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

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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 models[1]. Upon implantation, the trophectoderm of the blastocyst rapidly proliferates and generates the trophoblast, the unique cell type of the placenta. *In vivo*, the 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 arteries[2]. 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 criteria[3]. 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; TGFβ 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 TGFβ/SMAD inhibitor) and Rspondin-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

90 value≤0.05) between placental villi, trophoblast organoids and stromal cells highlights other
91 genes of interest, such as *PGF, CCNE1, ERBB3* and *FOLR1*; translation of CCNE1 in
92 trophoblast was validated by IHC (Extended Data Fig. 4e). Imprinted genes, *PEG3* and
93 *PEG10* are also highly expressed in the trophoblast organoids (Extended Data Fig. 4d).
94 Amongst transcription factors, beside known genes, *GATA3* and *TFAP2C*, new genes
95 emerged such as *ELF3* (Extended Data Fig. 5a,b). Genome-wide methylation analysis
96 revealed a high degree of correlation between trophoblast organoids and first-trimester
97 placental villi across different genomic elements compared to blood and brain (Fig. 2g). The
98 hypomethylation of *ELF5* promoter was also confirmed (Extended Data Fig. 5c,d). Analysis
99 of the promoter regions of genes with similar methylation patterns to *Elf5* in mouse TS cells
100 shows that *ELF5, EZR, TINAGL1* and *LASP1* are similarly hypomethylated in placental villi
101 and trophoblast organoids (Extended Data Fig. 5e,f)[12]. Gene ontology analysis of DEGs,
102 represented by Chord plot, shows terms describing metabolic processes and cell-cell
103 organization converging on epithelial, developmental and hormonal pathways, *FZD5* (WNT
104 signalling), *INSIG1* (insulin signalling), *DHCR7* (cholesterol synthesis) and *OCLN*
105 (polarity)(Extended Data Fig. 5g).

106 Trophoblast organoids grow as complex structures closely recapitulating the structure
107 of placental villi *in vivo* where VCT stain for EPCAM and CDH1 (Fig. 3a,b). The basement
108 membrane is on the outside in contact with Matrigel with syncytial masses lining the central
109 cavity (Extended Data Fig. 6a). Similar to *in vivo*, VCT cells are Ki67+ and TP63+ (Fig. 3b,
110 Extended Data Fig. 6b,c). Following incubation with EdU, ~30-40% of cells are proliferating
111 when the organoids are small (100-200µm diameter), with a notable decrease as they enlarge
112 and differentiate (Extended Data Fig. 6d). Expression of SCT markers (CD71, CD46) is
113 found inside the organoids (Extended Data Fig. 6e). These characteristic features of SCT
114 were confirmed by electron microscopy (EM): multinucleated with abundant secretory
115 organelles and surface microvilli (Fig. 3c). Lacunae present within the syncytial areas
116 resemble those found *in vivo* (Extended Data Fig. 6f,g). *GCM1* drives fusion of VCT into
117 SCT by upregulating *ERVW-1* (*SYNCYTIN-1*)[13,14]. Using qRT-PCR, we detect high levels of
118 expression of *GCM1* and *ERVW-1*, comparable to those in the placenta (Fig. 3d; Source Data
119 File 2). Thus, trophoblast organoids closely mimic the villous placenta both structurally and
120 phenotypically. SCT secretes proteins and hormones into the maternal systemic circulation,
121 inducing maternal adaptations to pregnancy. We explored the secretory activity of trophoblast
122 organoids through an unbiased peptidomic analysis of the organoid medium and LC MS/MS
123 technology (Fig. 4a). Among the most abundant peptides are placental-specific PSGs and
124 INSL4, whose functions are unknown (Extended Data Table 3, Supplementary Table 2a-h).
125 Aldose reductase, which converts glucose to sorbitol is also detected. High concentrations of
126 sorbital are present in first trimester placentas[15]. Hence, the organoids also mimic the villous
127 placenta metabolically as well as endocrinologically. Peptides that induce physiological and
128 metabolic adaptations during pregnancy, including hCG, KISS1 and CSH1 are all abundant
129 as well as GDF15, implicated in hyperemesis gravidarum[16]. GDF15 and hCG are detected by
130 ELISA, showing that full-length and appropriately folded hormones are secreted by
131 trophoblast organoids (Fig. 4b,c; Source Data Files 3-4). Indeed, the 'pregnant' secretome of
132 the organoids is evident using an "over-the-counter" pregnancy test kit (Fig. 4d).

133 Human trophoblast also differentiates to EVT, a process crucial for proper
134 placentation. EVT express HLA-G and invade decidual tissue to transform the spiral
135 arteries[17]. In TOM our trophoblast organoids show only sporadic HLA-G+ cells (Extended
136 Data Fig. 7a). Recently, long-term, 2D monolayer cultures of human trophoblast cells derived
137 from first-trimester placentas that can differentiate into SCT and EVT were described[18]. By
138 adapting their EVT differentiation protocol and culturing both our trophoblast organoids and
139 primary villous explants in their EVT medium (EVTM), HLA-G+ cells appear that migrate
140 out of the organoids, digest the Matrigel to form tracks, and eventually adhere to the plastic
141 (Fig. 5a-e, Extended Data Fig. 7b, Supplementary Videos 1-6). *In vivo* EVT are generated at
142 the base of CCCs where cells express ITGA2[10]. We used flow cytometry to confirm that,
143 after exposure of organoids to EVTM, HLA-G+ EVT appear and ITGA2+ cells disappear
144 (Fig. 5f).

145 In summary, we describe the generation of human trophoblast organoids that grow as
146 complex 3D structures with fusion of VCT to hCG-secreting SCT, anatomically and
147 functionally closely resembling the villous placenta *in vivo*. In addition, we show
148 differentiation to HLA-G+ EVT that vigorously invade and digest the Matrigel in 3D.
149 Following the submission of our paper, there is a report describing the generation of
150 trophoblast organoids from pooled patient samples; however these cannot be cultured long-
151 term and have not been fully characterised[19]. Our findings complement those of Okae *et al.*
152 and mean that there are now two culture systems (2D and 3D) for human trophoblast[18]. Our
153 3D model has the advantage of organising into complex structures generating both SCT and
154 EVT. The retention of the normal villous structure will allow analysis of morphogenetic
155 events. We anticipate that these two different models will provide valuable tools across a
156 range of disciplines. They can be used to study maternal-fetal transmission of xenobiotics,
157 drugs and pathogens, and the proteins and hormones derived from the SCT[20]. Analysis of
158 CCC formation and EVT differentiation *in vitro* will allow investigation of the decidual
159 microenvironment on trophoblast function, such as the effect of glandular histotrophic
160 nutrition and the influence of the distinctive uterine natural killer (NK) cells[21,22]. Major
161 unexplained disorders of pregnancy such as pre-eclampsia, stillbirth and fetal growth
162 restriction have their origins in aberrant placental development in the first trimester[23].
163 Trophoblast organoids can be used to study maternal-fetal interactions following
164 implantation, and the maternal physiological, metabolic and hormonal changes occurring
165 during pregnancy.
166
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232 A.McW. and S.G.E.M. performed and analysed the STR and HLA typing; M.Ho. performed electron
233 microscopy and confocal analysis; R.F. and H.S. assisted with IHC and ELISA experiments; L.E. performed
234 qPCR experiments; A.S, F.G, F.R, M.He. and S.O. provided intellectual input to the study; M.He. performed the

