Article

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Table 1

Gene	Primer sequences (5'-3')
Cdx2	CAAGGACGTGAGCATGTATCC
	GTAACCACCGTAGTCCGGGTA
Gata3	CTCGGCCATTCGTACATGGAA
	GGATACCTCTGCACCGTAGC
Eomes	GCGCATGTTTCCTTTCTTGAG
	GGTCGGCCAGAACCACTTC
Hand1	CTACCAGTTACATCGCCTACTTG
	ACCACCATCCGTCTTTTTGAG
Gata2	CACCCCGCCGTATTGAATG
	CCTGCGAGTCGAGATGGTTG
Hes1	CCAGCCAGTGTCAACACGA
	AATGCCGGGAGCTATCTTTCT
Gapdh	AGGTCGGTGTGAACGGATTTG
	GGGGTCGTTGATGGCAACA
Ywhaz	TTGATCCCCAATGCTTCGCAA
	CAGCAACCTCGGCCAAGTAA

Figure 1

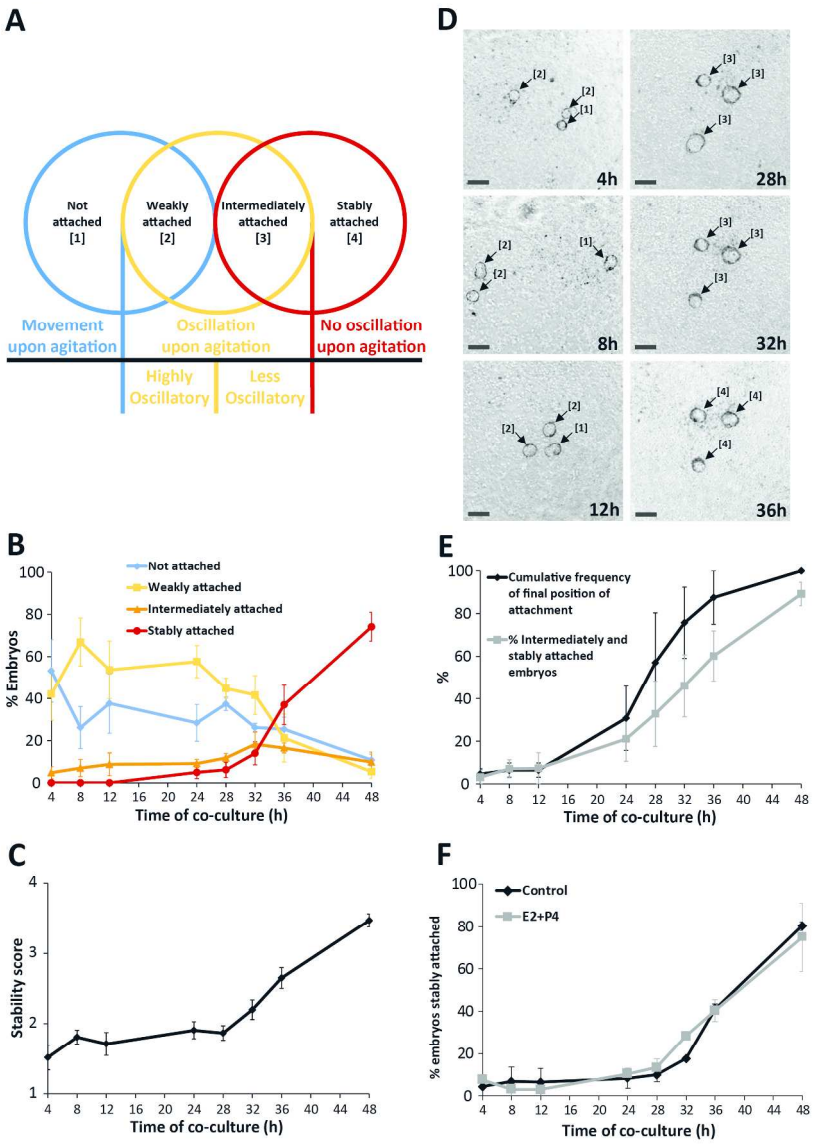

Figure 1 A Schematic to illustrate the criteria for the 4-point grading of the stability of mouse blastocyst attachment to Ishikawa cells; [1] = not attached, [2] = weakly attached, [3] = intermediately attached, [4] = stably attached. B Hatched E4.5 mouse blastocysts were co-cultured with Ishikawa cells for 48h and the stability of attachment was scored (1-4) every 4h from 0-12h, 24-36h, and at 48h. The mean attachment stability scores, from 4 independent experiments using 102 blastocysts, are presented. Error bars +/- standard error of the mean (SEM). C The mean attachment stability score was calculated from the data in B. Error bars +/- SEM. D Phase contrast images of the mouse blastocyst-Ishikawa cell co-culture at 4h, 8h, 12h, 28h, 32h and 36h. Blastocysts are indicated by arrows and the attachment score for each blastocyst is displayed. Scale bars 200μm. E The cumulative frequency of the time at which the final position of attachment was reached was plotted alongside the percentage of blastocysts scored as intermediately or stably attached (mean +/- SEM from 3 independent experiments using 84 blastocysts). F Ishikawa cells were pre-treated with 10nM 17β-estradiol 48h prior to co-culture, then with 10nM 17β-estradiol and 1μM progesterone 24h prior to co-culture and during co-culture with hatched E4.5 mouse blastocysts. The

attachment stability of mouse blastocysts was scored every 4h from 0-12h, 24-36h, and at 48h. Blastocysts scored as intermediately and stably attached were added together and the mean from 3 independent experiments using 72 blastocysts was plotted +/- SEM.

