

## Self-Renewing Trophoblast Organoids Recapitulate the Developmental Program of the Early Human Placenta

Sandra Haider,<sup>1</sup> Gudrun Meinhardt,<sup>1</sup> Leila Saleh,<sup>1</sup> Viktoria Kunihs,<sup>1</sup> Magdalena Gamperl,<sup>2</sup> Ulrich Kaindl,<sup>2</sup> Adolf Ellinger,<sup>2</sup> Thomas R. Burkard,<sup>3</sup> Christian Fiala,<sup>4</sup> Jürgen Pollheimer,<sup>1</sup> Sasha Mendjan,<sup>3</sup> Paulina A. Latos,<sup>2,\*</sup> and Martin Knöfler<sup>1,\*</sup>

<sup>1</sup>Department of Obstetrics and Gynaecology, Medical University of Vienna, Reproductive Biology Unit, Währinger Gürtel 18-20, 5Q, 1090 Vienna, Austria

<sup>2</sup>Center for Anatomy and Cell Biology, Medical University of Vienna, Schwarzspanierstrasse 17, 1090 Vienna, Austria

<sup>3</sup>Institute of Molecular Biotechnology, Austrian Academy of Sciences, 1030 Vienna, Austria

<sup>4</sup>Gynmed Clinic, 1150 Vienna, Austria

\*Correspondence: paulina.latos@meduniwien.ac.at (P.A.L.), martin.knoefler@meduniwien.ac.at (M.K.)

<https://doi.org/10.1016/j.stemcr.2018.07.004>

### SUMMARY

Defective placentation is the underlying cause of various pregnancy complications, such as severe intrauterine growth restriction and preeclampsia. However, studies on human placental development are hampered by the lack of a self-renewing *in vitro* model that would recapitulate formation of trophoblast progenitors and differentiated subtypes, syncytiotrophoblast (STB) and invasive extravillous trophoblast (EVT), in a 3D orientation. Hence, we established long-term expanding organoid cultures from purified first-trimester cytotrophoblasts (CTBs). Molecular analyses revealed that the CTB organoid cultures (CTB-ORGs) express markers of trophoblast stemness and proliferation and are highly similar to primary CTBs at the level of global gene expression. Whereas CTB-ORGs spontaneously generated STBs, withdrawal of factors for self-renewal induced trophoblast outgrowth, expressing the EVT progenitor marker NOTCH1, and provoked formation of adjacent, distally located HLA-G<sup>+</sup> EVTs. In summary, we established human CTB-ORGs that grow and differentiate under defined culture conditions, allowing future human placental disease modeling.

### INTRODUCTION

During placentation two types of differentiated epithelial trophoblasts arise. In floating villi, bathed in maternal blood, cytotrophoblasts (CTBs) undergo cell fusion thereby forming syncytiotrophoblasts (STBs). The latter represent the brush border between maternal and fetal circulation, transport nutrients and oxygen to the developing embryo, and produce hormones maintaining pregnancy (Aplin, 2010; Evain-Brion and Malassine, 2003). In villi, anchoring to the maternal decidua, proliferative cell columns give rise to extravillous trophoblasts (EVTs) invading maternal uterine stroma and the spiral arteries (Pijnenborg et al., 1980). Remodeling of the latter promotes low-pressure blood flow to the developing placenta, thereby adapting oxygen and nutrient supply for fetal growth (Burton et al., 2010; Pijnenborg et al., 2006). Abnormal changes in EVT invasion and remodeling have been detected in different pregnancy disorders such as severe forms of intrauterine growth restriction, preeclampsia, miscarriage, recurrent abortion, and preterm labor (Hustin et al., 1990; Khong et al., 1987; Pijnenborg et al., 1991; Romero et al., 2011; Sheppard and Bonnar, 1976; Windsperger et al., 2017). Defects in EVT progenitor formation and differentiation could represent an underlying cause (Lim et al., 1997; Zhou et al., 1997). However, human trophoblast models, that adequately mimic these early developmental processes, have not been established. Primary CTBs, isolated from first-trimester placenta, can

hardly be manipulated since they rapidly cease proliferation in culture. Alternative models, such as trophoblast cell lines, choriocarcinoma cells, and bone morphogenetic protein 4 (BMP4)-treated human embryonic stem cells (hESCs) have been established. However, global gene expression profiles of BMP4-treated hESCs and trophoblast-specific markers, as well as human leucocyte antigen (HLA) status of the cell lines, diverge considerably from primary tissues (Apps et al., 2009; Bilban et al., 2010; Jain et al., 2017; Lee et al., 2016; Yabe et al., 2016). Thus, localization and characteristics of human trophoblast stem cells (TSCs) and progenitors during pregnancy remain largely elusive (Chang and Parast, 2017; Gamage et al., 2016). Previous investigations suggested the presence of bipotential as well as distinct progenitors for syncytialization and EVT formation (Baczyk et al., 2006; Haider et al., 2016; Horii et al., 2016; James et al., 2007). Very recently, bipotential human TSCs have been isolated from blastocysts and CTB preparations showing long-term, 2D proliferation under defined culture conditions (Okoe et al., 2018). However, development of a system allowing studying 3D cell column formation, growth, and differentiation, as it occurs during *in vivo* placentation, has not been reported.

Establishment of self-renewing 3D epithelial organoids, closely resembling structure and physiology of the original organ, has been achieved from various tissues containing multipotent stem cells (Boretto et al., 2017; Kessler et al., 2015; Kretzschmar and Clevers, 2016; Turco et al., 2017).





However, organoids of the human placenta have not been generated so far. Therefore, we herein established human CTB organoid cultures (CTB-ORGs) from purified first-trimester CTB preparations that are capable of self-renewal and expansion under defined culture conditions. Whereas STBs were spontaneously generated in this system, removal of factors, promoting long-term expansion, provoked development of EVT progenitors and their differentiation. The present model provides evidence for a TSC niche in the villous epithelium of the human placenta. Further, it will allow studying discrete steps of EVT formation in a sequential manner.

