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Trophoblast organoids as a model for maternal-fetal interactions during human placentation

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Abstract

The placenta is the extraembryonic organ that supports the fetus during intrauterine life. Although placental dysfunction results in major disorders of pregnancy with immediate and lifelong consequences for both mother and child, our knowledge of the human placenta is limited due to lack of functional experimental models¹. Upon implantation, the trophoctoderm of the blastocyst rapidly proliferates and generates the trophoblast, the unique cell type of the placenta. *In vivo*, the

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Data availability. Microarray data for Figure 2, Extended Data Figures 4 and 5 have been deposited in the ArrayExpress database at EMBL-EBI under accession number E-MTAB-6683 (<https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6683>). Illumina EPIC Methylation array data for Figure 2 and Extended Data Figure 5 have been deposited in the ArrayExpress database at EMBL-EBI under accession number E-MTAB-7204. (<https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-7204>). Code used to analyse microarray data and EPIC array samples is available at <https://github.com/CTR-BFX/2018-Turco-Moffett>. The mass spectrometry proteomics data for Extended Data Table 3 and Supplementary Table 2 have been deposited to the ProteomeXchange Consortium via the PRIDE36 partner repository (www.ebi.ac.uk/pride/archive/) with the dataset identifier PXD009118 and 10.6019/PXD009118. All source data for graphs are in Supplementary Table 3. All other data supporting the findings of this study are available from the corresponding authors upon reasonable request.

Author Contributions M.Y.T. and L.G. designed and carried out all the core experiments and analysed the data; R.K. performed LCS-MS analysis; R.S.H. and M.P. performed microarray and DNA methylation analysis; A.McW. and S.G.E.M. performed and analysed the STR and HLA typing; M.Ho. performed electron microscopy and confocal analysis; R.F. and H.S. assisted with IHC and ELISA experiments; L.E. performed qPCR experiments; A.S., F.G., F.R., M.He. and S.O. provided intellectual input to the study; M.He. performed the ELF5 methylation experiment; G.J.B. and A.M. assisted in analysis and interpretation of results and jointly supervised the project. M.Y.T., G.J.B. and A.M. wrote the manuscript.

Competing interests The authors declare no competing interests.

proliferative villous cytotrophoblast cells (VCT) differentiate into two main sub-populations: syncytiotrophoblast (SCT), the multinucleated epithelium of the villi responsible for nutrient exchange and hormone production, and extravillous trophoblast (EVT) that anchor the placenta to the maternal decidua and transform the maternal spiral arteries². Here, we describe the generation of long-term, genetically-stable organoid cultures of trophoblast cells that can differentiate to SCT and EVT. We used HLA-typing to confirm the organoids are fetally-derived, and verified their identity against four trophoblast-specific criteria³. The cultures organise into villous-like structures, and we detected secretion of placental-specific peptides and hormones, including hCG, GDF15, PSGs and PAPPa, by mass spectrometry. The organoids also differentiate to HLA-G+ EVT that vigorously invade in 3D. Analysis of the methylome reveals the organoids closely resemble normal first-trimester placentas. This organoid model will be transformative for studying human placental development and for investigating trophoblast interactions with the local and systemic maternal environment.

To devise an organoid culture system suitable for trophoblast, we focussed on maternal and placental products that might signal to the stem/progenitor cells that reside in areas of Ki67+ VCT proliferation and/or at the base of the cytotrophoblast cell columns (CCC) that give rise to EVT (Fig. 1a,b)^{4–9}. We investigated signalling pathways between 6–8 weeks g.a when proliferation is high: WNT through β -catenin; TGF β through SMAD2/3; MAPK through Erk1/2 and STAT3. Our findings led to empirical trials of agonists/antagonists, along with other agents, which resulted in a basal Trophoblast Organoid Medium (TOM) composed of EGF, FGF2, CHIR99021 (a WNT activator), A83-01 (a TGF β /SMAD inhibitor) and Rspodin-1 (Extended Data Fig. 1a–d). To prepare isolates of trophoblast cells, first trimester placentas (6–9 weeks g.a) were enzymatically digested to enrich for cell clusters containing EPCAM+ cells; EPCAM marks proliferative trophoblast (Fig. 1c)¹⁰. Cell clusters were seeded into Matrigel drops and grown in TOM. Although some growth was seen, we also tested factors used in other organoid systems and/or present in the first trimester microenvironment (Extended Data Fig. 2a, Supplementary Table 1a). HGF, PGE2 and Y-27632 (a ROCK inhibitor) increase cell viability and growth and, when they are combined with TOM, there is rapid growth of cells within a week (Fig. 1d, Supplementary Table 1b). After the first passage, organoid structures appear and homogeneous trophoblast organoids are established within 2 passages (10–14 days) with an efficiency of 91% (20/22 patient samples). To confirm their fetal origin, we used microsatellite analysis and HLA typing (Extended Data Table 1). Derivation of the organoids in TOM in the absence of each factor reveals that EGF is the most important, with effects also seen with Y-27632, A83-01 and CHIR99021. We have now derived trophoblast organoids that are genetically stable after many passages (Extended Data Fig. 2b,c); three randomly selected cultures are still growing after a year (Supplementary Table 1c), are healthy and show active mitochondrial function (Extended Data Fig. 2d). Maternal epithelial cells are always detectable by flow cytometry in placental cell isolates. Nicotinamide enriches for cystic structures resembling glandular organoids at early stages of derivation (Extended Data Fig. 2a), and microsatellite analysis and HLA typing confirmed their maternal origin (Extended Data Table 2). By selecting the appropriate media, we can derive both decidual glandular and trophoblast organoid cultures from the same pregnancy (Extended Data Fig. 2e)¹¹. This highlights the importance of

verifying the maternal or fetal origin of any cultures derived from decidual or placental cell isolates.

The trophoblast identity of the organoids was verified on the basis of our previously defined criteria: they express *GATA3*, *KRT7*, *EGFR*, *TFAP2A* and *TFAP2C*; they lack expression of HLA class I molecules; they express *ELF5* and its promoter is hypomethylated; and they express microRNAs from the C19MC miRNA complex at similar or higher levels to choriocarcinoma lines, JEG-3 and JAR (Fig. 2a-d; Extended Data Fig. 3a-f; Source data Files 1-2)³. To assess how well the trophoblast organoids recapitulate their tissue of origin in an unbiased approach, we performed a microarray analysis of established organoids and compared them to first-trimester placental villi (also containing stromal, Hofbauer and endothelial cells) and cultured villous stromal cells. To check for maternal cell contamination, decidual glandular organoids were included. The results were analysed by PCA and hierarchical clustering (Fig 2e,f, Extended Data Fig. 4a,b). PC1 shows trophoblast organoids cluster closely to the placenta with enrichment for trophoblast-specific genes, *CGB3*, *GATA3* and *PSG6*, compared to the stromal cells and glandular organoids. PC2 highlights epithelial genes (*CLDN3*, *TACSTD2* and *KRT23*), specific for trophoblast compared to other placental cells (Extended Data Fig. 4b). IHC confirms that *KRT23* is a novel trophoblast-specific keratin placental villi and in trophoblast organoids (Extended Data Fig. 4c). Comparison of differentially expressed genes (fold change ≥ 2 , adjusted P value ≤ 0.05) between placental villi, trophoblast organoids and stromal cells highlights other genes of interest, such as *PGF*, *CCNE1*, *ERBB3* and *FOLR1*; translation of *CCNE1* in trophoblast was validated by IHC (Extended Data Fig. 4e). Imprinted genes, *PEG3* and *PEG10* are also highly expressed in the trophoblast organoids (Extended Data Fig. 4d). Amongst transcription factors, beside known genes, *GATA3* and *TFAP2C*, new genes emerged such as *ELF3* (Extended Data Fig. 5a,b). Genome-wide methylation analysis revealed a high degree of correlation between trophoblast organoids and first-trimester placental villi across different genomic elements compared to blood and brain (Fig. 2g). The hypomethylation of *ELF5* promoter was also confirmed (Extended Data Fig. 5c,d). Analysis of the promoter regions of genes with similar methylation patterns to *Elf5* in mouse TS cells shows that *ELF5*, *EZR*, *TINAGL1* and *LASP1* are similarly hypomethylated in placental villi and trophoblast organoids (Extended Data Fig. 5e,f)¹². Gene ontology analysis of DEGs, represented by Chord plot, shows terms describing metabolic processes and cell-cell organization converging on epithelial, developmental and hormonal pathways, *FZD5* (WNT signalling), *INSIG1* (insulin signalling), *DHCR7* (cholesterol synthesis) and *OCN* (polarity)(Extended Data Fig. 5g).

Trophoblast organoids grow as complex structures closely recapitulating the structure of placental villi *in vivo* where VCT stain for EPCAM and CDH1 (Fig. 3a,b). The basement membrane is on the outside in contact with Matrigel with syncytial masses lining the central cavity (Extended Data Fig. 6a). Similar to *in vivo*, VCT cells are Ki67+ and TP63+ (Fig. 3b, Extended Data Fig. 6b,c). Following incubation with EdU, ~30-40% of cells are proliferating when the organoids are small (100-200 μ m diameter), with a notable decrease as they enlarge and differentiate (Extended Data Fig. 6d). Expression of SCT markers (CD71, CD46) is found inside the organoids (Extended Data Fig. 6e). These characteristic features of SCT were confirmed by electron microscopy (EM): multinucleated with abundant

secretory organelles and surface microvilli (Fig. 3c). Lacunae present within the syncytial areas resemble those found *in vivo* (Extended Data Fig. 6f,g). *GCM1* drives fusion of VCT into SCT by upregulating *ERVW-1 (SYNCYTIN-1)*^{13,14}. Using qRT-PCR, we detect high levels of expression of *GCM1* and *ERVW-1*, comparable to those in the placenta (Fig. 3d; Source Data File 2). Thus, trophoblast organoids closely mimic the villous placenta both structurally and phenotypically. SCT secretes proteins and hormones into the maternal systemic circulation, inducing maternal adaptations to pregnancy. We explored the secretory activity of trophoblast organoids through an unbiased peptidomic analysis of the organoid medium and LC MS/MS technology (Fig. 4a). Among the most abundant peptides are placental-specific PSGs and INSL4, whose functions are unknown (Extended Data Table 3, Supplementary Table 2a-h). Aldose reductase, which converts glucose to sorbitol is also detected. High concentrations of sorbitol are present in first trimester placentas¹⁵. Hence, the organoids also mimic the villous placenta metabolically as well as endocrinologically. Peptides that induce physiological and metabolic adaptations during pregnancy, including hCG, KISS1 and CSH1 are all abundant as well as GDF15, implicated in hyperemesis gravidarum¹⁶. GDF15 and hCG are detected by ELISA, showing that full-length and appropriately folded hormones are secreted by trophoblast organoids (Fig. 4b,c; Source Data Files 3-4). Indeed, the ‘pregnant’ secretome of the organoids is evident using an “over-the-counter” pregnancy test kit (Fig. 4d).

Human trophoblast also differentiates to EVT, a process crucial for proper placentation. EVT express HLA-G and invade decidual tissue to transform the spiral arteries¹⁷. In TOM our trophoblast organoids show only sporadic HLA-G+ cells (Extended Data Fig. 7a). Recently, long-term, 2D monolayer cultures of human trophoblast cells derived from first-trimester placentas that can differentiate into SCT and EVT were described¹⁸. By adapting their EVT differentiation protocol and culturing both our trophoblast organoids and primary villous explants in their EVT medium (EVTM), HLA-G+ cells appear that migrate out of the organoids, digest the Matrigel to form tracks, and eventually adhere to the plastic (Fig. 5a-e, Extended Data Fig. 7b, Supplementary Videos 1-6). *In vivo* EVT are generated at the base of CCCs where cells express ITGA2¹⁰. We used flow cytometry to confirm that, after exposure of organoids to EVTM, HLA-G+ EVT appear and ITGA2+ cells disappear (Fig. 5f).

In summary, we describe the generation of human trophoblast organoids that grow as complex 3D structures with fusion of VCT to hCG-secreting SCT, anatomically and functionally closely resembling the villous placenta *in vivo*. In addition, we show differentiation to HLA-G+ EVT that vigorously invade and digest the Matrigel in 3D. Following the submission of our paper, there is a report describing the generation of trophoblast organoids from pooled patient samples; however these cannot be cultured long-term and have not been fully characterised¹⁹. Our findings complement those of Okae *et al.* and mean that there are now two culture systems (2D and 3D) for human trophoblast¹⁸. Our 3D model has the advantage of organising into complex structures generating both SCT and EVT. The retention of the normal villous structure will allow analysis of morphogenetic events. We anticipate that these two different models will provide valuable tools across a range of disciplines. They can be used to study maternal-fetal transmission of xenobiotics, drugs and pathogens, and the proteins and hormones derived from the SCT²⁰. Analysis of CCC formation and EVT differentiation *in vitro* will allow investigation of the decidual

microenvironment on trophoblast function, such as the effect of glandular histotrophic nutrition and the influence of the distinctive uterine natural killer (NK) cells^{21,22}. Major unexplained disorders of pregnancy such as pre-eclampsia, stillbirth and fetal growth restriction have their origins in aberrant placental development in the first trimester²³. Trophoblast organoids can be used to study maternal-fetal interactions following implantation, and the maternal physiological, metabolic and hormonal changes occurring during pregnancy.

Methods

Patient samples

All tissue samples used for this study were obtained with written informed consent from all participants in accordance with the guidelines in The Declaration of Helsinki 2000. Elective terminations of normal pregnancies were performed at Addenbrooke's Hospital (6-9 weeks gestation) under ethical approval from the Cambridge Local Research Ethics Committee (04/Q0108/23). Human peripheral blood was collected from a healthy donor in a BD vacutainer following informed consent and in accordance with the ethical approval of the Human Biology Research Ethics Committee, University of Cambridge (HBREC.2016.03).

Derivation of trophoblast organoids from human placental tissue

To obtain trophoblast-enriched cell suspensions, villi from first trimester placental tissue were sequentially digested with 0.2% trypsin-250 (Pan Biotech P10-025100P)/0.02% EDTA (Sigma #E9884)/PBS then collagenase V at 1.0mg/ml (Sigma #C9263) in Hams F12/10% FBS. Both digests were pooled, washed in Advanced DMEM/F12 medium (Gibco #12634-010) and re-suspended in ~10x volume growth-factor-reduced Matrigel (Corning #356231) on ice. 25µL drops were plated per well into a 48-well culture plate (Costar #3548), set at 37°C for 15min and overlaid with 250µL Trophoblast Organoid Medium (TOM, Supplementary Table 1b). Cultures were maintained in 5% CO₂ in a humidified incubator at 37°C. Medium was replaced every 2-3d. Small organoid clusters became visible by ~7d and were passaged when at least 50% had reached a diameter of 200-300µm (usually between 7-10d). Mechanical disruption was achieved with Eppendorf Explorer Plus automatic pipettes on a mix cycle of 99 rounds (x4-5), maximum speed. Organoids from the same sample were initiated and maintained in the absence of each individual component to test its importance. Frozen stocks of organoids were made in 70% TOM, 20% FBS and 10% DMSO freeze medium and stored in liquid nitrogen. A step-by-step protocol of the derivation and maintenance of human trophoblast organoid cultures can be found at Nature Protocol Exchange²⁴.

Generation of EVT from trophoblast organoids

Trophoblast organoids were passaged and plated into 35mm dishes or ibidi µ-dishes (Thermo Scientific #81156). Differentiation was achieved through a modification of the protocol used by Okae et al¹⁸. After passaging, organoids were maintained in TOM for 3-4d and switched to EVT medium (EVTM: advanced DMEM/F12, 0.1mM 2-mercaptoethanol (Gibco #31350), 0.5% Penicillin-streptomycin, 0.3% BSA (Sigma #A8412), 1% ITS-X supplement (Gibco #51500-056), 100ng/mL NRG1 (Cell Signaling #5218SC), 7.5µM

A83-01 (Tocris Biotechnique #2939), 4% Knockout serum replacement (ThermoFisher #10828010). When organoids showed outgrowth of cells (typically 7-10d), the medium was changed to EVTm without NRG1 for a further 7-10d. For comparison, fresh placental villi were embedded into 300 μ L Matrigel in ibidi μ -dishes and grown under the same conditions.

Isolation of placental stromal cells

Placental villous stromal cells were isolated by digesting the tissue remaining after the initial trypsin/collagenase digests in 10-15mL collagenase V in Hams F12/10% FBS with gentle shaking at 37°C for 5-10min. The cell suspension was filtered through gauze, washed and pelleted. Cells were resuspended in Advanced DMEM/F12/10% FBS with additional L-Glutamine, non-essential amino acids (Gibco #11140-035) and primocin (Invivogen #ant-pm-1) and seeded into tissue culture flasks. They were cultured to 80-90% confluency and passaged once before use.

Peripheral blood monocytes (PBMC) isolation

PBMC were isolated from blood by Pancoll-based (Pan-Biotech #P04-60500) density gradient separation. PBMC viability was >95% by Trypan Blue exclusion. PBMC were resuspended in *QIAzol lysis* reagent (Qiagen #79306) for total RNA extraction following the supplier's protocol.

Cell lines

Human choriocarcinoma cell lines, JEG-3 and JAR, were obtained from the American Type Culture Collection (ATCC) and cultured as previously described³.