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237
238 **Competing interests** The authors declare no competing interests.
239
240 **Additional information**
241 **Extended data** is available for this paper at
242 **Supplementary information** is available for this paper at
243 **Reprints and permissions information** is available at
244 **Correspondence and requests for materials** should be addressed to M.Y.T., G.J.B or A.M.
245

246 **METHODS**

247 **Patient samples**. All tissue samples used for this study were obtained with written informed consent
248 from all participants in accordance with the guidelines in The Declaration of Helsinki 2000. Elective
249 terminations of normal pregnancies were performed at Addenbrooke's Hospital (6-9 weeks gestation)
250 under ethical approval from the Cambridge Local Research Ethics Committee (04/Q0108/23). Human
251 peripheral blood was collected from a healthy donor in a BD vacutainer following informed consent
252 and in accordance with the ethical approval of the Human Biology Research Ethics Committee,
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254 **Derivation of trophoblast organoids from human placental tissue.** To obtain trophoblast-enriched
255 cell suspensions, villi from first trimester placental tissue were sequentially digested with 0.2%
256 trypsin-250 (Pan Biotech P10-025100P)/0.02% EDTA (Sigma #E9884)/PBS then collagenase V at
257 1.0mg/ml (Sigma #C9263) in Hams F12/10% FBS. Both digests were pooled, washed in Advanced
258 DMEM/F12 medium (Gibco #12634-010) and re-suspended in ~10x volume growth-factor-reduced
259 Matrigel (Corning #356231) on ice. 25µL drops were plated per well into a 48-well culture plate
260 (Costar #3548), set at 37°C for 15min and overlaid with 250µL Trophoblast Organoid Medium
261 (TOM, Supplementary Table 1b). Cultures were maintained in 5% CO_2 in a humidified incubator at
262 37°C. Medium was replaced every 2-3d. Small organoid clusters became visible by ~7d and were
263 passaged when at least 50% had reached a diameter of 200-300µm (usually between 7-10d).
264 Mechanical disruption was achieved with Eppendorf Explorer Plus automatic pipettes on a mix cycle
265 of 99 rounds (x4-5), maximum speed. Organoids from the same sample were initiated and maintained
266 in the absence of each individual component to test its importance. Frozen stocks of organoids were
267 made in 70% TOM, 20% FBS and 10% DMSO freeze medium and stored in liquid nitrogen. A step-
268 by-step protocol of the derivation and maintenance of human trophoblast organoid cultures can be
269 found at Nature Protocol Exchange[24].

270 **Generation of EVT from trophoblast organoids.** Trophoblast organoids were passaged and plated
271 into 35mm dishes or ibidi µ-dishes (Thermo Scientific #81156). Differentiation was achieved through
272 a modification of the protocol used by Okae et al[18]. After passaging, organoids were maintained in
273 TOM for 3-4d and switched to EVT medium (EVTM: advanced DMEM/F12, 0.1mM 2-
274 mercaptoethanol (Gibco #31350), 0.5% Penicillin-streptomycin, 0.3% BSA (Sigma #A8412), 1%
275 ITS-X supplement (Gibco #51500-056), 100ng/mL NRG1 (Cell Signaling #5218SC), 7.5µM A83-01
276 (Tocris Biotechne #2939), 4% Knockout serum replacement (ThermoFisher #10828010). When
277 organoids showed outgrowth of cells (typically 7-10d), the medium was changed to EVTM without
278 NRG1 for a further 7-10d. For comparison, fresh placental villi were embedded into 300uL Matrigel
279 in ibidi µ-dishes and grown under the same conditions.

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

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

291 **Cell lines**. Human choriocarcinoma cell lines, JEG-3 and JAR, were obtained from the American
292 Type Culture Collection (ATCC) and cultured as previously described[3].

293 **Immunohistochemistry (IHC).** Organoids were formalin-fixed and embedded as previously
294 described[11]. IHC on sections of organoids and first-trimester placentas was performed using
295 A.Menarini heat-induced epitope retrieval buffers and Vectastain avidin-biotin-HRP reagents as
296 previously described[11]. Primary antibodies (Supplementary Table 3) were replaced with equivalent
297 concentrations of isotype-matched mouse or rabbit IgG for controls. Images were captured with a
298 Zeiss Axiovert Z1 microscope and Axiovision imaging software SE64 V4.8.
299 **Immunofluorescence (IF) and confocal microscopy.** Trophoblast organoids were grown in 4-5
300 20μL Matrigel drops in 35mm ibidi μ-dishes and EdU and/or antibody labelling was performed as
301 previously described[11]. EdU incubation was for 1h at 37°C in TOM containing 10μM EdU. For
302 primary antibodies and secondary antibodies used see Supplementary Table 1d. Imaging was with the
303 ZEISS 700 Confocal microscope and ZEN Microscope Software.
304 **Mitotracker staining.** Mitochondrial function was evaluated by Mitotracker Red-CMXRos
305 (Thermofisher #M7512). Organoids were released from Matrigel with Cell Recovery Solution
306 (Corning #354253) and incubated in 500nM of Mitotracker Red in TOM in suspension at 37°C for 30
307 mins. The organoids were washed in basal medium, resuspended and plated into a thin layer of
308 Matrigel in ibidi μ-dishes for imaging on a ZEISS 700 Confocal microscope with ZEN Microscope
309 Software.
310 **Time-lapse microscopy.** Trophoblast organoids or placental villous explants embedded in 300uL of
311 Matrigel in 35mm ibidi μ-dishes were imaged in phase-contrast and across several z-stacks on a Zeiss
312 Axiovert Z1 microscope with the multidimensional imaging function of the Axio Observer software
313 Axiovision image software V4.8. The images were compiled into a single movie by using the
314 extended focus wavelet function.
315 **ELISA.** Conditioned media were harvested from organoid cultures and centrifuged to remove debris
316 and stored at -80°C until use. Human Chorionic Gonadotropin β (hCG-β) ELISA (Abcam #ab108638)
317 was performed on 50μL supernate with 100μL sample buffer in duplicate alongside hCG-β standards
318 following the manufacturer's instructions. Concentration of hCG-β in the supernates was calculated
319 from the line formula of the standard plots in Microsoft Office Excel. Supernatants were also tested
320 with Clear&Simple Digital Pregnancy Test following manufacturer's instructions. Image reproduced
321 with the permission of SPD Swiss Precision Diagnostics GmbH (SPD). GDF-15 was measured by in-
322 house electrochemiluminescence immunoassay on the MesoScale Discovery assay platform (MSD)
323 using BioTechne DuoSet antibodies and standard (BioTechne #DY957). For further details see
324 Supplementary Methods.
325 **Flow cytometry.** Organoids were removed from Matrigel with Cell Recovery Solution and
326 dissociated with 0.2% trypsin 250 (Pan Biotech P10-025100P)/0.02% EDTA (Sigma #E9884) in PBS
327 at 37°C for 5 min. Cells were washed in medium containing FBS and passed through a 40μm cell
328 strainer (Falcon #2340). Cells were blocked with human IgG (Sigma #I4506) in DPBS (ThermoFisher
329 Scientific #14190136)/1%FBS prior to labelling with W6/32-Alexa-488 anti-HLA-A,B,C antibody,
330 HLA-G-PE, ITGA2-PE or isotype-matched controls (Supplementary Table 1d). LIVE/DEAD™
331 Fixable Far Red Dead Cell Stain (Life Technologies #L10119) was used for live/dead discrimination.
332 Data were acquired using Cytek Development DxP8 (488/637/561). Data were analysed in FlowJo
333 (Tree Star) and all compensation was applied digitally after acquisition.
334 **In Situ Hybridization (ISH) Assays.** ISH for *LGR5* was performed on 4μm paraffin sections with
335 RNAscope 2.0 High definition assay (Advanced Cell Diagnostics) following the manufacturer's
336 instructions. Briefly, tissue sections were baked at 60°C for 1h, dewaxed with xylene, cleared in
337 100% ethanol and air-dried prior to the standard protocol: 10min in Pretreat buffer 1, 15min in
338 Pretreat buffer 2 and 30min at 37°C in Pretreat buffer 3 followed by incubation with *LGR5* probe
339 (#311021), positive control probe *UBC* (#310041) or negative control probe dapB (#310043) for 2h at
340 40°C. Signal was visualized with the amplification kit and DAB for 10min. Sections were dehydrated,