## RESULTS

### Establishment of Long-Term Expanding Human Trophoblast Organoids

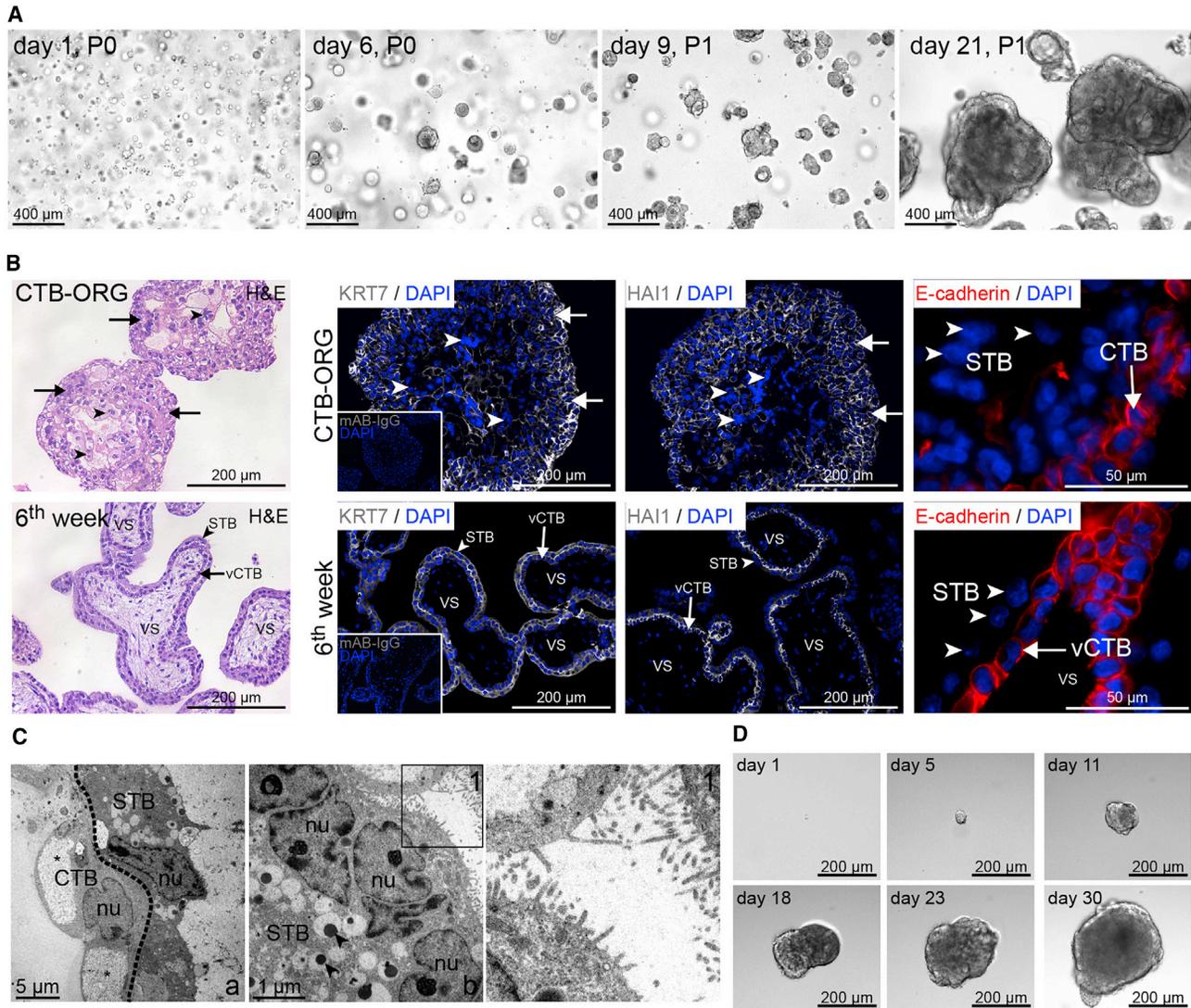
To generate human trophoblast organoids, villous cytotrophoblasts (vCTBs), purified from pooled first-trimester placental tissues of the 6th to 7th week of gestation, were embedded in Matrigel containing a mixture of growth factors and signaling inhibitors, previously shown to promote stemness and formation of organoids (Kretzschmar and Clevers, 2016). Inhibition of transforming growth factor  $\beta$  (TGF- $\beta$ ) and BMP signaling with A83-01 and Noggin, respectively, supplementation of epidermal growth factor (EGF), and activation of Wnt signaling, using R-spondin, the glycogen synthase kinase 3 $\alpha$  and 3 $\beta$  (GSK-3 $\alpha/\beta$ ) inhibitor CHIR99021 and prostaglandin E<sub>2</sub>, have been successfully applied for establishing gut and other types of human epithelial organoids (Dutta et al., 2017; Kretzschmar and Clevers, 2016). Using this defined cocktail of factors, small cell clusters formed within several days of culture, which rapidly grew and developed into organoids with irregular structures after 2–3 weeks (Figure 1A). Currently, 16 different organoid cultures were established from early placental tissues (sixth to seventh week) with 100% derivation efficiency, which could be cryo-preserved and re-cultivated. However, we could not generate organoids from five different vCTB preparations of pooled placental tissues of the 10th to 12th week of gestation (data not shown). Organoids were passaged approximately every 14 days and could be expanded for more than 5 months (passage 10 [P10]–P11). At this stage, growth rates were decreasing and organoids could not be reformed after P13. A video showing time-dependent growth (between day 2 and 6) of a representative organoid culture at P2 is depicted (Video S1).

Immunofluorescence revealed that the outer layers of the organoids harbor multiple rows of mononuclear, cytokeratin 7 (KRT7)<sup>+</sup> cells expressing the CTB marker hepatocyte

growth factor activator inhibitor type 1 (Figure 1B). The spheroid-like structures also expressed the STB marker chorionic gonadotrophin  $\beta$  (CG $\beta$ ), but completely lacked vimentin, confirming their trophoblast identity (Figures S1A and S1B). In contrast to the architecture of the placental villous epithelium, the CTB-ORGs show an inverse organization with multinuclear, E-cadherin<sup>-</sup> cells in the inner part, indicating that the CTBs undergo cell fusion toward the center (Figure 1B). As organoids grew in size, CG $\beta$  expression markedly increased (Figures S1C and S1D). Electron microscopy unraveled formation of a micro-villous membrane on the multinucleated structures indicating that these cells could be very similar to *in vivo* STBs (Figure 1C). Indeed, prominent numbers of transport vesicles in these cells (Figure 1C) and detection of CG $\beta$  in the supernatants of CTB-ORGs (Figure S1E) suggested that the multinucleated cells also exhibit secretory activity. The presence of EGF and of the TGF- $\beta$  signaling inhibitor A83-01, respectively, was found to be critical for long-term expansion of CTB-ORGs (Figure S2A), whereas absence of the Wnt inducers (R-spondin and CHIR99021) promoted trophoblast outgrowth and differentiation (Figures S5A and S5B). Clonogenic assays revealed that large organoids are formed from single cells within 3–4 weeks (Figures 1D and S2B).

### Human Trophoblast Organoids Express Markers of Human CTB Stemness, Proliferation, and Cell Fusion

To further assess trophoblast origin and maintenance of CTB-ORGs, we analyzed widely accepted transcription factors of human CTB identity, stemness, and differentiation (Hemberger et al., 2010; Lee et al., 2016; Li et al., 2014) (Figure 2). In agreement with their expression in vCTBs of the 6th-week placenta, we observed that caudal-type homeobox protein 2 (CDX2), tumor protein p63 (TP63), and TEA-domain transcription factor 4 (TEAD4) were expressed in the outer CTB layers of CTB-ORGs (Figure 2A). The respective mRNAs were also detectable in CTB-ORGs. Moreover, other regulators of trophoblast development, AP-2 $\alpha$ , AP-2 $\gamma$ , and GATA3, expressed in STBs and/or CTBs of first-trimester placenta (Biadasiewicz et al., 2011; Paul et al., 2017), were also present in CTBs of organoids (Figures 2A and 2B). Cyclin A (CCNA) and KI67, marking proliferative vCTBs in 6th-week placenta, were expressed in the outer CTB layers of CTB-ORGs, but not in the inner STBs (Figure 2C). Expression of the *ELF5* gene and demethylation of its promoter region were shown to be indicative for human CTB origin (Hemberger et al., 2010; Lee et al., 2016). The proximal *ELF5* promoter was largely hypomethylated in CTB-ORGs and vCTBs, whereas 74% of the CpG sites were methylated in placental fibroblasts (Figure 2D). Accordingly, *ELF5* mRNA was detected in CTB-ORGs, but absent from the villous stromal cells (Figure 2B).