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Figure 2

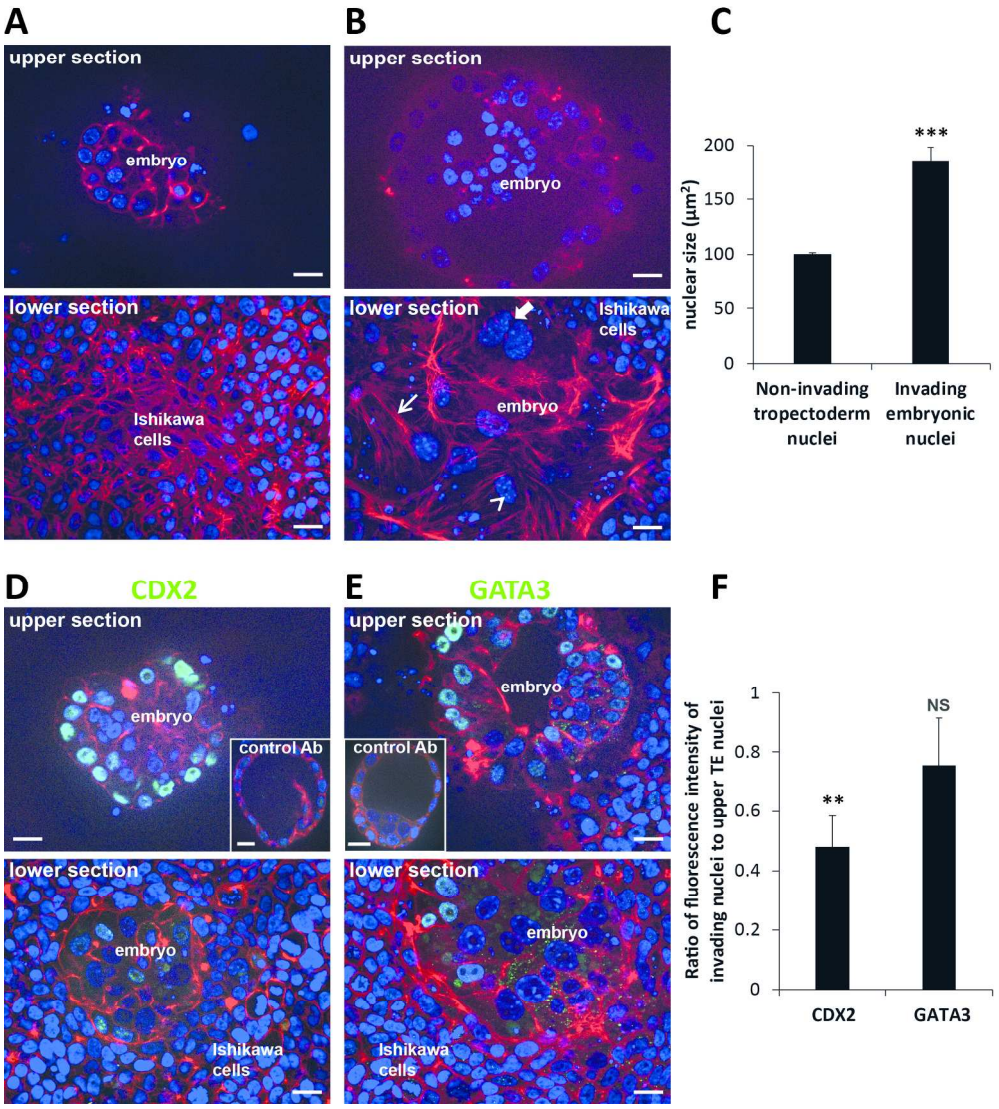

Figure 2 Attached embryos not breaching (A) and breaching (B) Ishikawa cell layers were optically sectioned after PFA fixation and incubation with phalloidin to label actin fibres (red) and DAPI to label nuclei (blue). Invasive cells of breaching embryos exhibit binuclearity (closed arrow), prominent nucleosomes (arrowhead), and actin stress fibres (open arrow). Upper/lower section refers to depth of the optical section in the Z-axis. Scale bars 20µm. C The size of invasive embryonic nuclei in the lower optical section was measured and compared to the size of outer, peripheral embryonic nuclei, presumed to be TE and not inner cell mass, in the corresponding upper section. 309 nuclei were measured in 12 embryos and mean nuclear size was plotted +/- SEM. *** t-test $p<0.001$. Breaching embryos were fixed and labelled with anti-CDX2 antibody (D), rabbit isotype control antibody (D inset), anti-GATA3 antibody (E), or mouse isotype control antibody (E inset), as well as phalloidin and DAPI. Upper/lower section refers to depth of the optical section in the Z-axis. Scale bars 20µm. F The intensity of CDX2 and GATA3 fluorescence labelling was measured in invasive embryonic nuclei in the lower optical section and compared to that of outer embryonic nuclei, presumed to be TE and not inner cell mass, in the corresponding upper section. 158 nuclei were measured in 5 embryos labelled with anti-CDX2 and 226 nuclei were measured in 5 embryos labelled with anti-GATA3. The mean ratio of invasive nuclei to upper TE nuclei fluorescence intensity was plotted +/- SEM. ** t-test

p<0.01, NS not significant.

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Figure 3

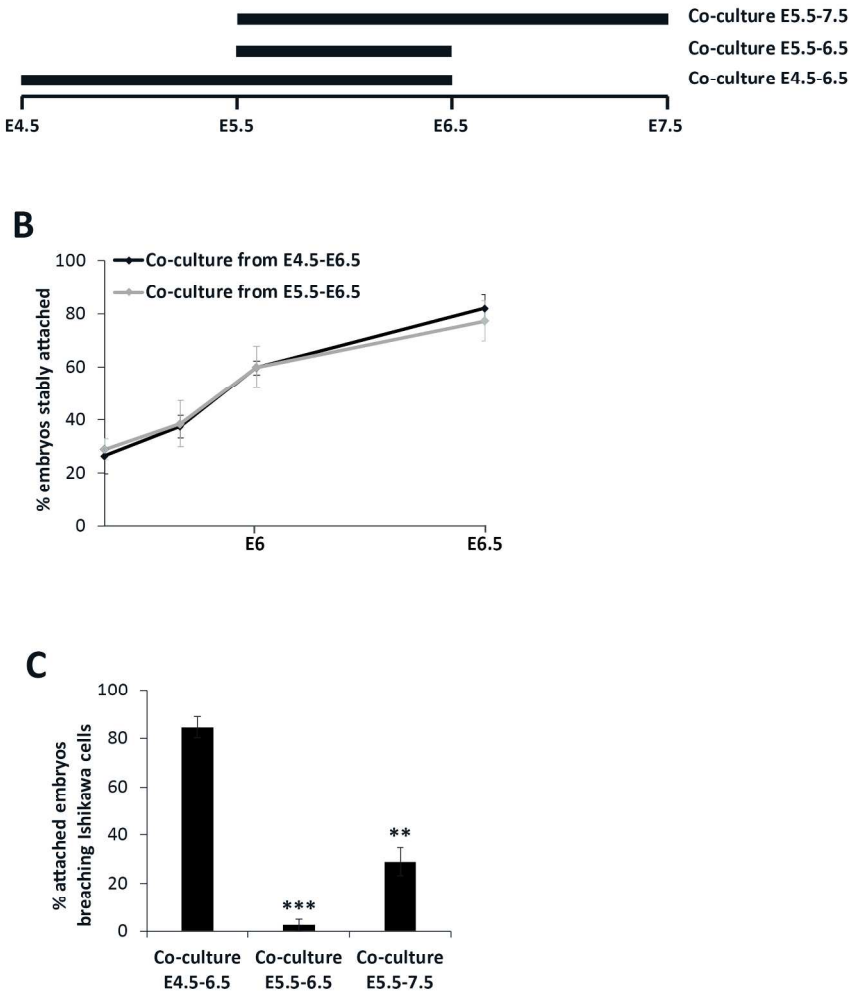

Figure 3 A Schematic representing embryo-Ishikawa cell co-culture experimental design. B Hatched E4.5 mouse blastocysts were either co-cultured with Ishikawa cells to E6.5 or cultured alone in co-culture media to E5.5 before co-culture with Ishikawa cells to E6.5 or E7.5. The stability of attachment was scored every 4h from E5.5-6 and at E6.5. Mean percentage blastocysts scored as intermediately or stably attached from 6 independent experiments using 149 blastocysts was plotted +/- SEM. C Attached embryos co-cultured from E4.5-6.5, E5.5-6.5 and E5.5-7.5, were fixed, labelled with phalloidin and DAPI and optically sectioned to determine whether embryonic cells had breached the Ishikawa cells. The mean proportion of attached embryos to have breached the Ishikawa cells was plotted +/- SEM. ** ANOVA $p<0.01$, *** ANOVA $p<0.001$, demonstrating significant difference from co-culture E4.5-6.5.