341 mounted in DPX (Sigma #44581) and imaged on a Zeiss Axiovert Z1 microscope with Axiovision
342 imaging software SE64 V4.8.
343 **Electronic microscopy (EM).** Trophoblast organoids were directly fixed in 35mm dishes with 0.5%
344 glutaraldehyde/0.2 M sodium cacodylate buffer (pH 7.2) for 30min and reduced with osmium
345 tetroxide as previously described[11]. Ultrathin sections were examined in an FEI Tecnai G2 TEM at
346 80Kv. Images were acquired with MegaView III CCD and Soft Imaging Systems program. Samples
347 from human placentas were fixed by immersion in 3% glutaraldehyde-0.3% hydrogen peroxide in
348 0.1mol/L 1,4-piperazine diethane sulfonic acid (PIPES) buffer (pH 7). After 2h at RT, tissue was
349 washed for 30min in 0.1 mol/L PIPES buffer. Secondary fixation was by immersion in 1% osmium
350 tetroxide in PIPES buffer for 1h at RT. After washing, specimens were dehydrated in graded ethanol
351 and embedded in Araldite epoxy resin. Ultrathin sections (50nm) were cut on a Reichert-Jung Ultracut
352 S (Reichert-Jung). Sections were counterstained with uranyl acetate, followed by lead citrate, before
353 viewing in a Philips CM100 electron microscope (Philips Electronics).
354 **DNA extraction and quantification.** QIAamp DNA blood Mini kit (Qiagen #51104) was used to
355 extract genomic DNA from patients' blood for Short tandem repeat analysis, HLA tissue typing and
356 Bisulphite sequencing. DNA was extracted from trophoblast organoids, decidual and placental tissues
357 by digestion with ATL buffer (Qiagen #19076) and Proteinase K (Sigma #P4850), followed by
358 purification steps with RNase A (Sigma #R6513) and Protein Precipitation Solution (Qiagen
359 #158910). DNA was precipitated with isopropanol and washed with 70% ethanol. DNA quality and
360 concentration were determined in a Nanodrop ND-1000 Spectrophotometer.
361 **Comparative Genomic Hybridization (CGH) analysis.** DNA from two independently derived
362 trophoblast organoid samples at early and late passages was analysed with Agilent Sureprint G3
363 unrestricted CGH ISCA 8x60K array (Agilent #G4450A). DNA samples from late passage organoids
364 were compared to early passage organoids (hybridization control). DNA was diluted to 50ng/uL and
365 labelled using the Agilent kit following manufacturer's instructions. Data analysis for segmentation
366 and copy number calls was performed at a genome-wide resolution of 500kb by the default analysis
367 method – CGH v2 from the Agilent CytoGenomics software Edition 2.5.8.11 (Build 37).
368 **Short tandem repeat analysis and HLA typing.** Microsatellite analysis was performed with the
369 GenePrint® PowerPlex™16 System (Promega) involving fluorescent labelled multiplexed PCR
370 amplification of 15 short tandem repeat (STR) loci and Amelogenin sex determining fragments. PCR
371 fragment size resolution was achieved with capillary electrophoresis on a 3730XL DNA Analyzer
372 (Applied Biosystems) before analysis of the raw data and STR allele calling with GeneMapper
373 Versions 4 and 5 (Life technologies) fragment analysis sizing and genotyping software. All typing
374 was performed blind. The Promega PowerPlex 16 kit was designed for Forensic testing and has a
375 sensitivity capable of detecting down to 5% contamination. It is used by for monitoring of post-
376 haematopoietic stem cell transplant chimerism[25,26]. The DNA for HLA genotyping was processed via
377 the workflows of the EFI accredited Clinical Histocompatibility Laboratory. Low resolution typing of
378 the HLA-A, -B and -C genes was achieved with LABType kits (One Lambda) which rely on reaction
379 patterns observed when sequence specific DNA probes immobilised on fluorescent X-MAP
380 polystyrene beads (Luminex) hybridise to biotin labelled multiplexed gene specific PCR amplicons.
381 The hybrids were detected with a Liquichip 200 fluorimeter (QIAgen) and HLA allele assignment
382 was from HLA Fusion software (One Lambda). Ultra High resolution typing of HLA-A, -B, -C, -
383 DRB1, - DQB1 and -DPB1 was achieved with an 'in house' Third Generation Sequencing pipeline
384 utilising Pacific Biosciences' Single Molecule Real-Time DNA sequencing technology as previously
385 described[27].
386 **Bisulphite sequencing.** Approximately 300ng of DNA was used for bisulphite conversion performed
387 with the EpiTect Bisulfite Kit (Qiagen #59110), according to the manufacturer's protocol. The *ELF5*

