evolutionarily conserved network of maternal genes that is responsive to embryonic signals and contributes to post-implantation development. By contrast, the response to DIE signals had the hallmarks

of a stress response (Fig. S2). This was confirmed by Western blot analysis demonstrating strong uterine expression of Xbp1, Chop and Lc3b in response to DIE signals (Fig. S3). Notably, a modest stress-like response was also apparent upon flushing of the uterine lumen with medium conditioned by DCEs, which may point towards the induction of a decidual response and associated UPR.

The role of embryo-derived trypsin in maternal embryo recognition. A surprising observation was the induction of *Prss28* (68-fold) and *Prss29* (6-fold) mRNAs in the mouse uterus in response to DCE signals (Table S3). *Prss28* and *Prss29* are implantation-specific serine proteases that exhibit trypsin-like substrate specificity^{30,31}. Embryonic trypsinases activate Ca^{2+} signaling and upregulate COX-2 levels in murine endometrial epithelial cells (EECs), leading to prostaglandin production required for implantation²⁶. *Prss28* and *Prss29* have no functional homologs in humans³², suggesting a compensatory role for other serine proteases or, perhaps, that tryptic activity is no longer involved in embryo-EEC signaling. Comparative analysis of gene expression data showed that (Fig. S4 & S5), unlike their murine counterparts, developing human embryos up- and down-regulate the expression of *TMPRSS15* and *AMBP* coding for the trypsinogen activator (enterokinase) and trypsin inhibitor (trypstatin/bikunin precursor), respectively (Fig. S5). Trypsin activity was detectable in culture medium conditioned by human embryos (Fig. 3B). Furthermore, exposure of Ishikawa cells (a cell line model for human EECs) to spent embryo medium induced



oscillatory increases in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$). Interestingly, $[\text{Ca}^{2+}]_i$ oscillations were discrete, lasting approximately 5 min, in response to DCE medium (Fig. 3C, upper panel). This contrasted to more pronounced and much longer oscillations when cells were exposed to DIE signals (Fig. 3C, lower panel). The $[\text{Ca}^{2+}]_i$ fluxes induced by embryonic cues bore a striking similarity to $[\text{Ca}^{2+}]_i$ oscillations induced upon application of trypsin (Fig. S6A and D). Soybean trypsin inhibitor dramatically decreased $[\text{Ca}^{2+}]_i$ signals induced by spent embryo medium (Fig. S6B and D). Conversely, embryo-induced $[\text{Ca}^{2+}]_i$ oscillations greatly attenuated the subsequent $[\text{Ca}^{2+}]_i$ responses to trypsin (Fig. S6C and D). Finally, short exposure to trypsin (5 min) was sufficient to upregulate COX-2 expression in Ishikawa cells (Fig. 3D). These data indicate that EECs serve to amplify discrete embryonic protease signals that induce a supportive maternal environment.

Discussion

Conflict between parent and offspring is thought to drive reproductive evolution and innovation³³. This hypothesis predicts that the embryonic genome evolves to extract as much as possible from the mother to ensure its propagation whereas maternal genes will adapt to safeguard the success of current as well as future offspring. Thus, reproductive success in different species depends on balancing evolving embryonic and maternal traits^{34–36}. A singular feature of the reproductive cycle in humans, shared with very few other mammalian species, is menstruation, a process triggered by ‘spontaneous’ decidualization of the endometrium in an embryo-independent manner^{36,37}. When placed in the context of fetal-maternal conflict^{33,38}, our findings indicate that cyclic decidualization coupled to menstruation emerged as a strategy for early detection and active rejection of developmentally abnormal embryos that have breached the luminal epithelium. We show that decidual cells mount an extraordinarily polarized transcriptional response to embryonic signals, ranging from being exceptionally discrete in case of a competent embryo to extensive and complex in the presence of a low-quality embryo. We further demonstrate that *HSPA8* is particularly sensitive to signals from DCEs. Down-regulation of this molecular chaperone in decidual cells converts the differentiation-associated UPR into an overt ER stress response, which in turn compromises secretion of decidual factors, including PRL and IGFBP1, essential for placental formation and fetal development.