Immunohistochemistry (IHC)

Organoids were formalin-fixed and embedded as previously described¹¹. IHC on sections of organoids and first-trimester placentas was performed using A.Menarini heat-induced epitope retrieval buffers and Vectastain avidin-biotin-HRP reagents as previously described¹¹. Primary antibodies (Supplementary Table 3) were replaced with equivalent concentrations of isotype-matched mouse or rabbit IgG for controls. Images were captured with a Zeiss Axiovert Z1 microscope and Axiovision imaging software SE64 V4.8.

Immunofluorescence (IF) and confocal microscopy

Trophoblast organoids were grown in 4-5 20 μ L Matrigel drops in 35mm ibidi μ -dishes and EdU and/or antibody labelling was performed as previously described¹¹. EdU incubation was for 1h at 37°C in TOM containing 10 μ M EdU. For primary antibodies and secondary antibodies used see Supplementary Table 1d. Imaging was with the ZEISS 700 Confocal microscope and ZEN Microscope Software.

Mitotracker staining

Mitochondrial function was evaluated by Mitotracker Red-CMXRos (ThermoFisher #M7512). Organoids were released from Matrigel with Cell Recovery Solution (Corning #354253) and incubated in 500nM of Mitotracker Red in TOM in suspension at 37°C for 30 mins. The organoids were washed in basal medium, resuspended and plated into a thin layer

of Matrigel in ibidi μ -dishes for imaging on a ZEISS 700 Confocal microscope with ZEN Microscope Software.

Time-lapse microscopy

Trophoblast organoids or placental villous explants embedded in 300 μ L of Matrigel in 35mm ibidi μ -dishes were imaged in phase-contrast and across several z-stacks on a Zeiss Axiovert Z1 microscope with the multidimensional imaging function of the Axio Observer software Axiovision image software V4.8. The images were compiled into a single movie by using the extended focus wavelet function.

ELISA

Conditioned media were harvested from organoid cultures and centrifuged to remove debris and stored at -80°C until use. Human Chorionic Gonadotropin β (hCG- β) ELISA (Abcam #ab108638) was performed on 50 μ L supernate with 100 μ L sample buffer in duplicate alongside hCG- β standards following the manufacturer's instructions. Concentration of hCG- β in the supernates was calculated from the line formula of the standard plots in Microsoft Office Excel. Supernatants were also tested with Clear&Simple Digital Pregnancy Test following manufacturer's instructions. Image reproduced with the permission of SPD Swiss Precision Diagnostics GmbH (SPD). GDF-15 was measured by in-house electrochemiluminescence immunoassay on the MesoScale Discovery assay platform (MSD) using BioTechne DuoSet antibodies and standard (BioTechne #DY957). For further details see Supplementary Methods.

Flow cytometry

Organoids were removed from Matrigel with Cell Recovery Solution and dissociated with 0.2% trypsin 250 (Pan Biotech P10-025100P)/0.02% EDTA (Sigma #E9884) in PBS at 37°C for 5 min. Cells were washed in medium containing FBS and passed through a 40 μ m cell strainer (Falcon #2340). Cells were blocked with human IgG (Sigma #I4506) in DPBS (ThermoFisher Scientific #14190136)/1%FBS prior to labelling with W6/32-Alexa-488 anti-HLA-A,B,C antibody, HLA-G-PE, ITGA2-PE or isotype-matched controls (Supplementary Table 1d). LIVE/DEADTM Fixable Far Red Dead Cell Stain (Life Technologies #L10119) was used for live/dead discrimination. Data were acquired using Cytex Development DxP8 (488/637/561). Data were analysed in FlowJo (Tree Star) and all compensation was applied digitally after acquisition.

In Situ Hybridization (ISH) Assays

ISH for *LGR5* was performed on 4 μ m paraffin sections with RNAscope 2.0 High definition assay (Advanced Cell Diagnostics) following the manufacturer's instructions. Briefly, tissue sections were baked at 60°C for 1h, dewaxed with xylene, cleared in 100% ethanol and air-dried prior to the standard protocol: 10min in Pretreat buffer 1, 15min in Pretreat buffer 2 and 30min at 37°C in Pretreat buffer 3 followed by incubation with *LGR5* probe (#311021), positive control probe *UBC* (#310041) or negative control probe *dapB* (#310043) for 2h at 40°C . Signal was visualized with the amplification kit and DAB for 10min. Sections were

dehydrated, mounted in DPX (Sigma #44581) and imaged on a Zeiss Axiovert Z1 microscope with Axiovision imaging software SE64 V4.8.

Electronic microscopy (EM)

Trophoblast organoids were directly fixed in 35mm dishes with 0.5% glutaraldehyde/0.2 M sodium cacodylate buffer (pH 7.2) for 30min and reduced with osmium tetroxide as previously described¹¹. Ultrathin sections were examined in an FEI Tecnai G2 TEM at 80Kv. Images were acquired with MegaView III CCD and Soft Imaging Systems program. Samples from human placentas were fixed by immersion in 3% glutaraldehyde-0.3% hydrogen peroxide in 0.1mol/L 1,4-piperazine diethane sulfonic acid (PIPES) buffer (pH 7). After 2h at RT, tissue was washed for 30min in 0.1 mol/L PIPES buffer. Secondary fixation was by immersion in 1% osmium tetroxide in PIPES buffer for 1h at RT. After washing, specimens were dehydrated in graded ethanol and embedded in Araldite epoxy resin. Ultrathin sections (50nm) were cut on a Reichert-Jung Ultracut S (Reichert-Jung). Sections were counterstained with uranyl acetate, followed by lead citrate, before viewing in a Philips CM100 electron microscope (Philips Electronics).

DNA extraction and quantification

QIAamp DNA blood Mini kit (Qiagen #51104) was used to extract genomic DNA from patients' blood for Short tandem repeat analysis, HLA tissue typing and Bisulphite sequencing. DNA was extracted from trophoblast organoids, decidual and placental tissues by digestion with ATL buffer (Qiagen #19076) and Proteinase K (Sigma #P4850), followed by purification steps with RNase A (Sigma #R6513) and Protein Precipitation Solution (Qiagen #158910). DNA was precipitated with isopropanol and washed with 70% ethanol. DNA quality and concentration were determined in a Nanodrop ND-1000 Spectrophotometer.

Comparative Genomic Hybridization (CGH) analysis

DNA from two independently derived trophoblast organoid samples at early and late passages was analysed with Agilent Sureprint G3 unrestricted CGH ISCA 8x60K array (Agilent #G4450A). DNA samples from late passage organoids were compared to early passage organoids (hybridization control). DNA was diluted to 50ng/uL and labelled using the Agilent kit following manufacturer's instructions. Data analysis for segmentation and copy number calls was performed at a genome-wide resolution of 500kb by the default analysis method – CGH v2 from the Agilent CytoGenomics software Edition 2.5.8.11 (Build 37).

Short tandem repeat analysis and HLA typing

Microsatellite analysis was performed with the GenePrint® PowerPlex™16 System (Promega) involving fluorescent labelled multiplexed PCR amplification of 15 short tandem repeat (STR) loci and Amelogenin sex determining fragments. PCR fragment size resolution was achieved with capillary electrophoresis on a 3730XL DNA Analyzer (Applied Biosystems) before analysis of the raw data and STR allele calling with GeneMapper Versions 4 and 5 (Life technologies) fragment analysis sizing and genotyping software. All

typing was performed blind. The Promega PowerPlex 16 kit was designed for Forensic testing and has a sensitivity capable of detecting down to 5% contamination. It is used by for monitoring of post-haematopoietic stem cell transplant chimerism^{25,26}. The DNA for HLA genotyping was processed via the workflows of the EFI accredited Clinical Histocompatibility Laboratory. Low resolution typing of the HLA-A, -B and -C genes was achieved with LABType kits (One Lambda) which rely on reaction patterns observed when sequence specific DNA probes immobilised on fluorescent X-MAP polystyrene beads (Luminex) hybridise to biotin labelled multiplexed gene specific PCR amplicons. The hybrids were detected with a Lighthouse 200 fluorimeter (QIAGEN) and HLA allele assignment was from HLA Fusion software (One Lambda). Ultra High resolution typing of HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 was achieved with an 'in house' Third Generation Sequencing pipeline utilising Pacific Biosciences' Single Molecule Real-Time DNA sequencing technology as previously described²⁷.

Bisulphite sequencing

Approximately 300ng of DNA was used for bisulphite conversion performed with the EpiTect Bisulfite Kit (Qiagen #59110), according to the manufacturer's protocol. The *ELF5* promoter region was amplified as described previously²⁸. PCR products were cloned and sequenced, confirming representation of distinct alleles.

Global DNA methylation analysis

Genomic DNA bisulfite (BS) was performed with the CEGX TrueMethyl kit (Cambridge Epigenetics/NuGEN) and used for microarray-based DNA methylation analysis, performed at GenomeScan (GenomeScan B.V., Leiden, The Netherlands), on the HumanMethylation850 BeadChip (Illumina) and were scanned on the Illumina iScan system. The resulting iDAT files were imported and analysed by ChAMP (v2.9.10)^{29,30}. Samples were processed filtering for a probe detection p-value ≤ 0.01 , probes with a bead count < 3 in at least 5% of samples, no CpG and known SNPs at probe starts, probes aligning to multiple locations, and QC using the on array control probes³¹. Of the total probes on the array 755577 passed the filtering and QC steps. The BMIQ method was used to normalise the two probe types present on the array. Beta methylation values from the EPIC array range from 0 (unmethylated) to 1 (methylated) and are equivalent of percentage methylation³². Genomic annotations were imported from FDb.InfiniumMethylation.hg19 and IlluminaHumanMethylationEPICmanifest³³. Genomic features in Fig. 2g comprise the following numbers of assayed CpGs: CpG Islands, 48799, 48799, 13576; Promoters, 151270, 151270, 88621; Gene Bodies, 280,284,280,284,117,138; LINE1, 48799, 48799, 13576. Boxplots comprise min: 1.5 x inter-quartile, lower: 1st quartile, middle: median, upper: 3rd quartile, max: 1.5 x inter-quartile range (Extended Data Figures 2G and 5e). Density/violin plots are scaled to area. LINE1 elements were downloaded as tables from UCSC Genome browser for hg19³⁰. Maternal blood samples (normal) are taken from E-GEOD-66210.

RNA extraction, quantification and quality control

Total RNA was isolated using the miRNeasy isolation kit (Qiagen #217004) with on-column DNase digestion (Qiagen #79254). Quantitation of RNA was performed with the Quant-

iTTM RiboGreenTM RNA Assay Kit (Thermo Fisher Scientific #R11490) by measuring the intensity of fluorescence at 528nm with a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments) according to manufacturer's instructions. RNA quality was assessed on the Agilent 2100 bioanalyzer (Thermo Fisher Scientific). RNA Integrity Number (RIN) of each tested sample was greater or equal to 8.

Reverse transcription and Real-time quantitative RT-PCR (qRT-PCR)

The expression of *ELF5*, *ERVW-1* and *GCM1* was analysed with Taqman Gene expression assays (Applied Biosystems). 500ng-1µg of total RNA was reverse transcribed with Superscript VILO Reverse Transcriptase (Thermo Fisher Scientific #11754050) in the presence of random hexamers and RNase inhibitor following supplier's instructions. qRT-PCR was performed on 7900HT Fast Real-Time PCR system (Applied Biosystems) as previously described¹¹. Relative expression levels were normalised to the geometric mean of three housekeeping genes *HPRT1*, *TOP1* and *TBP* and the 2^{-Ct} method. The expression of C19MC miRNAs *hsa-miR-517-5p*, *hsa-miR-517(a, b)-3p*, *hsa-miR-526b-3p*, *hsa-miR-525-3p* and reference gene *RNU48* were analysed by TaqMan miRNA assays (Applied Biosystems). 10ng of total RNA were reverse transcribed using miRNA specific stem-loop RT primers and TaqMan microRNA reverse transcription kit (Applied Biosystems #4366596) according to the supplier's instructions. QRT-PCR assays were run with qPCRBIO Probe Mix Lo-ROX (PCR Biosystems) containing specific probes on an Eppendorf Mastercycler® RealPlex 2 instrument. Ct data were normalised to the RNU48 internal control by the 2^{-Ct} method. All qPCR reactions included no-template controls and minus RT controls (-RT). For further details and for Taqman Assay IDs for each gene see Supplementary Methods.

Microarray expression profiling and data analysis

The microarray experiment was performed at Cambridge Genomic Services at University of Cambridge with a species specific Gene 2.1 ST Array Plate (Affymetrix) according to manufacturer's instructions. Briefly, 100ng of total RNA was amplified for each sample with inline PolyA spike-in control and the WT PLUS amplification kit (Affymetrix). By using the in line hybridization controls, we successfully amplified samples with the GeneChip WT terminal labelling kit (Affymetrix). Plate arrays were processed on the GeneTitan instrument (Affymetrix) with the GeneTitan Hybridization, Wash and Stain kit (Affymetrix). Samples were hybridized to the array, washed, stained and scanned. CEL files generated were loaded in R using the oligo package from Bioconductor. The raw data were then processed after quality controls using the Robust Multichip Analysis method. The limma package (3.34.8) was used to make the comparisons, and results were corrected for multiple testing using False Discovery Rate. Microarray probes without gene identifier (ensembl gene id) were filtered out. Initial QC included PCA and MDS plots. Finally the quality of the data was assessed, and the correlation of the samples in the groups compared. Heatmaps were generated with the R package 'pheatmap' (1.0.8), which uses the Euclidean method. For the gene heatmaps, the input is the normalized intensity matrix. GO terms enrichment was obtained with R package 'clusterProfiler' (3.6.0) with function `enrichGO`, and chord plots were generated with the R package 'GOplot' (1.0.2).

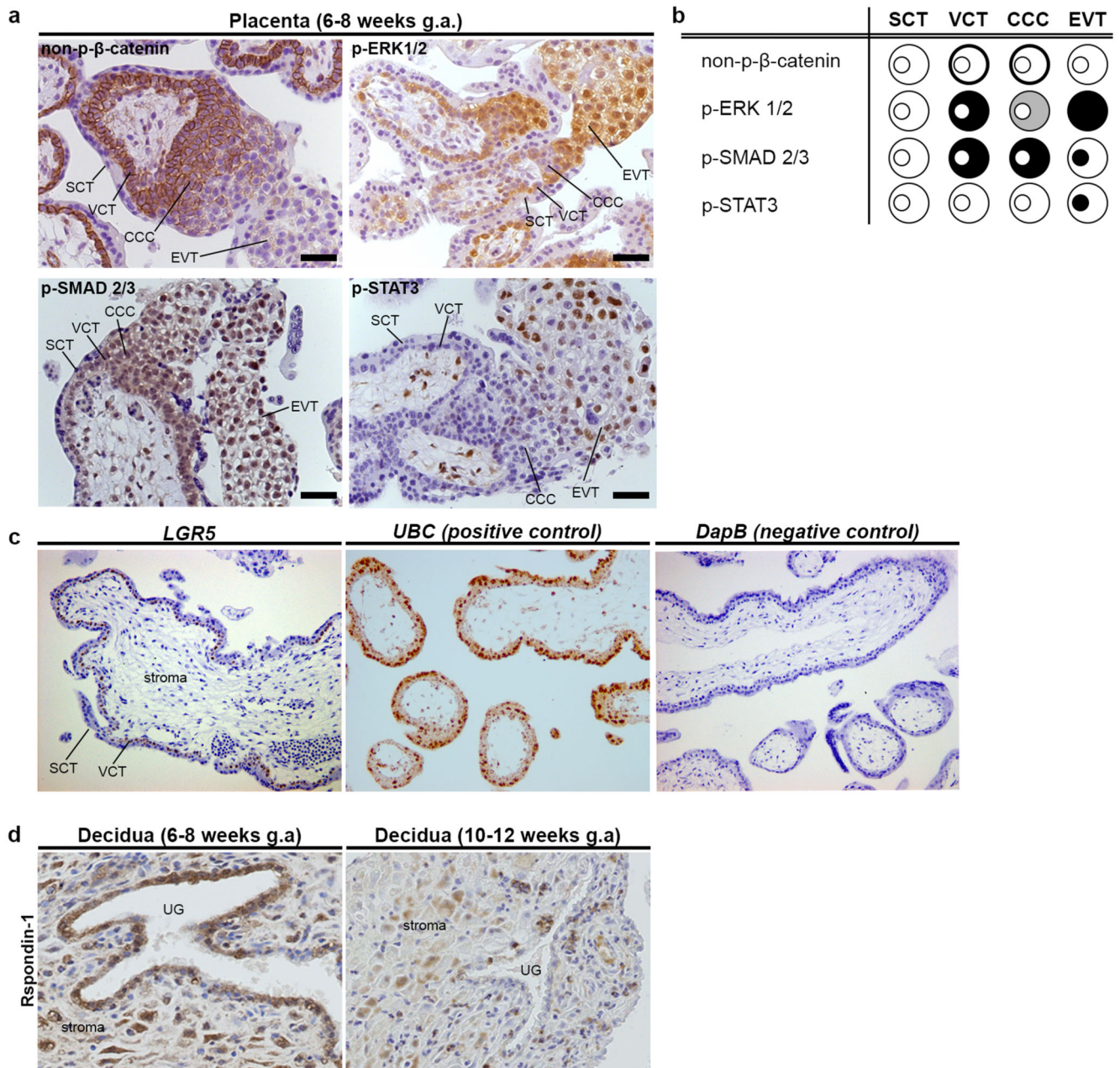
LC-MS analysis of trophoblast organoid supernatants

Trophoblast organoids (day 10 after passaging) were grown in trophoblast organoid medium and supernatants collected after overnight incubation. The supernatants (500 μ L) and an aliquot of growth media were acidified with 50 μ L of 1% formic acid in water (v/v) and loaded directly onto an Oasis Prime μ -elution 96 well SPE plate (Waters #186008052) and extracted as described previously³⁴. The eluant was evaporated under oxygen free nitrogen at 40°C and reconstituted into 75 μ L of 50mM ammonium bicarbonate in water with 10mM dithiothreitol. Protein digests were prepared and analysed using an Ultimate 3000 nano LC system coupled to a Q Exactive Plus Orbitrap mass spectrometer (ThermoScientific) as described previously³⁵. The nano LC/MS files obtained from the six different extracts were combined and searched using Peaks 8.5 software (BSI) against the human Swissprot database (Downloaded on 26-Oct-2017). A tryptic digest setting was used and precursor and product ion tolerances were set at 10 ppm and 0.05 Da respectively. The search parameters included a fixed modification of a carboxyamidomethylation on cysteine residues and variable modifications such as methionine oxidation, N-terminal pyro-glutamate, N-terminal acetylation and C-terminal amidation. A false discovery rate (FDR) value of 1% was applied at the peptide and a minimum of 1 unique peptide also was required. For further details see Supplementary Methods.