### Figure 1. Establishment of Human CTB Organoids from Isolated First-Trimester Villous CTBs

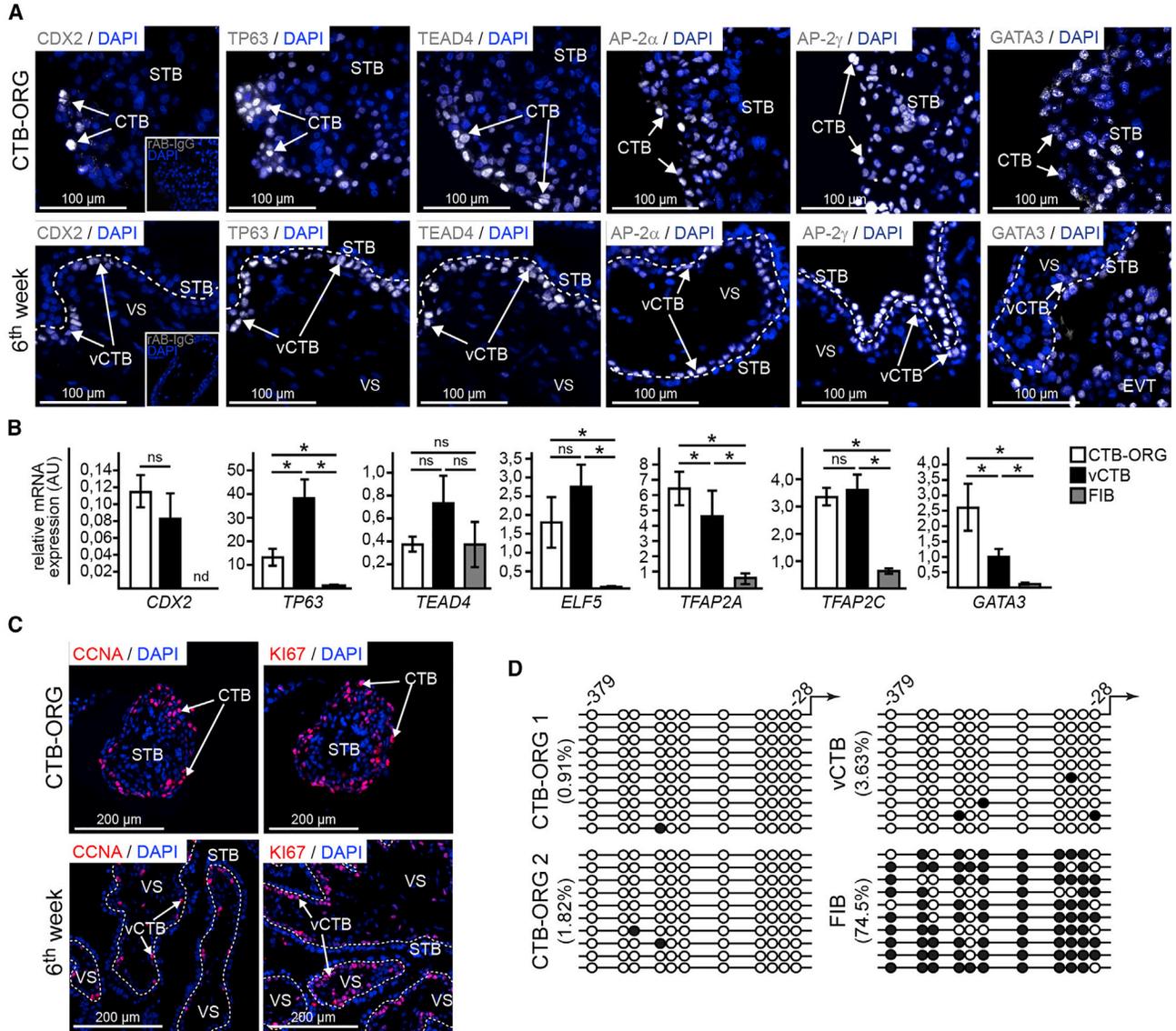
(A) Representative phase contrast images of CTB-ORGs ( $n = 8$ ) before (P0) and after first passaging (P1). Small spheroid-like structures are visible after 4–7 days and reach a diameter of approximately 300–800  $\mu$ m within 3 weeks.

(B) H&E staining and immunofluorescence of tissue sections of CTB-ORGs revealed densely packed cell clusters of epithelial origin with fused regions in the center. Representative pictures of CTB-ORGs ( $n = 8$  different cultures between P2 and 4) and first-trimester placenta ( $n = 6$ ; 6th to 7th week of gestation) showing cytokeratin 7 (KRT7) (white), hepatocyte growth factor activator inhibitor type 1 (HAI1) (white), E-cadherin (red), and nuclei (DAPI, blue) are shown. Arrows mark mononuclear CTBs in organoids and villous cytotrophoblasts (vCTBs), whereas arrowheads depict multinuclear structures and STBs in organoids and placental tissues, respectively. VS, villous stroma. In negative controls primary antibodies were replaced with mouse monoclonal isotype IgG (mAB-IgG).

(C) Representative electron transmission microscopy images of a CTB-ORG at P3 out of three different cultures (P2 and P3) analyzed. Stippled line in (a) indicates boundary between CTB and syncytiotrophoblasts (STB). \*Glycogen deposits in CTBs; nu, nucleus; (b) illustrates nuclei and transport vesicles (marked by arrowheads) in magnified STBs. Inset picture (1), showing the micro-villous surface of STBs, is depicted at a higher magnification on the right hand side.

(D) Time-dependent growth (phase contrast images) of a representative clonal organoid line, generated from a single cell of an organoid culture at P3.

See also [Figures S1](#) and [S2](#).