Conversely, this study shows that competent pre-implantation human embryos have retained the ability to actively enhance the uterine environment for implantation. This response to DCE signals is characterized by the induction of 29 known implantation factors, including COX-2, as well as various metabolic enzymes involved in lipid accumulation, glucose uptake, and energy expenditure (Table S3). Interestingly, several of these metabolic genes (e.g. *Cidec*, *Plin1*, *Adipoq*, *Retn* and *Fabp4*) are transcriptionally regulated by peroxisome proliferator-activated receptor gamma (PPAR γ) in adipocytes^{39–41}, suggesting that embryonic signals activate this nuclear receptor in the endometrium, perhaps indirectly via induction of COX-2-dependent prostaglandin production⁴². In mice, initiation of implantation requires release of embryonic serine proteases, which in turn activate epithelial Na^+ channel (ENaC) in EECs, triggering Ca^{2+} influx and induction of COX-2²⁶. Several lines of evidence indicate that this implantation pathway is not only conserved in humans but also important for embryo sensing. We found that tryptic activity is detectable in medium conditioned by human embryos even before hatching. Incubation of EECs with ECM elicited $[\text{Ca}^{2+}]_i$ oscillations, a response markedly blunted by soybean trypsin inhibitor and recapitulated upon treatment of cells with low concentrations of trypsin. Further, brief exposure of EECs to trypsin was sufficient to induce COX-2 expression. *In silico* analysis showed that progression to the blastocyst stage is associated with gene changes predictive of increased expression and activation of various

proteases implicated in ENaC activation, although the pattern of expression of individual genes during pre-implantation development frequently differs between mouse and human embryos (Fig. S4 & S5 and data not shown). Remarkably, cues from competent human embryos strongly induced two non-conserved implantation-specific serine proteases, Prss28 (also known as implantation serine protease 1 or ISP1) and Prss29 (ISP2), in the mouse uterus. These proteases are also co-expressed in pre-implantation murine embryos^{30,31}. ISP1 and 2 heterodimerize and form an enzymatic complex essential for blastocyst hatching, outgrowth and implantation in mice. Taken together, these observations suggest that endometrial protease production accelerates as the embryo approaches the surface epithelium, perhaps aligning hatching of the blastocyst with implantation. By contrast, low-quality human embryos triggered prolonged and disorganized $[\text{Ca}^{2+}]_i$ oscillations in EECs and an uterine stress response *in vivo*. The mechanism that couples these events warrants further investigations, although it is likely to involve illicit, excessive or unopposed activation of embryonic proteases, leading to proteotoxic stress in both the conceptus and surrounding maternal cells. This conjecture is supported by the observation that DCE but not DIE signals strongly enhance uterine expression of *Spink3*, which codes for the secreted serine protease inhibitor Kazal type 3 (SPINK3). The human homolog SPINK1 critically protects the pancreas from auto-digestion by preventing premature protease activation^{43,44}.

In summary, reproductive success in humans depends on sustained maternal investment in one - occasionally two - implanting embryos. Genomic instability, giving rise to a vast array of chromosomal errors of variable complexity³, is prevalent in human embryos throughout all stages of pre-implantation development. This engrained diversity in embryo quality poses an obvious maternal challenge. Our observations show that both positive and negative selection mechanisms govern implantation (Fig. 4), rendering this process intrinsically dynamic and adaptable to individual embryos.

Methods

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

Primary cultures. Endometrial samples were obtained during the secretory phase at the time of hysterectomy for benign indications or as an outpatient procedure using a Wallach Endocell™ sampler (Wallach, USA) under ultrasound guidance. HESC cultures were established, passaged once and decidualized with 0.5 mM 8-Bromo-cAMP (Sigma, UK) and 10^{-6} M medroxyprogesterone acetate (MPA; Sigma, UK) as previously described⁴⁵.