Statistics and Reproducibility

All experiments reported in this study have been reproduced with similar results derived from independent samples (tissues and organoids) from multiple patients. The number of times the experiments were repeated with independently-derived trophoblast organoid cultures are reported in figure legends as (n =) and summarized in Supplementary Table 1c. Given the descriptive nature of the work and biological variation between human samples, the experimental data points for each patient sample are shown separately unless stated otherwise. Trophoblast organoid culture protocols were independently replicated by four scientists. Statistical analyses used to analyse microarray, methylation and LC-MS data are reported in Methods above.

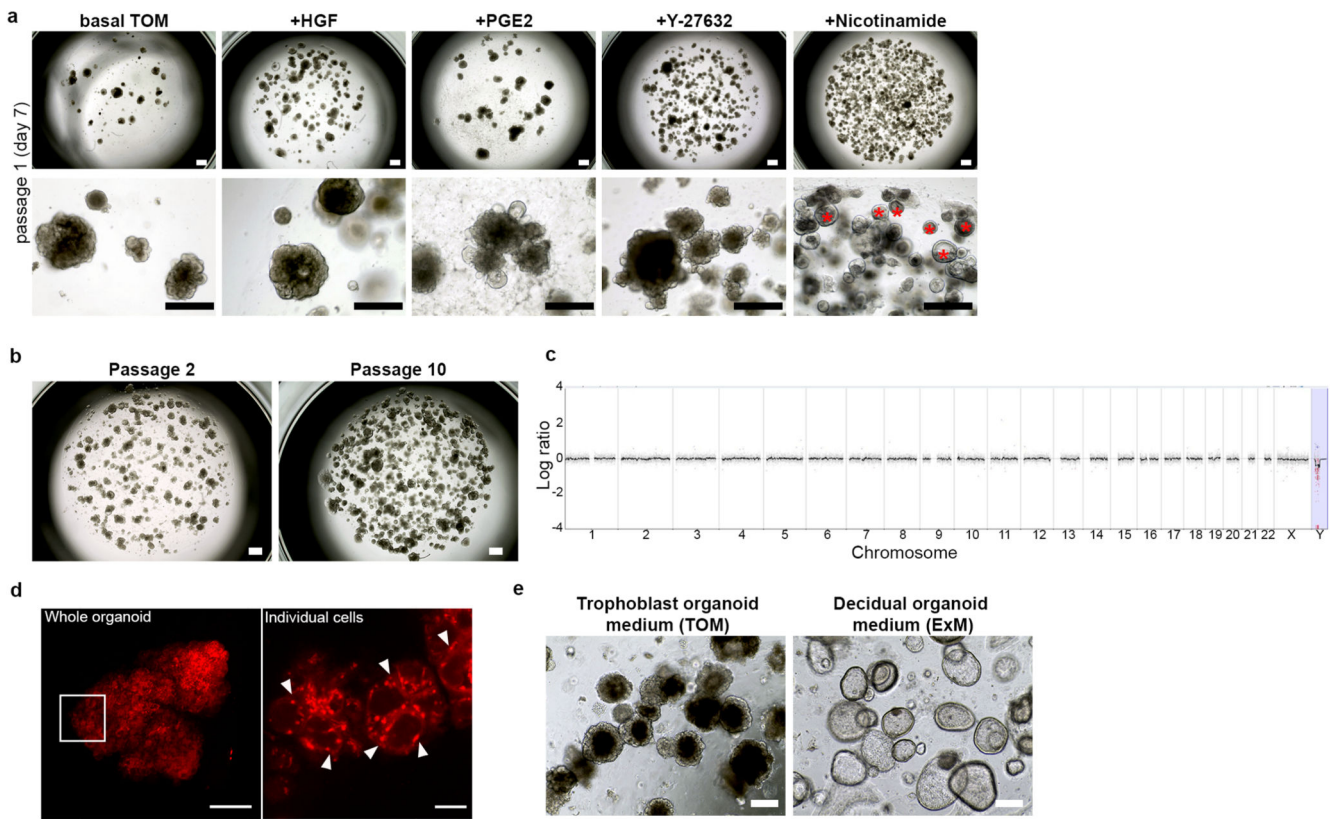
Extended Data



Extended Data Figure 1. Staining for signalling pathways in first trimester placenta and decidua.

a, IHC of first trimester placenta (6-8 weeks g.a.) for effectors of major signalling pathways (i) WNT signalling, non-phosphorylated (p)-β-catenin (S33/S37/T41), (ii) TGFβ signalling through p-SMAD 2 (S465/467)/Smad3 (S423/425), (iii) MAPK signalling through p-ERK 1/2 (T202/Y204) and (iv) p-STAT3 (Y705) signalling. Scale bars, 50μm. Representative images from $n=8$, for each antibody. BMP signalling through SMAD1/5/8 was not possible to assess by IHC. VCT and CCC displayed membrane-localised staining of non-p β-catenin, whereas p-Erk1/2 was mostly cytoplasmic in both cell types. Cytoplasmic and nuclear signals for p-Erk1/2 were detected in EVT. P-SMAD 2/3 staining also showed stronger nuclear signals in EVT, suggesting a role for TGFβ signalling in differentiation in

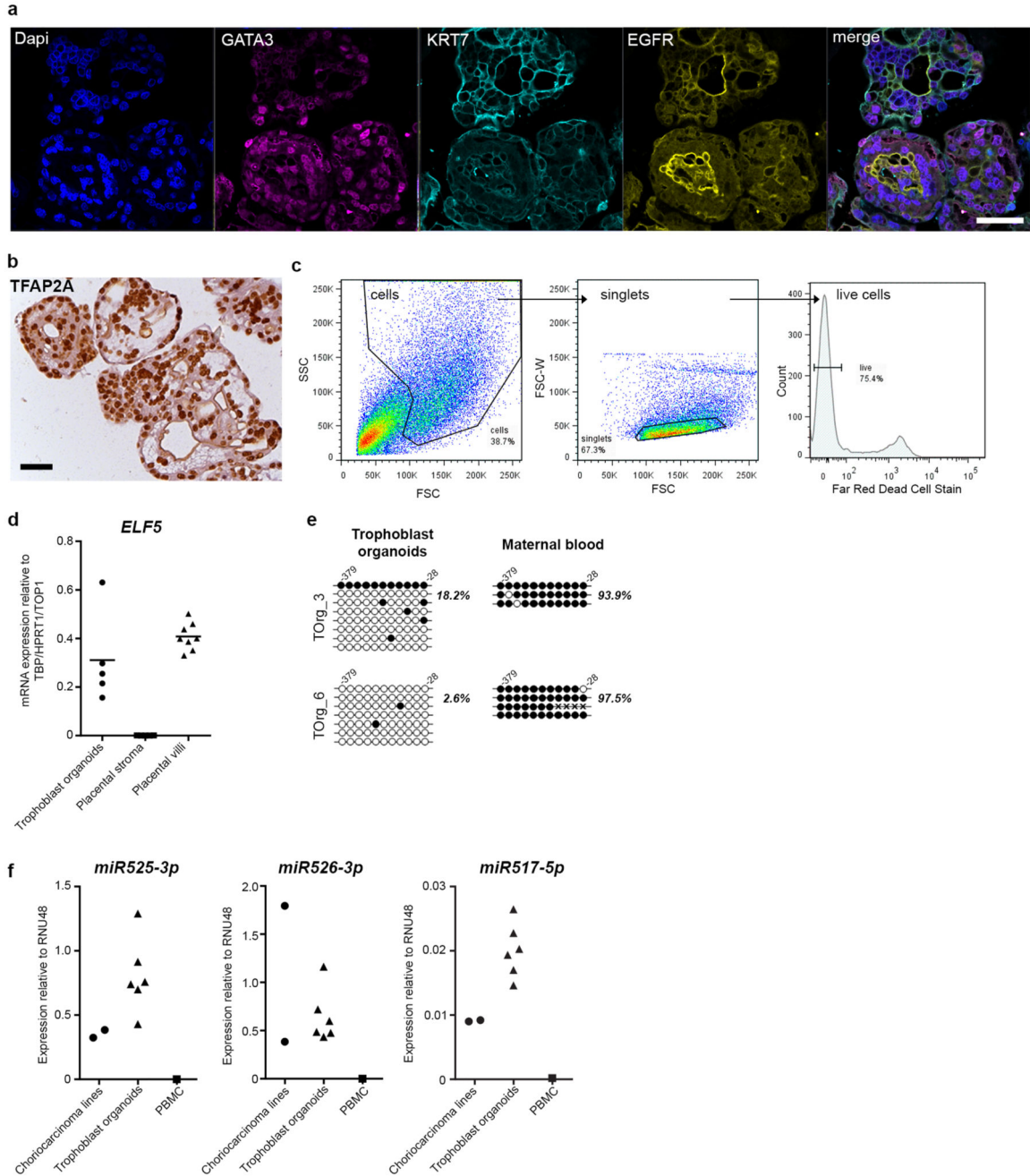
accordance with previous report⁹. Phosphorylated nuclear STAT3 was detected only in EVT, again indicating involvement in their differentiation. SCT was negative for all these signals. **b**, Summary of findings from **a**. Trophoblast cells from different regions of the placenta are represented as a circle with nucleus (small inner circle). Black indicates strong staining, grey indicates faint staining and white not detected. Thicker circles indicate staining localized to cell membrane. **c**, In situ hybridization for *LGR5* on first trimester placental villi. *LGR5* transcripts are detected in VCT. Stroma is negative. Positive control probe is for *UBIQUITIN (UBC)*. Negative control probe is for the bacterial gene *dapB*. Nuclei are counterstained in Haematoxylin. Images are at x10 magnification. Representative images from $n=2$. **d**, IHC for Rspodin-1 on early first trimester (6-8 weeks g.a.) and late first trimester (10-12 weeks g.a.) decidual samples. Representative images from $n=2$ for each tissue type. Images are at x20 magnification. SCT, syncytiotrophoblast; VCT, villous cytotrophoblast; CCC, cytotrophoblast cell columns; EVT, extravillous trophoblast; UG, uterine glands.



Extended Data Figure 2. Culture components tested for the establishment of long-term organoid cultures of trophoblast from human placentas.

a, Growth factors (HGF, PGE2, Y-27632, Nicotinamide) were added as supplements to basal trophoblast organoid medium (TOM) that contains EGF, CHIR99021, Rspondin-1, A83-01, FGF2 (Supplementary Table 1a,b). Bright field images of placental digests at passage 1, day 7. The cystic structures that appear with the addition of Nicotinamide (red asterisks) are contaminating maternal glandular organoids. Representative images from $n=2$. Conditions containing factors that did not show growth are not included. Scale bars in upper and lower rows, 500 μ m. **b**, Trophoblast organoid cultures at passage 2 and at passage 10 with continuous culture. Representative images from $n=3$. Scale bars, 500 μ m. **c**, Analysis of genetic stability of cultures ($n=2$) with comparative genomics hybridization (CGH) array. Shown is a representative whole-genome array CGH plot generated with Agilent Cytogenomics software. Genomic DNA from late passage (p8) trophoblast organoids is compared to genomic DNA from early passage (p2). Each spot is a single probe. Plotted are the log ratios of the average signal intensity of each probe on the Y-axis along its position on the chromosomes (1-22, X and Y) on the x-axis. A log signal ratio of 0 represents equivalent copy number the samples. No significant DNA copy number abnormalities were identified. **d**, Live imaging of trophoblast organoid cultures ($n=2$) passaged >6 months and then frozen/thawed and exposed to Mitotracker Red. Functional mitochondria are visible showing that the cells are healthy (white arrowheads). Scale bars, whole organoid 50 μ m; Individual cells 10 μ m. **e**, Organoids derived from the same placental cell isolate using either trophoblast organoid medium (TOM) or Decidual organoid medium (ExM) demonstrating that matched

placental (fetal) and decidual (maternal) organoids can be derived from the one sample. Representative bright field images from $n=3$. Scale bars, 500 μ m.

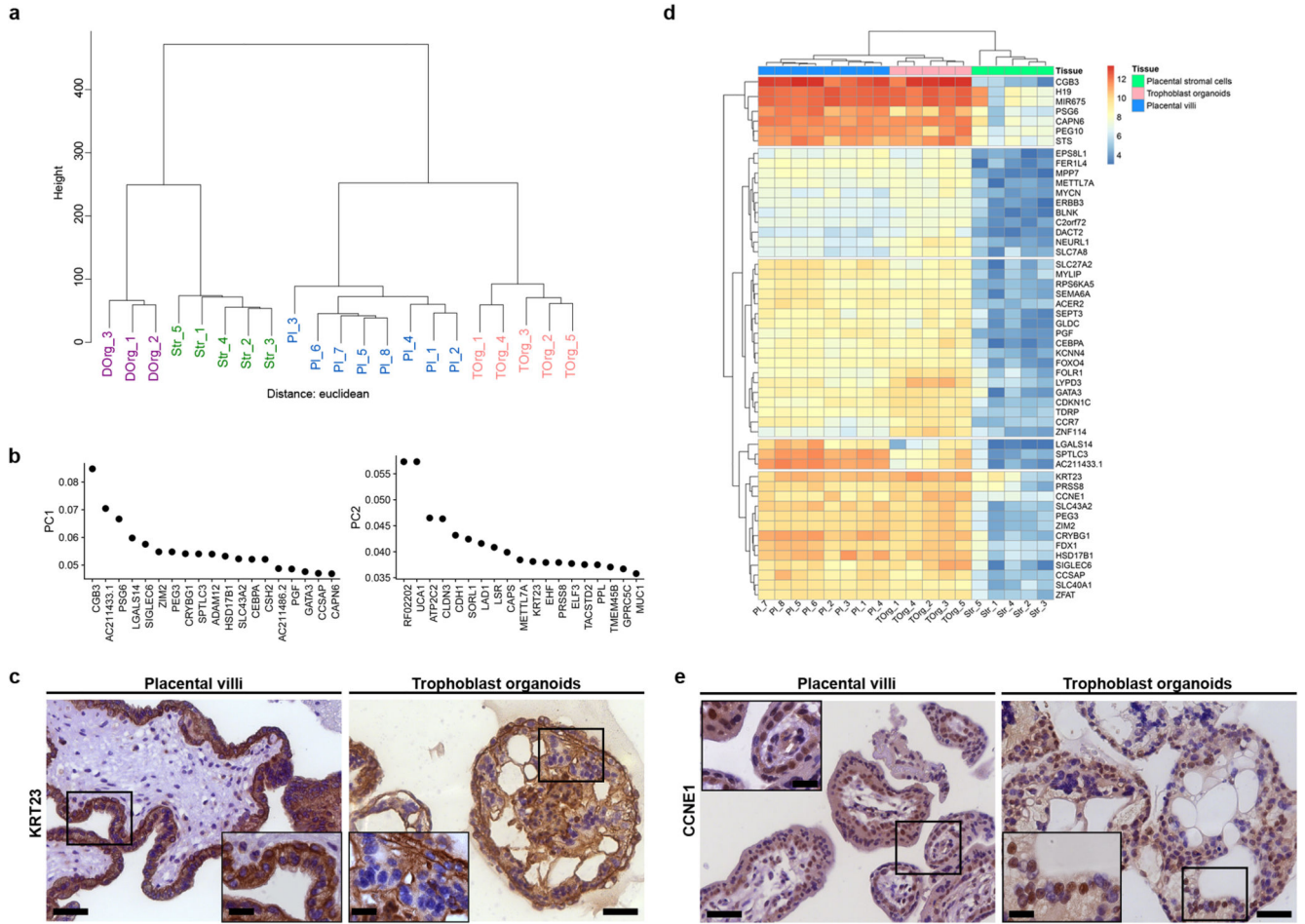


Extended Data Figure 3. Trophoblast organoids retain characteristic features of first trimester trophoblast *in vivo*.

a, Representative images of positive staining for GATA3, KRT7, EGFR and DAPI on trophoblast organoids by confocal microscopy (representative image from $n=3$). EGFR stains both VCT and the surface of SCT as *in vivo*. The basement membrane is around the outside of the organoids with formation of syncytial masses in the centre. Scale bar, 50 μ m.

b, IHC for transcription factor TFAP2A shows uniform expression on trophoblast organoids (representative image from $n=20$). Scale bars, 50 μ m. **c**, Gating strategy used for flow