209x234mm (300 x 300 DPI)

Figure 4

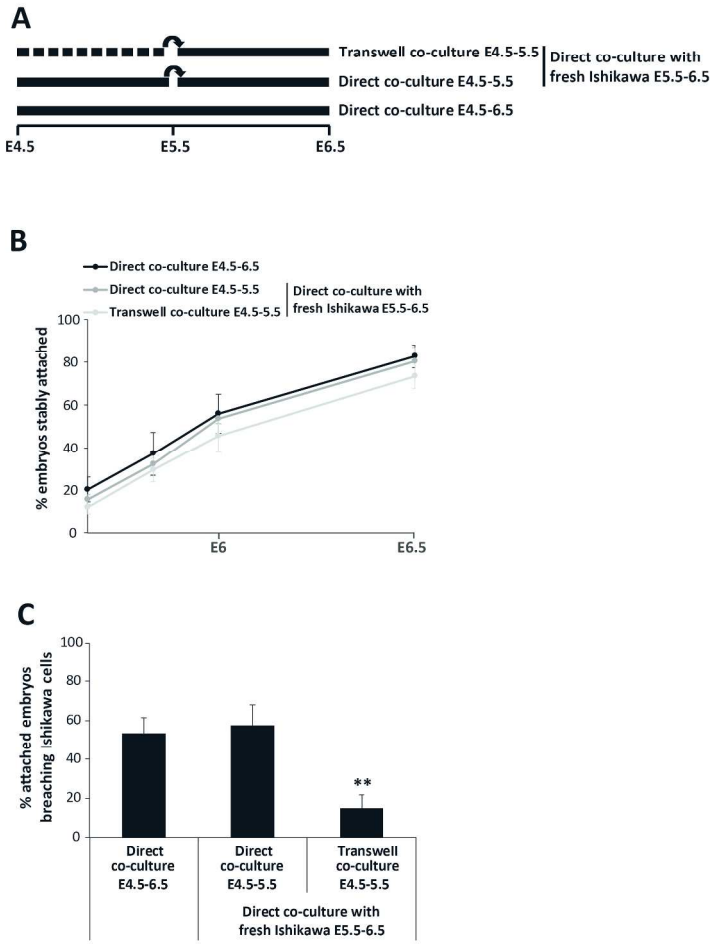

Figure 4 A Schematic representing embryo-Ishikawa cell co-culture experimental design. B Hatched E4.5 mouse blastocysts were co-cultured directly with Ishikawa cells from E4.5-6.5, co-cultured directly with Ishikawa cells to E5.5 before transfer to fresh Ishikawa cells and co-cultured to E6.5, or co-cultured in a transwell above Ishikawa cells to E5.5 before transfer to fresh Ishikawa cells and co-cultured to E6.5. The stability of attachment was scored every 4h from E5.5-6 and at E6.5. Mean percentage blastocysts scored as intermediately or stably attached from 5 independent experiments using 157 blastocysts was plotted +/- SEM. C Attached embryos were fixed, labelled with phalloidin and DAPI and optically sectioned to determine whether embryonic cells have breached the Ishikawa cells. The mean proportion of attached embryos to have breached the Ishikawa cells +/- SEM. ** ANOVA $p<0.01$, demonstrating significant difference from direct co-culture E4.5-6.5.

254x250mm (300 x 300 DPI)

Figure 5

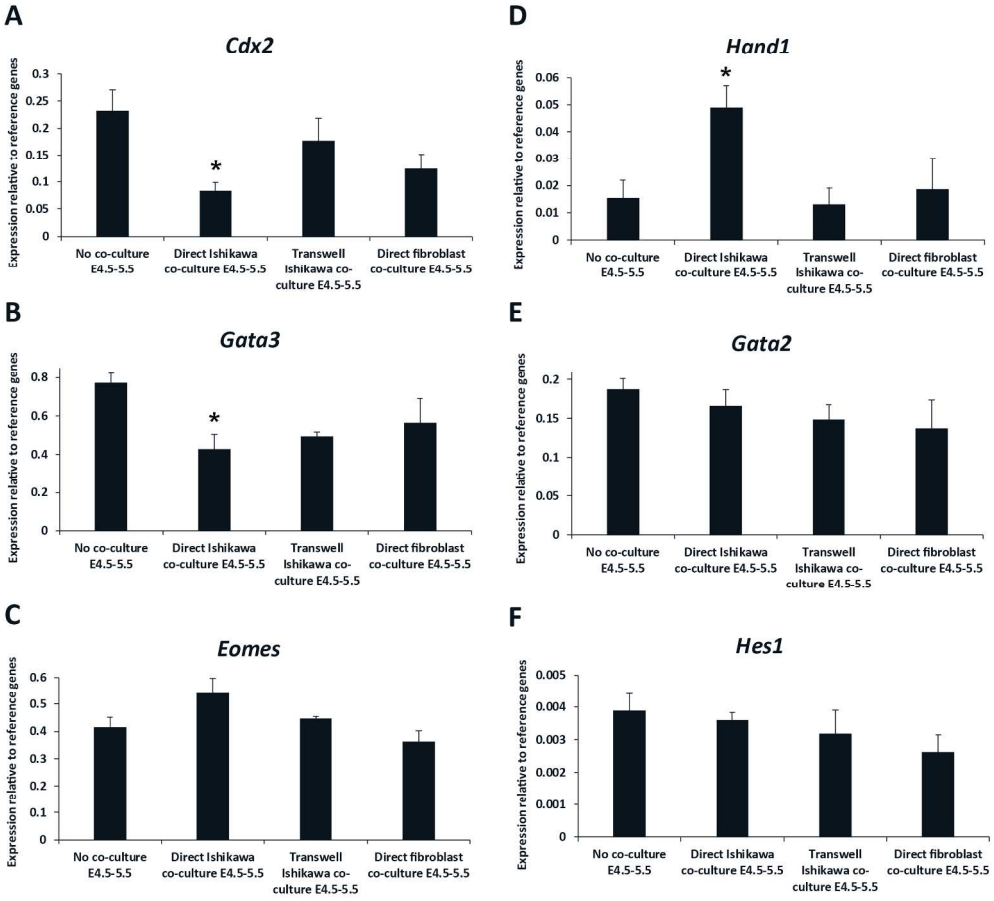

Figure 5 Hatched E4.5 mouse blastocysts were cultured alone, co-cultured directly with Ishikawa cells, co-cultured in a transwell above Ishikawa cells or co-cultured directly with human fibroblasts as a control cell type, to E5.5 before retrieval and lysis for RNA isolation and gene expression analysis by qPCR. Data is presented as expression relative to reference genes (2-ct, relative to geometric mean of Gapdh and Ywhaz) of A Cdx2, B Gata3, C Eomes, D Hand1, E Gata2, and F Hes1 in embryos cultured in indicated conditions (mean +/- SEM of 5 independent experiments using 200 embryos). * ANOVA $p<0.05$, demonstrating significant difference from no co-culture E4.5-5.5.