Embryo conditioned media. All patients underwent ovarian stimulation with recombinant FSH and final oocyte maturation was triggered with hCG. Human embryos were cultured in microdroplets (30 μ l) of Human Tubal Fluid medium, supplemented with 5% GPO (40 g/l pasteurized plasma protein, containing 95% albumin) under mineral oil from the second day after oocyte retrieval until day 4 (morula stage). The supernatants were collected from individually cultured embryo that resulted in pregnancy after transfer of a single fresh embryo ($n = 40$) and from embryos deemed of poor quality ($n = 49$), and unsuitable for embryo transfer based on standard morphological criteria¹⁵. In parallel, microdroplets not containing embryos were collected for control experiments.

Microarray analysis of primary HESC cultures and gene ontology. Primary HESCs were plated in 48-well tissue culture-grade plates, decidualized with 8-Bromo-cAMP and MPA for 5 d, and then incubated for 12 h with 100 μ l of separately pooled supernatants, each derived from 10 individually expressed embryos. Three pools of conditioned media used were from DCEs and three from DIEs. Total RNA from HESCs in individual wells was subjected to microarray analysis. Human 70-mer oligos (Operon, Human V2 AROS) spotted onto Codelink Activated slides (Surmodics USA) were used for genome-wide expression profiling. RNA amplifications, labeling and hybridizations were performed as described⁴⁶. Briefly, 500 ng of each amplified cRNA was coupled to Cy3 or Cy5 fluorophores (Amersham, UK) and subsequently hybridized on a Tecan HS4800PRO and scanned on an Agilent G2565BA microarray scanner. After data extraction using Imagen 8.0 (BioDiscovery), print-tip Loess normalization was performed on mean spot-intensities without background subtraction 30. Data were analyzed using ANOVA (R version 2.2.1/MAANOVA version 0.98-7) (<http://www.r-project.org/>). Genes with a