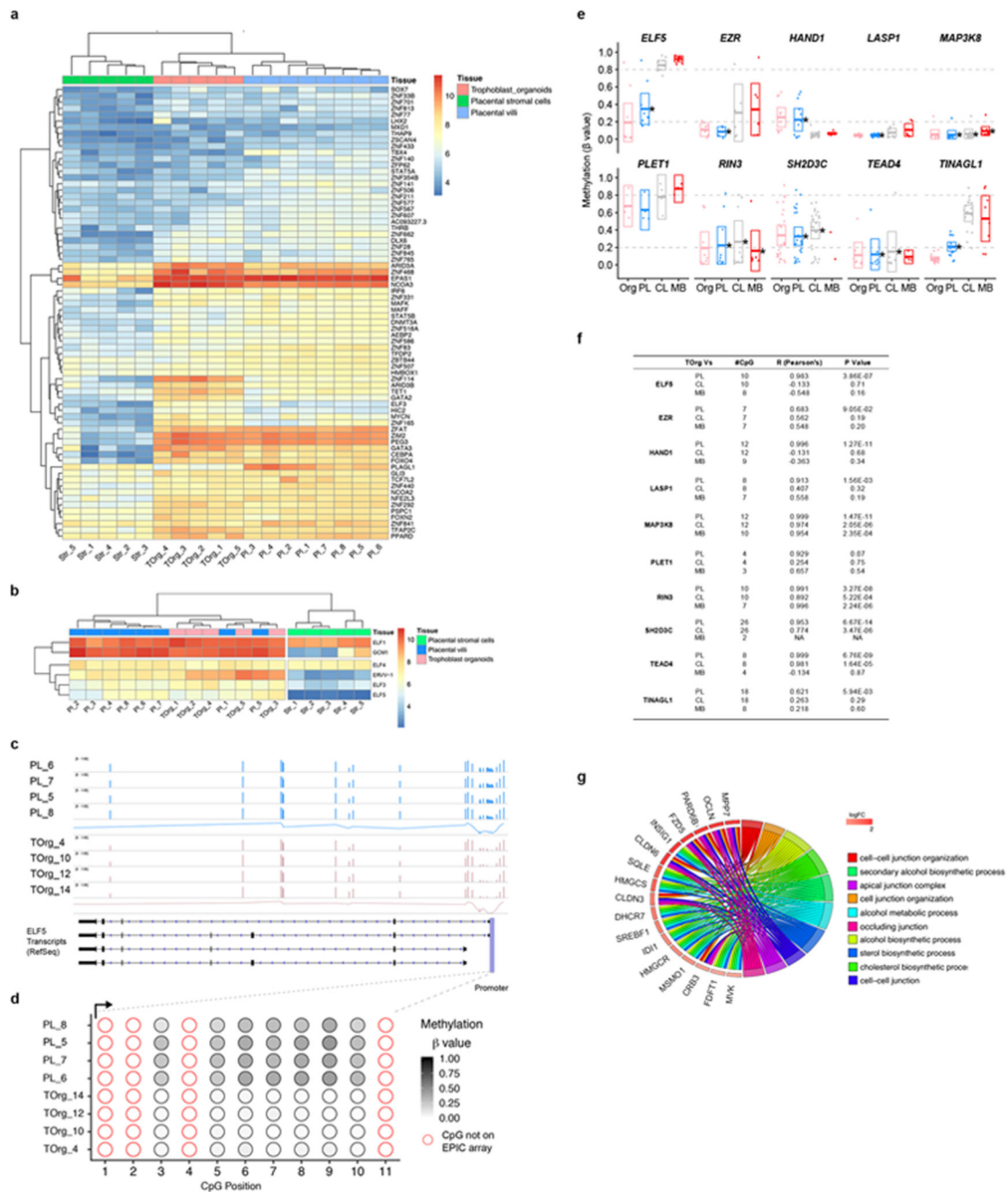
cytometric analysis of single, live cells from trophoblast organoids. **d**, Quantitative RT-PCR (qPCR) analysis of *ELF5* in trophoblast organoids ($n=5$) compared to whole placental villi ($n=8$) and stromal cells isolated from the placenta ($n=5$). Graph shows expression levels relative to the geometric mean of three housekeeping genes *TBP*, *TOP1* and *HPRT1*. The mean *ELF5* expression is shown for each sample group. Source Data File 2. **e**, Bisulfite sequencing of the *ELF5* promoter region (-379bp to -28bp upstream of the transcription start site) of trophoblast organoids from two different placentas (TOrg 3, 6) and matched maternal blood leukocytes (positive control). The relative % of methylated cytosine residues (filled circles) are indicated. **f**, qPCR analysis for *miR525-3p*, *miR526-3p* and *miR517-5p* from C19MC miRNA cluster on trophoblast organoids ($n=6$), JEG-3 and JAR (positive controls) and peripheral blood monocytes (PBMC, low expression/negative control). Graph shows relative expression levels of each organoid culture to housekeeping gene *RNU48*. Source Data File 1.



Extended Data Figure 4. Hierarchical clustering of microarray data comparing placental villi, trophoblast organoids and placental stromal cells.

a, Unsupervised hierarchical clustering analysis of global gene expression profiles by microarray of first trimester placental villi (PI)(n=8), trophoblast organoids (TOrg)(n=5), placental stromal cells (Str)(n=5) and decidual organoids (DOrg)(n=3). Analysis was based on 12673 probes. The expression profiles of trophoblast organoids cluster with first trimester placental samples whilst decidual organoids and placental stromal cells cluster in a separate tree. **b**, Top 20 genes contributing to PC1 and PC2 in the PCA plot from Fig. 2e. The top genes contributing to PC1 are all trophoblast-specific genes such as *CGB3*, *GATA3* and *PSG6* indicating that these genes separate the trophoblast organoid and placental villous samples from the two potentially-contaminating, non-trophoblast samples (decidual organoids and placental stroma). The top genes contributing to PC2 are epithelial genes such as *CLDN3*, *TACSTD2* and *KRT23*. The organoids only contain trophoblast, but cells of the villous core (stromal, Hofbauer and endothelial cells) are also present in the placental samples. **c**, IHC of placental villi and trophoblast organoids stained for KRT23 showing expression in all trophoblast cells *in vivo* and *in vitro*. Experiment repeated independently 3 times. Scale bar, 50µm. Higher power in insets scale bar, 20µm. **d**, Clustered heatmap of differentially expressed genes between trophoblast organoids, placental villi and placental stroma with an absolute log₂ fold change of 2 (adjusted p.value < 0.05). **e**, IHC of placental

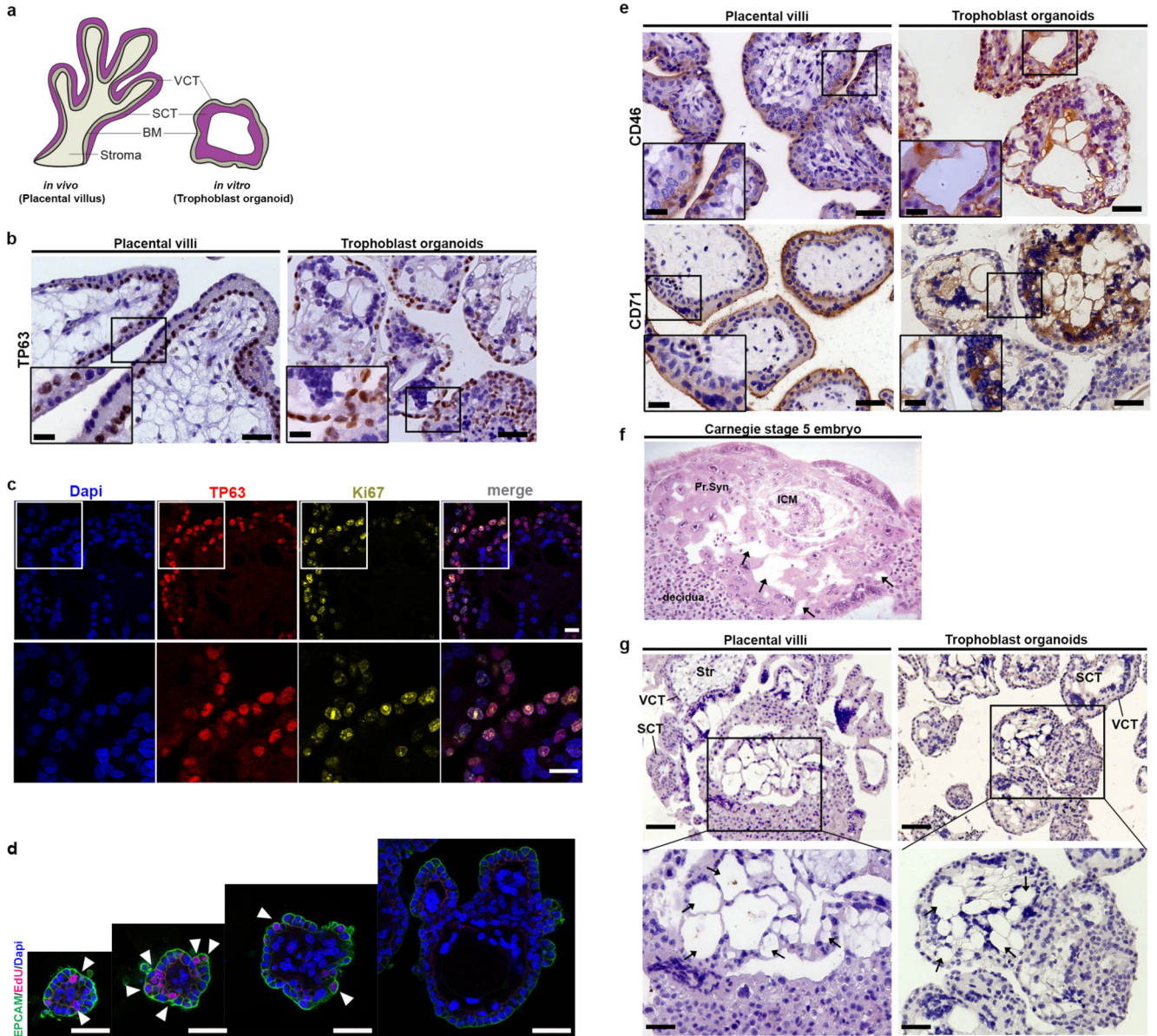
villi and trophoblast organoids stained for CCNE1 showing expression in trophoblast cells *in vivo* and *in vitro*. Scale bar, 50 μ m. Higher power in insets scale bar, 20 μ m. Experiment repeated independently 3 times.



Extended Data Figure 5. Transcription factor expression profiles of trophoblast organoids and placental villi.

a, Heatmap highlighting transcription factors from the differentially expressed genes between placental villi, trophoblast organoids and placental stromal cells. **b**, Heatmap of genes from the *ELF* family of transcription factors and syncytial genes, *GCM1* and *ERVW-1*. *ELF3* and *ELF5* both show moderate expression levels across the organoids and placental samples, and very low/no expression in the stromal samples. *ELF4* and *ELF1* are similar in all samples. There is very high expression of *ELF1* in placentas and organoids.

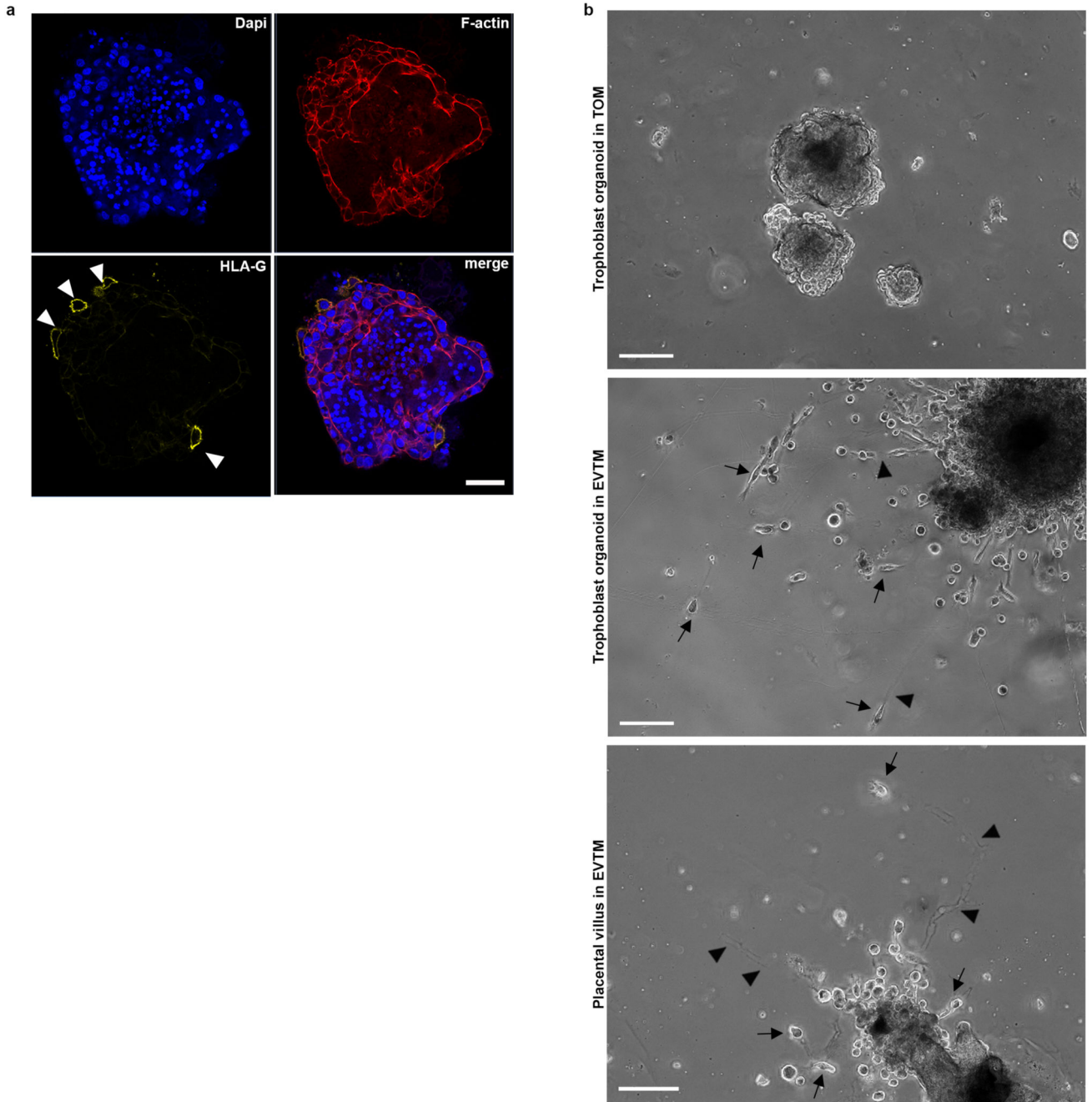
Similarly, both *ERVW-1* and especially *GCM1* are expressed at higher levels in placentas and organoids in agreement with qPCR data (Fig. 3d). **c**, Genomic mapping of the methylation array probes to the *ELF5* gene. The height of the bars indicates methylation level from 0, unmethylated to 1.0, fully methylated. **d**, Methylation of the *ELF5* promoter shows hypomethylation in the organoid and placenta samples. **e**, Distribution of methylcytosine across the promoters of the 10 murine trophoblast gatekeeper genes¹². The organoids (Org) and placenta (PL) samples show very similar methylation patterns across all 10 gene promoters that are distinct from the control/brain (CL) and maternal-blood (MB) in the majority of the genes. Boxplots comprise min: 1.5 x inter-quartile, lower: 1st quartile, middle: median, upper: 3rd quartile, max: 1.5 x inter-quartile range. Significant correlations (<0.01) are indicated (*). **f**, Table showing Pearson's correlation coefficient (R), number of CpG/probes compared and p-values for **e**. **g**, Chord plot representing terms from the gene ontology analysis of upregulated genes in trophoblast organoids.



Extended Data Figure 6. Structure and proliferation in trophoblast organoids.

a, A schematic diagram of a normal placental villus *in vivo* compared to a trophoblast organoid. The basement membrane (BM) beneath the VCT is contiguous with the stromal villous core *in vivo* and with the Matrigel *in vitro*. The SCT contacts maternal blood in the intervillous space *in vivo*. SCT forms in the centre of the organoids. **b**, IHC for TP63 in first trimester placenta and trophoblast organoids (representative images from $n=14$). TP63 is expressed in VCT. Scale bar, 50 μ m. Higher power insets scale bar, 20 μ m. **c**, Representative images of TP63, Ki67 and DAPI staining on trophoblast organoids by confocal microscopy ($n=3$). Cells on the outside of the organoids are TP63+ and Ki67+. Scale bars, 20 μ m. **d**, Confocal microscopy images of trophoblast organoid stained for EdU, EPCAM and DAPI showing fewer proliferating cells (white arrowheads) as the organoids enlarge. Scale bar, 50 μ m. Representative images from $n=3$. **e**, IHC for markers of SCT, CD46 and CD71, in

first trimester placenta and trophoblast organoids (representative images from $n=20$). CD46 and CD71 stain the syncytial brush border. Scale bar, 50 μ m. Higher power insets scale bar, 20 μ m. **f**, Carnegie stage 5b embryo (~9 days post-fertilization) from Carnegie Collection at the early lacunar stage (number 8171). Courtesy of Prof. Enders and the Centre for Trophoblast Research (<https://www.trophoblast.cam.ac.uk/Resources/enders>). Arrows point to examples of cavities that appear in the primitive syncytium due to fluid accumulation before the coelomic cavity and the embryo have fully developed. **g**, Similar cavities in placental tissue samples from first trimester (6-9 weeks g.a) and in syncytium in centre of trophoblast organoids. Boxed areas are shown at higher magnification (bottom). Scale bars, 200 μ m (top); 50 μ m (bottom). Similar morphology seen in at least 5 early placental villi and in all organoids. ICM, inner cell mass. Pr.Syn. primitive syncytium; VCT, villous cytotrophoblast; SCT, syncytiotrophoblast; Str, stromal core.