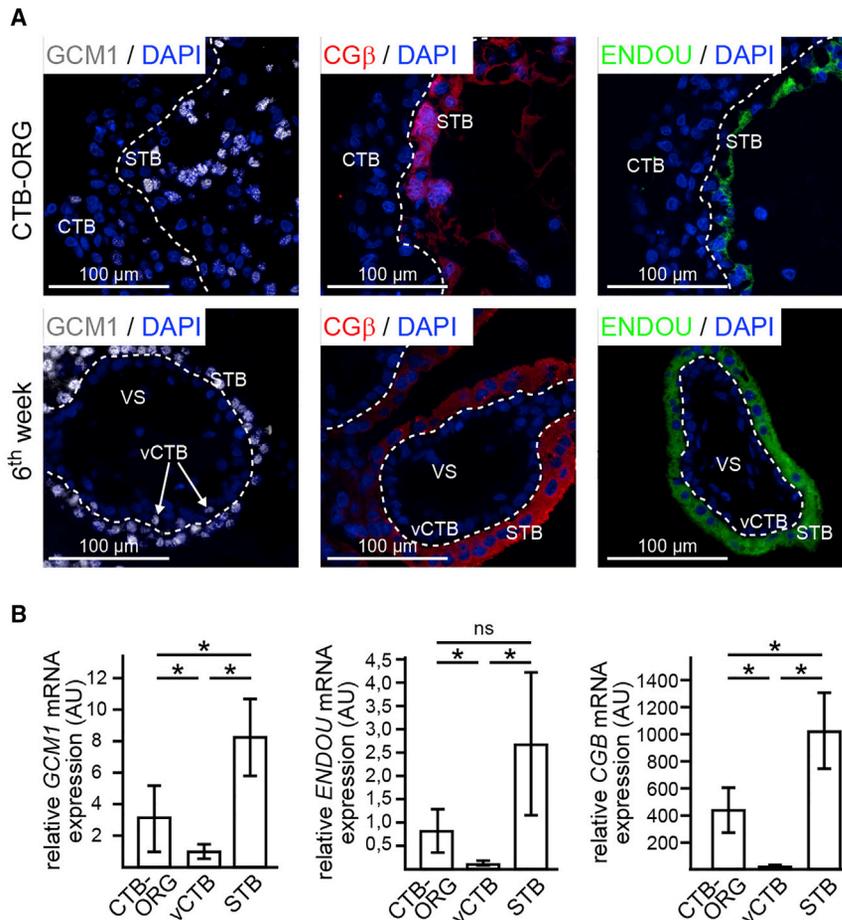


**Figure 2. Human Trophoblast Organoids Express Markers of CTB Identity, Stemness, and Proliferation**

(A–C) Representative immunofluorescence images of first-trimester placentae ( $n = 4$ ) and CTB-ORGs between P2 and P5 ( $n = 6$  different cultures) are shown. EVT, extravillous trophoblast; vCTB, villous cytotrophoblast; STB, syncytiotrophoblasts; VS, villous stroma; nuclei were counterstained with DAPI. Trophoblasts expressing markers of stemness or proliferation are marked with arrows. Stippled line in pictures of 6th-week placentae demarcates STBs from underlying vCTBs. (A) Immunofluorescence of markers of trophoblast stemness and identity in serial sections of CTB-ORGs and 6th-week placenta. In negative controls primary antibodies were replaced with rabbit isotype IgG (rAB-IgG). (B) qPCR showing mRNA expression of CTB identity and stemness markers in CTB-ORGs (P2–P4;  $n = 6$  different cultures), primary vCTBs ( $n = 4$ ), and placental fibroblasts (FIB) ( $n = 4$ ). Mean values  $\pm$  SD measured in duplicates (normalized to *TBP*) are shown \* $p < 0.05$ ; ns, not significant; nd, not detectable; AU, arbitrary units; *TFAP2A* and *TFAP2C* mRNAs encode AP-2 $\alpha$  and AP-2 $\gamma$ , respectively. (C) Immunofluorescence of cyclin A (CCNA) and KI67 in CTB-ORGs and 6th-week placenta.

(D) Methylation status of individual CpG sites in the proximal *ELF5* promoter region (–379 to –28) in two different CTB-ORGs (P1 and P3), vCTB and FIB isolated from 6th-week placenta. Percentages indicate proportion of methylated (filled circles) to unmethylated (open circles) CpG residues.

See also Figure S3.



**Figure 3. CTB Organoids Express Markers of Syncytiotrophoblasts in the Inside Region**

(A) Immunofluorescence of different STB markers in serial sections of a representative CTB-ORG culture ( $n = 4$  different cultures analyzed between P2 and P5) and in 6th-week placental tissue ( $n = 3$ ). Stippled line depicts the border between CTB and STB. ENDOU, poly(U)-specific endoribonuclease; GCM1, glial cell missing 1; CG $\beta$ , chorionic gonadotrophin  $\beta$ . Nuclei were stained with DAPI. Note that GCM1 is predominantly expressed in STBs, but can also be detected in a small number of vCTBs (marked by arrows). (B) qPCR showing mRNA expression of STB markers in CTB-ORGs ( $n = 6$  different cultures, P2–P4), vCTBs ( $n = 4$ ), and STBs ( $n = 3$ ), generated *in vitro* by differentiating vCTBs for 72 hr on fibronectin. Mean values  $\pm$  SD, measured in duplicate, are shown. \* $p < 0.05$ ; AU, arbitrary units. CG $\beta$ , CG $\beta$  mRNA. See also Figure S4.

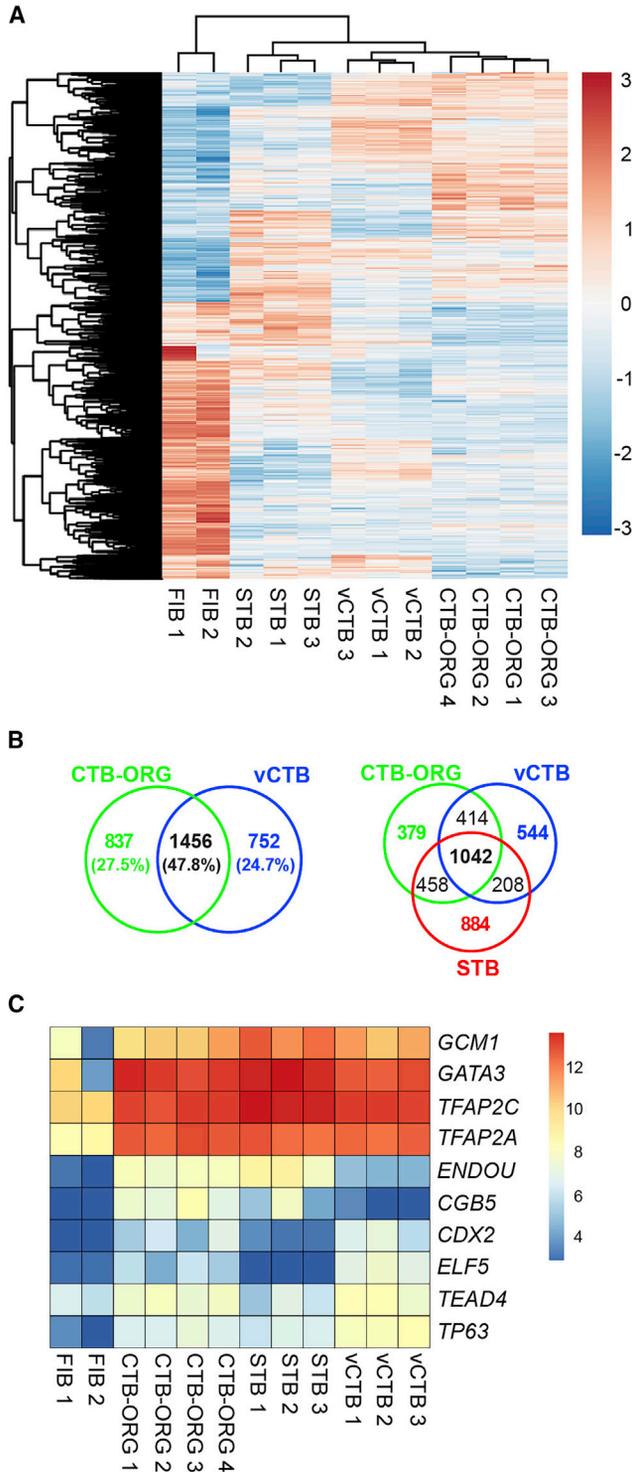
To assess long-term self-renewing capacity of CTB-ORGs, mRNA expression of markers of trophoblast identity and stemness were analyzed at low, middle, and high passage numbers (Figure S3). *TFAPC*, encoding AP-2 $\gamma$ , *TP63*, and *ELF5* mRNAs were expressed at similar levels between P1 and P3, P4 and P7, and P8 and P11. *TEAD4* and *CDX2* transcript levels tended to decrease at P8–P11, whereas *GATA3* increased. *CCNA* mRNA also decreased between P8 and P11, coinciding with the decelerating growth of CTB-ORGs. Vimentin (*VIM*) mRNA was undetectable in CTB-ORGs, indicating that CTB-ORGs do not undergo an abnormal epithelial-to-mesenchymal transition (EMT) upon long-term passaging.