207x198mm (300 x 300 DPI)

Apposition to endometrial epithelial cells activates mouse blastocysts for implantation

Running title: Blastocyst apposition to endometrial cells

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Abstract

Study question: How do interactions between blastocyst-stage embryos and endometrial epithelial cells regulate the early stages of implantation in an in-vitro model?

Summary Answer: Mouse blastocyst apposition with human endometrial epithelial cells initiates trophectoderm differentiation to trophoblast, which goes on to breach the endometrial epithelium.

What is known already: In-vitro models using mouse blastocysts and human endometrial cell lines have proven invaluable in the molecular characterisation of embryo attachment to endometrial epithelium at the onset of implantation. Genes involved in embryonic breaching of the endometrial epithelium have not been investigated in such in-vitro models.

Study design, size, duration: This study used an established in-vitro model of implantation to examine cellular and molecular interactions during blastocyst attachment to endometrial epithelial cells.

Participants/materials, setting, methods: Mouse blastocysts developed from embryonic day (E) 1.5 in vitro were hatched and co-cultured with confluent human endometrial adenocarcinoma-derived Ishikawa cells in serum-free medium. A scale of attachment stability based on blastocyst oscillation upon agitation was devised. Blastocysts were monitored for 48h to establish the kinetics of implantation, and optical sectioning using fluorescence microscopy revealed attachment and invasion interfaces. Quantitative PCR was used to determine blastocyst gene expression. Data from a total of 680 mouse blastocysts are reported, with 3-6 experimental replicates. T-test and ANOVA analyses established statistical significance at $p<0.05$, $p<0.01$ and $p<0.001$.

Main results and the role of chance: Hatched E4.5 mouse blastocysts exhibited weak attachment to confluent Ishikawa cells over the first 24h of co-culture, with intermediate and stable attachment occurring from 28h (E5.5+4h) in a hormone-independent manner.

Attached embryos fixed after 48h (E6.5) frequently exhibited outgrowths, characterised morphologically and with antibody markers as trophoblast giant cells (TGCs), which had breached the Ishikawa cell layer. Beginning co-culture at E5.5 also resulted in intermediate and stable attachment from E5.5+4h, however these embryos did not go on to breach the Ishikawa cell layer, even when co-culture was extended to E7.5 ($p<0.01$). Blastocysts cultured from E4.5 in permeable transwell inserts above Ishikawa cells before transfer to direct co-culture at E5.5 went on to attach but failed to breach the Ishikawa cell layer by E6.5 ($p<0.01$). Gene expression analysis at E5.5 demonstrated that direct co-culture with Ishikawa cells from E4.5 resulted in downregulation of trophectoderm transcription factors *Cdx2* ($p<0.05$) and *Gata3* ($p<0.05$) and upregulation of the trophoblast giant cell transcription factor *Hand1* ($p<0.05$). Co-culture with non-endometrial human fibroblasts did not alter the expression of these genes.

Large scale data: N/A

Limitations, reasons for caution: The in-vitro model used here combines human carcinoma-derived endometrial cells with mouse embryos, in which the cellular interactions observed may not fully recapitulate those in-vivo. The data gleaned from such models can be regarded as hypothesis-generating, and research is now needed to develop more sophisticated models of human implantation combining multiple primary endometrial cell types with surrogate and real human embryos.

Wider implications of the findings: This study implicates blastocyst apposition to endometrial epithelial cells as a critical step in trophoblast differentiation required for implantation. Understanding this maternal regulation of the embryonic developmental programme may lead to novel treatments for infertility.

Study funding and competing interest(s): This work was supported by funds from the charities Wellbeing of Women (RG1442) and Diabetes UK (15/0005207), and studentship support for SCB from the Anatomical Society. No conflict of interest is declared.

Keywords: implantation, endometrium, embryo development, trophoblast, transcription factors

Introduction

Implantation begins with attachment of the trophectoderm (TE) of a blastocyst-stage embryo to the hormonally-responsive receptive endometrial luminal epithelium (LE), followed by breaching of this barrier and invasion of TE-derived trophoblast into the underlying decidua (Aplin & Kimber 2004, Aplin & Ruane 2017). Implantation failure remains a bottleneck in human assisted reproduction treatment, with only ~25% of treatment cycles resulting in a live birth (Ferraretti *et al.* 2012), despite efforts to select developmentally-competent embryos and receptive endometrium (Brison *et al.* 2004, Aplin 2006, Dominguez *et al.* 2008, Glujovsky *et al.* 2012, Ruiz-Alonso *et al.* 2013, Salamonsen *et al.* 2013, Armstrong *et al.* 2015, Harbottle *et al.* 2015, Simon *et al.* 2015). A comprehensive understanding of this founding stage of pregnancy is necessary to improve treatments for infertility.

Initial maternal-embryo interactions are mediated through the trophic and paracrine action of uterine fluid, secreted by uterine glands, which promotes embryo development to blastocyst and subsequent implantation (Filant & Spencer 2014). There is now considerable evidence that bidirectional signalling between human blastocysts and endometrium elicits responses which modulate implantation receptivity (Evans *et al.* 2016). For example, blastocyst-dependent local reduction in the anti-adhesive glycocalyx of the LE (Meseguer *et al.* 2001), and LE microRNA regulation of TE gene expression (Vilella *et al.* 2015), are suggested to promote embryo attachment. Molecular dialogue has been observed upon blastocyst apposition to the endometrium in mice, with both paracrine and juxtacrine activation of ErbB4 by soluble and membrane-bound HB-EGF, respectively, promoting expression of integrins at the surface of LE and TE, leading to blastocyst attachment and invasion (Wang *et al.* 2000, Wang *et al.* 2002). In humans, the homophilic receptor, trophinin, may feed into the HB-EGF axis at apposition to promote TE proliferation in readiness for invasion while

triggering LE apoptosis to allow epithelial breaching (Sugihara *et al.* 2007, Tamura *et al.* 2011).

After blastocyst attachment to the LE in humans and rodents, embryonic invasion results in haemochorial placentation (Rossant & Cross 2001). The initial invasive cell type in the mouse embryo is the trophoblast giant cell (TGC) (Dickson 1963), whereas in human and macaque this is thought to be primary syncytium (Hertig *et al.* 1956, Enders 2007). Significant progress has been made in understanding the regulatory networks governing the formation of these cell types (Knott & Paul 2014). Recently trophoblast lineages have been shown to arise from embryos attaching to culture surfaces in the absence of maternal cells (Bedzhov & Zernicka-Goetz 2014, Deglincerti *et al.* 2016, Shahbazi *et al.* 2016), however the contribution of endometrial-embryo interactions to the development of the pioneering invasive embryonic cells remains unknown.