Extended Data Figure 7. Trophoblast organoids grown in TOM and EVTm.

a, Confocal image of organoid stained for F-actin, DAPI and HLA-G. A few isolated cells stain for HLA-G (white arrowheads) at the periphery of the organoid. Scale bar, 50 μ m. Representative image from $n=3$. **b**, Phase contrast images from time-lapse videos of 16h of trophoblast organoids grown in TOM when EVT differentiation does not occur (top). No invasive cells are visible. Shown for comparison is an organoid (middle) and a placental villous explant exposed to EVTm (bottom). Black arrows indicate cells migrating and

arrowheads show visible tracks made as the cells invade through the Matrigel. For time-lapse videos of these cultures see Supplementary Videos 1, 4 and 5. Scale bars, 200 μ m.

Extended Data Table 1
Microsatellite analysis and HLA typing of organoids from placental digests in TOM show that they are of fetal origin

a, PowerPlex 16 Short Tandem Repeat (STR) genotyping of DNA from matched maternal-fetal samples (maternal blood, decidua and placenta) to identify origin of organoids. Shown in top row are the 15 STR loci analysed. Numbered STR alleles observed for each DNA at a particular locus are listed within the relevant column. As expected in most cases a maximum of two alleles were seen. In cases where there was evidence of an additional allele from a fetal derived STR haplotype the allele number appears in brackets. This is consistent with the decidua containing fetal extravillous trophoblast. The results at informative loci where fetus and trophoblast organoids match are underlined. **b**, HLA genotyping with Third Generation SMRT sequencing of DNA from matched maternal blood and trophoblast organoid to confirm STR analysis. HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 loci were investigated. The two HLA alleles at each locus defined at high resolution are shown for each sample. For each locus, the fetal trophoblast will share one allele with the mother but the other allele will be derived from the father and is likely to be different. Not all loci are informative as some paternal and non-inherited maternal alleles are the same.

Sample ID	D3S1358	TH01	D21S11	D18S51	Penta E	D5S818	D13S317	D7S820	DI6S539	CSFIPO	Penta D	Amelo.	vWA	D8S1179	FGA
Maternal Blood_2	16,17	7,9,3	28,30	12,14	15,15	12,13	10,15	11,11	12,13	10,10	9,9	x,x	15,18	12,13	22,2,23
Decidua_2	16,17	7,9,3	28,30	12,14	15,15	12,13	10,15	11,11	12,13	10,10	9,9	x,x	15,18	12,13	22,2,23
Placenta_2	<u>14,16</u>	<u>6,9,3</u>	28,30	<u>14,15</u>	<u>12,15</u>	<u>11,13</u>	<u>10,14</u>	<u>10,11</u>	<u>12,12</u>	10,10	<u>9,13</u>	x,x	<u>15,17</u>	<u>13,13</u>	<u>22,23</u>
Trophoblast Organoid_2	<u>14,16</u>	<u>6,9,3</u>	28,30	<u>14,15</u>	<u>12,15</u>	<u>11,13</u>	<u>10,14</u>	<u>10,11</u>	<u>12,12</u>	10,10	<u>9,13</u>	x,x	<u>15,17</u>	<u>13,13</u>	<u>22,23</u>
Maternal Blood_3	16,17	6,9	29,31,2	12,14	7,12	11,12	10,11	9,10	11,11	10,12	10,13	x,x	18,18	12,13	20,25
Decidua_3	16,17	6,9 (9,3)	29,31,2 (28)	12,14	7,12 (19)	11,12	10,11 (14)	9,10	11,11	10,12 (11)	10,13 (14)	x,x	18,18 (19)	12,13	20,25 (21)
Placenta_3	<u>16,16</u>	<u>9,9,3</u>	<u>28,31,2</u>	12,14	<u>7,19</u>	<u>11,11</u>	<u>10,14</u>	9,10	11,11	<u>11,12</u>	<u>13,14</u>	x,x	<u>18,19</u>	<u>12,12</u>	<u>21,25</u>
Trophoblast Organoid_3	<u>16,16</u>	<u>9,9,3</u>	<u>28,31,2</u>	12,14	<u>7,19</u>	<u>11,11</u>	<u>10,14</u>	9,10	11,11	<u>11,12</u>	<u>13,14</u>	x,x	<u>18,19</u>	<u>12,12</u>	<u>21,25</u>
Maternal Blood_6	14,15	7,9,3	30,30	12,16	16,19	11,11	11,13	9,12	11,13	10,11	9,9	x,x	15,15	10,10	21,26
Decidua_6	14,15	7,9,3	30,30	12,16	16,19	11,11	11,13	9,12	11,13	10,11	9,9	x,x	15,15	10,10	21,26
Placenta_6	<u>15,17</u>	<u>6,7</u>	<u>28,30</u>	<u>16,17</u>	<u>5,16</u>	<u>10,11</u>	<u>12,13</u>	<u>9,11</u>	11,13	<u>10,10</u>	<u>9,13</u>	<u>x,y</u>	<u>15,17</u>	<u>10,11</u>	<u>24,26</u>
Trophoblast Organoid_6	<u>15,17</u>	<u>6,7</u>	<u>28,30</u>	<u>16,17</u>	<u>5,16</u>	<u>10,11</u>	<u>12,13</u>	<u>9,11</u>	11,13	<u>10,10</u>	<u>9,13</u>	<u>x,y</u>	<u>15,17</u>	<u>10,11</u>	<u>24,26</u>

a

Sample ID	D3S1358	TH01	D21S11	D18S51	Penta E	D5S818	D13S317	D7S820	D16S539	CSFIPO	Penta D	Amelo.	vWA	D8S1179	FGA
Maternal Blood_7	15,16	6,6	29,30,2	16,16	7,18	9,13	12,12	9,10	13,13	11,11	10,11	x,x	17,18	12,14	21,21
Decidua_7	15,16 (17)	6,6	29,30,2	16,16	7,18	9,13	12,12 (10)	9,10	13,13 (10)	11,11	10,11	x,x (y)	17,18	12,14 (13)	21,21 (24)
Placenta_7	<u>15,17</u>	6,6	<u>29,29</u>	16,16	7,18	<u>13,13</u>	<u>10,12</u>	9,10	<u>10,13</u>	11,11	10,11	<u>x,y</u>	17,18	<u>13,14</u>	<u>21,24</u>
Trophoblast Organoid_7	<u>15,17</u>	6,6	<u>29,29</u>	16,16	7,18	<u>13,13</u>	<u>10,12</u>	9,10	<u>10,13</u>	11,11	10,11	<u>x,y</u>	17,18	<u>13,14</u>	<u>21,24</u>
Maternal Blood_8	17,18	7,9	30,30	16,20	11,12	12,12	11,12	9,10	11,14	11,12	11,13	x,x	15,18	13,14	22,22
Decidua_8	17,18 (15)	7,9 (8)	30,30	16,20 (12)	11,12 (7)	12,12 (10)	11,12	9,10 (11)	11,14	11,12	11,13	x,x (y)	15,18	13,14	22,22 (28)
Placenta_8	<u>15,18</u>	<u>7,8</u>	30,30	<u>12,16</u>	<u>7,12</u>	<u>10,12</u>	<u>11,11</u>	<u>9,11</u>	<u>9,14</u>	<u>11,11</u>	<u>11,11</u>	<u>x,y</u>	<u>15,17</u>	13,14	<u>22,28</u>
Trophoblast Organoid_8	<u>15,18</u>	<u>7,8</u>	30,30	<u>12,16</u>	<u>7,12</u>	<u>10,12</u>	<u>11,11</u>	<u>9,11</u>	<u>9,14</u>	<u>11,11</u>	<u>11,11</u>	<u>x,y</u>	<u>15,17</u>	13,14	<u>22,28</u>
Maternal Blood_9	15,18	9,3,9,3	33,2,34,2	12,17	5,5	12,13	12,12	10,11	11,11	10,12	11,12	x,x	15,16	14,15	19,22
Decidua_9	15,18	<u>9,3,9,3</u> <u>(10)</u>	33,2,34,2 (31)	12,17 (13)	5,5 (14)	12,13	12,12	10,11(8)	11,11	10,12	11,12 (10)	x,x (y)	15,16	14,15 (12)	19,22 (21)
Placenta_9	<u>15,15</u>	<u>9,3,10</u>	<u>31,33,2</u>	<u>13,17</u>	<u>5,14</u>	12,13	12,12	<u>8,10</u>	11,11	10,12	<u>10,12</u>	<u>x,y</u>	<u>16,16</u>	<u>12,14</u>	<u>19,21</u>
Trophoblast Organoid_9	<u>15,15</u>	<u>9,3,10</u>	<u>31,33,2</u>	<u>13,17</u>	<u>5,14</u>	12,13	12,12	<u>8,10</u>	11,11	10,12	<u>10,12</u>	<u>x,y</u>	<u>16,16</u>	<u>12,14</u>	<u>19,21</u>

b

Sample ID	HLA-A*	HLA-A*	HLA-B*	HLA-B*	HLA-C*	HLA-C*	HLA-C*	HLA-DRB1*	HLA-DRB1*	HLA-DQB1*	HLA-DQB1*	HLA-DRB1*	HLA-DRB1*	HLA-DQB1*	HLA-DQB1*	HLA-DPBI*	HLA-DPBI*
Maternal Blood_2	02:01:01:01/16	02:01:01:01/16	35:01:01:02/04/05/06	44:02:01:03	07:04:01:01/03	03:03:01:01	03:03:01:01	11:01:01	08:01/77	03:01:01	04:02:01	04:01:01	04:01:01	04:02:01	04:01:01	04:01:01	04:01:01
Trophoblast Organoid_2	02:01:01:01/16	11:01:01:01	35:01:01:02/04/05/06	39:01:01:03/05	12:03:01:01	03:03:01:01	03:03:01:01	11:01:01	08:01/77	03:01:01	04:02:01	04:01:01	04:01:01	04:02:01	04:01:01	04:01:01	04:01:01
Maternal Blood_3	02:01:01:01/16	24:02:01:01	15:01:01:01	40:01:02:01/04	03:04:01:01	03:03:01:01	03:03:01:01	04:04:01	01:01:01	03:02:01	05:01:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01
Trophoblast Organoid_3	02:01:01:01/16	24:02:01:01	15:01:01:01	15:01:01:01	03:04:01:01	03:03:01:01	03:03:01:01	04:04:01	04:01:01	03:02:01	03:02:01	04:01:01	04:01:01	03:02:01	04:01:01	04:01:01	04:01:01

b

Sample ID	HLA-A*	HLA-A*	HLA-B*	HLA-B*	HLA-C*	HLA-C*	HLA-DRB1*	HLA-DRB1*	HLA-DQB1*	HLA-DQB1*	HLA-DPBI*	HLA-DPBI*
Maternal Blood_6	03:01:01:01	30:02:01:01	07:02:01:01/03	18:01:01:01/06	05:01:01:01	07:02:01:03	01:03:01	01:03:01	03:01:01	05:01:01	02:01:02/19	04:01:01
Trophoblast Organoid_6	68:01:02:02	30:02:01:01	27:05:02:01	18:01:01:01/06	05:01:01:01	07:04:01:01/03	01:03:01	08:01:77	04:02:01	05:01:01	02:01:02/19	04:01:01
Maternal Blood_7	32:01:01:01	33:01:01:01	14:02:01:01	44:02:01:01	05:01:01:02	08:02:01:01	01:01:01	01:02:01	05:01:01	05:01:01	13:01/107:01	04:01:01
Trophoblast Organoid_7	24:02:01:04	33:01:01:01	14:02:01:01	44:02:01:01	05:01:01:02	08:02:01:01	13:01:01	01:02:01	05:01:01	06:03:01	02:01:02/19	04:01:01
Maternal Blood_8	24:02:01:01	25:01:01:01	18:01:01:02/05	27:05:02:01	01:02:01:01	12:03:01:01	15:01:01	13:03:01	03:01:01	06:02:01	04:01:01	03:01:01
Trophoblast Organoid_8	24:02:01:01	02:01:01:01/16	49:01:01	27:05:02:01	01:02:01:01	07:02:01:01/15	11:02:01	13:03:01	03:01:01	03:19:01	10:01:01	03:01:01
Maternal Blood_9	26:01:01:01	03:01:01:01	07:02:01:01/03	27:05:02:01	02:02:01:01	07:02:01:03	04:04:01	04:04:01	03:02:01	03:02:01	04:01:01	02:01:02/19
Trophoblast Organoid_9	26:01:01:01	02:01:01:01/16	44:02:01:01	27:05:02:01	02:02:01:01	05:01:01:02	04:01:01	04:04:01	03:01:01	03:02:01	04:02:01	02:01:02/19

Extended Data Table 2 Microsatellite analysis and HLA typing of organoids from placental digests derived in ExM show that they are of maternal origin

a, PowerPlex16 STR genotyping of DNA from matched maternal-fetal samples (decidua, maternal blood, placenta) to identify origin of organoids. Shown in the top row are the 15 STR loci analysed. Numbered STR alleles observed for each DNA at a particular locus are listed within the relevant column. As expected in most cases a maximum of two alleles were seen but where there was lesser evidence of an additional allele from a fetal derived STR haplotype the allele number appears in brackets. The results at informative loci where maternal blood, decidua and organoids match are underlined. **b**, HLA genotyping with LABType Reverse SSO of DNA from 4 pregnancies with matched decidua, blood, placenta and organoids. Each pair of HLA alleles defined, at low resolution, for each sample at a particular locus are listed within the relevant column. The maternal origin of the organoids is clear from HLA-A, -B and -C alleles in 3 of the 4 pregnancies. The data was inconclusive for pregnancy 2 but all 4 organoids are clearly maternal in origin when using both methods (see above).

Sample ID	D3S1358	TH01	D21S11	D18S51	Penta E	D5S818	D13S317	D7S820	D16S539	CSFIPO	Penta D	Amelo.	vWA	D8S1179	FGA
Maternal Blood_1	15, 15	<u>7.9</u>	<u>30, 30</u>	<u>14, 17</u>	<u>11, 12</u>	<u>12, 13</u>	11, 12	<u>8, 8</u>	<u>11, 12</u>	<u>12, 12</u>	<u>9, 12</u>	xx	<u>17, 19</u>	13, 15	<u>22, 25</u>
Decidua_1	15, 15	<u>7.9</u>	<u>30, 30</u>	<u>14, 17</u>	<u>11, 12</u>	<u>12, 13</u>	11, 12	<u>8, 8</u>	<u>11, 12</u>	<u>12, 12</u>	<u>9, 12</u>	xx	<u>17, 19</u>	13, 15	<u>22, 25</u>
Placenta_1	15, 15	7, 8	29, 30	17, 17	10, 12	11, 12	11, 12	8, 10	12, 12	10, 12	10, 12	xx	16, 17	13, 15	<u>22, 25</u>
Decidual Organoid_1	15, 15	<u>7.9</u>	<u>30, 30</u>	<u>14, 17</u>	<u>11, 12</u>	<u>12, 13</u>	11, 12	<u>8, 8</u>	<u>11, 12</u>	<u>12, 12</u>	<u>9, 12</u>	xx	<u>17, 19</u>	13, 15	<u>22, 25</u>
Maternal Blood_2	16, 17	<u>6.9.3</u>	<u>28, 28</u>	15, 19	<u>12, 12</u>	11, 12	<u>8, 11</u>	<u>12, 14</u>	<u>12, 12</u>	10, 11	11, 12	xx	16, 17	<u>12, 15</u>	<u>20, 21</u>
Decidua_2	16, 17	<u>6.9.3</u>	<u>28, 28</u>	15, 19	<u>12, 12</u>	11, 12	<u>8, 11</u>	<u>12, 14</u>	<u>12, 12</u>	10, 11	11, 12	xx	16, 17	<u>12, 15</u>	<u>20, 21</u>
Placenta_2	16, 17	6, 8	28, 31.2	15, 19	12, 15	11, 12	8, 9	10, 12	12, 13	10, 11	11, 12	xx	16, 17	10, 15	<u>20, 23</u>
Decidual Organoid_2	16, 17	<u>6.9.3</u>	<u>28, 28</u>	15, 19	<u>12, 12</u>	11, 12	<u>8, 11</u>	<u>12, 14</u>	<u>12, 12</u>	10, 11	11, 12	xx	16, 17	<u>12, 15</u>	<u>20, 21</u>
Maternal Blood_3	<u>14, 17</u>	6, 6	<u>28, 31.2</u>	<u>12, 12</u>	<u>7, 13</u>	<u>12, 12</u>	11, 12	<u>10, 12</u>	<u>11, 13</u>	<u>10, 11</u>	<u>9, 10</u>	xx	15, 17	<u>13, 13</u>	<u>23, 24</u>
Decidua_3	<u>14, 17</u>	6, 6	<u>28, 31.2</u>	<u>12, 12</u>	<u>7, 13</u>	<u>12, 12</u>	11, 12	<u>10, 12, (9)</u>	<u>11, 13</u>	<u>10, 11, (12)</u>	<u>9, 10, (13)</u>	xx(y)	15, 17	<u>13, 13, (14)</u>	<u>23, 24, (22)</u>
Placenta_3	17, 17	6, 6	28, 28	12, 15	7, 12	12, 13	11, 11	9, 10	13, 13	11, 12	10, 13	xy	15, 17	13, 14	<u>22, 24</u>
Decidual Organoid_3	<u>14, 17</u>	6, 6	<u>28, 31.2</u>	<u>12, 12</u>	<u>7, 13</u>	<u>12, 12</u>	11, 12	<u>10, 12</u>	<u>11, 13</u>	<u>10, 11</u>	<u>9, 10</u>	xx	15, 17	<u>13, 13</u>	<u>23, 24</u>
Maternal Blood_4	<u>15, 15</u>	<u>9.3, 9.3</u>	<u>30, 32.2</u>	<u>18, 22</u>	<u>12, 14</u>	11, 12	8, 12	<u>10, 12</u>	<u>12, 13</u>	<u>9, 10</u>	<u>12, 16</u>	xx	<u>15, 16</u>	<u>13, 13</u>	<u>19, 22</u>