To further characterize inner cells of CTB-ORGs, several markers of syncytialization were investigated (Figure 3). In agreement with the inside-out architecture of CTB-ORGs, poly(U)-specific endoribonuclease (*ENDOU*), identified as an STB-specific mRNA in the RNA sequencing (RNA-seq) analyses (see below), glial cell missing 1 (*GCM1*), and CG $\beta$  were expressed in the outer STB layer of the 6th-week placenta and in the inner layer of CTB-ORGs (Figure 3A). The respective transcripts *GCM1*, *ENDOU*, and

*CGB* could be identified in CTB-ORGs and in STBs, generated by *in vitro* cell fusion, but were weakly expressed (*GCM1*) or largely absent (*ENDOU*, *CGB*) from purified placental vCTBs (Figure 3B). Apoptotic cells were detected in a very small percentage of cells of CTB-ORGs and resided in E-cadherin<sup>+</sup> CTBs as well as in E-cadherin<sup>-</sup> STBs (Figure S4).

#### Gene Expression Profiles of Trophoblast Organoids and Primary Human Trophoblasts Are Highly Similar

To unravel similarities between gene expression profiles, CTB-ORGs and primary vCTBs preparations, freshly isolated (0 hr) or *in vitro* differentiated for 72 hr (STB), were subjected to RNA-seq and bioinformatic analyses (Figure 4). Hierarchical clustering revealed close similarities between the different CTB-ORGs, vCTBs, and *in-vitro*-generated STBs, whereas the gene expression profiles of placental fibroblasts considerably diverged from the trophoblast cultures (Figure 4A). Closer inspection indicated that 47.8% of all trophoblast-upregulated genes (>2-fold change relative to fibroblasts) were common to CTB-ORGs and primary vCTBs, including all markers of CTB identity and



**Figure 4. RNA-Seq and Bioinformatic Analyses of Placental Organoids and Primary Trophoblasts**

(A) Hierarchical clustering of genome-wide mRNA expression profiles. A total of 7,417 genes, differentially expressed between CTB-ORG cultures (P2 and P4) and FIBs ( $n = 2$  from 6th week), and expression of these genes in vCTB ( $n = 3$  from 6th- to 7th-week

stemness, *TFAP2A*, *TFAP2C*, *CDX2*, *GATA3*, *TP63*, *TEAD4*, and *ELF5* (Figures 4B and 4C). Upon comparison of CTB-ORGs, primary vCTBs, and STBs, 1,042 (26.5%) commonly expressed genes were identified. A heatmap shows relative mRNA levels of trophoblast markers in the different cultures (Figure 4C). Gene expression signatures, established herein, are accessible at the GEO database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109976>).

### Loss of R-Spondin and CHIR99021 Changes the Cell Fate of Human Trophoblasts

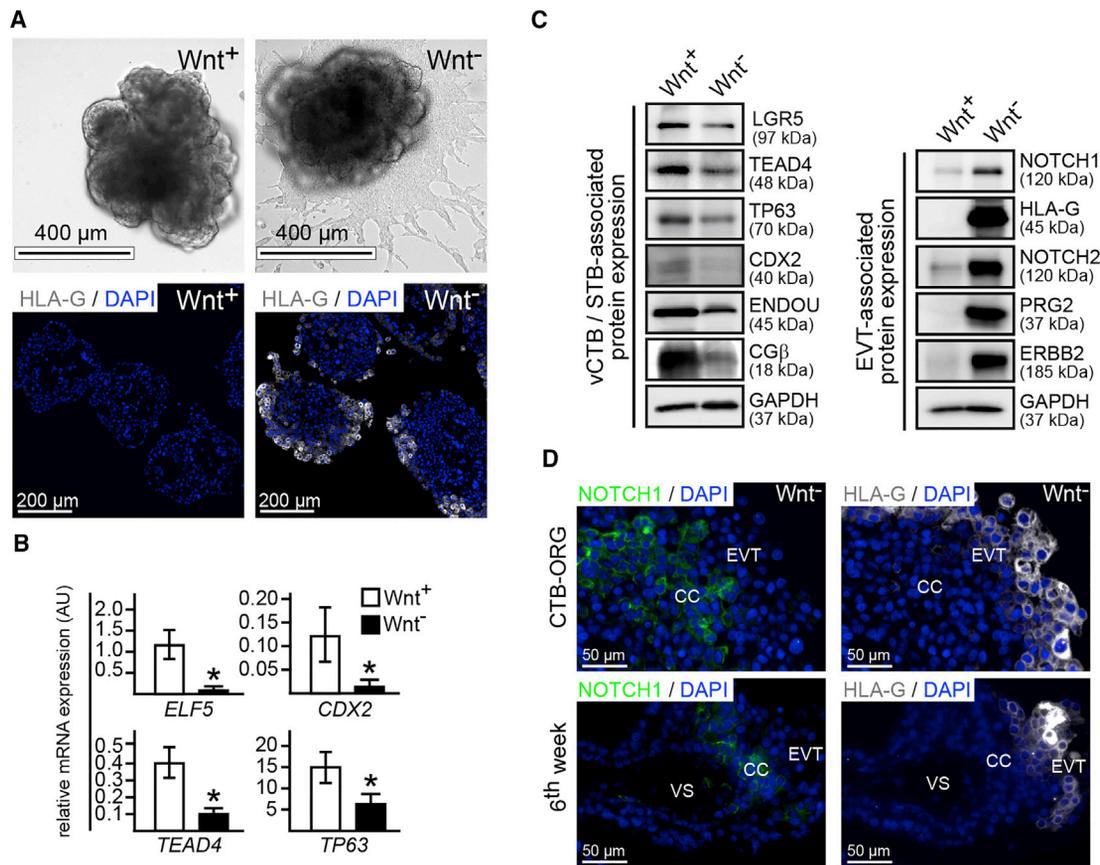
Wnt signaling maintains stemness and proliferation of epithelial cells in different organoid culture systems (Kretzschmar and Clevers, 2016). To assess its role in CTB-ORG self-renewal, R-spondin and the GSK-3 $\alpha/\beta$  inhibitor, CHIR99021, were omitted from the organoid cultures (Figure 5). Microscopy and immunofluorescence analyses revealed that withdrawal of the Wnt stimulators (indicated as Wnt<sup>-</sup> condition) promoted trophoblast outgrowth from the outer CTB layers and expression of HLA-G at distal sites of CTB-ORGs (Figure 5A). Outgrowth in Wnt<sup>-</sup> CTB-ORGs continued for up to at least 14 days, whereas organoids became smaller at subsequent stages (Figures S5A and S5B). Incubation of Wnt<sup>-</sup> CTB-ORGs with recombinant Wnt3a largely compensated for the loss of R-spondin/CHIR99021, since it reduced the average area of outgrowth to 11% (Figure S5C). Further, the Wnt<sup>-</sup> condition decreased expression of the Wnt stem cell receptor LGR5 and the CTB self-renewal markers *ELF5*, *TEAD4*, *CDX2*, and *TP63* (Figures 5B and 5C). In contrast, the EVT markers *HLA-G* (Kovats et al., 1990; Yelavarthi et al., 1991), *NOTCH2* (Plessl et al., 2015), *PRG2* (Windsperger et al., 2017), and *ERBB2* (Fock et al., 2015) were induced in the absence of Wnt activators (Figure 5C). *In vitro* development of the EVT lineage occurred at the expense of cell fusion since the STB markers *ENDOU* and *CGB* decreased (Figure 5C).

