Evolution of placental invasion and cancer metastasis are causally linked

Kshitiz1,2,3*, Junaid Afzal4, Jamie D. Maziarz1,5, Archer Hamidzadeh1,2, Cong Liang1, Eric M. Erkenbrack1,5, Hong Nam Kim6, Jan-Dirk Haeger7, Christiane Pfarrer7, Thomas Hoang2, Troy Ott8, Thomas Spencer9, Mihaela Pavličev10, Douglas F. Antczak11, Andre Levchenko1,5,12,13* and Günter P. Wagner1,5,12,13*

Among mammals, placental invasion is correlated with vulnerability to malignancy. Animals with more invasive placentaion (for example, humans) are more vulnerable to malignancy. To explain this correlation, we propose the hypothesis of ‘Evolved Levels of Invasibility’ proposing that the evolution of invasibility of stromal tissue affects both placental and cancer invasion. We provide evidence for this using an in vitro model. We find that bovine endometrial and skin fibroblasts are more resistant to invasion than are their human counterparts. Gene expression profiling identified genes with high expression in human but not in bovine fibroblasts. Knocking down a subset of them in human fibroblasts leads to stronger resistance to cancer cell invasion. Identifying the evolutionary determinants of stromal invasibility can provide important insights to develop rational antimetastatic therapeutics.

Placental invasion into the maternal endometrium of the uterus shows substantial similarities to early cancer dissemination into stroma1–4. These similarities have inspired the hypothesis of antagonistic pleiotropy5,6. According to this hypothesis, trophoblasts evolved the capacity to invade the endometrium, leading to invasive placentaion. These mechanisms can become reactivated in cancer cells, leading to a predisposition to metastasis. This implies that cancer malignancy should be limited to placental mammals where invasive placentaion first evolved. This prediction, however, is inconsistent with the fact that poossums, with ancestrally non-invasive placentaion5,6, get invasive skin cancers6. Here, we explore an alternative scenario in which stromal cells of the uterus evolved to either resist or permit invasion, determining the outcome of placental invasiveness6.

The likelihood that the evolution of the stromal environment is driving the evolution of cancer malignancy is enhanced by the fact that the molecular mechanisms used by cancer cells to metastasize are shared with other biological processes. For instance, the mechanisms regulating gastrulation, wound healing, extravasation by leukocytes and so on, are shared with both trophoblast and cancer invasion5,6,10. This implies that invading cancer cells use mechanisms that evolved much earlier than placental invasion and, therefore, the evolution of invasive placentaion per se cannot be responsible for the origin of malignant cancer. It is important to note, however, that the invasiveness of the placenta continued to evolve after its origin. Placental invasion reverted to a non-invasive phenotype in several lineages of placental mammals, as well as evolving an even higher degree of invasiveness in the great apes, which includes humans12–14, A complete loss of placental invasion evolved in hoofed mammals, such as cows and horses and their relatives, and these animals have lower malignancy rates for a variety of cancers1. In contrast, humans (with very invasive placentas) are highly vulnerable to melanoma malignancy.

On the basis of the arguments outlined above, we suggest that evolutionary changes in the permissiveness or resistance of uterine stromal cells to placental invasion are mechanistically linked to the vulnerability to cancer malignancy5. We term this ‘Evolved Levels of Invasibility’ (ELI; Fig. 1a) and experimentally test ELI using an in vitro model (Fig. 1b). We demonstrate that human fibroblasts are more permissive to invasion by trophoblasts, as well as cancer cells, compared to their bovine counterparts. We then identify factors responsible for the resistance to invasion, paving a way for therapeutic interventions. This study highlights how investigating evolutionary processes may lead to the identification of therapeutic targets, pointing to the clinical potential of evolutionary analysis.

Results

Collective cell invasion behaviour can be modelled in an ECM-mimetic co-culture system. To test ELI, we developed a method for measuring collective cell invasion into a lawn of stromal cells. The collective cell invasion into stroma is a complex process15–17. During infiltration and spread, invading cells interact with stroma, which modulates their invasive capacity while experiencing contact guidance from the underlying extracellular matrix (ECM) fibres18,19. On the basis of these considerations, we sought to mimic the stromal invasion process through a patterned
co-culture of stromal and invasive cells on structured adhesion substrata. This approach was validated in previous cancer–stroma interaction studies\(^{22,23}\). The extent of invasion was measured as the average distance over which invasive cells have penetrated the stromal monolayer. The platform also enabled the sensitive measurement of cell invasion (Fig. 1c).

We first compared the spread of malignant (1205Lu) and non-malignant (WM35) melanoma cells into a monolayer of human skin fibroblasts (BJ5ta) on substrata reproducing the nanotopographic features of aligned ECM fibres or on flat surfaces. Aligned matrix fibres are a common feature in many tissues\(^{24}\). We found that mimicking aligned ECM matrix microenvironment allowed malignant 1205Lu melanoma cells to more extensively invade the skin fibroblast monolayer than non-malignant WM35 cells did (Fig. 1d,e and Extended Data Fig. 1a). Furthermore, when 1205Lu and WM35 cells were compared on a flat surface, no statistically significant difference could be detected. In contrast, a significant difference between 1205Lu and WM35 was detectable in the ECM-mimetic platform (Fig. 1e and Extended Data Fig. 1b,c), indicating that this assay has higher sensitivity in measuring stroma invasion compared to experiments on a flat surface.

**Bovine endometrial stroma is resistant to trophoblast invasion.**

To explore the causes of species differences in placental invasion, we measured the invasion of a human choriocarcinoma (J3) cell line into human endometrial stromal fibroblasts (hESFs) and compared them to bovine trophoblasts (F3) invading bovine endometrial stromal fibroblasts (bESFs). At 48h J3 cells invaded deeply into hESF layer, while the F3 invasion into bESFs was much more limited (Fig. 1f–h). These results recapitulate the in vivo species differences during embryo implantation\(^{25–27}\).