a

Sample ID	D3S1358	TH01	D21S11	D18S51	Penta E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta D	Amelo.	vWA	D8S1179	FGA
Decidua_4	<u>15,15</u>	<u>9,3,9,3</u>	<u>30,32,2</u>	<u>18,22</u>	<u>12,14</u>	<u>11,12</u>	<u>8,12</u>	<u>10,12</u>	<u>12,13</u>	<u>9,10</u>	<u>12,16</u>	xx	<u>15,16</u>	<u>13,13</u>	<u>19,22</u>
Placenta_4	<u>15,16</u>	<u>7,9,3</u>	<u>30,30</u>	<u>14,18</u>	<u>11,12</u>	<u>11,12</u>	<u>12,14</u>	<u>9,12</u>	<u>13,14</u>	<u>10,10</u>	<u>9,16</u>	xx	<u>16,16</u>	<u>13,14</u>	<u>20,22</u>
Decidual Organoid_4	<u>15,15</u>	<u>9,3,9,3</u>	<u>30,32,2</u>	<u>18,22</u>	<u>12,14</u>	<u>11,12</u>	<u>8,12</u>	<u>10,12</u>	<u>12,13</u>	<u>9,10</u>	<u>12,16</u>	xx	<u>15,16</u>	<u>13,13</u>	<u>19,22</u>

b

Sample ID	HLA-A	HLA-B	HLA-C
Maternal Blood_1	01,02	<u>41,52</u>	<u>12,17</u>
Decidua_1	01,02	<u>41,52</u>	<u>12,17</u>
Placenta_1	01,02	27,52	01,12
Decidual Organoid_1	<i>Fail x2</i>	<u>41,52</u>	<u>12,17</u>
Maternal Blood_2	02,02	07,44	05,07
Decidua_2	02,02	07,44	05,07
Placenta_2	02,02	07,44	05,07
Decidual Organoid_2	02,02	07,44	05,07
Maternal Blood_3	<u>25,29</u>	18,44	<u>12,16</u>
Decidua_3	<i>Fail x2</i>	18,44	<u>12,16</u>
Placenta_3	23,25	18,44	04,12
Decidual Organoid_3	<u>25,29</u>	18,44	<u>12,16</u>
Maternal Blood_4	<u>23,24</u>	<u>13,44</u>	<u>04,04</u>
Decidua_4	<u>23,24</u>	<u>13,44</u>	<u>04,04</u>
Placenta_4	11,24	13,40	02,04
Decidual Organoid_4	<u>23,24</u>	<u>13,44</u>	<u>04,04</u>

Extended Data Table 3 Liquor chromatography-mass spectrometry (LC-MS) analysis of the secretome of trophoblast organoids.

Supernatants from independently-derived trophoblast organoid cultures from six different placental samples were analysed: TOrg_2 (p23), TOrg_3 (p20), TOrg_5 (p6), TOrg_10 (p12), TOrg_12 (p4) and TOrg_14 (p5). Table shows the glycoproteins, classical peptides/protein hormones, placental specific peptides and proteins enriched in placental tissue identified in the secretome data by LC-MS. The -10lgP value is the statistical significance assigned to a peptide/protein match by the PEAKS software³⁷. Coverage% refers to the proportion of the primary amino acid sequence of each protein that is identified in the experiment. #Peptides refers to the number of peptide matches assigned to a protein, whilst #Unique refers to the number of peptides that are assigned solely to that protein group. #Spec refers to the number of peptide MS/MS spectra matched against a particular protein. The columns also indicate whether these are unique products produced by the placenta and/or whether they are products produced by other tissues but highly enriched in the placenta (among the top 10 organs producing that protein based on RNA expression levels). Tissue location data was compared to data from the Human Protein Atlas, <https://www.proteinatlas.org/>. All protein identification data are in Supplementary Table 2.

Accession	Description	-10lgP	Coverage (%)	Peptides	Unique	Spec	Placental specific	Placental enriched
Glycoproteins								
Q16557 PSG3_HUMAN	Pregnancy-specific beta-1-glycoprotein 3 GN=PSG3	272.19	28	10	1	25	Yes	
P11465 PSG2_HUMAN	Pregnancy-specific beta-1-glycoprotein 2 GN=PSG2	259.79	34	8	2	30	Yes	
Q00888 PSG4_HUMAN	Pregnancy-specific beta-1-glycoprotein 4 GN=PSG4	236.62	21	7	1	17	Yes	
P11464 PSG1_HUMAN	Pregnancy-specific beta-1-glycoprotein 1 GN=PSG1	228.57	21	8	1	15	Yes	
Classical peptides/hormones								
P01233 CGHB_HUMAN	Choriogonadotropin subunit beta GN=CGB	340.98	48	20	20	218	Yes	
P01215 GLHA_HUMAN	Glycoprotein hormones alpha chain GN=CGA	167.67	44	3	3	33	Yes	
Q15726 KISS1_HUMAN	Metastasis-suppressor KISS-1 GN=KISS1	268.26	62	11	11	59	Yes	
Q99988 GDF15_HUMAN	Growth/differentiation factor 15 GN=GDF15	236.31	34	7	7	42	Yes	Yes
Q1464 INSL4_HUMAN	Early placenta insulin-like peptide GN=INSL4	154.72	27	2	2	4	Yes	
P35318 ADML_HUMAN	Adrenomedullin GN=ADM	114.05	10	1	1	8	Yes	Yes
P0DML2 CSH1_HUMAN	Chorionic somatomammotropin hormone 1 GN=CSH1	85.82	6	1	1	1	Yes	
P01344 IGF2_HUMAN	Insulin-like growth factor II GN=IGF2	59.42	4	1	1	1	Yes	
P01303 NPY_HUMAN	Pro-neuropeptide Y GN=NPY	50.4	8	1	1	1	unknown	unknown
Placental specific peptides (not hormones)								
P35556 FBN2_HUMAN	Fibrillin-2 GN=FBN2	157.65	1	2	2	3	Yes	
Q9BXP8 PAPP2_HUMAN	Pappalysin-2 GN=PAPPA2	124.88	1	1	1	1	Yes	
O60829 PAGE4_HUMAN	P antigen family member 4 GN=PAGE4	112.65	37	2	2	2	Yes	

Accession	Description	-10lgP	Coverage (%)	Peptides	Unique	Spec	Placental specific	Placental enriched
P14061 DHB1_HUMAN	Estradiol 17-beta-dehydrogenase 1 GN=HSD17B1 PE=1 SV=3	83.47	5	1	1	1	Yes	Yes
P02771 FETA_HUMAN	Alpha-fetoprotein GN=AFP PE=1 SV=1	74.38	2	2	1	19	Yes	Yes
Q96GT9 XAGE2_HUMAN	X antigen family member 2 GN=XAGE2	62.57	23	1	1	1	Yes	Yes
Proteins enriched in placental tissue								
P09486 SPRC_HUMAN	SPARC GN=SPARC	310.3	59	13	13	29		Yes
Q12805 FBLN3_HUMAN	EGF-containing fibulin-like extracellular matrix protein 1 GN=EFEMP1	270.65	25	7	7	17		Yes
P15121 ALDR_HUMAN	Aldose reductase GN=AKR1B1	264.8	49	8	8	21		Yes
P80723 BASPI_HUMAN	Brain acid soluble protein 1 GN=BASPI	234.76	36	4	4	13		Yes
P02751 FINC_HUMAN	Fibronectin GN=FNI	233.91	5	7	7	7		Yes
O95633 FSTL3_HUMAN	Follistatin-related protein 3 GN=FSTL3	222.26	29	5	5	27		Yes
P23142 FBLN1_HUMAN	Fibulin-1 GN=FBLN1	218.6	10	5	5	10		Yes
P10451 OSTP_HUMAN	Osteopontin GN=SPP1	178.67	32	5	5	5		Yes
P14543 NID1_HUMAN	Nidogen-1 GN=NID1	162.21	4	4	4	21		Yes
Q14118 DAG1_HUMAN	Dystroglycan GN=DAG1	148.59	5	2	2	7		Yes
P27487 DPP4_HUMAN	Dipeptidyl peptidase 4 GN=DPP4	115.06	3	1	1	1		Yes
P21980 TGM2_HUMAN	Protein-glutamine gamma-glutamyltransferase 2 GN=TGM2	106.05	6	2	2	2		Yes
P24593 IBP5_HUMAN	Insulin-like growth factor-binding protein 5 GN=IGFBP5	100.63	5	1	1	1		Yes
P09601 HMOX1_HUMAN	Heme oxygenase 1 GN=HMOX1	100.13	8	1	1	1		Yes
Q15582 BGH3_HUMAN	Transforming growth factor-beta-induced protein ig-h3 GN=TGFB1	99.46	3	1	1	1		Yes
O76061 STC2_HUMAN	Stanniocalcin-2 GN=STC2	94.72	8	1	1	2		Yes
P05111 INH1A_HUMAN	Inhibin alpha chain GN=INH1A	80.59	5	1	1	2		Yes
P25815 S100P_HUMAN	Protein S100-P GN=S100P	70.28	40	2	2	2		Yes
P17936 IBP3_HUMAN	Insulin-like growth factor-binding protein 3 GN=IGFBP3	58.22	6	1	1	1		Yes

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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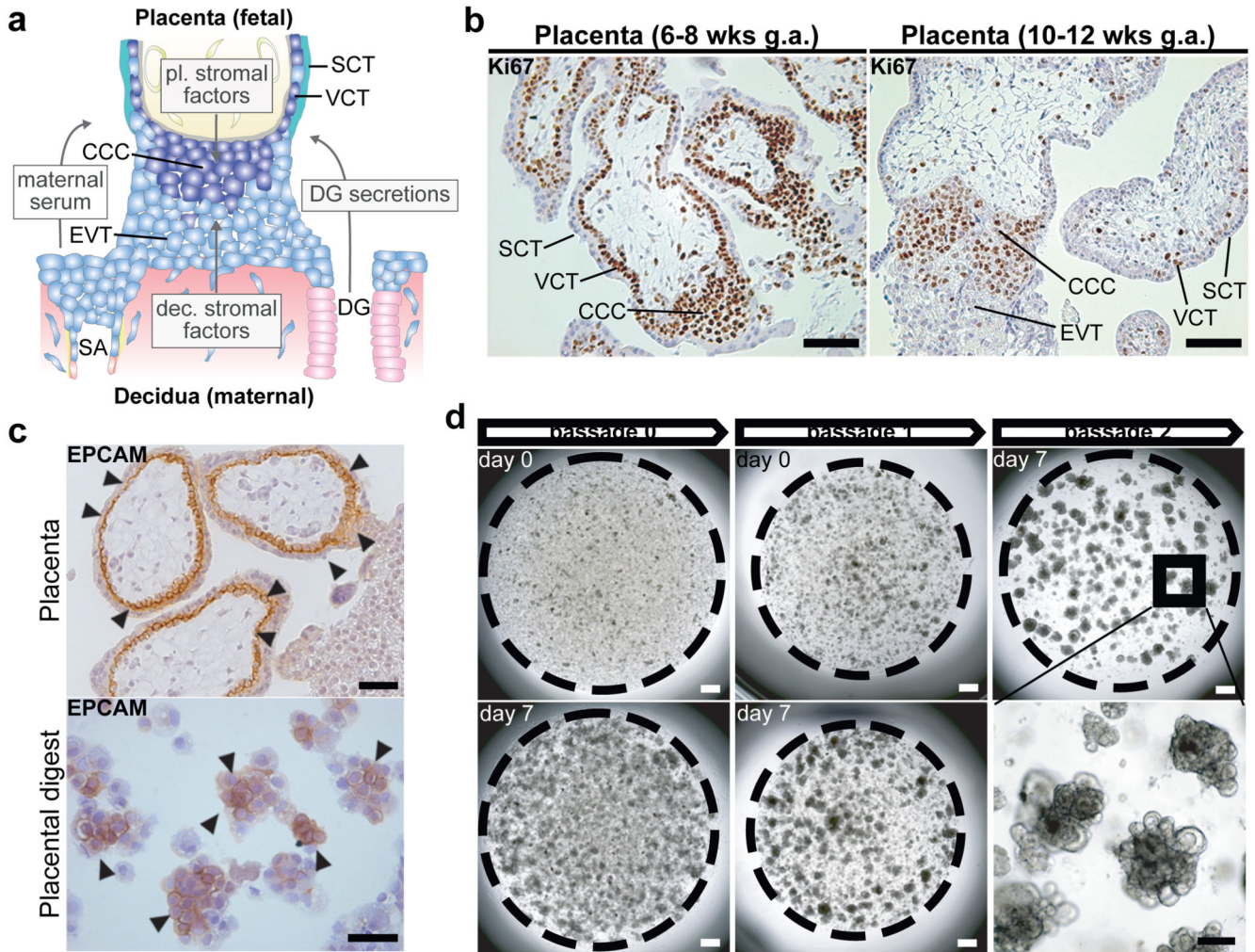


Figure 1. Establishment of long-term organoid cultures of trophoblast from human placentas.

a, A placental villus at the maternal-fetal interface in the first trimester of pregnancy showing the different trophoblast subsets: SCT, VCT, CCC and EVT. Sources of the intrinsic and extrinsic signals that could signal to proliferative Ki67+ trophoblast cells (dark blue) are shown. **b**, IHC for Ki67 in early first trimester placenta (6-8 weeks g.a.) compared to late first trimester (10-12 weeks g.a.). The proportion of proliferative cells is greatly reduced towards the end of first trimester and the cells are localized mostly in the CCC. Representative images from $n=6$ for each tissue type. Scale bars, 100 μ m. **c**, IHC for EPCAM on first trimester placenta (6-8 weeks g.a.) and cell clusters from placental digests. Experiment independently repeated twice with similar results. Arrowheads show VCT and CCC are EPCAM+ and these cells are present in the cell clumps from the placental digests. Scale bars, 50 μ m (placenta) and 200 μ m (placental digest). **d**, Time course for derivation of trophoblast organoids from one placental isolate. Bright field images of Matrigel drops after seeding placental digests starting from passage 0 day 0, until generation of homogenous trophoblast organoids (passage 2, day 7). For passages 0 and 1, timepoints at day 0 and 7 are shown. Passage 2, day 7 is shown together with the zoom of the boxed area in the lower panel. Experiment independently repeated for all organoid cultures with similar results.

Scale bars, 500 μ m (Matrigel droplet images) and 200 μ m (zoom in on trophoblast organoids). Gestational age, g.a.; SCT, syncytiotrophoblast; VCT, villous cytotrophoblast; CCC, cytotrophoblast cell column; EVT, extravillous trophoblast; DG, decidual gland; SA, spiral artery; pl, placental; dec, decidual.