Notably, we also observed upregulation of the cell column marker *NOTCH1*, a critical regulator of EVT progenitor formation and survival (Haider et al., 2016), in Wnt<sup>-</sup>

placentae) and differentiated STB ( $n = 3$ ; 6th- to 7th-week placentae) are shown. Analysis is based on  $p < 0.05$ .

(B) Venn diagram on the left shows that of 2,293 of trophoblast-upregulated mRNAs (>2-fold change relative to FIB;  $p < 0.05$ ), 1,456 were common to CTB-ORG and vCTB. CTB-ORGs also express 1,500 STB-specific mRNAs (Venn diagram on the right-hand side) and share 1,042 common genes with both vCTB and STB.

(C) Clustered heatmap depicting selected expression patterns of markers of CTB stemness and cell fusion. *TFAP2A* and *TFAP2C* mRNAs encode AP-2 $\alpha$  and AP-2 $\gamma$ , respectively. *CGB5*, mRNA of the chorionic gonadotrophin  $\beta 5$  gene; *ENDOU*, poly(U)-specific endoribonuclease.



### Figure 5. Loss of Wnt Activators Promotes Formation of EVT and Their Progenitors in Trophoblast Organoid Cultures

(A) Upper pictures show representative phase contrast images of (CTB-ORG cultures) at P2 in the absence (4 days, Wnt<sup>-</sup>) or presence (Wnt<sup>+</sup>) of R-spondin and CHIR99021. Lower immunofluorescence images depict HLA-G expression in representative CTB-ORGs (P2). In total n = 10 different CTB-ORGs (P2–P5) were analyzed. Note that EVTs, which have deeply migrated into Matrigel, are lost during the fixation procedure. Hence, HLA-G<sup>+</sup> cells of Wnt<sup>-</sup> CTB-ORGs, shown in (A and D), likely represent EVTs of the distal cell column. Nuclei are stained with DAPI. RNA (B) and protein (C) expression of vCTB lineage/stem cell-specific genes, and of STB and EVT markers in Wnt<sup>+</sup> and Wnt<sup>-</sup> CTB-ORGs.

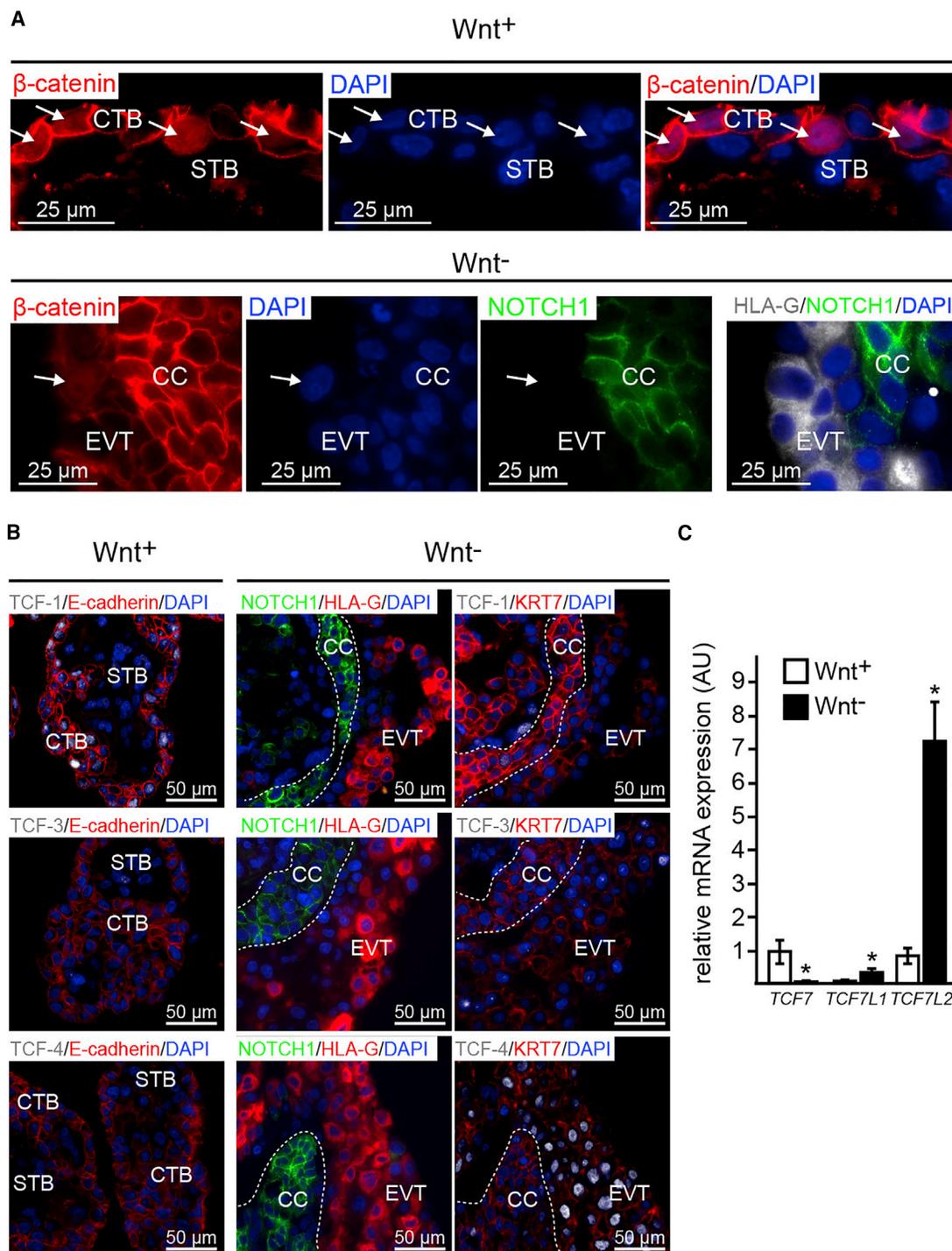
(B) qPCR analyses in different CTB-ORGs (n = 5; P2–P5). Expression was normalized to *TBP*. Mean values ± SD, measured in duplicate, are shown, \*p < 0.05; AU, arbitrary units.