Characterisation of this early stage of implantation is particularly tractable in vitro. The human endometrial adenocarcinoma Ishikawa cell line (Nishida *et al.* 1985), offers a model epithelial system for investigating interactions with rodent and human embryos (Singh *et al.* 2010, Kaneko *et al*. 2011, Kang *et al.* 2014). Ishikawa cells exhibit moderate epithelial polarisation and surface glycoprotein composition comparable with LE in vivo (Heneweer *et al*. 2005, Singh & Aplin 2014, Buck *et al*. 2015). They also mount transcriptional responses to estrogen and progesterone, but do not require steroid hormones for receptivity to mouse embryos (Lessey *et al.* 1996, Castelbaum *et al.* 1997, Singh *et al.* 2010, Tamm-Rosenstein *et al.* 2013). Ishikawa cell-rodent embryo co-cultures are recognised as a useful model to investigate molecular pathways of attachment (Kaneko *et al*. 2011, Kaneko *et al.* 2012, Kang *et al.* 2014, Green *et al.* 2015, Kang *et al.* 2015), however a thorough dissection of attachment and invasion in this model system has not been performed.

Here, we have characterised the kinetics of attachment and invasion of mouse embryos on Ishikawa cells. We show that prior to stable attachment, apposition is required for

subsequent TGC invasion. Moreover, apposition leads to changes in embryonic gene expression consistent with TE differentiation to invasive TGCs. Our data suggests that the differentiation of trophoblast required for implantation of mouse blastocysts is maternally regulated, and implicates a conserved system in human LE.

Materials and Methods

Cell culture

Ishikawa cells (ECACC 99040201) and primary human foreskin fibroblasts were maintained at 37ºC, 95% air and 5% CO_2 in growth medium (1:1 Dulbecco's modified Eagle's medium:Ham's-F12 (Sigma) containing 10% fetal bovine serum (Sigma) supplemented with 2mM L-glutamine, 100µg/ml streptomycin and 100IU/ml penicillin (Sigma)). Cells were cultured to confluency in 24-well plates (Greiner) on 13mm glass coverslips coated with 2% growth factor-reduced Matrigel (Sigma).

Mouse embryos

All experiments were conducted and licensed under the Animal Act, 1986, and had local ethical approval for care and use of laboratory animals and standards of humane animal care. CD1 strain mice (Charles River) were maintained by the Biological Services Unit at the University of Manchester and kept under standard environmental conditions of 12h light and 12h dark at 20–22°C and 40–60% humidity with food and water provided *ad libitum*. 8-10-week old female mice were superovulated by intraperitoneal injection with 5IU pregnant mare serum gonadotrophin (Intervet), followed by 5IU human chorionic gonadotrophin (Intervet) 46h later, then housed overnight with ≤9-month-old stud males for mating. Midday the following day was designated embryonic day (E) 0.5. Embryos were collected at E1.5 by flushing dissected oviducts with M2 medium (Millipore) containing 0.4% w/v BSA (Sigma). All embryo manipulation was performed using a Flexipet with 140µm and 300µm pipettes (Cook). E1.5 embryos were cultured for 72h in KSOM medium (Millipore) containing 0.4%

BSA at 37ºC, 95% air and 5% CO_2. E4.5 blastocysts were hatched from the zona pellucida (ZP) using acid Tyrode's (pH 2.5) (Sigma).

In-vitro implantation model

Ishikawa cells were grown to full confluence in 24-well plates, washed and replenished with serum-free co-culture medium (1:1 DMEM:F12 supplemented with 2mM L-glutamine, 100 µg/ml streptomycin and 100IU/ml penicillin) 24h before transfer of three hatched mouse blastocysts per well and co-culture at 37ºC, 95% air and 5% CO_2. The stability of mouse blastocyst attachment to Ishikawa cells was assessed using a four-point scale of blastocyst behaviour upon agitation of the sample; translocation (not attached), major oscillation about an attachment point (weakly attached), minor oscillation (intermediately attached), and no oscillation (stably attached) (Figure 1A, Movie 1) (Kang *et al.* 2014, Kang *et al.* 2015). The stability of blastocyst attachment was assessed at 4h intervals from 0-12h, 24-36h and 48h using an inverted phase contrast microscope (Evos). At termination, co-cultures were washed in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) in PBS for 20 minutes. For hormone treatments, Ishikawa cells were maintained in growth medium containing 10nM 17β-estradiol (Sigma) for 24h, then co-culture medium containing 10nM 17β-estradiol and 1µM progesterone (Sigma) for a further 24h, before addition of embryos. To separate embryos from Ishikawa cells during co-culture, 3µm transwell filters (Costar) were used. Blastocysts were collected from co-cultures and transwells using a 300µm Flexipet.

Fluorescence staining and microscopy

Fixed attached embryo samples were washed with PBS and quenched with 50mM ammonium chloride solution before permeabilisation with 0.5% Triton-X100 PBS. Samples were incubated with primary antibody (rabbit anti-CDX2 or mouse anti-GATA3, both Cell Signalling Technologies) in PBS for 2h or overnight, followed by alexa568-phalloidin (Life Technologies), 4',6-diamidino-2-phenylindole (DAPI) (Sigma), and alexa488 secondary

antibodies (Life Technologies) for 1h before mounting in a chamber of 3% 1,4-diazabicyclo[2.2.2]octane (Sigma) in PBS. Fluorescence microscopy was carried out using a Zeiss Axiophot microscope equipped with an Apotome module for optical sectioning. Images were analysed and processed using Zeiss Zen software and ImageJ.

Blastocyst RNA extraction and quantitative polymerase chain reaction

RNA from 10 blastocysts per treatment was isolated using the RNeasy Micro Kit (Qiagen), according to the manufacturer's instructions. Samples of 25ng RNA were added to reverse transcription (RT) reactions with random 9mer primers (Agilent) using the Sensiscript RT kit (Qiagen), according to the manufacturer's instructions. Quantitative polymerase chain reactions (qPCR) were carried out using the RT reactions along with 0.25μM primers (Table 1) and QuantiTect SYBR green PCR kit (Qiagen). qPCRs were run on a Stratagene Mx3000p machine with thermocycle parameters according to QuantiTect instructions (35 cycles using 58ºC annealing temperature for all primers), and analysed with Stratgene MxPro to yield cycle threshold (Ct) values. RT reactions without sample RNA and without reverse transcriptase enzyme were used as controls in qPCR reactions with all primer pairs, and all yielded no Ct value. Dissociation curves were run with each sample to rule out the presence of non-specific PCR products.