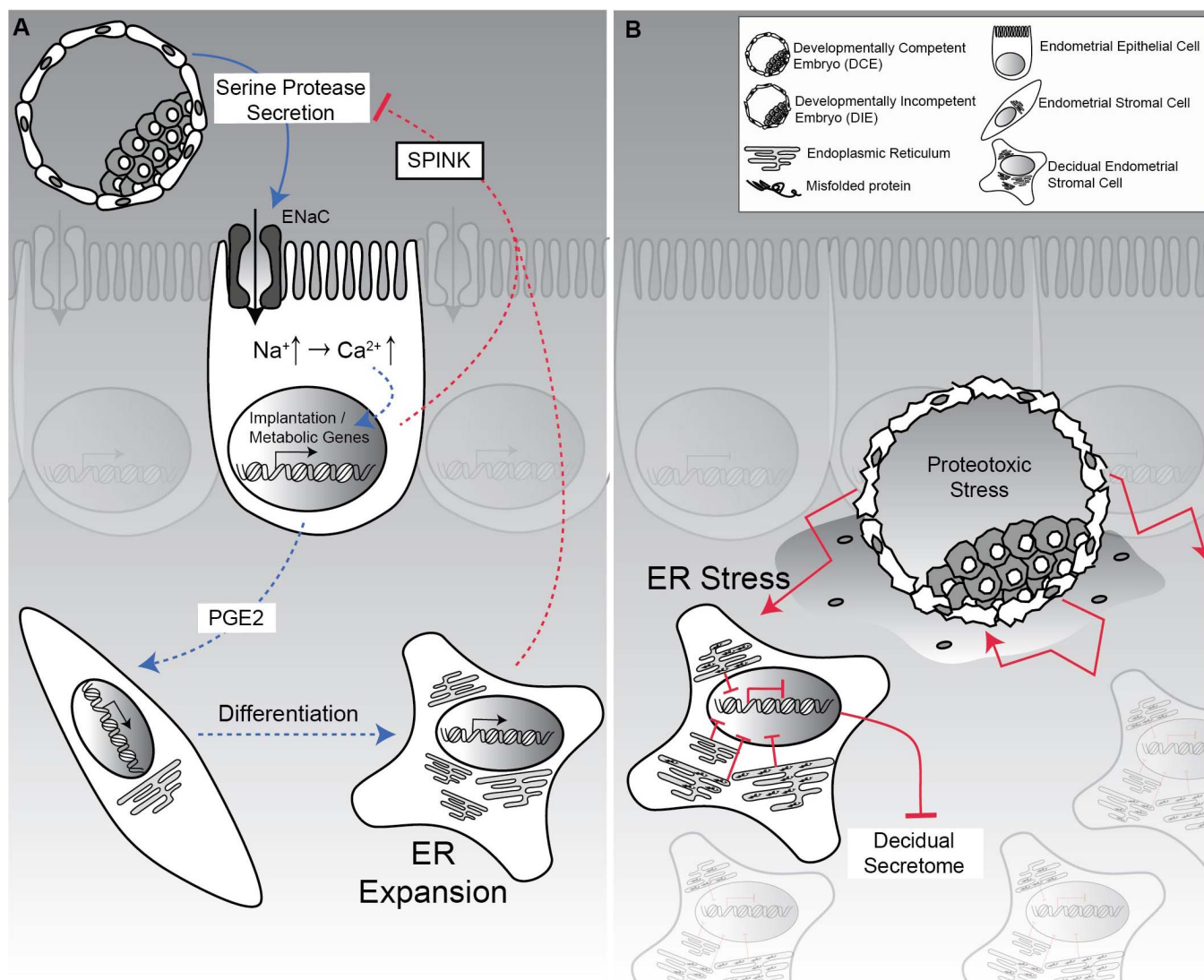


Figure 4 | Positive and negative mechanisms contribute to active selection of human embryos at implantation. (A) Developmentally competent human embryos secrete evolutionary conserved serine proteases that activate epithelial Na^+ channel (ENaC) expressed on luminal epithelial cells²⁶, triggering Ca^{2+} signalling and, ultimately, induction of genes involved in implantation and post-implantation embryo development. In concert, the decidualizing endometrium secretes serine protease inhibitors, such as murine SPINK3 and the human homolog SPINK1, to limit embryo-derived proteolytic activity. Note that acquisition of a secretory phenotype upon decidualization depends on massive expansion of the ER in HESCs. (B) By contrast, excessive protease activity emanating from developmentally compromised embryos that have breached the luminal epithelium down-regulates the expression of molecular chaperones in surrounding decidual cells, leading to accumulation of misfolded proteins and ER stress. This in turn compromises decidual cell functions and triggers tissue breakdown and early maternal rejection.

$P < 0.01$ after false discovery rate correction were considered significant. In addition, a 1.2 fold-change cutoff was applied and the resulting gene lists used for gene ontology (GO) analysis. Regulated genes were mapped to GO-slim categories according to the Gene Ontology Consortium: (http://www.geneontology.org/GO_slims/goslim_generic.obo). Microarray data have been submitted to ArrayExpress under accession number E-TABM-1064.

Animal experiments. C57BL/6 mice were purchased from Charles River Ltd (Margate, UK) and all experiments were carried out in accordance with the UK Home Office Project Licence (PPL70/6867). Immature female (3-week old) mice received a single dose of 1 mg progesterone and 10 $\mu\text{g}/\text{kg}/\text{day}$ β -estradiol (Sigma) for a total of 3 d to prime the uterus for embryo transfer. The uterine horns of control and study mice were injected with an equal volume (50 μl) of either unconditioned embryo culture medium (ECM), serving as controls, or pooled conditioned media from DCE ($n = 9$) or DIE embryos ($n = 18$). The cervix was not clamped. Then, the incision was closed to allow recovery of the mice. The control and treatment groups each consisted of three animals. The mice were sacrificed 24 h later and uteri either fixed in formalin or snap-frozen and stored at -80°C for RNAseq and protein analyses. Both uterine horns of each animal were analyzed individually.