(C and D) Representative western blots performed with different CTB-ORGs (n = 4; P2–P5) are shown (C). GAPDH was used as loading control. PRG2, proteoglycan 2; (D) immunofluorescence showing NOTCH1 and adjacent HLA-G expression in a CTB-ORG (P3) and in 6th-week placenta. Representative images of CTB-ORGs (n = 10 different cultures; P2–P5) and first-trimester placental tissues (n = 4) are depicted. CC, cell column; EVT, extravillous trophoblast; VS, villous stroma; See also Figures S5 and S6.

CTB-ORGs (Figures 5C and 5D). Similar to the *in vivo* situation, NOTCH1<sup>+</sup> CTBs developed in proximal regions of trophoblast outgrowth, adjacent to distally located HLA-G<sup>+</sup> EVTs (Figure 5D). Notably, *CCNA* mRNA expression transiently increased upon loss of R-spondin and CHIR99021 (Figure S6A). Like KI67, *CCNA* protein was predominantly detected in NOTCH1<sup>+</sup> CTBs in the absence of Wnt stimulators (Figure S6B). These data suggest that Wnt<sup>-</sup> organoids mirror *in vivo* cell column/EVT progenitor formation and EVT differentiation in an equivalent 3D orientation.

### Expression of Key Components of Canonical Wnt Signaling in Human First-Trimester Placenta and Organoids Differs between Trophoblast Subtypes

Although previous studies suggested a role for Wnt signaling in EVT function and differentiation (Meinhardt et al., 2014; Pollheimer et al., 2006), the present data also indicated its importance in vCTB stemness. To further delineate the possible dual role of Wnt in placental development, we evaluated the expression patterns of key components of canonical Wnt signaling in first-trimester placental tissues (Figure S7) and CTB-ORGs (Figure 6). In



**Figure 6. Differential Expression Patterns of Nuclear  $\beta$ -Catenin and T Cell Factors in Distinct Trophoblast Subtypes of Organoids and the Effects of Wnt Inhibition on EVT Formation**

(A and B) Representative pictures of  $n = 6$  (A) and  $n = 4$  (B) different CTB-ORGs cultures (analyzed between P2 and P4) are depicted. CC, cell column; CTB, cytotrophoblast; EVT, extravillous trophoblast; STB, syncytiotrophoblast; Nuclei are visualized with DAPI. (A) Localization of  $\beta$ -catenin in CTB-ORGs in the absence ( $Wnt^{-}$ ) or presence ( $Wnt^{+}$ ) of R-spondin and CHIR99021. Trophoblasts displaying nuclear  $\beta$ -catenin are marked with arrows. (B) Expression of T cell factors (TCFs) in  $Wnt^{+}$  and  $Wnt^{-}$  CTB-ORGs, co-stained with trophoblast subtype markers. Stippled line indicates NOTCH1<sup>+</sup> progenitor population. Serial sections are shown in the  $Wnt^{-}$  condition.

(legend continued on next page)



6th-week placenta nuclear  $\beta$ -catenin was detected in a small number of vCTBs and EVT of the distal cell column, but absent from NOTCH1<sup>+</sup> progenitors (Figure S7A). Similarly, a minor fraction of proliferative CTBs (Wnt<sup>+</sup>) and HLA-G<sup>+</sup> EVTs (Wnt<sup>-</sup>) of CTB-ORGs revealed nuclear  $\beta$ -catenin expression (Figure 6A). In contrast, NOTCH1<sup>+</sup> CTB nuclei of Wnt<sup>-</sup> CTB-ORGs were negative for  $\beta$ -catenin.

Moreover, distinct expression patterns of T cell factors (TCFs), the key transcription factors of canonical Wnt signaling, could be observed in the different trophoblast subtypes. In first-trimester placenta, TCF-1 was expressed in a subset of E-cadherin<sup>+</sup> vCTBs, but undetectable in NOTCH1<sup>+</sup> progenitors and EVTs of the distal cell column (Figure S7B). TCF-3 and -4 were absent from vCTBs and proximal cell column trophoblasts of early placental tissues, but present in EVTs adjacent to the NOTCH1<sup>+</sup> cells (Figure S7B). Expression patterns of *TCF* mRNAs in purified vCTBs and EVTs reflected protein distribution in tissues (Figure S7C). In Wnt-stimulated CTB-ORGs, TCF-1 was expressed in outer nuclei, but undetectable in NOTCH1<sup>+</sup> progenitors and EVTs under Wnt<sup>-</sup> conditions (Figures 6B and 6C). In contrast, TCF-3 and TCF-4 protein expression could be neither observed in CTBs of Wnt<sup>+</sup> CTB-ORGs nor in NOTCH1<sup>+</sup> progenitors (Wnt<sup>-</sup> condition), but was induced in EVTs of Wnt<sup>-</sup> CTB-ORGs (Figures 6B and 6C). Hence, expression and localization of  $\beta$ -catenin and TCFs in Wnt<sup>+</sup> and Wnt<sup>-</sup> CTB-ORGs mimic the *in vivo* expression pattern in the distinct trophoblast subtypes.

### Inhibition of Wnt Response Blocks Extravillous Trophoblast Formation in Human Placental Organoids

To further assess the putative dual role of Wnt signaling in CTB-ORGs, cultures were treated with inhibitor of Wnt response 1 (IWR-1), 2 days after withdrawal of the Wnt stimulators (Figure 7). The chemical inhibitor IWR-1 was shown to specifically increase Axin protein levels, thereby enhancing  $\beta$ -catenin phosphorylation and degradation (Chen et al., 2009). As expected, incubation with IWR-1 did not affect growth of Wnt<sup>+</sup> CTB-ORGs, since elevated Axin levels cannot abolish CHIR99021-mediated inhibition of GSK-3 $\beta$  and, as a consequence,  $\beta$ -catenin stabilization (Figure 7A). However, the Wnt<sup>-</sup> condition provoked trophoblast outgrowth, as shown above (Figures 5 and S5), and subsequent incubation with IWR-1 prevented EVT formation. Notably, NOTCH1<sup>+</sup> progenitors accumulated upon supplementation of Wnt<sup>-</sup> organoids with IWR-1, whereas only few HLA-G<sup>+</sup> EVTs could be detected