In human placenta, extravillous trophoblasts (EVTs) constitute the most invasive cell type\(^{28–30}\). We therefore measured the invasion of human chorioblastoma cell line BeWo, the EVT cell line HTR8 and the bovine F3 cells invading the layer of human or bovine stromal fibroblasts in all combinations (Fig. 2a). BeWo cells, although being a carcinoma cell line, have been extensively used as model of human cytotrophoblast cells\(^{31}\). The results showed that, while hESF were invaded by all trophoblast cell lines, both human and bovine, bESF were more resistant to invasion (Fig. 2b). The largest differences were found in the case of F3 invasion; with F3 invading rapidly into hESF monolayers but being nearly completely halted by bESFs. These cross-species invasion experiments confirmed that the limited invasion in bESF is, to a large extent, a property of the stroma cells rather than the trophoblast cells (Fig. 2b).

In summary, the degree of trophoblast invasion is mostly controlled by the identity of the stromal cells rather than the invasive capacity of trophoblast cells, as assumed for ELI.

**Bovine skin fibroblasts resist melanoma invasion.**

The findings above leave open the possibility that the observed effects could be specific to the fetal–maternal interface rather than reflecting species differences in stromal invasibility in general. In humans, melanoma invasion into the surrounding stroma is a strong predictor of malignancy\(^{32}\). Would stromal compartments in other tissues than the uterus (for example, the skin) show similar species-dependent properties to the uterine stromal cells? To address this question, we investigated whether gene expression profiles of human and bovine skin fibroblasts are more similar to those of their corresponding endometrial fibroblasts in the same species than they are to their homologous cell type in the other species.

The null hypothesis of independent transcriptome evolution of cell types predicts that corresponding (homologous) cell types should be more similar to each other than they are to a different cell type in the same species\(^{33}\). This is because, for instance, the human and bovine lineages diverged more recently than skin and endometrial fibroblasts have differentiated in evolution. This is necessarily true because otherwise corresponding cell types would not be homologous\(^{34}\). On the other hand, if gene expression profiles in skin and endometrial fibroblasts co-evolved, the cells from the same species could be more similar than to the corresponding/homologous cell in the other species. ELI predicts that, in each species, endometrial fibroblasts and skin fibroblasts share the same level of invasion resistance because their gene expression profiles co-evolved, such that selection for higher or lower endometrial invasiveness has a parallel effect on the invasibility of skin fibroblasts. Therefore, we expect that the stromal cell types from the same species are more similar to each other than they are to corresponding cell types in the other species\(^{33}\).

To evaluate this expectation, we performed RNA sequencing on bovine and human skin fibroblasts and compared them to human and bovine endometrial fibroblasts. Principal component analysis showed that the gene expression distance between skin fibroblasts and endometrial stromal fibroblasts of the same species was less than the distance between corresponding cell types of different species (Fig. 2c). The observed pattern is consistent with the suggestion that gene expression in endometrial and skin fibroblasts indeed co-evolved due to pleiotropic effects of mutations on transcriptional regulation\(^{33,34}\). In other words, the biology of skin and endometrial fibroblasts can be expected to be similar within species even though they differ between species.

We then measured invasion of malignant human melanoma cells (A375) into the skin fibroblasts from human (BJ5ta) and bovine (bSkFb) and found that bSkFb indeed resisted A375 invasion more strongly than did human skin fibroblasts (Fig. 2d). This greater invasion resistance of bSkFb was supported by a similarly
high resistance of these cells to invasion by WM35, 1205Lu and SKMel28 melanoma cell lines (Fig. 2c). A similar assay with bovine melanoma cells was precluded owing to lack of bovine melanoma cell lines34. Overall, these results indicate that bovine skin fibroblasts can resist cell invasion better than their human counterparts can.

**Human and bovine fibroblasts respond differently to trophoblast co-culture.** To understand the genetic underpinnings of ELI in bovine stromal cells, we collected RNA sequencing data from human and bovine endometrial fibroblasts, with and without co-culture with the respective trophoblasts. We focused on differentially
expressed genes in stromal cells, both basally and upon interaction with species-specific trophoblasts. Endometrial fibroblasts of both species were labelled with DiI fluorescent stain and co-cultured with equal number of unlabelled trophoblast cells, HTR8 and F3 for human and bovine cells respectively (Extended Data Fig. 3a). Co-cultures were maintained for 72 h and the cells were sorted using fluorescence-activated cell sorting (FACS)20. Henceforth, the bovine cells will be referred to as bESFs and bESFco-F3 and human cells as hESFs and hESFco-HTR8.

Many genes were differentially expressed in bovine versus human endometrial fibroblasts (Extended Data Fig. 3b) and ESFs responded strongly to co-culture with their cognate trophoblast cells (Extended Data Fig. 3c,d). Furthermore, human and bovine ESFs responded differently to trophoblast co-culture (Fig. 3a,b and Extended Data Fig. 3e,f). Human and bovine endometrial stromal cells differed substantially with respect to the number of genes affected by co-culture, with human cells showing more differentially expressed genes (5,349 genes changed at \( P \leq 0.01 \); false discovery rate, FDR = 3.1 \times 10^{-3} \)) compared to bovine cells (3,101 genes at \( P \leq 0.01 \); FDR = 7.01 \times 10^{-3} \)). Gene-set analysis revealed genes belonging to chemotactic activity, cell motility and metastasis ontologies at higher relative abundance in hESFco-HTR8 compared to bESFco-F3 (Fig. 3b). These results suggest that species differences in invasibility may be caused by differential gene expression among the stromal cells in response to trophoblast cells.