388 promoter region was amplified as described previously[28]. PCR products were cloned and sequenced,
389 confirming representation of distinct alleles.
390 **Global DNA methylation analysis.** Genomic DNA bisulfite (BS) was performed with the CEGX
391 TrueMethyl kit (Cambridge Epigenetix /NuGEN) and used for microarray-based DNA methylation
392 analysis, performed at GenomeScan (GenomeScan B.V., Leiden, The Netherlands), on the
393 HumanMethylation850 BeadChip (Illumina) and were scanned on the Illumina iScan system. The
394 resulting iDAT files were imported and analysed by ChAMP (v2.9.10)[29,30]. Samples were processed
395 filtering for a probe detection p-value <= 0.01, probes with a bead count <3 in at least 5% of samples,
396 no CpG and known SNPs at probe starts, probes aligning to multiple locations, and QC using the on
397 array control probes[31]. Of the total probes on the array 755577 passed the filtering and QC steps. The
398 BMIQ method was used to normalise the two probe types present on the array. Beta methylation
399 values from the EPIC array range from 0 (unmethylated) to 1 (methylated) and are equivalent of
400 percentage methylation[32]. Genomic annotations were imported from FDb.InfiniumMethylation.hg19
401 and IlluminaHumanMethylationEPICmanifest[33]. Genomic features in Fig. 2g comprise the following
402 numbers of assayed CpGs: CpG Islands, 48799, 48799, 13576; Promoters, 151270, 151270, 88621;
403 Gene Bodies, 280,284,280,284,117,138; LINE1, 48799, 48799, 13576. Boxplots comprise min: 1.5 x
404 inter-quartile, lower: 1st quartile, middle: median, upper: 3rd quartile, max: 1.5 x inter-quartile range
405 (Extended Data Figures 2G and 5e). Density/violin plots are scaled to area. LINE1 elements were
406 downloaded as tables from UCSC Genome browser for hg1930. Maternal blood samples (normal) are
407 taken from E-GEOD-66210.
408 **RNA extraction, quantification and quality control.** Total RNA was isolated using the miRNeasy
409 isolation kit (Qiagen #217004) with on-column DNAse digestion (Qiagen #79254). Quantitation of
410 RNA was performed with the Quant-iT™ RiboGreen™ RNA Assay Kit (Thermo Fisher Scientific
411 #R11490) by measuring the intensity of fluorescence at 528nm with a Synergy HT Multi-Mode
412 Microplate Reader (BioTek Instruments) according to manufacturer's instructions. RNA quality was
413 assessed on the Agilent 2100 bioanalyzer (Thermo Fisher Scientific). RNA Integrity Number (RIN) of
414 each tested sample was greater or equal to 8.
415 **Reverse transcription and Real-time quantitative RT-PCR (qRT-PCR).** The expression of *ELF5*,
416 *ERVW-1* and *GCM1* was analysed with Taqman Gene expression assays (Applied Biosystems).
417 500ng-1μg of total RNA was reverse transcribed with Superscript VILO Reverse Transcriptase
418 (Thermo Fisher Scientific #11754050) in the presence of random hexamers and RNAse inhibitor
419 following supplier's instructions. qRT-PCR was performed on 7900HT Fast Real-Time PCR system
420 (Applied Biosystems) as previously described[11]. Relative expression levels were normalised to the
421 geometric mean of three housekeeping genes *HPRT1*, *TOP1* and *TBP* and the $2^{-\Delta Ct}$ method. The
422 expression of C19MC miRNAs *hsa-miR-517-5p*, *hsa-miR-517(a, b)-3p*, *hsa-miR-526b-3p*, *hsa-miR-*
423 *525-3p* and reference gene *RNU48* were analysed by TaqMan miRNA assays (Applied Biosystems).
424 10ng of total RNA were reverse transcribed using miRNA specific stem-loop RT primers and
425 TaqMan microRNA reverse transcription kit (Applied Biosystems #4366596) according to the
426 supplier's instructions. QRT-PCR assays were run with qPCRBIO Probe Mix Lo-ROX (PCR
427 Biosystems) containing specific probes on an Eppendorf Mastercycler® RealPlex 2 instrument. Ct
428 data were normalised to the RNU48 internal control by the $2^{-\Delta Ct}$ method. All qPCR reactions included
429 no-template controls and minus RT controls (–RT). For further details and for Taqman Assay IDs for
430 each gene see Supplementary Methods.
431 **Microarray expression profiling and data analysis.** The microarray experiment was performed at
432 Cambridge Genomic Services at University of Cambridge with a species specific Gene 2.1 ST Array
433 Plate (Affymetrix) according to manufacturer's instructions. Briefly, 100ng of total RNA was
434 amplified for each sample with inline PolyA spike-in control and the WT PLUS amplification kit
435 (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 (500µL) and an aliquot of growth media were acidified with 50µL 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 previously[34]. The eluant was evaporated under oxygen free nitrogen at 40ºC and reconstituted into 75µL 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 previously[35]. 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.

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 PRIDE[36] 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.

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523 **FIGURE LEGENDS**

524 **Figure 1. Establishment of long-term organoid cultures of trophoblast from human placentas. a,**
525 A placental villus at the maternal-fetal interface in the first trimester of pregnancy showing the
526 different trophoblast subsets: SCT, VCT, CCC and EVT. Sources of the intrinsic and extrinsic signals
527 that could signal to proliferative Ki67+ trophoblast cells (dark blue) are shown. **b,** IHC for Ki67 in
528 early first trimester placenta (6-8 weeks g.a) compared to late first trimester (10-12 weeks g.a.). The
529 proportion of proliferative cells is greatly reduced towards the end of first trimester and the cells are
530 localized mostly in the CCC. Representative images from *n*=6 for each tissue type. Scale bars, 100μm**.**
531 **c,** IHC for EPCAM on first trimester placenta (6-8 weeks g.a.) and cell clusters from placental digests.
532 Experiment independently repeated twice with similar results. Arrowheads show VCT and CCC are
533 EPCAM+ and these cells are present in the cell clumps from the placental digests. Scale bars, 50μm
534 (placenta) and 200μm (placental digest). **d,** Time course for derivation of trophoblast organoids from
535 one placental isolate. Bright field images of Matrigel drops after seeding placental digests starting
536 from passage 0 day 0, until generation of homogenous trophoblast organoids (passage 2, day 7). For
537 passages 0 and 1, timepoints at day 0 and 7 are shown. Passage 2, day 7 is shown together with the
538 zoom of the boxed area in the lower panel. Experiment independently repeated for all organoid
539 cultures with similar results. Scale bars, 500μm (Matrigel droplet images) and 200μm (zoom in on
540 trophoblast organoids). Gestational age, g.a.; SCT, syncytiotrophoblast; VCT, villous cytotrophoblast;
541 CCC, cytotrophoblast cell column; EVT, extravillous trophoblast; DG, decidual gland; SA, spiral
542 artery; pl, placental; dec, decidual.

543 **Figure 2. Trophoblast organoids retain characteristic features of first trimester trophoblast *in***
544 ***vivo,* and similar transcriptome and global methylation profiles. a,** IHC for TFAP2C shows
545 uniform expression (representative images from *n*=20). Scale bars, 50μm. **b,** FACS analysis of three
546 trophoblast organoids (TOrg10, 12, 14) and JEG (positive control) with mAb W6/32 .Gating strategy
547 see Extended Data Fig. 3c. Experiment independently repeated 3 times. **c,** Bisulfite sequencing of the
548 *ELF5* promoter region of trophoblast organoids (TOrg 6) and matched maternal leukocytes (positive
549 control). Relative % of methylated cytosine residues (filled circles) are indicated. **d,** qPCR analysis
550 for *miR517-3p* from C19MC cluster on trophoblast organoids (*n*=6), JEG-3 and JAR choriocarcinoma
551 (Chc.) lines (positive controls) and peripheral blood monocytes (PBMC). Graph shows relative
552 expression levels to housekeeping gene *RNU48.* Source Data File 1. **e,** PCA of placental villi (*n*=8);
553 trophoblast organoids derived from different placentas, TOrg_1 (passage p4), TOrg_2 (p7), TOrg_3
554 (p6), TOrg_4 (p4) and TOrg_5 (p6) (*n*=5); placental stromal cells (*n*=5); and decidual organoids
555 (*n*=3). Analysis based on 12673 probes. Organoids cluster more closely with placental villi on PC1
556 axis. **f,** Clustered heatmap of differentially expressed genes in first trimester placental villi
557 (*n=8*)(blue), trophoblast organoids (*n=5*)(pink) and cultured placental villous stromal cells
558 (*n=5*)(green). **g,** Distribution of methyl-cytosine across genomic features is similar between
559 trophoblast organoids (*n=4*) and placental samples (*n=4*). In contrast, the brain (*n=1*) and maternal
560 blood (*n=5*) samples show distinct patterns, especially across CpG islands, gene bodies and LINE1
561 elements. Pearson's correlation coefficient (R) indicated for each comparison against trophoblast
562 organoid samples (all p values <2.2e-16). Density/violin plots are scaled to area.