RNAseq analysis of mouse uteri. Uterine mRNA profiles of 25-day old wild-type (WT) mice were generated by deep sequencing, in triplicate, using Illumina HiSeq

2000 platform. The sequence reads that passed quality filters were analyzed with the following methods: Bowtie Alignment followed by TopHat (splice junctions mapper) and Cufflinks (transcript abundance). Sequence data have been submitted to GEO (GSE47019).

Transfections of primary endometrial cells. Primary HESCs at 80% confluency were transfected with DNA vectors or small interfering RNA (siRNA) oligonucleotides by the calcium phosphate co-precipitation method using the Profection mammalian transfection kit (Promega, Madison, WI) according to the manufacturer's instructions. Reporter assays were done in 96-well plates. The X-box binding protein 1 (pcDNA3/XBP1-luc) reporter construct was a kind gift from Dr. Etsu Tashiro (Keio University, Tokyo, Japan). A constitutively active renilla expression vector (pRL-sv40) served as an internal transfection control. The plates were washed twice in phosphate-buffered saline (PBS) and firefly and Renilla activities were measured using the Lucite luciferase reporter assay system (Lucite, PerkinElmer, Boston, MA) and the luminescence was measured on a Victor II plate reader (PerkinElmer). For gene-silencing studies, HESCs were cultured in 6-well plates until 80% confluency and transiently transfected with 100 nM of the following siRNA reagents (Dharmacon, Lafayette, CO): siCONTROL non-targeting (NT) siRNA pool and HSPA8 siGENOME SMARTpool. All experiments were performed on three or more primary cultures from different endometrial biopsies.



Western blot analysis. Protein extracts were prepared by lysing cells in RIPA buffer. Protein yield was quantified using the Bio-Rad DC protein assay kit (Bio-Rad, USA). Equal amounts of protein were separated by 10% SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) before wet-transfer onto PVDF membrane (Amersham Biosciences, UK). Nonspecific binding sites were blocked by overnight incubation with 5% nonfat dry milk in Tris-buffered saline with 1% Tween (TBS-T; 130 mmol/L NaCl, 20 mmol/L Tris, pH7.6 and 1% Tween). Primary antibodies used were anti-HSC70 (Abcam, UK), anti-BiP, anti-Calnexin, anti-ERo1 α , anti-CHOP, anti-PERK, anti-PDI, anti-LC3B, anti-COX2 (Cell Signaling, USA) and β -ACTIN (Abcam, UK) which was used as a loading control. All primary antibodies were diluted 1 : 1000; except for the anti- β -ACTIN, which was diluted 1 : 100,000. Full length scans are presented as supplementary information. Note that some images were reflected for consistency in the sequence of presentation.

PRL, IGFBP1, and trypsin activity measurements. PRL and IGFBP-1 levels in the HESC culture media were determined using an amplified two-step sandwich-type immunoassay (R&D Systems, UK) according to the manufacturer's protocol. The Trypsin Activity Assay Kit (ABCAM) was used according to the manufacturer's instructions to measure trypsin activity in undiluted ECM and unconditioned culture medium (control). Trypsin activity in ECM was measured between day 4–6 of embryo development in 16 cultures (containing a total of 163 embryos). The ECM and unconditioned control medium were stored at -80°C until analysis.

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

Confocal microscopy and immunohistochemistry. Primary HESCs cultured on chamber slides were transfected with either siRNA targeting *HSPA8* or non-targeting siRNA and then either remained untreated or were decidualized with 8-Bromo-cAMP and MPA for 5 d. ER stress in control cultures was induced by treating cells with thapsigargin for 12 h (Sigma, UK). Cells were then fixed in 4% paraformaldehyde and permeabilized in 0.5% Triton. Primary antibodies, incubated overnight at 4°C in a humidified chamber (anti-LC3B 1 : 400). The secondary antibody used was labeled with Alexa Fluor® 488 (1 : 200; Invitrogen). Slides were mounted with proGOLD (Invitrogen) and stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Images were captured using a Leica SP5 II confocal microscope.