We found that human and bovine ESFs expressed markedly different sets of chemokine ligands (Fig. 3c) and chemokine receptors (Fig. 3d), although both were enriched in transcripts associated with angiogenesis. Genes showing elevated expression in hESFs included fibroblast growth factors, vascular endothelial growth factors, semaphorins, members of the transforming growth factor (TGF) family, as well as NRPs and ROBO1, whereas bESFs showed high expression of endothelin (END1), plasminogen activator (PLAU), thrombopoietin (THPO) and notably, transforming growth factor β2 (TGFB2). In contrast, genes belonging to ontologies GO_adherens junction and endothelial barrier did not show systematic expression differences in favour of either hESF or bESF (Extended Data Fig. 4a,b). Finally, the genes related to GO_fibroblast migration tend to be expressed at lower levels in bESF versus hESF (Extended Data Fig. 4c). These data point to the possibility that stromal response to...
trophoblast co-culture varies strongly between the species for genes that could play a role in the interaction between invading trophoblast cells and stromal cells, as opposed to genes that regulate basal stromal integrity. Differential expression of these genes may affect the invasibility phenotype in either species.

These analyses suggested that differences in the interactions between the stromal and invading cells were drivers of ELI in human and bovine. For instance, transforming growth factor β (TGF-β) and WNT signalling pathways are mediators of cancer stromal interaction and thus we explored the effect of trophoblast co-culture on the activation of these signalling pathways to see whether there are parallels between cancer–stroma and trophoblast–endometrial stroma interactions. Expression of genes in non-canonical WNT and TGF-β pathways were indeed higher in human and bovine ESFs with and without co-culture with HTR8 and F3 trophoblast cells, respectively.

**Fig. 3 | Transcriptomic analysis of bovine and human ESFs reveals differential response to trophoblasts.**

- **a.** Heatmap of gene expression differences of human and bovine ESFs with and without co-culture with HTR8 and F3 trophoblast cells, respectively.
- **b.** Volcano plot showing fold differences in the TPM values of bESFs versus hESFs co-cultured with the respective trophoblasts, colour-coded for relevant gene ontologies.
- **c, d.** Heatmaps showing expression of genes in non-canonical WNT and TGF-β pathways were indeed higher in human and bovine ESFs with and without co-culture with HTR8 and F3, respectively, belonging to the gene-sets chemokine and chemokine receptors (d). **e, f.** TPM values of genes significantly different in hESFs and bESFs for KEGG pathways for TGF-β (e) and non-canonical WNT signalling (f). For each gene, TPM values before and after co-culture are shown as blue and red dots, respectively. Arrows show the change in TPM values due to trophoblast co-culture.
Articles Nature ecology & evolution

where TPM is transcripts per million).

BJ5ta (resistance to invasion following gene knockdowns in both hESF and detected a weak but significant correlation in the increase of stromal

√ the inferred boreoeutherian ancestor (TPMspecies

uterus and in tumour lesion, prompting us to further explore the role of the signalling networks in stromal invasion.

Selected gene knockdown in human fibroblasts increases resistance to melanoma invasion. We hypothesized that modulating the differentially expressed genes in human stromal cells could induce them to become more resistant to invasion in human cells. We focused on genes related to WNT and TGF-β signalling. Specifically, we selected 16 genes from the TGF-β and WNT pathways that had higher expression in hESF or were upregulated in hESF in co-culture with trophoblast cells (Fig. 4a). We then targeted these genes using a battery of small interfering RNAs (siRNAs) in hESFs and BJ5ta to test whether they could modulate stromal invasibility.

Comparison of each siRNA-transfected stromal cell population was made with appropriate untransfected controls, as well as with cells transfected with control non-targeting siRNA (Extended Data Fig. 5a,b). HTR8 invasion into hESF was significantly reduced after 24 h in eight out of 16 genes (Fig. 4b). A375 invasion into BJ5ta skin fibroblast monolayer was observed for 18 h and 36 h after gene knockdown. Nine of these gene knockdowns significantly decreased invasion into the skin fibroblast monolayer (Fig. 4c), including members of WNT superfamily, TGF-β ligands, as well as less-established targets and effectors of WNT signalling, for example STARD7 (ref. 10), LPIN1 (refs. 42–46) and YAP1 (ref. 47). Furthermore, we detected a weak but significant correlation in the increase of stromal resistance to invasion following gene knockdowns in both hESF and BJ5ta (R² = 0.25, P = 0.02), indicating that gene silencing enhances resistance to invasion in a similar manner in both human endometrial and skin fibroblasts (Fig. 4d). Moreover, the average response for all gene knockdowns was an increase in resistance to invasion (Fig. 4c). These results suggested that human skin fibroblasts could be induced to resist melanoma invasion and that knowledge of gene expression in cow fibroblasts can tell us how to achieve this.

Opposite evolutionary trends of vulnerability to malignancy in humans and bovines. To assess the evolutionary history of the expression of the genes identified above as important for ELI, we cultured the ESFs from rabbit, rat, guinea pig, cat, horse and sheep to extend our analysis (Fig. 4f). This taxon sample represents two clades of eutherian mammals, the Euarchontoglires and the Laurasiatheria, together forming the clade of Boreoeutheria. We plotted the phylogenetic tree of these eight species48, along with the expression of these genes in skin fibroblasts compared to the inferred boreoeutherian ancestor (√TPMspecies − √TPMspeciesancestor where TPM is transcripts per million).

We found, surprisingly, that humans evolved higher expression levels of invasibility enhancing genes (TGFβ1, ACVR1, DDR2, LPIN1, CD44, MMP1), compared to the inferred boreoeutherian ancestor. The expression levels of these genes in human skin fibroblasts is higher than that of rabbit, rat and guinea pig. All of these species have haemochorial placentation and, thus, the higher expression level of these genes in humans probably evolved in the primate lineage. The limited data on a selected group of genes, therefore, supports ELI amongst mammals and points to the possibility of increased stromal vulnerability in humans and decreased stromal vulnerability in bovines.