563 **Figure 3. Trophoblast organoids form complex structures resembling placental villi with**
564 **formation of syncytiotrophoblast (SCT). a,** Confocal microscopy images of trophoblast organoid
565 stained for F-actin, EPCAM, DAPI merged with phase image (representative image from *n*=5). The
566 EPCAM+ cells are surrounding the organoid. Scale bar, 100μm. **b,** IHC for CDH1 and Ki67 of first
567 trimester placenta and trophoblast organoids (representative images from *n*=6 for Ki67 and *n*=20 for

568 trophoblast organoids). VCT stains positively for CDH1. Ki67 is present in the inner VCT layer in
569 villi and the outer layer in organoids. Scale bar, 50µm. Higher power in insets scale bar, 20µm. **c,**
570 Electron micrograph images of first trimester SCT compared to the centre of a trophoblast organoid.
571 Surface microvilli (arrowheads) and multinucleated areas can be seen (arrows). Scale bars, 5µm
572 (placenta), 1µm (trophoblast organoids, microvilli) and 2.5µm (trophoblast organoids, nuclei).
573 Representative images from *n*=2. **d,** qPCR analysis of genes *ERVW-1* and *GCM1* in trophoblast
574 organoids (*n*=5) compared to whole placental villi (*n*=8) and stromal cells isolated from the placenta
575 (*n*=5). Graphs show expression levels relative to geometric mean of three housekeeping genes *TBP*,
576 *TOP1* and *HPRT1*. The mean *ERVW-1* and *GCM1* expression are shown for each sample group.
577 Source Data File 2.
578
579 **Figure 4. Secretome of trophoblast organoids contains placental hormones and proteins. a,**
580 Experimental work-flow for Liquid chromatography-mass spectrometry (LC-MS) analysis of the
581 secretome of trophoblast organoids. Supernatants from six trophoblast organoid cultures derived
582 independently from six different placental samples were analysed: TOrg_2 (p23), TOrg_3 (p20),
583 TOrg_5 (p6), TOrg_10 (p12), TOrg_12 (p4) and TOrg_14 (p5). **b,** ELISA for GDF15 secreted by
584 trophoblast organoids (*n*=6). Shown is amount of GDF15 (ng/mL) produced by trophoblast organoids
585 (between day 7-10 after passaging) in 48h. Source Data File 3. **c,** ELISA for HCGβ secreted by
586 trophoblast organoids (*n*=5). Shown is amount of HCGβ (ng/mL) produced by trophoblast organoids
587 (between day 7-10 after passaging) in 48h. Source Data File 4. **d,** Over-the-counter pregnancy stick
588 denoting "Pregnant" after being placed into dish containing cultures of trophoblast organoids. Image
589 reproduced with the permission of SPD Swiss Precision Diagnostics GmbH (SPD). Experiment
590 independently repeated twice.
591
592 **Figure 5. Generation of migratory and invasive HLA-G+ extravillous trophoblast (EVT) cells**
593 **from trophoblast organoids. a,** Phase-contrast images taken across several z-stacks combined into a
594 single image by the extended focus module from Zeiss Axiovision of a trophoblast organoid and **b,** a
595 placental villous explant plated into Matrigel after 7-10 days in EVT differentiation medium (EVTM).
596 Regions of interest in samples are boxed in white and corresponding higher power snapshots are
597 shown with relative time-lapse intervals in yellow (h:min). Migratory cells are labelled with yellow
598 arrowhead. In EVTM, cells from both the organoids and primary tissue show random migration. See
599 also control image for organoid in TOM (Extended Data Fig. 7b and time-lapse videos
600 (Supplementary Videos 1-6). Scale bars, large images in **a** and **b** 200µm; images shown from insets,
601 50µm. **c,** Phase contrast images of trophoblast organoids plated in Matrigel drop and exposed to either
602 TOM or EVTM. Cells stream out of organoids, digesting the Matrigel and eventually adhering to the
603 plastic only when cultured in EVTM. Scale bars, 200µm. **d,** Live cells growing out from trophoblast
604 organoids in EVTM stained with HLA-G monoclonal antibody, G233. Scale bar, 50µm. **e,** Flow
605 cytometry of trophoblast organoids cultured in TOM or EVTM and double-stained with monoclonal
606 antibodies W6/32 (binds all HLA class I molecules) and MEMG9 (specific for HLA-G). In TOM
607 virtually all cells lack HLA class I expression but become HLA-G+ after culturing in EVTM. **f,**
608 Histogram showing organoids cultured in TOM or EVTM stained for ITGA2, that marks cells at the
609 base of the cytotrophoblast cell columns. Before exposure to EVTM, 23% cells are ITGA2+ but very
610 few are present after differentiation to HLA-G+ EVT. All experiments (a-f) have been repeated
611 independently at least 3 times.
612
613 **EXTENDED DATA LEGENDS**

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 report[9]. 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 Rspondin-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, 500µm. **b,** Trophoblast organoid cultures at passage 2 and at passage 10 with continuous culture. Representative images from *n=3*. Scale bars, 500µm. **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 50µm; Individual cells 10µm. **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, 500µm.