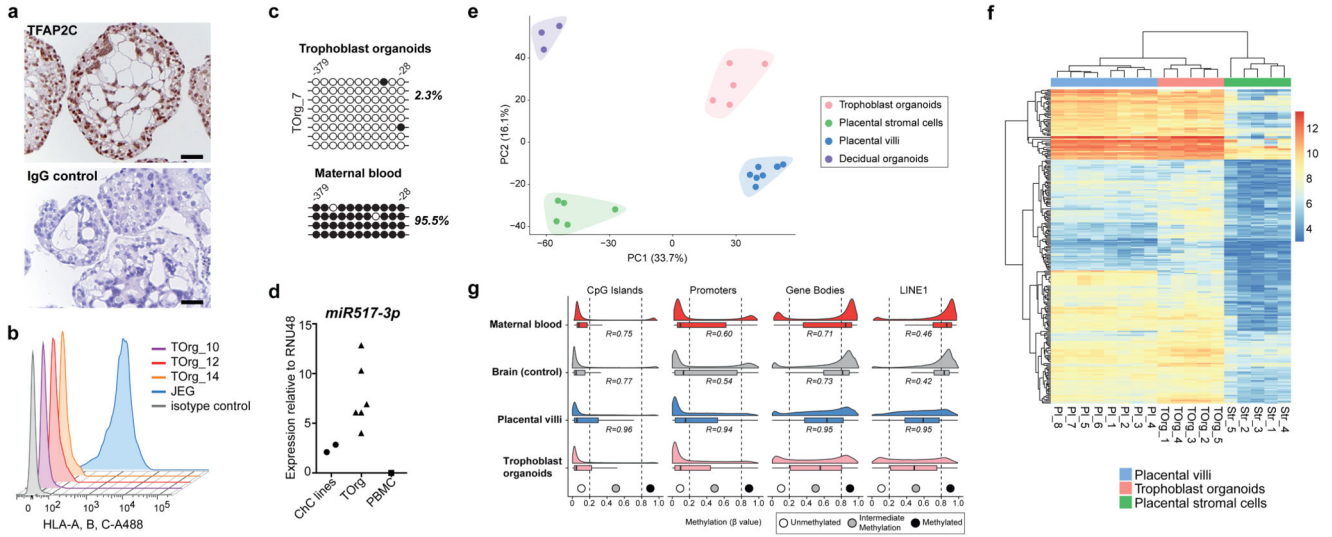


Figure 2. Trophoblast organoids retain characteristic features of first trimester trophoblast *in vivo*, and similar transcriptome and global methylation profiles.

a, IHC for TFAP2C shows uniform expression (representative images from $n=20$). Scale bars, 50 μ m. **b**, FACS analysis of three trophoblast organoids (TOrg10, 12, 14) and JEG (positive control) with mAb W6/32. Gating strategy see Extended Data Fig. 3c. Experiment independently repeated 3 times. **c**, Bisulfite sequencing of the *ELF5* promoter region of trophoblast organoids (TOrg 6) and matched maternal leukocytes (positive control). Relative % of methylated cytosine residues (filled circles) are indicated. **d**, qPCR analysis for *miR517-3p* from C19MC cluster on trophoblast organoids ($n=6$), JEG-3 and JAR choriocarcinoma (Chc.) lines (positive controls) and peripheral blood monocytes (PBMC). Graph shows relative expression levels to housekeeping gene *RNU48*. Source Data File 1. **e**, PCA of placental villi ($n=8$); trophoblast organoids derived from different placentas, TOrg_1 (passage p4), TOrg_2 (p7), TOrg_3 (p6), TOrg_4 (p4) and TOrg_5 (p6) ($n=5$); placental stromal cells ($n=5$); and decidual organoids ($n=3$). Analysis based on 12673 probes. Organoids cluster more closely with placental villi on PC1 axis. **f**, Clustered heatmap of differentially expressed genes in first trimester placental villi ($n=8$)(blue), trophoblast organoids ($n=5$)(pink) and cultured placental villous stromal cells ($n=5$)(green). **g**, Distribution of methyl-cytosine across genomic features is similar between trophoblast organoids ($n=4$) and placental samples ($n=4$). In contrast, the brain ($n=1$) and maternal blood ($n=5$) samples show distinct patterns, especially across CpG islands, gene bodies and LINE1 elements. Pearson's correlation coefficient (R) indicated for each comparison against trophoblast organoid samples (all p values $<2.2e-16$). Density/violin plots are scaled to area.

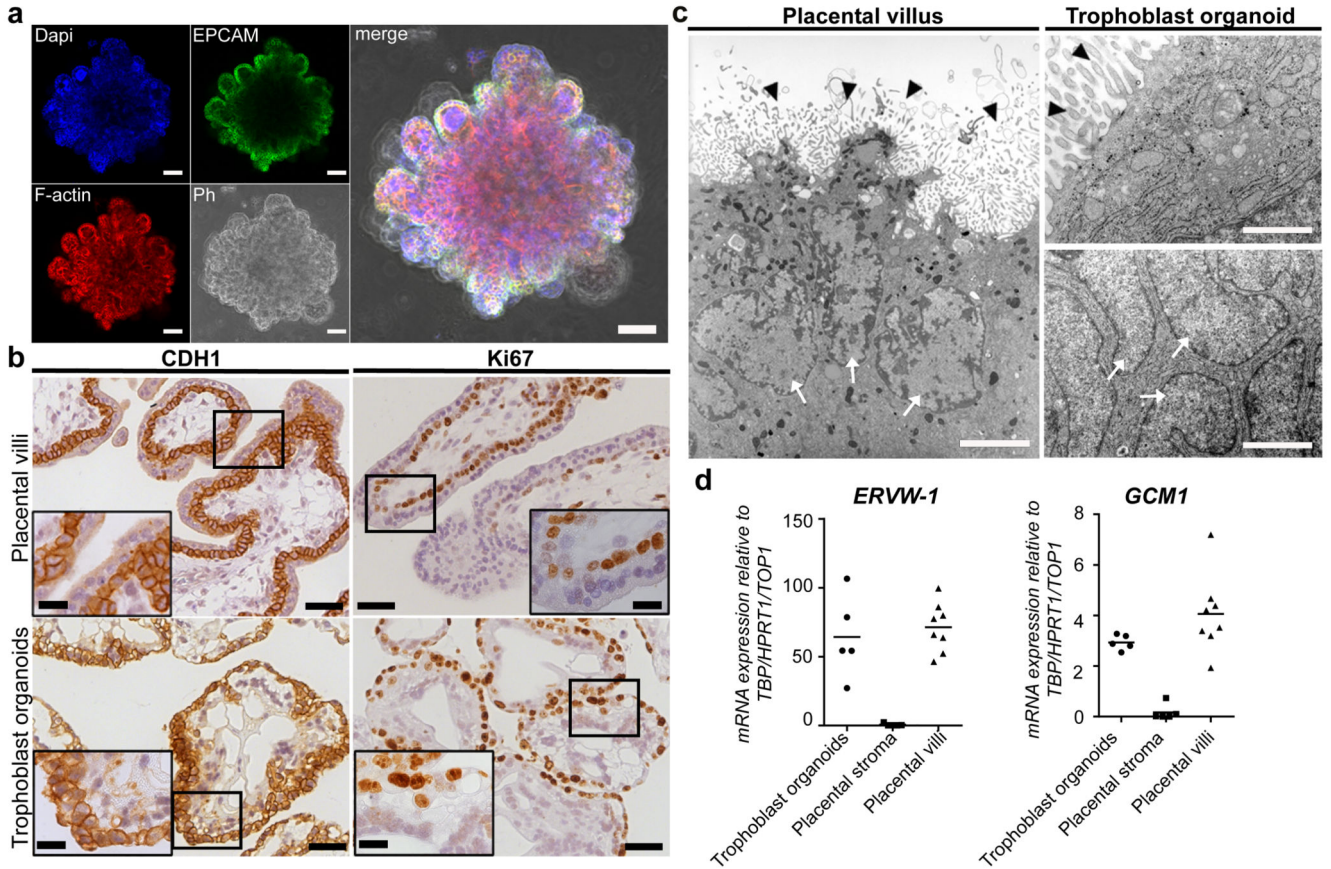


Figure 3. Trophoblast organoids form complex structures resembling placental villi with formation of syncytiotrophoblast (SCT).

a, Confocal microscopy images of trophoblast organoid stained for F-actin, EPCAM, DAPI merged with phase image (representative image from $n=5$). The EPCAM+ cells are surrounding the organoid. Scale bar, 100 μ m. **b**, IHC for CDH1 and Ki67 of first trimester placenta and trophoblast organoids (representative images from $n=6$ for Ki67 and $n=20$ for trophoblast organoids). VCT stains positively for CDH1. Ki67 is present in the inner VCT layer in villi and the outer layer in organoids. Scale bar, 50 μ m. Higher power in insets scale bar, 20 μ m. **c**, Electron micrograph images of first trimester SCT compared to the centre of a trophoblast organoid. Surface microvilli (arrowheads) and multinucleated areas can be seen (arrows). Scale bars, 5 μ m (placenta), 1 μ m (trophoblast organoids, microvilli) and 2.5 μ m (trophoblast organoids, nuclei). Representative images from $n=2$. **d**, qPCR analysis of genes *ERVW-1* and *GCM1* in trophoblast organoids ($n=5$) compared to whole placental villi ($n=8$) and stromal cells isolated from the placenta ($n=5$). Graphs show expression levels relative to geometric mean of three housekeeping genes *TBP*, *TOP1* and *HPRT1*. The mean *ERVW-1* and *GCM1* expression are shown for each sample group. Source Data File 2.

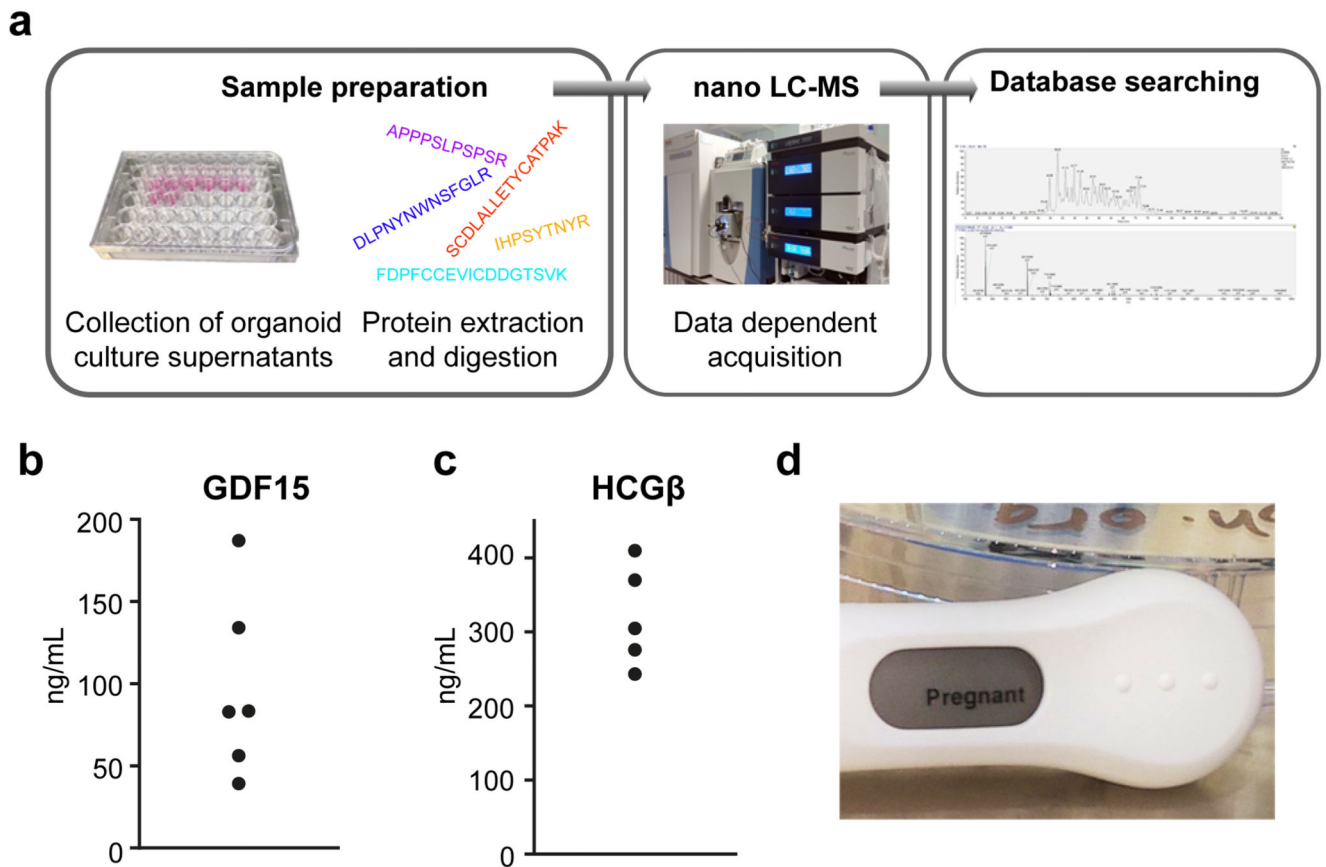


Figure 4. Secretome of trophoblast organoids contains placental hormones and proteins.
a, Experimental work-flow for Liquid chromatography-mass spectrometry (LC-MS) analysis of the secretome of trophoblast organoids. Supernatants from six trophoblast organoid cultures derived independently from six different placental samples were analysed: TOrg_2 (p23), TOrg_3 (p20), TOrg_5 (p6), TOrg_10 (p12), TOrg_12 (p4) and TOrg_14 (p5). **b**, ELISA for GDF15 secreted by trophoblast organoids ($n=6$). Shown is amount of GDF15 (ng/mL) produced by trophoblast organoids (between day 7-10 after passaging) in 48h. Source Data File 3. **c**, ELISA for HCG β secreted by trophoblast organoids ($n=5$). Shown is amount of HCG β (ng/mL) produced by trophoblast organoids (between day 7-10 after passaging) in 48h. Source Data File 4. **d**, Over-the-counter pregnancy stick denoting “Pregnant” after being placed into dish containing cultures of trophoblast organoids. Image reproduced with the permission of SPD Swiss Precision Diagnostics GmbH (SPD). Experiment independently repeated twice.

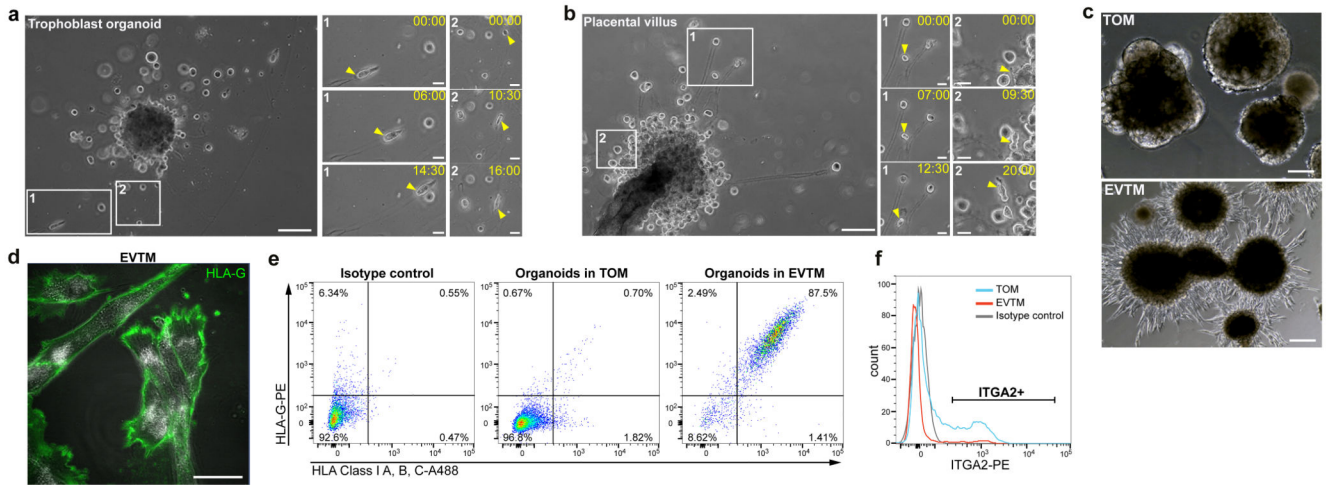


Figure 5. Generation of migratory and invasive HLA-G⁺ extravillous trophoblast (EVT) cells from trophoblast organoids.

a, Phase-contrast images taken across several z-stacks combined into a single image by the extended focus module from Zeiss Axiovision of a trophoblast organoid and **b**, a placental villous explant plated into Matrigel after 7-10 days in EVT differentiation medium (EVTM). Regions of interest in samples are boxed in white and corresponding higher power snapshots are shown with relative time-lapse intervals in yellow (h:min). Migratory cells are labelled with yellow arrowhead. In EVT, cells from both the organoids and primary tissue show random migration. See also control image for organoid in TOM (Extended Data Fig. 7b and time-lapse videos (Supplementary Videos 1-6)). Scale bars, large images in **a** and **b** 200 μ m; images shown from insets, 50 μ m. **c**, Phase contrast images of trophoblast organoids plated in Matrigel drop and exposed to either TOM or EVT. Cells stream out of organoids, digesting the Matrigel and eventually adhering to the plastic only when cultured in EVT. Scale bars, 200 μ m. **d**, Live cells growing out from trophoblast organoids in EVT stained with HLA-G monoclonal antibody, G233. Scale bar, 50 μ m. **e**, Flow cytometry of trophoblast organoids cultured in TOM or EVT and double-stained with monoclonal antibodies W6/32 (binds all HLA class I molecules) and MEMG9 (specific for HLA-G). In TOM virtually all cells lack HLA class I expression but become HLA-G⁺ after culturing in EVT. **f**, Histogram showing organoids cultured in TOM or EVT stained for ITGA2, that marks cells at the base of the cytotrophoblast cell columns. Before exposure to EVT, 23% cells are ITGA2⁺ but very few are present after differentiation to HLA-G⁺ EVT. All experiments (a-f) have been repeated independently at least 3 times.

Extended Data Table 1
Microsatellite analysis and HLA typing of organoids from placental digests in TOM show that they are of fetal origin

a, PowerPlex16 Short Tandem Repeat (STR) genotyping of DNA from matched maternal-fetal samples (maternal blood, decidua and placenta) to identify origin of organoids. Shown in top row are the 15 STR loci analysed. Numbered STR alleles observed for each DNA at a particular locus are listed within the relevant column. As expected in most cases a maximum of two alleles were seen. In cases where there was evidence of an additional allele from a fetal derived STR haplotype the allele number appears in brackets. This is consistent with the decidua containing fetal extravillous trophoblast. The results at informative loci where fetus and trophoblast organoids match are underlined. **b**, HLA genotyping with Third Generation SMRT sequencing of DNA from matched maternal blood and trophoblast organoid to confirm STR analysis. HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 loci were investigated. The two HLA alleles at each locus defined at high resolution are shown for each sample. For each locus, the fetal trophoblast will share one allele with the mother but the other allele will be derived from the father and is likely to be different. Not all loci are informative as some paternal and non-inherited maternal alleles are the same.