Discussion

Mammalian species differ in their potential for tumourigenesis, as well as their vulnerability to cancer metastasis49. The comparative biology of cancer incidence across different animals has identified species-specific tumour suppressor mechanisms explaining the variation in occurrence of tumours between species50–55. In a recent review, Constanzo and collaborators made a convincing case for a model where cancer progression in humans includes the reactivation of embryonic gene expression normally controlling placenta development and immune evasion56. Here, we focus on differences across species in the rates of cancer malignancy rather than differences in rates of tumourigenesis. For instance, melanoma does occur in bovines and equines but remains largely benign13–19; while it is highly malignant in humans. This correlates with the phenotype of the fetal–maternal interface (the degree of placental invasion during pregnancy). In human skin fibroblasts, siRNA-guided knockdown revealed genes that impart stromal invasibility by melanoma and trophoblast cells (Fig. 4g). These genes include TGF-β ligands, consistent with results from colorectal cancer49. Other genes enhancing stromal invasibility are members of the non-canonical WNT pathway, as well as WNT signalling modulators (for example, LPIN1, ref. 42), by directly regulating β-catenin levels (for example, YAP1/TAZ, ref. 47) or by being regulated by β-catenin-induced transcription (for example, CD44, ref. 48). Of these, YAP1, a Hippo signalling pathway target promoting tumour growth60, can also be regulated by non-canonical WNT signalling and can, in turn, inhibit canonical WNT signalling49. Both TGF-β and non-canonical WNT signalling49 are known to affect tumour progression and metastasis. TGF-β1 and TGF-β3, expressed at relatively higher levels in hESF, are also reported to regulate β-catenin activity, indicating that human stromal vulnerability to invasion may be influenced by paracrine signalling to invading cells through secreted ligands. Also of interest, many of the genes identified in our comparative gene expression screen have connections to metabolic regulation. These include genes encoding LPIN1, which regulates triglyceride metabolism48, and MGAT5, known to regulate glucose uptake in tumour cells54.

The results presented here show that species differences in malignancy rates may, in part, be caused by species differences in invasibility of stromal cells. We found that bovine stroma evolved lower invasibility probably via decreased paracrine signalling through TGF-β and WNT pathways. In particular, these results argue that TGF-β secretion and high non-canonical WNT signalling in stromal cells are causal factors explaining the high vulnerability of human stromal tissues to cancer invasion, at least in the case of melanoma. Comparative transcriptomic data across multiple additional species further suggests that the human lineage evolved higher expression of these genes enhancing tumour and trophoblast invasion and, thus, probably has evolved higher malignancy rates than in the common ancestor of boreoeutherian mammals.

Our data support the ELI hypothesis, suggesting that differences in stromal gene expression between species are causal in determining the degree of embryo implantation as well as stromal resistance to early cancer dissemination. In Eutheria epitheliochorial placentation has evolved several times from the pre-existing invasive placentaion, suggesting that evolutionary advantages of non-invasive placenta12–14 may drive corresponding differences in malignancy resistance. We further found that apes may also have evolved increased stromal receptivity to trophoblast invasion and

1748

NATURE ECOLOGY & EVOLUTION | VOL 3 | DECEMBER 2019 | 1743–1753 | www.nature.com/natecolevol
**Fig. 4 | Induced resistance to invasion in human stroma by evolutionarily inspired gene silencing.**

**a.** Heatmap showing selected set of genes used for gene silencing in human stromal fibroblasts. Shown are $Z$-scores of TPM values for each transcript.

**b–e.** siRNA-based gene knockdown in human stroma to induce expression similar to bESF increases resistance to invasion. Each dot in b and c reflects an individual invasion observation. Error bars show s.e.m. Statistical comparisons made with scrambled siRNA using Student’s $t$-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

**d.** Average extent of invasion in human stromal cells. Each dot in b and c reflects an individual invasion observation. Error bars show s.e.m. Statistical comparisons made with scrambled siRNA using Student’s $t$-test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

**e.** Correlation shown between the extent of invasion of HTR8 into hESF and A375 into BJ5ta for each siRNA-based gene silencing.

**f.** Relative transcript levels of the selected genes in various boaroeutherian species compared to their most recent common ancestor; red-coloured bars denote decrease and blue bars show increase in $\sqrt{\text{TPM}}$ values versus the common ancestor; Ma, million years ago.

**g.** Network of WNT signalling and its interaction with TGF-β pathway showing previously known interactions, mapped with the gene-silencing-induced resistance in hESF and BJ5ta stromal cells.
correlatively have evolved higher vulnerability to cancer malignancy. As EVTs have evolved recently in great apes, the endometrial stroma may also have increased invasiveness to accommodate the more invasive trophoblast types. Our ELI hypothesis, and its experimental validation, suggest that studying the genetic basis for evolved resistance to invasion can identify factors determining the stromal control of cancer progression and identify therapeutic targets.

Methods
Cell sourcing. The study used a variety of cell sources for comparative analyses. Human ESFs were immortalized by the Charles Lockwood laboratory and obtained from Gil Mor group. The J3, HTR8/SVNeo, BeWo and BJ5ta cells were obtained from ATCC. Skin fibroblasts bSkFb bovine (Bos taurus), as well as those from guinea pig (Cavia porcellus), sheep (Ovis aries), pig (Sus scrofa) and rabbit (Oryctolagus cuniculus) were obtained from fresh skin tissue. A small piece of skin was collected, hair removed and the sample was washed in PBS buffer and cut into strips approximately 1 cm². Dermis was separated from epidermis by enzymatic digestion (30 min in 0.25% Trypsin buffer at 37 °C, followed by dissociation buffer (1 mg ml⁻¹ collagenase, 1 mg ml⁻¹ Dispase, 400 μg ml⁻¹ DNase I) for 45 min at 37 °C). Epidermis was removed and 2 mm pieces were cut from the dermis and transferred to a 12-well plate and covered with media. Fibroblasts emerged from the explants and grew to confluence in growth media. Extra tissue was removed and cells were subcultured a few times using a cell scraper. Bovine ESFs were obtained as follows. Uterine tissues were collected from each species and primary ESF were obtained by enzymatic digestion. Uterus fragments, 2-3 mm in size, were created using a scalpel and digested with 0.25% Trypsin-EDTA for 35 min at 37 °C, followed by dissociation buffer (1 mg ml⁻¹ collagenase, 1 mg ml⁻¹ Dispase, 400 μg ml⁻¹ DNase I) for 45 min at 37 °C. Cell clumps were homogenized by passage through a 22-gauge syringe followed by passage through a 40-μm nylon mesh filter to remove remaining clumps. A single-cell suspension was obtained from the lysate, transferred to fresh growth medium and cultured in T25 flasks. To facilitate enrichment of fibroblasts versus epithelial cells, media were exchanged in each well after 15 min to remove floating cells that had not yet attached while stromal fibroblasts had attached. Cells were grown to confluence and sub-passaged by scraping the cells off the surface to be split into two T25 flasks. At confluence, cells were split through one more round of differential attachment. We used immunohistochemistry to test for abundance of vimentin (Santa Cruz, sc-6260) and cytokeratin (Abcam, ab3877) to validate fibroblast subtype in the isolated cells. Isolates of hESF, bSkFb and hESFs were tested for potential mycoplasma contamination and were found to be free from it.