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

661 stains both VCT and the surface of SCT as *in vivo*[7]. The basement membrane is around the outside of
662 the organoids with formation of syncytial masses in the centre. Scale bar, 50µm. **b,** IHC for
663 transcription factor TFAP2A shows uniform expression on trophoblast organoids (representative
664 image from *n*=20). Scale bars, 50µm. **c,** Gating strategy used for flow cytometric analysis of single,
665 live cells from trophoblast organoids. **d,** Quantitative RT-PCR (qPCR) analysis of *ELF5* in
666 trophoblast organoids (*n*=5) compared to whole placental villi (*n*=8) and stromal cells isolated from
667 the placenta (*n*=5). Graph shows expression levels relative to the geometric mean of three
668 housekeeping genes *TBP*, *TOP1* and *HPRT1*. The mean *ELF5* expression is shown for each sample
669 group. Source Data File 2. **e,** Bisulfite sequencing of the *ELF5* promoter region (-379bp to -28bp
670 upstream of the transcription start site) of trophoblast organoids from two different placentas (TOrg 3,
671 6) and matched maternal blood leukocytes (positive control). The relative % of methylated cytosine
672 residues (filled circles) are indicated. **f,** qPCR analysis for *miR525-3p*, *miR526-3p* and *miR517-5p*
673 from C19MC miRNA cluster on trophoblast organoids (*n*=6), JEG-3 and JAR (positive controls) and
674 peripheral blood monocytes (PBMC, low expression/negative control). Graph shows relative
675 expression levels of each organoid culture to housekeeping gene *RNU48.* Source Data File 1.
676
677 **Extended Data Figure 4. Hierarchical clustering of microarray data comparing placental villi,**
678 **trophoblast organoids and placental stromal cells. a,** Unsupervised hierarchical clustering analysis
679 of global gene expression profiles by microarray of first trimester placental villi (Pl)(n=8), trophoblast
680 organoids (TOrg)(n=5), placental stromal cells (Str)(n=5) and decidual organoids (DOrg)(n=3).
681 Analysis was based on 12673 probes. The expression profiles of trophoblast organoids cluster with
682 first trimester placental samples whilst decidual organoids and placental stromal cells cluster in a
683 separate tree. **b,** Top 20 genes contributing to PC1 and PC2 in the PCA plot from Fig. 2e. The top
684 genes contributing to PC1 are all trophoblast-specific genes such as *CGB3*, *GATA3* and *PSG6*
685 indicating that these genes separate the trophoblast organoid and placental villous samples from the
686 two potentially-contaminating, non-trophoblast samples (decidual organoids and placental stroma).
687 The top genes contributing to PC2 are epithelial genes such as *CLDN3*, *TACSTD2* and *KRT23*. The
688 organoids only contain trophoblast, but cells of the villous core (stromal, Hofbauer and endothelial
689 cells) are also present in the placental samples. **c,** IHC of placental villi and trophoblast organoids
690 stained for KRT23 showing expression in all trophoblast cells *in vivo* and *in vitro*. Experiment
691 repeated independently 3 times. Scale bar, 50µm. Higher power in insets scale bar, 20µm. **d,**
692 Clustered heatmap of differentially expressed genes between trophoblast organoids, placental villi and
693 placental stroma with an absolute log2 fold change of 2 (adjusted p.value < 0.05). **e,** IHC of placental
694 villi and trophoblast organoids stained for CCNE1 showing expression in trophoblast cells *in vivo* and
695 *in vitro*. Scale bar, 50µm. Higher power in insets scale bar, 20µm. Experiment repeated independently
696 3 times.
697
698 **Extended Data Figure 5. Transcription factor expression profiles of trophoblast organoids and**
699 **placental villi. a,** Heatmap highlighting transcription factors from the differentially expressed genes
700 between placental villi, trophoblast organoids and placental stromal cells. **b,** Heatmap of genes from
701 the *ELF* family of transcription factors and syncytial genes, *GCM1* and *ERVW-1*. *ELF3* and *ELF5*
702 both show moderate expression levels across the organoids and placental samples, and very low/no
703 expression in the stromal samples. *ELF4* and *ELF1* are similar in all samples. There is very high
704 expression of *ELF1* in placentas and organoids. Similarly, both *ERVW-1* and especially *GCM1* are
705 expressed at higher levels in placentas and organoids in agreement with qPCR data (Fig. 3d). **c,**
706 Genomic mapping of the methylation array probes to the *ELF5* gene. The height of the bars indicates
707 methylation level from 0, unmethylated to 1.0, fully methylated. **d,** Methylation of the *ELF5* promoter
708 shows hypomethylation in the organoid and placenta samples. **e,** Distribution of methyl-cytosine

709 across the promoters of the 10 murine trophoblast gatekeeper genes[12]. The organoids (Org) and
710 placenta (PL) samples show very similar methylation patterns across all 10 gene promoters that are
711 distinct from the control/brain (CL) and maternal-blood (MB) in the majority of the genes. Boxplots
712 comprise min: 1.5 x inter-quartile, lower: 1st quartile, middle: median, upper: 3rd quartile, max: 1.5 x
713 inter-quartile range. Significant correlations (<0.01) are indicated (*). **f,** Table showing Pearson's
714 correlation coefficient (R), number of CpG/probes compared and p-values for **e**. **g,** Chord plot
715 representing terms from the gene ontology analysis of upregulated genes in trophoblast organoids.
716
717 **Extended Data Figure 6. Structure and proliferation in trophoblast organoids. a,** A schematic
718 diagram of a normal placental villus *in vivo* compared to a trophoblast organoid. The basement
719 membrane (BM) beneath the VCT is contiguous with the stromal villous core *in vivo* and with the
720 Matrigel *in vitro*. The SCT contacts maternal blood in the intervillous space *in vivo*. SCT forms in the
721 centre of the organoids. **b,** IHC for TP63 in first trimester placenta and trophoblast organoids
722 (representative images from *n*=14). TP63 is expressed in VCT. Scale bar, 50µm. Higher power insets
723 scale bar, 20µm. **c,** Representative images of TP63, Ki67 and DAPI staining on trophoblast organoids
724 by confocal microscopy (*n*=3). Cells on the outside of the organoids are TP63+ and Ki67+. Scale
725 bars, 20µm. **d,** Confocal microscopy images of trophoblast organoid stained for EdU, EPCAM and
726 DAPI showing fewer proliferating cells (white arrowheads) as the organoids enlarge. Scale bar, 50µm.
727 Representative images from *n*=3. **e,** IHC for markers of SCT, CD46 and CD71, in first trimester
728 placenta and trophoblast organoids (representative images from *n*=20). CD46 and CD71 stain the
729 syncytial brush border. Scale bar, 50µm. Higher power insets scale bar, 20µm. **f,** Carnegie stage 5b
730 embryo (~9 days post-fertilization)from Carnegie Collection at the early lacunar stage (number 8171).
731 Courtesy of Prof. Enders and the Centre for Trophoblast Research
732 (https://www.trophoblast.cam.ac.uk/Resources/enders). Arrows point to examples of cavities that
733 appear in the primitive syncytium due to fluid accumulation before the coelomic cavity and the
734 embryo have fully developed.**g,** Similar cavities in placental tissue samples from first trimester (6-9
735 weeks g.a) and in syncytium in centre of trophoblast organoids. Boxed areas are shown at higher
736 magnification (bottom). Scale bars, 200µm (top); 50µm (bottom). Similar morphology seen in at least
737 5 early placental villi and in all organoids. ICM, inner cell mass. Pr.Syn. primitive syncytium; VCT,
738 villous cytotrophoblast; SCT, syncytiotrophoblast; Str, stromal core.
739
740 **Extended Data Figure 7. Trophoblast organoids grown in TOM and EVTM. a,** Confocal image
741 of organoid stained for F-actin, DAPI and HLA-G. A few isolated cells stain for HLA-G (white
742 arrowheads) at the periphery of the organoid. Scale bar, 50 µm. Representative image from *n*=3. **b,**
743 Phase contrast images from time-lapse videos of 16h of trophoblast organoids grown in TOM when
744 EVT differentiation does not occur (top). No invasive cells are visible. Shown for comparison is an
745 organoid (middle) and a placental villous explant exposed to EVTM (bottom). Black arrows indicate
746 cells migrating and arrowheads show visible tracks made as the cells invade through the Matrigel. For
747 time-lapse videos of these cultures see Supplementary Videos 1, 4 and 5. Scale bars, 200µm.
748
749 **Extended Data Table 1. Microsatellite analysis and HLA typing of organoids from placental**
750 **digests in TOM show that they are of fetal origin a,** PowerPlex16 Short Tandem Repeat (STR)
751 genotyping of DNA from matched maternal-fetal samples (maternal blood, decidua and placenta) to
752 identify origin of organoids. Shown in top row are the 15 STR loci analysed. Numbered STR alleles
753 observed for each DNA at a particular locus are listed within the relevant column. As expected in
754 most cases a maximum of two alleles were seen. In cases where there was evidence of an additional
755 allele from a fetal derived STR haplotype the allele number appears in brackets. This is consistent
756 with the decidua containing fetal extravillous trophoblast. The results at informative loci where fetus