Statistical Analysis

Independent t-test and ANOVA with Dunnett's post-hoc test were performed using SPSS (IBM), with significance at $p<0.05$.

Results

E4.5 mouse blastocysts weakly and reversibly attach to Ishikawa cells over 24h before stably attaching in a hormone-independent manner

E4.5 mouse blastocysts hatched from the ZP barrier were introduced into co-culture with confluent Ishikawa cells in serum-free medium and monitored at 4h intervals from 0-12h, 24-

36h, and at 48h, to characterise the kinetics of attachment (Figure 1B). Only weak attachment was observed during the first 24h of co-culture (E4.5-5.5), with intermediate and stable attachment increasing from 28-48h (E5.5+4h-E6.5), as demonstrated by plotting the average stability of attachment at each time point (Figure 1C). Weak attachment was reversible since blastocysts cycled between weakly attached and not attached, and positional analysis demonstrated that weak attachment could occur at multiple successive sites (Figure 1D). Later attached blastocysts did not change position over time (Figure 1D), and cumulative frequency of the final position of blastocyst attachment correlated with intermediate and stable attachment scores (Figure 1E). These scores of attachment stability therefore represent irreversible attachment, with the lag between final position of attachment and intermediate-stable attachment likely reflecting a weak attachment phase prior to more stable attachment.

This process of prolonged, reversible weak attachment followed by initiation of stable attachment after 28h co-culture appears to mirror the apposition and attachment stages, respectively, of implantation in vivo (Enders & Schlafke 1969). Thus, the summation of intermediate and stable attachments was used for all subsequent analyses. Additionally, the kinetics of attachment were not altered by treating Ishikawa cells with estradiol and progesterone (Figure 1F), therefore further experiments were performed in the absence of steroid hormones.

Mouse blastocysts produce trophoblast giant cells to breach Ishikawa cell layers

Blastocysts attached to Ishikawa cells were fixed after 48h co-culture, fluorescently labelled and optically sectioned to visualise the interface. Although embryos that had attached to the apical surface of the Ishikawa cells but not breached the cell layer were observed (Figure 2A), outgrowths from attached embryos that had breached the Ishikawa cell layer were frequently seen (Figure 2B).

These outgrowths contained spread cells with large nuclei, clear nucleoli (arrowheads) and prominent actin stress fibres (thin arrows), and were occasionally bi- and tri-nucleate (thick arrows), reminiscent of trophoblast giant cells (TGCs) (Figure 2B). When compared to the TE nuclei above the plane of the Ishikawa cells (nuclei on the periphery of the embryo), the nuclei of embryonic cells invading into the Ishikawa cell layer were significantly larger (Figure 2C). Moreover, antibodies to the TE transcription factors CDX2 and GATA3, which are downregulated upon trophoblast differentiation to TGCs (Knott & Paul 2014), appeared to preferentially label the upper, non-invasive TE nuclei (Figure 2D, E), although only CDX2 labelling exhibited significantly lower intensity in the invasive nuclei (Figure 2F). Altogether, these observations demonstrate that mouse embryos can breach and invade Ishikawa cell layers and implicate TGCs in this process.

Co-culture from E4.5-5.5 is required for embryos to progress beyond attachment to breach the Ishikawa cell layer

We asked whether early weak attachments influence later embryo-Ishikawa interactions by comparing the attachment kinetics of blastocysts cultured with or without Ishikawa cells during E4.5-5.5 (Figure 3A). Embryos without prior co-culture were still able to stably attach to Ishikawa cells during E5.5-E6.5 (Figure 3B), though such embryos exhibited very few breaching events (Figure 3C). Notably, continued co-culture of these embryos to E7.5 still did not yield the levels of breaching observed for embryos co-cultured from E4.5-6.5 (Figure 3C). This suggests that E4.5-5.5 blastocysts require a dialogue with Ishikawa cells in order to breach the Ishikawa cell layer at a later embryonic stage.

Physical apposition with Ishikawa cells from E4.5-5.5 promotes subsequent embryonic breaching

To characterise the dialogue between embryos and Ishikawa cells, we employed a separated co-culture system. Blastocysts were incubated in permeable transwell inserts above Ishikawa cells to allow paracrine crosstalk but prevent direct contact during E4.5-5.5.

The capacity of these embryos to attach and breach after transfer to fresh Ishikawa cells at E5.5 was compared to embryos which were co-cultured directly with Ishikawa cells from E4.5 before collection (all embryos, non-attached and attached, were collected) and transfer to fresh Ishikawa cells at E5.5 (Figure 4A). The E5.5-6.5 attachment kinetics of the two groups were indistinguishable and did not differ from a control group of embryos co-cultured directly with Ishikawa cells from E4.5-6.5 without transfer to fresh Ishikawa cells at E5.5 (Figure 4B). Conversely, compared to embryos in direct co-culture during E4.5-5.5, significantly fewer embryos co-cultured in transwells from E4.5-5.5 went on to breach Ishikawa cells at E6.5 (Figure 4C). These data rule out priming of Ishikawa cells by embryos from E4.5-5.5 as a mechanism contributing embryonic invasion, and suggest that physical apposition with Ishikawa cells from E4.5-5.5 activates blastocysts for invasion.

Apposition from E4.5-5.5 induces a TGC differentiation program in the embryo

The cells forming the embryonic outgrowths at Ishikawa cell breach sites bore hallmarks of TGCs (Figure 2), leading us to hypothesise that apposition during E4.5-5.5 initiates a TGC differentiation program in the blastocyst TE. Therefore, we assessed the expression of transcription factors associated with TGC differentiation (Simmons & Cross 2005, Liu *et al.* 2009, Knott & Paul 2014), in E5.5 embryos that had not been co-cultured, had been directly or indirectly co-cultured with Ishikawa cells for 24h, or had been directly co-cultured with human foreskin fibroblasts as an alternative cell type. We found significant changes in the expression of the TE transcription factors *Cdx2* and *Gata3* (0.36- and 0.55-fold downregulation, respectively) and the TGC transcription factor *Hand1* (upregulated 3.17-fold), but only in blastocysts that had been directly co-cultured with Ishikawa cells from E4.5-5.5 (Figure 5A, B, D, respectively). No change in the expression of other TE (*Eomes*) or TGC transcription factors (*Gata2* and *Hes1*) was detected (Figure 5C, E, F, respectively). Other TGC markers, such as *Bhlhe40*, *Mdfi* and *Prl3d1*, were not detected at this stage of embryonic development (data not shown).

Discussion

Dialogue between the embryo and endometrium is necessary to orchestrate implantation in both mouse and human (Wang & Dey 2006). Here, observations in an established in-vitro model of implantation lead to the proposal that blastocyst apposition with LE initiates changes in embryonic gene expression which result in the differentiation of TE to invasive trophoblast. Such maternal regulation of embryonic differentiation constitutes a novel and potentially critical stage in embryo development; a detailed mechanistic understanding of this process could offer targets for the treatment of infertility.