Cell culture. Human ESFs were grown in phenol-red free DMEM/F12 with high glucose (25 mM), supplemented with 10% charcoal-stripped calf serum (HyClone) and 1% antibiotic/antimycotic (Gibco). Decidualization was induced in ESFs with 0.5 mM of 8-Br-cAMP (Sigma) and 0.5 mM of progesterone analogue medroxyprogesterone acetate for 96 h in DMEM supplemented with 2% charcoal-stripped calf serum (HyClone). BJ5ta (ATCC) cells were cultured in 80% DMEM and 20% MEM supplemented with 10% FBS, 1% antibiotic/antimycotic and 0.01 mg ml⁻¹ hygromycin. F3 cells were obtained from Pfarrer's group and were cultured in DMEM with high glucose supplemented with 10% FBS, 1% antibiotic/antimycotic (Gibco). J3 cells were cultured in DMEM supplemented with 10% FBS, 1% antibiotic/antimycotic (Gibco), while HTR8/SVNeo (ATCC) were cultured in RPMI-1640 supplemented with 10% FBS and 1% antibiotic/antimycotic (Gibco).

Fabrication of polyurethane acrylate mould. Photore sist was spin-coated on silicon wafers and electron-beam lithography was used to pattern the wafers (JRX-9300FS, JEOL). After the photoresist was developed, exposed silicon was etched by deep-reactive ion etcher (STS ICP Etcher) resulting in formation of silicon master previously prepared and contacted with polyethylene terephthalate (PET) film. Application of UV light (wavelength λ = 200–400 nm, 100 μm cm⁻²) for 1 min was used to cure PUA, after which the mould was peeled off with tweezers and cured overnight under UV light to terminate the residual acrylate groups. The process resulted in a PUA mould of ~50 μm thickness.

Fabrication of nanotextured substrate. Previously prepared PET mould was used as a replica mould to perform the topographic pattern onto glass substrate using the technique of capillary force lithography. Briefly, glass substrate was cleaned using NaOH (0.1 M for 1 h), washed with deionized water and dried under N₂ stream. Primer (polyethylene glycol monomethyl ether acrylate in a ratio of 1:10) was spin-coated on the cover slip as a thin layer and baked for 20–30 min at 70 °C. Then 150 μl of PEG-DA (molecular weight = 575) precursor was dispensed onto the primed cover slip and the PET mould was placed reversibly. After the PUA precursor filled the submicrometre cavities by capillary action, the substrate was cured in UV light (λ = 250–400 nm, 100 μJ cm⁻²) for 1 min. The mould was peeled after polymerization using tweezers. The substrate resultant from the method consisted of nanotextured grooves with an expected elastic modulus of ~70 MPa. The substrate was cured again for 1 h under UV light to terminate residual active acrylate groups.

Cell patterning for stromal invasion assay. For cell patterning, we used a stereolithographic plastic mould to create a polydimethylsiloxane (PDMS) stencil. The PDMS stencil was cast by mixing monomer and cross-linker in a ratio of 10:1 and cured at 80 °C for 4 h by placing it on a pre-designed plastic mould. Stencils were washed using isopro pyl alcohol and dried using N₂ stream, placed on the nanotopographic substrate coated with laminin (25 μg ml⁻¹) and the device kept in a vacuum to allow air under the stencil to be removed to avoid chance of leakage. DiI-labelled invasive cells were seeded onto the stencil at a density of 10⁶ cells per 100 μl and allowed to attach and polarize for 8 h. Unattached cells were washed off twice with PBS and the stencil removed carefully using blunt-end tweezers. Unlabelled stromal cells were seeded at a density of 10⁶ cells per 100 μl to attach to the area previously covered by PDMS stencil. After 6 h of attachment, unattached cells were washed off and the substrate mounted for live-cell microscopy. To ensure selection of definitive fibroblasts, and to avoid selection of trophoblasts taking up dye from dead stromal cells and occasional heterotypic cell-fusion events, we only collected DiI⁺ cells. All experiments were conducted in similar culture conditions by 1:1 mixture of the media in either comparable conditions.

Live-cell fluorescence microscopy. Phase-contrast and epifluorescence microscopy was performed using a Leica DMi8 model, with an HC PL Fluotar ×10, 0.30 dry objective and images were acquired using Leica LASX Software and processed using Fiji image analysis software. Lumencor SpectraX was used as a source of light for excitation and routed through a rhodamine excitation filter cube consisting of excitation 546 nm per 10 nm (band-pass filter), dichroic 560 nm (long-pass filter) and emission 585 nm per 40 nm (band-pass filter) and acquired using an Andor EMCCD iXon Ultra 888 camera.