757 and trophoblast organoids match are underlined. **b,** HLA genotyping with Third Generation SMRT
758 sequencing of DNA from matched maternal blood and trophoblast organoid to confirm STR analysis.
759 HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 loci were investigated. The two HLA alleles at each locus
760 defined at high resolution are shown for each sample. For each locus, the fetal trophoblast will share
761 one allele with the mother but the other allele will be derived from the father and is likely to be
762 different. Not all loci are informative as some paternal and non-inherited maternal alleles are the
763 same.

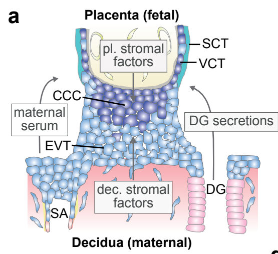
764 **Extended Data Table 2. Microsatellite analysis and HLA typing of organoids from placental**
765 **digests derived in ExM show that they are of maternal origin a,** PowerPlex16 STR genotyping of
766 DNA from matched maternal-fetal samples (decidua, maternal blood, placenta) to identify origin of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

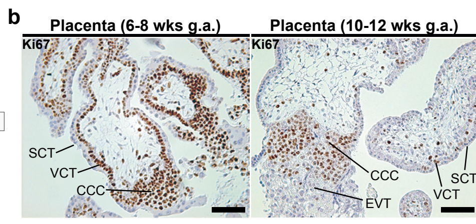
777 **Extended Data Table 3. Liquid chromatography-mass spectrometry (LC-MS) analysis of the**
778 **secretome of trophoblast organoids.** Supernatants from independently-derived trophoblast organoid
779 cultures from six different placental samples were analysed: TOrg_2 (p23), TOrg_3 (p20), TOrg_5
780 (p6), TOrg_10 (p12), TOrg_12 (p4) and TOrg_14 (p5). Table shows the glycoproteins, classical
781 peptides/protein hormones, placental specific peptides and proteins enriched in placental tissue
782 identified in the secretome data by LC-MS. The -10lgP value is the statistical significance assigned to
783 a peptide/protein match by the PEAKS software[37]. Coverage% refers to the proportion of the primary
784 amino acid sequence of each protein that is identified in the experiment. #Peptides refers to the
785 number of peptide matches assigned to a protein, whilst #Unique refers to the number of peptides that
786 are assigned solely to that protein group. #Spec refers to the number of peptide MS/MS spectra
787 matched against a particular protein. The columns also indicate whether these are unique products
788 produced by the placenta and/or whether they are products produced by other tissues but highly
789 enriched in the placenta (among the top 10 organs producing that protein based on RNA expression
790 levels). Tissue location data was compared to data from the Human Protein Atlas,
791 https://www.proteinatlas.org/. All protein identification data are in Supplementary Table 2.

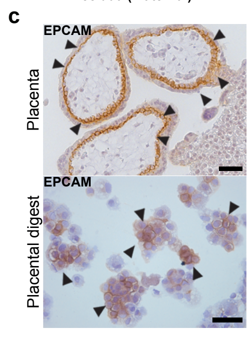
792

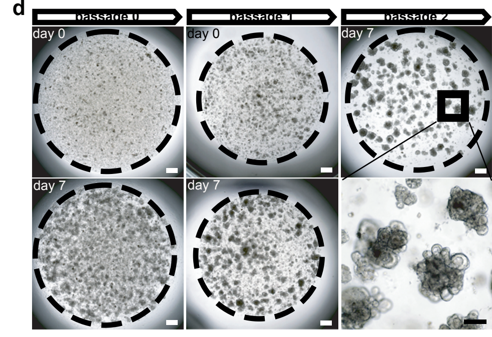
793

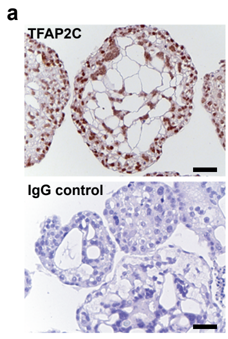
794

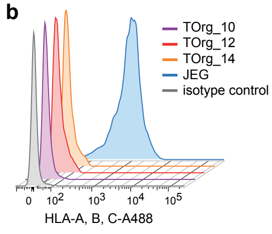

a
Placenta (fetal)
pl. stromal factors
SCT
VCT
CCC
maternal serum
DG secretions
EVT
dec. stromal factors
DG
SA
Decidua (maternal)

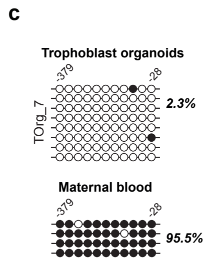

b
Placenta (6-8 wks g.a.)
Placenta (10-12 wks g.a.)
Ki67
Ki67
SCT
VCT
CCC
CCC
EVT
VCT
SCT