Combining a categorical scale of mouse blastocyst attachment stability with microscopic analysis to determine embryonic breaching of the Ishikawa cell layer has revealed progressive stages of weak reversible attachment, stable irreversible attachment and subsequent breaching of the epithelium. The rates of stable attachment and breaching in our in-vitro model correlate with in-vitro-matured blastocyst implantation rates in CD1 mice invivo (Schwarzer *et al*., 2012; Hemkemeyer *et al*., 2014). Kinetic analysis demonstrated that in-vitro-matured, chemically hatched mouse blastocysts are competent for stable attachment to Ishikawa cells between E5.5 and E6.5, independent of prior co-culture. These findings are consistent with those of a previous study which found that in-vivo-matured, naturally hatched mouse blastocysts co-cultured with Ishikawa cells from E4.5-6.5 attached only after E5.5 and did not require prior co-culture (attachment was scored using a binary scale in the face of medium aspiration by pipette, likely to identify only intermediate and stably attached embryos) (Green *et al.* 2015). Our data also imply that Ishikawa cells are constitutively receptive to mouse blastocyst attachment, as neither co-culture with blastocysts prior to E5.5 nor hormonal stimulation was required to induce an adhesive Ishikawa cell phenotype. Increased mouse blastocyst stable attachment to Ishikawa cells in response to estrogen and progesterone has been reported (Singh *et al.* 2010), however subsequent studies have not used hormones to induce receptivity to rodent and human embryos (Kaneko *et al.* 2011, Kang *et al.* 2014, Green *et al.* 2015). The steroid hormone-mediated shift from non-receptive

to receptive endometrium seen in vivo is not replicated by Ishikawa cells in these culture conditions, despite expression of estrogen and progesterone receptors and responsiveness to steroid hormones at the level of gene expression (Tamm-Rosenstein *et al.* 2013).

The reversible weak attachment we observed from E4.5-5.5 may mimic the apposition stage of mouse implantation in vivo, which is thought to occur from E4-5 and entail embryo-maternal dialogue leading to locally adhesive LE (Cha & Dey 2014, Aplin & Ruane 2017). Our finding that apposition is required for embryonic breaching of Ishikawa cells suggests that juxtacrine signalling prior to attachment promotes trophoblast penetration of the LE. In contrast, apposition from E4.5-5.5 is not necessary for stable attachment to be achieved in the period from E5.5-6.5. This also suggests that initiating TE differentiation to invasive trophoblast is not required for blastocyst attachment. It is possible that LE-derived signals, such as HB-EGF, trophinin and microRNA hsa-mIR-30d (Wang et al. 2002, Sugihara et al. 2007, Vilella et al. 2015), promote stable attachment just prior to and during the process. Importantly, this model shows that the attachment competence of mouse blastocysts is regulated during the window of attachment but progress to invasion requires maternal input during the developmental window prior to attachment.

We characterised the invasive embryonic cells in our model as TGCs, as they were occasionally bi-/tri-nucleate, exhibited large nuclei with prominent nucleoli, contained pronounced actin stress fibres, and had reduced CDX2 levels (Simmons *et al.* 2007). This mimics the pioneering invasive trophoblast observed in vivo (Dickson 1963), indicating that mouse embryos respond to human Ishikawa cells in ways that resemble implantation in vivo. Moreover, the finding that Ishikawa cells, but not human fibroblasts, can induce specific changes in embryo gene expression suggests that human and mouse LE may share some phenotypic properties, and that Ishikawa cells retain receptive LE-like functions despite being derived from an endometrial adenocarcinoma (Nishida *et al.* 1985).

The mechanism of breaching the Ishikawa cells is not yet clear, but our observations are suggestive of an initial narrow penetration, possibly between cells, followed by trophoblast outgrowth into the surrounding cell layer. LE apoptosis at the embryo attachment site has been proposed as a mechanism of LE penetration in mouse and human (Parr *et al.* 1987, Galan *et al.* 2000, Tu *et al.* 2015), and a recent in-vivo study in mice suggested entosis of LE cells by TE as the mechanism of penetration (Li *et al.* 2015). We did not detect clear morphological signs of either process at sites of embryonic breaching, however induction of TE differentiation to TGC by apposition with LE is not incompatible with these mechanisms. Furthermore, LE induction of TE differentiation to invasive trophoblast recalls human LE-TE interactions from in-vitro studies, whereby homophilic trophinin engagement promotes TE invasion by concomitantly driving LE apoptosis and TE proliferation (Sugihara *et al.* 2007, Tamura *et al.* 2011).

Our investigation implicates the TE differentiation to TGC induced by apposition to LE as a critical step in mouse embryo implantation. Rodent trophoblast differentiation has been widely studied, particularly using blastocyst-derived trophoblast stem cells (TSCs), and transcription factor hierarchies that lead to distinct trophoblast lineages have been determined (Simmons & Cross 2005). *Cdx2*, *Gata3* and *Eomes* are essential to the first lineage allocation to TE, and are associated with TSC-like states, as their downregulation is required for differentiation into all trophoblast lineages (Guzman-Ayala *et al.* 2004, Ralston *et al.* 2010). TGC differentiation from TE/TSCs also requires upregulation of *Hand1*, *Bhlhe40* and *Mdfi* (Cross *et al.* 1995, Kraut *et al.* 1998, Scott *et al.* 2000, Hughes *et al.* 2004). We detected downregulation of both *Cdx2* and *Gata3*, and upregulation of *Hand1* in E5.5 mouse blastocysts specifically after apposition with Ishikawa cells. In addition, anti-CDX2 antibody staining was significantly reduced in embryonic cells breaching the Ishikawa cell layer. We propose that a maternally-derived juxtacrine signal impinges on TE gene expression during apposition to downregulate TSC-like transcription factors and upregulate *Hand1*. We could

not detect *Bhlhe40* or *Mdfi*, suggesting that *Hand1* is an early, maternally-regulated TGC transcription factor.

In humans, the pioneering invasive trophoblast at implantation is thought to be primary syncytium (Hertig *et al.* 1956, Aplin & Ruane 2017), the formation of which is controlled by the expression of the syncytium regulator, transcription factor GCM1 (Yu *et al.* 2002, Liang *et al.* 2010). The existence of an Ishikawa cell-derived signal that promotes mouse TE differentiation in this in-vitro model implicates maternally-regulated induction of trophoblast differentiation as an important mechanism in human embryo implantation. Understanding such events may lead to the development of novel treatments for implantation failure in human ART.

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Authors' roles

PTR, SJK, DRB, MW and JDA designed the study, and SJK, DRB, MW and JDA obtained funding. PTR, SCB, RK and JW carried out the experimental work. PTR wrote the paper, which was edited by SJK, DRB, MW and JDA.

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Conflict of interest

We declare no conflicts of interest.

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Table, movie and figure legends

Table 1 Mouse gene names and corresponding forward and reverse primer sequences. Primer sequences were obtained from PrimerBank (Spandidos et al. 2010), with the following references: *Cdx2* 31560722a1; *Gata3* 6679951a1; *Eomes* 26354683a1; *Hand1* 118130896c3; *Gata2* 226530725c1; *Hes1* 6680205a1; *Gapdh* 126012538c1; *Ywhaz* 359385697c3.