Invasion analysis. Acquired sequential images for each condition were analysed using Fiji image analysis software after contrast enhancement. DiI-labelled invasive cell fronts (trophoblasts or melanoma cells) were identified manually for each time point. Area occupied by DiI- positive cells was measured at each time point. Total invasion was calculated as follows:

\[ \Delta A_{\text{area}}(t) = A_{\text{area}}(t) - A_{\text{area}}(t_0) \]

Extent of invasion per unit measurement of interface was determined by dividing \( \Delta A_{\text{area}} \) by the length of initial intercellular interface:

\[ \frac{\Delta A_{\text{area}}(t)}{L_{\text{interface}}} \]

Fluorescence-assisted flow sorting. Cells were detached from the substrate using TrypLE solution (Gibco), quenched with excess medium and washed thrice with PBS. Isolated cells were suspended in 1% Albumax (Gibco) dissolved in PBS and sorted using FACS. FACs was performed using BD FacsARIA II using PE.Cy5 channel and analysis performed using FACS Diva v.6.0. To increase the purity of ESF collected after the co-culture experiments and to account for possible uptake of dye from dead or dying stromal cells by the trophoblasts, as well as for other non-cell-cell fusion events, we only sorted DiI⁺ cells. Sorted cells were collected directly in RNASelect to limit RNA degradation. Even cells that were not in co-culture were subjected to the same sorting protocol.

siRNA transfection and characterization. For gene-silencing experiments, stromal cells (BJ5ta or hESFs) were cultured in 12-well plates and transfected with at least two siRNAs (shown in Table 1) at a concentration of 50 nmol per well. Lipofactamine RNAiMax was used to transfect siRNAs obtained from Integrated DNA Technologies (IDT).

RNA isolation and qRT–PCR. RNA was isolated using RNAasy micro kit (QIAGEN) and resuspended in 10 μl of water. SuperScript IV VARIO Master Mix (ThermoFisher) was used to synthesize cDNA from 1 μg of RNA and eDNAase was used to remove DNA after reverse transcription. Primers from IDT were used for qRT–PCR reaction using SybGreen (ThermoFisher) to evaluate the efficiency of siRNA-mediated knockdown. 2ΔM and 2ΔΔM were used as internal reference genes and both were found to be consistent within all samples after knockdown. Quantitative RT–PCR was performed in QuantiStudio3 (ThermoFisher) and relative quantitation was performed by comparative cycle number, Cₚ.
Table 1 | Sequences of siRNA used for the knockdown experiments in human stromal cells

<table>
<thead>
<tr>
<th>Gene name</th>
<th>siRNA name</th>
<th>Duplex sequence 5′</th>
<th>Duplex sequence 3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPIN1</td>
<td>Hs.R.LPIN1.13.1</td>
<td>GCGCUACUUGGAUGACUCACAGA</td>
<td>GCGCCAGGAUGACACUUGGAGUU</td>
</tr>
<tr>
<td>RAC2</td>
<td>Hs.R.RAC2.13.1</td>
<td>AAGCAUUUUGGUCCAAGAAGACAC</td>
<td>UCUUUGGAAAAAACAGUCCUUGGUC</td>
</tr>
<tr>
<td>MGAT5</td>
<td>Hs.R.MGAT5.13.1</td>
<td>CUCUGACUUGGAGUAUCAGUCA</td>
<td>GUGACAACUGGAAUGGAGG</td>
</tr>
<tr>
<td>WNT5B</td>
<td>Hs.R.WNT5B.13.1</td>
<td>GGGCUACUUGGAGUAUUGAAGA</td>
<td>UCUUUUGGAAAAAACAGUCCUUGGUC</td>
</tr>
<tr>
<td>STARD7</td>
<td>Hs.R.STARD7.13.2</td>
<td>GCUCUGCAACUUGGAAUGG</td>
<td>AGGCCAUAGAUGGAGAAUGCU</td>
</tr>
<tr>
<td>YAP1</td>
<td>Hs.R.YAP1.13.1</td>
<td>GUGACAGAUGAAUUGAAUUGAGU</td>
<td>GACCUUUGUAGAAUGGAGAAUGCU</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>Hs.R.TGFβ1.13.1</td>
<td>CAGCAAAAUUGCCGGAUCCTC</td>
<td>AAGCUGUUGUAGAAGGAGG</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>Hs.R.TGFβ3.13.1</td>
<td>CAACUUGAUGCCUAUUAGCATT</td>
<td>AGGUGAUAACUUGAUAUGCU</td>
</tr>
<tr>
<td>ACVR1</td>
<td>hs.R.ACVR1.13.1</td>
<td>GACUUGAAGCACAUAUUGAUAUCC</td>
<td>UUCUGAUAACUGGAAUUGAAG</td>
</tr>
<tr>
<td>Twist1</td>
<td>hs.R.Twist1.13.1</td>
<td>UUCUGAUGAAUUGGAAUCAGU</td>
<td>GAGAAUUAUAUUGAAUUGAAG</td>
</tr>
<tr>
<td>NCOA3</td>
<td>hs.R.NCOA3.13.1</td>
<td>CUGCAAAUUGGUAUUGAAGCAA</td>
<td>UAGACCGUUGAUAACUUGA</td>
</tr>
<tr>
<td>ERBB3</td>
<td>hs.R.ERBB3.13.1</td>
<td>GCCAUUUGUGGAAUGAUAUCC</td>
<td>AACGUUGAAGCAUGAUAUGAU</td>
</tr>
<tr>
<td>DDR2</td>
<td>hs.R.DDR2.13.1</td>
<td>CCACUUCACCUUGGAAUUGAUUAA</td>
<td>AGCCGGAGUUGAUAUUGAAG</td>
</tr>
<tr>
<td>BMP4</td>
<td>hs.R.BMP4.13.1</td>
<td>CAGCUUGGUGAAGGACUGAUA</td>
<td>CCGUGGAGGAGCAUGAUAUGA</td>
</tr>
<tr>
<td>MMP1</td>
<td>hs.R.MMP1.13.1</td>
<td>AGCAACUGAUAUGUAUUGGTC</td>
<td>GUGUUGGUGAUAACUUGGAG</td>
</tr>
<tr>
<td>CD44</td>
<td>hs.R.CD44.13.1</td>
<td>GCUCUGACUGAGGAAUUGAAGCCT</td>
<td>UUGACGACUGUGAACAUGGAA</td>
</tr>
</tbody>
</table>