Confocal imaging of intracellular calcium ($[\text{Ca}^{2+}]_i$). Experiments were performed on Ishikawa cells cultured in glass bottomed 35 mm Petri dishes as described. For imaging of $[\text{Ca}^{2+}]_i$, cells were incubated for 40 min at room temperature in physiological saline solution (PSS) containing 5 μM Fluo-4/AM (Invitrogen, UK). Non-ionic detergent Pluronic F127 (0.025%, w/v) was included to aid the dye loading. After incubation with Fluo-4/AM, the cells were washed with PSS and the dish was mounted in a temperature-controlled environmental chamber on the stage of a confocal microscope (LSM 510 META, Carl Zeiss, UK). The cells were superfused with pre-warmed (35°C) Krebs solution for 20–30 min to ensure complete de-esterification of Fluo-4/AM. For image acquisition, perfusion was stopped and solution volume in the Petri dish adjusted to 200 μL . Fluo-4 fluorescence was excited using 488 nm line of argon ion laser. Images were recorded at 1 frame per second through the C-Apochromat 63 \times /1.20 W objective lens. Two time series were acquired from each Petri dish. During the first time series, baseline activity was recorded for 2 min, then 10 μL of conditioned embryo culture medium was added to the Petri dish and the recording continued for another 5 min. The second time series was acquired from the same viewing field after 10 min break. In control experiments, 10 μL of unconditioned instead of conditioned ECM was added. Experiments with trypsin and trypsin inhibitor were conducted in a similar manner. Trypsin (TRLS, Cat# LS003734) and soybean trypsin inhibitor (SI, Cat# LS003570) were purchased from Worthington Biochemical Corp., Lakewood, NJ, USA). Off-line image analysis was performed using ImageJ (NIH, <http://imagej.nih.gov/ij/>). Regions of interest (ROI) were drawn around each cell within the field of view. The Multi Measure function in the ImageJ ROI Manager was used to extract intensity profiles over time for each ROI. Intensity profiles were imported into Origin 8.5 (OriginLab Corporation, USA) for further processing, graphing and statistical analysis. Each trace was normalized to its corresponding baseline to yield a self-ratio trace (F/F₀). The $[\text{Ca}^{2+}]_i$ signals induced by embryo-conditioned media were quantified as area under the curve (AUC) calculated above the base line ($\Delta\text{F}/\text{F}_0$) for the first and the last 5 min time periods of the conditioned medium treatment.

Statistical analysis. Statistical analysis was performed by ANOVA with Bonferroni correction, Student *t*-test or Mann Whitney U test, as appropriate.

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Author contributions

J.J.B., M.S.S. and N.S.M. designed the experiments. M.S.S., G.T., S.S., E.S.L. and A.S. performed the *in vitro* experiments. J.N., J.H.S. and M.C. performed the *in vivo* studies and were assisted in the tissue analysis by S.S. and B.M.-J. Embryo culture media and clinical data collection were performed by G.T., C.M.B., C.J.H. and N.S.M. Microarray analysis was performed and the data interpreted by M.J.G.K. and F.C.P.H. RNA sequencing data were analyzed by Y.-W.C., E.S.L., S.S. and J.D.M. Confocal imaging of calcium was performed by A.S., who also analyzed the data. S.Q. and G.M.H. provided clinical resources and contributed to data interpretation. E.S.L. and J.J.B. prepared Figure 4. J.J.B. wrote the paper and M.S.S., J.N., S.S., E.S.L., M.C., Y.-W.C., G.M.H., S.S., B.M.-J., S.Q., M.J.G.K., F.C.P.H., N.S.M. and A.S. edited the manuscript.

Additional information

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