Movie 1 Differential interference contrast imaging at 14 frames per second of E5.5+4h mouse embryos after 28h co-culture with Ishikawa cells exhibiting, upon agitation, weak attachment (major oscillation about an attachment point, left embryo), intermediate attachment (minor oscillation, right embryo) and stable attachment (no oscillation, middle embryo) to Ishikawa cells. Scale bar 100μm.

Figure 1 A Schematic to illustrate the criteria for the 4-point grading of the stability of mouse blastocyst attachment to Ishikawa cells; [1] = not attached, [2] = weakly attached, [3] = intermediately attached, [4] = stably attached. **B** Hatched E4.5 mouse blastocysts were co-cultured with Ishikawa cells for 48h and the stability of attachment was scored (1-4) every 4h from 0-12h, 24-36h, and at 48h. The mean attachment stability scores, from four

564 independent experiments using 102 blastocysts, are presented. Error bars +/- standard error
565 of the mean (SEM). **C** The mean attachment stability score was calculated from the data in
566 B. Error bars +/- SEM. **D** Phase contrast images of the mouse blastocyst-Ishikawa cell co-
567 culture at 4h, 8h, 12h, 28h, 32h and 36h. Blastocysts are indicated by arrows and the
568 attachment score for each blastocyst is displayed. Scale bars 200μm. **E** The cumulative
569 frequency of the time at which the final position of attachment was reached was plotted
570 alongside the percentage of blastocysts scored as intermediately or stably attached (mean
571 +/- SEM from three independent experiments using 84 blastocysts). **F** Ishikawa cells were
572 pre-treated with 10nM 17β-estradiol 48h prior to co-culture, then with 10nM 17β-estradiol
573 and 1μM progesterone 24h prior to co-culture and during co-culture with hatched E4.5
574 mouse blastocysts. The attachment stability of mouse blastocysts was scored every 4h from
575 0-12h, 24-36h, and at 48h. Blastocysts scored as intermediately and stably attached were
576 added together and the mean from three independent experiments using 72 blastocysts was
577 plotted +/- SEM.

578 **Figure 2** Attached embryos not breaching (**A**) and breaching (**B**) Ishikawa cell layers were
579 optically sectioned after PFA fixation and incubation with phalloidin to label actin fibres (red)
580 and DAPI to label nuclei (blue). Invasive cells of breaching embryos exhibit binuclearity
581 (thick arrow), prominent nucleosomes (arrowhead), and actin stress fibres (thin arrow).
582 Upper/lower section refers to depth of the optical section in the Z-axis. Scale bars 20μm. **C**
583 The size of invasive embryonic nuclei in the lower optical section was measured and
584 compared to the size of outer, peripheral embryonic nuclei, presumed to be TE and not inner
585 cell mass, in the corresponding upper section; 309 nuclei were measured in 12 embryos and
586 mean nuclear size was plotted +/- SEM. *** t-test $p<0.001$. Breaching embryos were fixed
587 and labelled with anti-CDX2 antibody (**D**), rabbit isotype control antibody (**D** inset), anti-
588 GATA3 antibody (**E**), or mouse isotype control antibody (**E** inset), as well as phalloidin and
589 DAPI. Upper/lower section refers to depth of the optical section in the Z-axis. Scale bars
590 20μm. **F** The intensity of CDX2 and GATA3 fluorescence labelling was measured in invasive

591 embryonic nuclei in the lower optical section and compared to that of outer embryonic nuclei,
592 presumed to be TE and not inner cell mass, in the corresponding upper section; 158 nuclei
593 were measured in five embryos labelled with anti-CDX2 and 226 nuclei were measured in
594 five embryos labelled with anti-GATA3. The mean ratio of invasive nuclei to upper TE nuclei
595 fluorescence intensity was plotted +/- SEM. ** t-test $p<0.01$, NS not significant.

596 **Figure 3 A** Schematic representing embryo-Ishikawa cell co-culture experimental design. **B**
597 Hatched E4.5 mouse blastocysts were either co-cultured with Ishikawa cells to E6.5 or
598 cultured alone in co-culture media to E5.5 before co-culture with Ishikawa cells to E6.5 or
599 E7.5. The stability of attachment was scored every 4h from E5.5-6 and at E6.5. Mean
600 percentage blastocysts scored as intermediately or stably attached from six independent
601 experiments using 149 blastocysts was plotted +/- SEM. **C** Attached embryos co-cultured
602 from E4.5-6.5, E5.5-6.5 and E5.5-7.5, were fixed, labelled with phalloidin and DAPI and
603 optically sectioned to determine whether embryonic cells had breached the Ishikawa cells.
604 The mean proportion of attached embryos to have breached the Ishikawa cells was plotted
605 +/- SEM. ** ANOVA $p<0.01$, *** ANOVA $p<0.001$, demonstrating significant difference from
606 co-culture E4.5-6.5.

607 **Figure 4 A** Schematic representing embryo-Ishikawa cell co-culture experimental design. **B**
608 Hatched E4.5 mouse blastocysts were co-cultured directly with Ishikawa cells from E4.5-6.5,
609 co-cultured directly with Ishikawa cells to E5.5 before transfer to fresh Ishikawa cells and co-
610 culture to E6.5, or co-cultured in a transwell above Ishikawa cells to E5.5 before transfer to
611 fresh Ishikawa cells and co-culture to E6.5. The stability of attachment was scored every 4h
612 from E5.5-6 and at E6.5. Mean percentage blastocysts scored as intermediately or stably
613 attached from five independent experiments using 157 blastocysts was plotted +/- SEM. **C**
614 Attached embryos were fixed, labelled with phalloidin and DAPI and optically sectioned to
615 determine whether embryonic cells have breached the Ishikawa cells. The mean proportion
616 of attached embryos to have breached the Ishikawa cells +/- SEM. ** ANOVA $p<0.01$,
617 demonstrating significant difference from direct co-culture E4.5-6.5.

618 **Figure 5** Hatched E4.5 mouse blastocysts were cultured alone, co-cultured directly with
619 Ishikawa cells, co-cultured in a transwell above Ishikawa cells or co-cultured directly with
620 human fibroblasts as a control cell type, to E5.5 before retrieval and lysis for RNA isolation
621 and gene expression analysis by qPCR. Data is presented as expression relative to
622 reference genes (2^{-ct}, relative to geometric mean of *Gapdh* and *Ywhaz*) of **A** *Cdx2*, **B** *Gata3*,
623 **C** *Eomes*, **D** *Hand1*, **E** *Gata2*, and **F** *Hes1* in embryos cultured in the indicated conditions
624 (mean +/- SEM of five independent experiments using 200 embryos). * ANOVA $p<0.05$,
625 demonstrating significant difference from no co-culture E4.5-5.5.