Sample ID	D5S1358	TH01	D21S11	D18S51	Penta E	D5S818	D13S317	D7S820	DI6S539	CSFIPO	Penta D	Amelo.	vWA	D8S1179	FGA
Maternal Blood_2	16,17	7,9,3	28,30	12,14	15,15	12,13	10,15	11,11	12,13	10,10	9,9	x,x	15,18	12,13	22,2,23
Decidua_2	16,17	7,9,3	28,30	12,14	15,15	12,13	10,15	11,11	12,13	10,10	9,9	x,x	15,18	12,13	22,2,23
Placenta_2	14,16	6,9,3	28,30	14,15	12,15	11,13	10,14	10,11	12,12	10,10	9,13	x,x	15,17	13,13	22,23
Trophoblast Organoid_2	14,16	6,9,3	28,30	14,15	12,15	11,13	10,14	10,11	12,12	10,10	9,13	x,x	15,17	13,13	22,23
Maternal Blood_3	16,17	6,9	29,31,2	12,14	7,12	11,12	10,11	9,10	11,11	10,12	10,13	x,x	18,18	12,13	20,25
Decidua_3	16,17	6,9 (9,3)	29,31,2 (28)	12,14	7,12 (19)	11,12	10,11 (14)	9,10	11,11	10,12 (11)	10,13 (14)	x,x	18,18 (19)	12,13	20,25 (21)
Placenta_3	16,16	9,9,3	28,31,2	12,14	7,19	11,11	10,14	9,10	11,11	11,12	13,14	x,x	18,19	12,12	21,25
Trophoblast Organoid_3	16,16	9,9,3	28,31,2	12,14	7,19	11,11	10,14	9,10	11,11	11,12	13,14	x,x	18,19	12,12	21,25
Maternal Blood_6	14,15	7,9,3	30,30	12,16	16,19	11,11	11,13	9,12	11,13	10,11	9,9	x,x	15,15	10,10	21,26
Decidua_6	14,15	7,9,3	30,30	12,16	16,19	11,11	11,13	9,12	11,13	10,11	9,9	x,x	15,15	10,10	21,26
Placenta_6	15,17	6,7	28,30	16,17	5,16	10,11	12,13	9,11	11,13	10,10	9,13	x,y	15,17	10,11	24,26
Trophoblast Organoid_6	15,17	6,7	28,30	16,17	5,16	10,11	12,13	9,11	11,13	10,10	9,13	x,y	15,17	10,11	24,26
Maternal Blood_7	15,16	6,6	29,30,2	16,16	7,18	9,13	12,12	9,10	13,13	11,11	10,11	x,x	17,18	12,14	21,21
Decidua_7	15,16 (17)	6,6	29,30,2	16,16	7,18	9,13	12,12 (10)	9,10	13,13 (10)	11,11	10,11	x,x (y)	17,18	12,14 (13)	21,21 (24)
Placenta_7	15,17	6,6	29,29	16,16	7,18	13,13	10,12	9,10	10,13	11,11	10,11	x,y	17,18	13,14	21,24
Trophoblast Organoid_7	15,17	6,6	29,29	16,16	7,18	13,13	10,12	9,10	10,13	11,11	10,11	x,y	17,18	13,14	21,24
Maternal Blood_8	17,18	7,9	30,30	16,20	11,12	12,12	11,12	9,10	11,14	11,12	11,13	x,x	15,18	13,14	22,22
Decidua_8	17,18 (15)	7,9 (8)	30,30	16,20 (12)	11,12 (7)	12,12 (10)	11,12	9,10 (11)	11,14	11,12	11,13	x,x (y)	15,18	13,14	22,22 (28)

a

Sample ID	D3S1358	TH01	D21S11	D18S51	Penta E	D5S818	D13S317	D7S820	D16S539	CSFIPO	Penta D	Amdo.	vWA	D8S1179	FGA
Placenta_8	<u>15.18</u>	<u>7.8</u>	30,30	<u>12.16</u>	<u>7.12</u>	<u>10.12</u>	<u>11.11</u>	<u>9.11</u>	<u>9.14</u>	<u>11.11</u>	<u>11.11</u>	<u>x,y</u>	<u>15.17</u>	13,14	<u>22.28</u>
Trophoblast Organoid_8	<u>15.18</u>	<u>7.8</u>	30,30	<u>12.16</u>	<u>7.12</u>	<u>10.12</u>	<u>11.11</u>	<u>9.11</u>	<u>9.14</u>	<u>11.11</u>	<u>11.11</u>	<u>x,y</u>	<u>15.17</u>	13,14	<u>22.28</u>
Maternal Blood_9	15,18	9,3,9,3	33,2,34,2	12,17	5,5	12,13	12,12	10,11	11,11	10,12	11,12	x,x	15,16	14,15	19,22
Decidua_9	15,18	<u>9.3,9.3(10)</u>	<u>33.2,34.2(31)</u>	<u>12.17(13)</u>	<u>5.5(14)</u>	<u>12.13</u>	<u>12.12</u>	<u>10,11(8)</u>	<u>11,11</u>	<u>10,12</u>	<u>11,12(10)</u>	<u>x,x(y)</u>	<u>15,16</u>	<u>14,15(12)</u>	<u>19,22(21)</u>
Placenta_9	<u>15.15</u>	<u>9.3.10</u>	<u>31.33.2</u>	<u>13.17</u>	<u>5.14</u>	<u>12.13</u>	<u>12.12</u>	<u>8.10</u>	<u>11,11</u>	<u>10,12</u>	<u>10.12</u>	<u>x,y</u>	<u>16.16</u>	<u>12.14</u>	<u>19.21</u>
Trophoblast Organoid_9	<u>15.15</u>	<u>9.3.10</u>	<u>31.33.2</u>	<u>13.17</u>	<u>5.14</u>	<u>12.13</u>	<u>12.12</u>	<u>8.10</u>	<u>11,11</u>	<u>10,12</u>	<u>10.12</u>	<u>x,y</u>	<u>16.16</u>	<u>12.14</u>	<u>19.21</u>

b

Sample ID	HLA-A*	HLA-A*	HLA-B*	HLA-B*	HLA-C*	HLA-C*	HLA-C*	HLA-C*	HLA-C*	HLA-DRB1*	HLA-DRB1*	HLA-DRB1*	HLA-DRB1*	HLA-DQB1*	HLA-DQB1*	HLA-DQB1*	HLA-DQB1*	HLA-DQB1*	HLA-DPB1*	HLA-DPB1*
Maternal Blood_2	02:01:01:01/16	02:01:01:01/16	02:01:01:02/04/05/06	44:02:01:03	07:04:01:01/03	03:03:01:01	03:03:01:01	03:03:01:01	03:03:01:01	11:01:01	08:01/77	03:01:01	04:02:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01
Trophoblast Organoid_2	02:01:01:01/16	11:01:01:01	35:01:01:02/04/05/06	39:01:01:03/05	12:03:01:01	03:03:01:01	03:03:01:01	03:03:01:01	03:03:01:01	11:01:01	08:01/77	03:01:01	04:02:01	04:02:01	04:02:01	04:02:01	04:02:01	04:02:01	04:02:01	04:02:01
Maternal Blood_3	02:01:01:01/16	24:02:01:01	15:01:01:01	40:01:02:01/04	03:04:01:01	03:03:01:01	03:04:01:01	03:03:01:01	03:03:01:01	04:04:01	01:01:01	03:02:01	05:01:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01
Trophoblast Organoid_3	02:01:01:01/16	24:02:01:01	15:01:01:01	15:01:01:01	03:04:01:01	03:03:01:01	03:04:01:01	03:03:01:01	03:03:01:01	04:04:01	04:01:01	03:02:01	03:02:01	03:02:01	03:02:01	03:02:01	03:02:01	03:02:01	03:02:01	03:02:01
Maternal Blood_6	03:01:01:01	30:02:01:01	07:02:01:01/03	18:01:01:01/06	05:01:01:01	07:02:01:03	05:01:01:01	07:02:01:03	01:03:01	01:03:01	01:03:01	03:01:01	05:01:01	02:01:02/19	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01
Trophoblast Organoid_6	68:01:02:02	30:02:01:01	27:05:02:01	18:01:01:01/06	05:01:01:01	07:04:01:01/03	05:01:01:01	07:04:01:01/03	01:03:01	01:03:01	08:01/77	04:02:01	05:01:01	02:01:02/19	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01
Maternal Blood_7	32:01:01:01	33:01:01:01	14:02:01:01	44:02:01:01	05:01:01:02	08:02:01:01	05:01:01:02	08:02:01:01	01:01:01	01:01:01	01:02:01	05:01:01	06:03:01	13:01/107:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01
Trophoblast Organoid_7	24:02:01:04	33:01:01:01	14:02:01:01	44:02:01:01	05:01:01:02	08:02:01:01	05:01:01:02	08:02:01:01	13:01:01	13:01:01	01:02:01	05:01:01	06:03:01	02:01:02/19	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01
Maternal Blood_8	24:02:01:01	25:01:01:01	18:01:01:02/05	27:05:02:01	01:02:01:01	12:03:01:01	01:02:01:01	12:03:01:01	15:01:01	15:01:01	13:03:01	03:01:01	06:02:01	04:01:01	03:01:01	03:01:01	03:01:01	03:01:01	03:01:01	03:01:01
Trophoblast Organoid_8	24:02:01:01	02:01:01:01/16	49:01:01	27:05:02:01	01:02:01:01	07:02:01:01/15	01:02:01:01	07:02:01:01/15	11:02:01	11:02:01	13:03:01	03:01:01	03:19:01	10:01:01	03:01:01	03:01:01	03:01:01	03:01:01	03:01:01	03:01:01
Maternal Blood_9	26:01:01:01	03:01:01:01	07:02:01:01/03	27:05:02:01	02:02:01:01	07:02:01:03	02:02:01:01	07:02:01:03	04:04:01	04:04:01	04:04:01	03:02:01	03:02:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01	04:01:01
Trophoblast Organoid_9	26:01:01:01	02:01:01:01/16	44:02:01:01	27:05:02:01	02:02:01:01	05:01:01:02	02:02:01:01	05:01:01:02	04:01:01	04:01:01	04:04:01	03:01:01	03:02:01	04:02:01	04:02:01	04:02:01	04:02:01	04:02:01	04:02:01	04:02:01

Extended Data Table 2
Microsatellite analysis and HLA typing of organoids from placental digests derived in ExM show that they are of maternal origin

a, PowerPlex16 STR genotyping of DNA from matched maternal-fetal samples (decidua, maternal blood, placenta) to identify origin of organoids. Shown in the top row are the 15 STR loci analysed. Numbered STR alleles observed for each DNA at a particular locus are listed within the relevant column. As expected in most cases a maximum of two alleles were seen but where there was lesser evidence of an additional allele from a fetal derived STR haplotype the allele number appears in brackets. The results at informative loci where maternal blood, decidua and organoids match are underlined. **b**, HLA genotyping with LABType Reverse SSO of DNA from 4 pregnancies with matched decidua, blood, placenta and organoids. Each pair of HLA alleles defined, at low resolution, for each sample at a particular locus are listed within the relevant column. The maternal origin of the organoids is clear from HLA-A, -B and -C alleles in 3 of the 4 pregnancies. The data was inconclusive for pregnancy 2 but all 4 organoids are clearly maternal in origin when using both methods (see above).

Sample ID	D3S1358	TH01	D21S11	D18S51	Penta E	D5S818	D13S317	D7S820	D16S539	CSF1PO	Penta D	Amelo.	vWA	D8S1179	FGA
Maternal Blood_1	15, 15	<u>7</u> , 9	<u>30</u> , <u>30</u>	14, 17	<u>11</u> , 12	<u>12</u> , 13	11, 12	8, 8	<u>11</u> , 12	<u>12</u> , 12	<u>9</u> , 12	xx	<u>17</u> , 19	13, 15	22, 25
Decidua_1	15, 15	<u>7</u> , 9	<u>30</u> , <u>30</u>	14, 17	<u>11</u> , 12	<u>12</u> , 13	11, 12	8, 8	<u>11</u> , 12	<u>12</u> , 12	<u>9</u> , 12	xx	<u>17</u> , 19	13, 15	22, 25
Placenta_1	15, 15	7, 8	29, 30	17, 17	10, 12	11, 12	11, 12	8, 10	12, 12	10, 12	10, 12	xx	16, 17	13, 15	22, 25
Decidual Organoid_1	15, 15	<u>7</u> , 9	<u>30</u> , <u>30</u>	14, 17	<u>11</u> , 12	<u>12</u> , 13	11, 12	8, 8	<u>11</u> , 12	<u>12</u> , 12	<u>9</u> , 12	xx	<u>17</u> , 19	13, 15	22, 25
Maternal Blood_2	16, 17	<u>6</u> , <u>9</u> , 3	<u>28</u> , <u>28</u>	15, 19	<u>12</u> , 12	11, 12	8, 11	<u>12</u> , 14	<u>12</u> , 12	10, 11	11, 12	xx	16, 17	<u>12</u> , 15	<u>20</u> , 21
Decidua_2	16, 17	<u>6</u> , <u>9</u> , 3	<u>28</u> , <u>28</u>	15, 19	<u>12</u> , 12	11, 12	8, 11	<u>12</u> , 14	<u>12</u> , 12	10, 11	11, 12	xx	16, 17	<u>12</u> , 15	<u>20</u> , 21
Placenta_2	16, 17	6, 8	28, 31, 2	15, 19	12, 15	11, 12	8, 9	10, 12	12, 13	10, 11	11, 12	xx	16, 17	10, 15	20, 23
Decidual Organoid_2	16, 17	<u>6</u> , <u>9</u> , 3	<u>28</u> , <u>28</u>	15, 19	<u>12</u> , 12	11, 12	8, 11	<u>12</u> , 14	<u>12</u> , 12	10, 11	11, 12	xx	16, 17	<u>12</u> , 15	<u>20</u> , 21
Maternal Blood_3	14, 17	6, 6	<u>28</u> , <u>31</u> , 2	12, 12	7, 13	<u>12</u> , 12	11, 12	10, 12	<u>11</u> , 13	<u>10</u> , 11	<u>9</u> , 10	xx	15, 17	13, 13	<u>23</u> , 24
Decidua_3	14, 17	6, 6	<u>28</u> , <u>31</u> , 2	<u>12</u> , 12, (15)	7, 13	<u>12</u> , 12, (13)	11, 12	10, 12, (9)	<u>11</u> , 13	<u>10</u> , 11, (12)	<u>9</u> , 10, (13)	xx(y)	15, 17	<u>13</u> , 13, (14)	<u>23</u> , 24, (22)
Placenta_3	17, 17	6, 6	28, 28	12, 15	7, 12	12, 13	11, 11	9, 10	13, 13	11, 12	10, 13	xy	15, 17	13, 14	22, 24
Decidual Organoid_3	14, 17	6, 6	<u>28</u> , <u>31</u> , 2	<u>12</u> , 12	7, 13	<u>12</u> , 12	11, 12	10, 12	<u>11</u> , 13	<u>10</u> , 11	<u>9</u> , 10	xx	15, 17	<u>13</u> , 13	<u>23</u> , 24
Maternal Blood_4	15, 15	<u>9</u> , 3, 9, 3	<u>30</u> , <u>32</u> , 2	18, 22	<u>12</u> , 14	11, 12	8, 12	10, 12	<u>12</u> , 13	9, 10	<u>12</u> , 16	xx	<u>15</u> , 16	13, 13	<u>19</u> , 22
Decidua_4	15, 15	<u>9</u> , 3, 9, 3	<u>30</u> , <u>32</u> , 2	18, 22	<u>12</u> , 14	11, 12	8, 12	10, 12	<u>12</u> , 13	9, 10	<u>12</u> , 16	xx	<u>15</u> , 16	13, 13	<u>19</u> , 22
Placenta_4	15, 16	7, 9, 3	30, 30	14, 18	11, 12	11, 12	12, 14	9, 12	13, 14	10, 10	9, 16	xx	16, 16	13, 14	20, 22
Decidual Organoid_4	15, 15	<u>9</u> , 3, 9, 3	<u>30</u> , <u>32</u> , 2	18, 22	<u>12</u> , 14	11, 12	8, 12	10, 12	<u>12</u> , 13	9, 10	<u>12</u> , 16	xx	<u>15</u> , 16	13, 13	<u>19</u> , 22

b

Sample ID	HLA-A	HLA-B	HLA-C
Maternal Blood_1	01,02	41,52	12,17
Decidua_1	01,02	41,52	12,17
Placenta_1	01,02	27,52	01,12
Decidual Organoid_1	<i>Fail</i> x2	41,52	12,17
Maternal Blood_2	02,02	07,44	05,07
Decidua_2	02,02	07,44	05,07
Placenta_2	02,02	07,44	05,07
Decidual Organoid_2	02,02	07,44	05,07
Maternal Blood_3	25,29	18,44	12,16
Decidua_3	<i>Fail</i> x2	18,44	12,16
Placenta_3	23,25	18,44	04,12
Decidual Organoid_3	25,29	18,44	12,16
Maternal Blood_4	23,24	13,44	04,04
Decidua_4	23,24	13,44	04,04
Placenta_4	11,24	13,40	02,04
Decidual Organoid_4	23,24	13,44	04,04