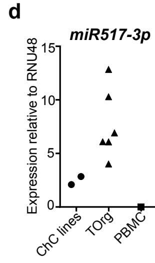

c
EPCAM
Placenta
EPCAM
Placental digest

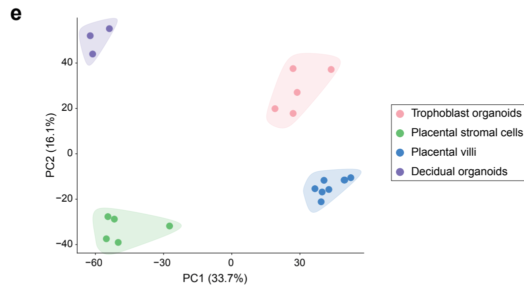

d
passage 0
passage 1
passage 2
day 0
day 0
day 7
day 7
day 7

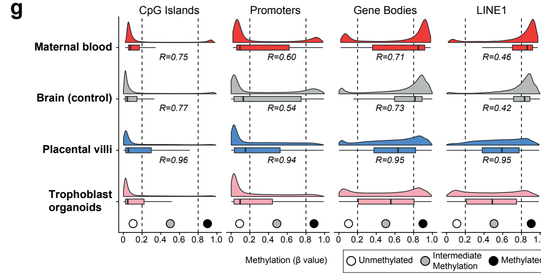

a
TFAP2C
IgG control

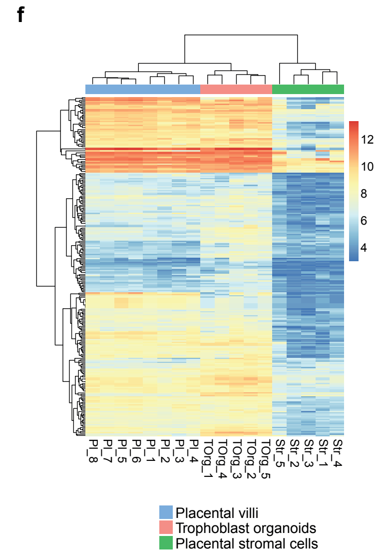

b
TOrg_10
TOrg_12
TOrg_14
JEG
isotype control
HLA-A, B, C-A488

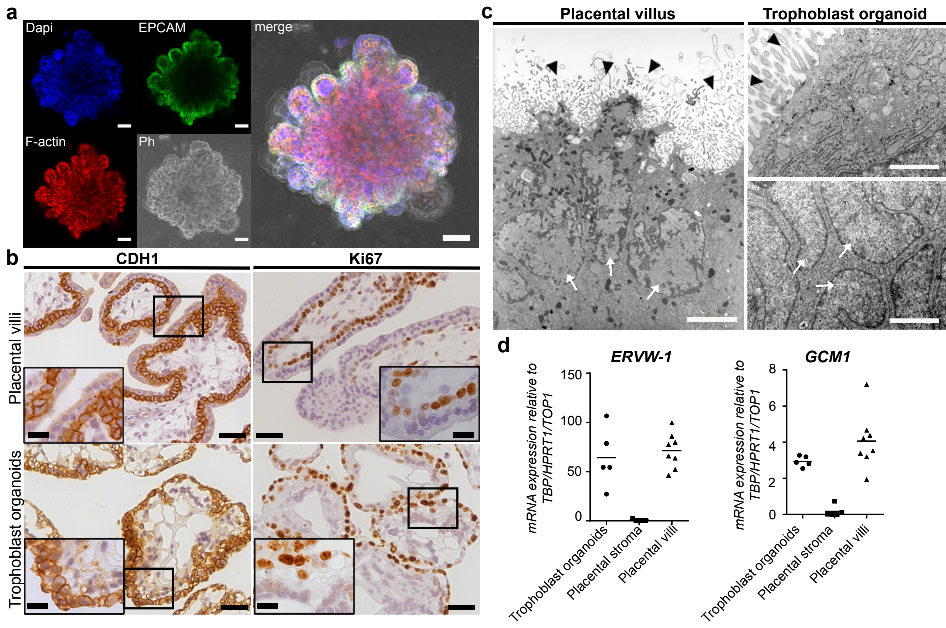

c
Trophoblast organoids
TOrg_7
2.3%
Maternal blood
95.5%


d
miR517-3p
Expression relative to RNU48
ChC lines
TOrg
PBMC


e
Trophoblast organoids
Placental stromal cells
Placental villi
Decidual organoids
PC1 (33.7%)
PC2 (16.1%)

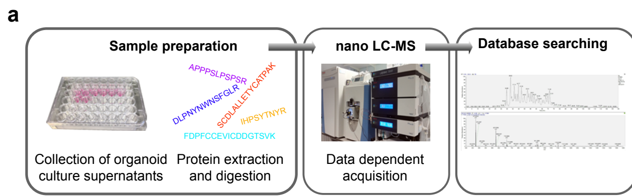

g
CpG Islands
Promoters
Gene Bodies
LINE1
Maternal blood
Brain (control)
Placental villi
Trophoblast organoids
Methylation (β value)
Unmethylated
Intermediate Methylation
Methylated


f
Placental villi
Trophoblast organoids
Placental stromal cells

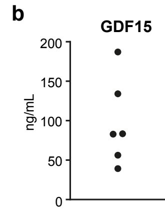


a
Dapi
EPCAM
merge
F-actin
Ph
b
CDH1
Ki67
Placental villi
Trophoblast organoids
c
Placental villus
Trophoblast organoid
d
ERVW-1
GCM1
mRNA expression relative to TBP/HPRT1/TOP1
150
100
50
0
8
6
4
2
0
Trophoblast organoids
Placental stroma
Placental villi

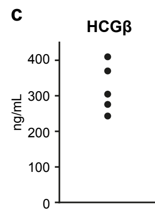
a



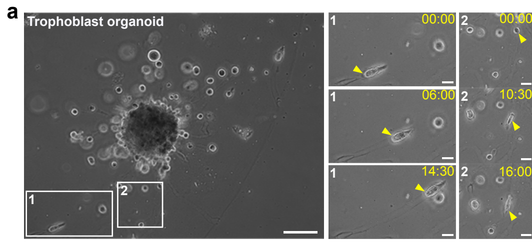
b

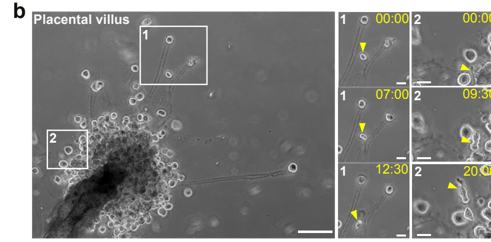


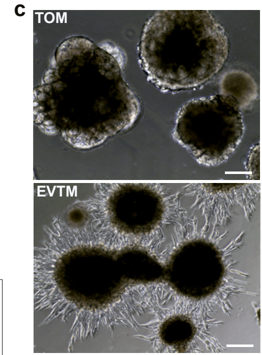
c

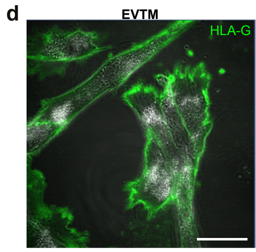


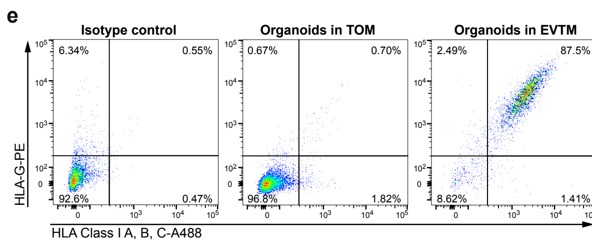
d

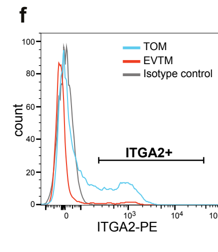

a
Trophoblast organoid
1
2
00:00
06:00
14:30
10:30
16:00


b
Placental villus
1
2
00:00
07:00
12:30
09:30
20:00


c
TOM
EVTM


d
EVTM
HLA-G


e
Isotype control
Organoids in TOM
Organoids in EVTM
6.34%
0.55%
92.6%
0.47%
0.67%
0.70%
96.8%
1.82%
2.49%
87.5%
8.62%
1.41%
HLA-G-PE
HLA Class I A, B, C-A488


f
TOM
EVTM
Isotype control
ITGA2+
count
ITGA2-PE