Partek Flow software v.5.0. Human cells data were aligned to reference genome (hg38) using STAR alignment (in Partek Flow) and bovine data were aligned using Bos Taurus assembly UMD_3.1.1/bosTaue. Quantification was performed using quantify to annotation model (Partek E/M) using Ensemble Transcripts release 85 (humans) or Ensemble-UMD3.1 gene annotation model. Normalization was performed using TFM. Gene-level comparison and statistical analysis was performed using analysis of variance and significance of (adjusted) P=0.05 or less was considered for analysis. Gene lists comparison was performed by obtaining ontology/pathway specific list from Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG). Comparative transcriptome analysis. Raw sequence reads (single-ended 75 base pairs) for ESIs from rabbit (Oryctolagus cuniculus), cat (Felis catus), horse (Equus caballus) and sheep (Ovis aries) were aligned to reference genome assemblies OryCun2.0, Rnor_6.0, Cavpor3.0, Felis_catus_8.0, EquCab2 and Oar_v3.1, respectively, using Tophat2 (v.2.1.1). Read counts for all genes were calculated using HTSeq (v.0.6.1p1, https://bitseq.readthedocs.io/en/release_0.11.1/index.html) with Python (v.2.7.2) according to Ensembl gene annotation release 92 (horse) or release 89 (other species). Then readthedocs.io/en/release_0.11.1/index.html) with Python (v.2.7.2) according to Ensembl gene annotation release 92 (horse) or release 89 (other species). Then}

References


**Acknowledgements**

This work was funded by National Cancer Institute Center grant no. U54-CA209992.

**Author contributions**


**Competing interests**

The authors declare no competing interests.

**Additional information**

Extended data is available for this paper at https://doi.org/10.1038/s41559-019-1046-4.

Supplementary information is available for this paper at https://doi.org/10.1038/s41559-019-1046-4.

Correspondence and requests for materials should be addressed to K., A.L. or G.P.W.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature Limited 2019
Extended Data Fig. 1 | Nanotextured stromal invasion assay quantitatively and sensitively measures collective intrusion of cells into stroma. (A) Time stamped images at 0 and 18 hours showing extent of invasion by Dil-labelled 1205Lu cells (red) into BJ5ta stromal fibroblast monolayer (unlabelled) on flat and nanotextured substrata; Quantification shown in Fig. 1d. (B-C) Sensitive measurement of differences in invasion by nanotextured stromal invasion platform; Time stamped images at 0, 6, 12 and 18 hours showing extent of invasion by Dil-labelled non-malignant WM35 (B), and malignant 1205Lu (C) cells into BJ5ta monolayer shown in both flat, and nanotextured cell patterned assay; Quantification shown in Fig. 1e.
Extended Data Fig. 2 | Human and bovine trophoblasts exhibit different invasive potential into endometrial stroma. Time stamped images showing extent of invasion of human choriocarcinoma-derived trophoblasts, J3 (red) (A), and bovine trophoblasts, F3 (red) (B) into their respective endometrial stromal fibroblast monolayers at 0, and 48 hours.
Extended Data Fig. 3 | Human and bovine ESFs respond differently to co-culture with trophoblasts. (A) Experimental plan to isolate endometrial cells after co-culture with respective trophoblast; (B) Volcano plot showing fold change in genes between bovine versus human endometrial stromal fibroblasts, along with their significance depicted in P value. (C) P-value distribution of t-tests comparing human ESFs with and without co-culture with HTR8 trophoblast cells. (D) P-value distribution of t-tests comparing bovine ESFs with and without co-culture with F3 trophoblast cells. In both C and D, note that thin right hand tail of the distribution, which indicates that a large number of genes are differentially expressed in response to the presence of the corresponding trophoblast cells. (E-F) Scatter plots showing relative TPM values of genes between (E) hESF and hESF co-cultured with HTR8, and (F) bESF and bESF co-cultured with F3; Red dots refer to individual gene transcripts abundance significantly different in between the compared conditions.
Extended Data Fig. 4 | Human and bovine endometrial stromal fibroblasts do not appear to differentially respond to their respective trophoblasts by regulating cell-cell adhesion. Gene ontology analysis of individual genes belonging to GO_Adherens (A), GO_Endothelial Barrier (B), GO_Fibroblast Migration (C), and Kegg ontology for Wnt Signalling (D) for hESF and bESF shown with their relative TPM values; Also are shown the coefficient of determinant, $R^2$ for the linear regression between TPM values of hESF and bESF in a given gene-sets, along with the standard deviation of the residuals, Sy.x. (E) Ingenuity Pathway Analysis of selected signalling pathways differentially activated in hESF and bESF; Shown are $-\log(p\text{-values})$ of genes in the respective pathways differentially activated, while the colour depicts the z-score.
Extended Data Fig. 5 | siRNA-induced gene knockdown reduces transcript levels in hESFs and BJ5ta cells. qRT-PCR results showing the percentage knockdown of transcript levels in hESF (A) and BJ5ta (B), calculated using $\Delta\Delta C_t$ method, compared using GAPDH as housekeeping gene with scrambled siRNA as control. For both A, and B, $n = 4$ biological replicates.
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection QuantStudio Software V1.3 (qRT-PCR),

Data analysis Partek® Flow® software, version 5.0 was used to analyze the RNA sequencing data. Fiji Image Analysis Software was used for Image analysis. Expression suite software was used for qRT-PCR. GraphPad Prism was used for the other statistical analysis

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data generated that supports this study will be available from the corresponding author upon request.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences
Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
For each invasion study, at least 6 (or up to 10) samples were chosen for a live/ end-point study.

Data exclusions
All samples were included in the final analysis, except those in which the cell layers ripped off during image acquisition. This occurred not due to the biological samples, but because of the swelling of the underlying nanopatterns due to manual plasma treatment.

Replication
All experiments were repeated at least 3 times.

Randomization
This was not relevant to our study.

Blinding
All experiments were analyzed by undergraduate assistants, who were not aware of the actual conditions. The analyses are objective, but were separated from the person performing image acquisition.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

<table>
<thead>
<tr>
<th>Involved in the study</th>
</tr>
</thead>
<tbody>
<tr>
<td>n/a</td>
</tr>
<tr>
<td>Antibodies</td>
</tr>
<tr>
<td>Eukaryotic cell lines</td>
</tr>
<tr>
<td>Palaeontology</td>
</tr>
<tr>
<td>Animals and other organisms</td>
</tr>
<tr>
<td>Human research participants</td>
</tr>
<tr>
<td>Clinical data</td>
</tr>
</tbody>
</table>

Methods

<table>
<thead>
<tr>
<th>Involved in the study</th>
</tr>
</thead>
<tbody>
<tr>
<td>n/a</td>
</tr>
<tr>
<td>ChIP-seq</td>
</tr>
<tr>
<td>Flow cytometry</td>
</tr>
<tr>
<td>MRI-based neuroimaging</td>
</tr>
</tbody>
</table>

Antibodies

Antibodies used
Describe all antibodies used in the study; as applicable, provide supplier name, catalog number, clone name, and lot number.

Validation
Describe the validation of each primary antibody for the species and application, noting any validation statements on the manufacturer’s website, relevant citations, antibody profiles in online databases, or data provided in the manuscript.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
BJSta (ATCC), HTR8/SVNeo (ATCC)

Authentication
The cell lines were not authenticated separately, it was bought from ATCC and directly used for this study within a few months. HTR8 was a newly available cell line in ATCC, and we waited for its initial authentication from ATCC.

Mycoplasma contamination
Cell lines tested negative for mycoplasma.

Commonly misidentified lines
(See ICLAC register)
Name any commonly misidentified cell lines used in the study and provide a rationale for their use.

Palaeontology

Specimen provenance
Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information).

Specimen deposition
Indicate where the specimens have been deposited to permit free access by other researchers.
Dating methods

If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.

Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Animals and other organisms

Policy information about studies involving animals. ARRIVE guidelines recommended for reporting animal research

Laboratory animals
For laboratory animals, report species, strain, sex and age OR state that the study did not involve laboratory animals.

Wild animals
Provide details on animals observed in or captured in the field, report species, sex and age where possible. Describe how animals were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method; if released, say where and when) OR state that the study did not involve wild animals.

Field-collected samples
For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field.

Ethics oversight
Identify the organization(s) that approved or provided guidance on the study protocol, OR state that no ethical approval or guidance was required and explain why not.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics
Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write “See above.”

Recruitment
Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.

Ethics oversight
Identify the organization(s) that approved the study protocol.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies
All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration
Provide the trial registration number from ClinicalTrials.gov or an equivalent agency.

Study protocol
Note where the full trial protocol can be accessed OR if not available, explain why.

Data collection
Describe the settings and locales of data collection, noting the time periods of recruitment and data collection.

Outcomes
Describe how you pre-defined primary and secondary outcome measures and how you assessed these measures.

ChIP-seq

Data deposition

Confirm that both raw and final processed data have been deposited in a public database such as GEO.

Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links
May remain private before publication.

For “Initial submission” or “Revised version” documents, provide reviewer access links. For your “Final submission” document, provide a link to the deposited data.

Files in database submission

Provide a list of all files available in the database submission.

Genome browser session

[e.g. UCSC]

Provide a link to an anonymized genome browser session for “Initial submission” and “Revised version” documents only, to enable peer review. Write “no longer applicable” for “Final submission” documents.

Methodology

Replicates
Describe the experimental replicates, specifying number, type and replicate agreement.
**Sequencing depth**

Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

**Antibodies**

Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

**Peak calling parameters**

Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.

**Data quality**

Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.

**Software**

Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

---

**Flow Cytometry**

**Plots**

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

**Methodology**

**Sample preparation**

Fibroblasts were labeled with DiI and mixed with trophoblasts, and separated based on fluorescence.

**Instrument**

BD FACSaria II

**Software**

BD FacsDIVA

**Cell population abundance**

The cell population isolated was > 25%

**Gating strategy**

Gating strategy is described in the figure. In brief, we were very conservative in selecting only DiI(hi) cells.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

---

**Magnetic resonance imaging**

**Experimental design**

**Design type**

Indicate task or resting state; event-related or block design.

**Design specifications**

Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.

**Behavioral performance measures**

State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).

**Acquisition**

**Imaging type(s)**


**Field strength**

Specify in Tesla

**Sequence & imaging parameters**

Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle.

**Area of acquisition**

State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined.

**Diffusion MRI**

- Used
- Not used
# Preprocessing

## Preprocessing software

Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.).

## Normalization

If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization.

## Normalization template

Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized.

## Noise and artifact removal

Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration).

## Volume censoring

Define your software and/or method and criteria for volume censoring, and state the extent of such censoring.

# Statistical modeling & inference

## Model type and settings

Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation).

## Effect(s) tested

Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used.

## Specify type of analysis:

- [ ] Whole brain
- [ ] ROI-based
- [ ] Both

## Statistic type for inference

(See Eklund et al. 2016)

Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.

## Correction

Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo).

# Models & analysis

<table>
<thead>
<tr>
<th>Involved in the study</th>
<th>Functional and/or effective connectivity</th>
<th>Graph analysis</th>
<th>Multivariate modeling and predictive analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

## Functional and/or effective connectivity

Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).

## Graph analysis

Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).

## Multivariate modeling and predictive analysis

Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.