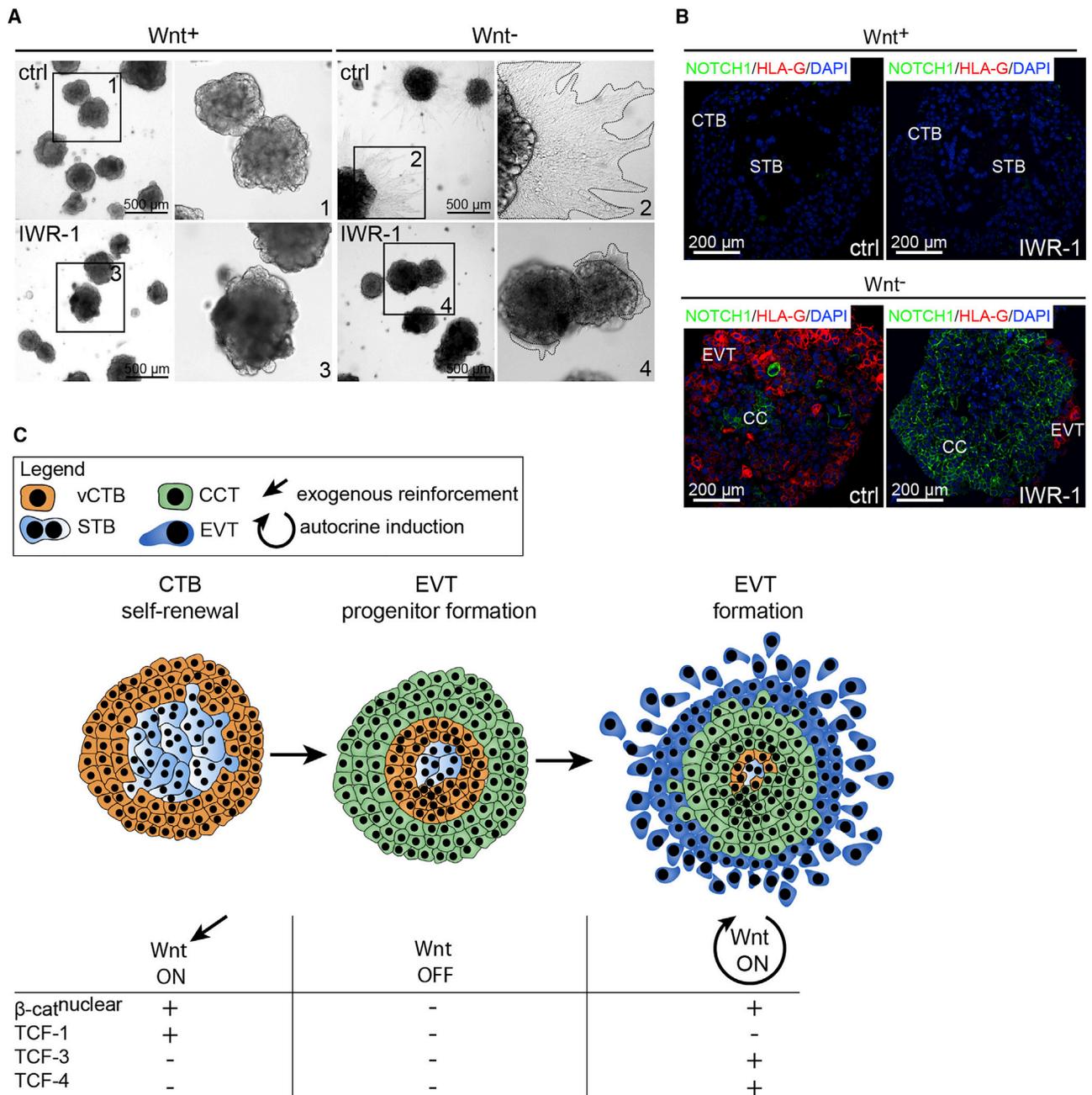
(Figure 7B). In summary, exogenous stimulation of Wnt signaling was necessary for self-renewal of CTB-ORGs, whereas removal of the Wnt activators promoted EVT progenitor development (Figure 7C). Upon differentiation of the latter endogenous Wnt signaling could be activated, promoting EVT formation in an autocrine fashion, as has been demonstrated in 2D cultures (Meinhardt et al., 2014; Pollheimer et al., 2006).

## DISCUSSION

Development of the human placenta and its different trophoblast subtypes remains poorly understood. Ethical constraints to obtain placental tissue from early pregnancy, as well as the lack of appropriate culture conditions, promoting trophoblast self-renewal, strongly hindered scientific progress. Therefore, localization of human trophoblast stem and progenitor cells in early placental tissues, and their specific features, are largely unknown (Chang and Parast, 2017; Gamage et al., 2016; Roberts and Fisher, 2011). Previously, human TSC-like line and trophoblast progenitors have been derived from single embryonic blastomeres of embryos and the chorionic mesenchyme, respectively, using 10% fetal bovine serum, fibroblast growth factor 2 (FGF2), and inhibition of TGF- $\beta$ /Activin/Nodal signaling (Genbacev et al., 2011; Zdravkovic et al., 2015). These data suggested that the TSC niche could be specified before blastocyst formation and might reside outside the placental epithelium at later stages of pregnancy. Supplementation of the above-mentioned ingredients was based on mouse studies showing that stimulation of FGF4 signaling promotes TSC growth, whereas inhibition of the TGF- $\beta$  pathway decreased expression of the pluripotency markers OCT4 and NANOG in hESCs and induced trophoblast differentiation (Tanaka et al., 1998; Wu et al., 2008). However, in contrast to mice, FGF receptors are absent from human blastocysts (Kunath et al., 2014), suggesting that other developmental signaling pathways could be necessary for maintenance of human TSCs. Indeed, recent data suggested that activation of EGF and Wnt as well as inhibition of histone deacetylases, Rho-associated kinase, and TGF- $\beta$  signaling was sufficient for long-term 2D self-renewal of TSCs from human blastocysts (Okae et al., 2018). These cells could also be derived from primary CTB preparations, providing evidence for localization of the TSC niche in the placental epithelium (Okae et al., 2018). The present study supports this view.

(C) qPCR analyses showing *TCF7* (encoding TCF-1), *TCF7L1* (encoding TCF-3), and *TCF7L2* (encoding TCF-4) mRNA expression (normalized to *TBP*) in different Wnt<sup>+</sup> and Wnt<sup>-</sup> CTB-ORGs (n = 5, P2–P4). Mean values  $\pm$  SD, measured in duplicates, are shown \*p < 0.05; AU, arbitrary units.

See also Figure S7.



**Figure 7. Inhibition of Wnt Response Prevents EVT Formation**

(A) Representative phase contrast images of different CTB-ORG cultures (n = 4, P2–P4) grown for 4 days under Wnt<sup>+</sup> or Wnt<sup>-</sup> conditions in the absence (ctrl) or presence of inhibitor of Wnt response 1 (IWR-1). The latter was supplemented 48 hr after removal of R-spondin and CHIR99021 for an additional 2 days. Inset pictures 1–4 (boxed areas) are depicted at a higher magnification. Trophoblast outgrowths are marked with stippled lines.

(B) Immunofluorescence images of representative Wnt<sup>+</sup> or Wnt<sup>-</sup> CTB-ORGs (n = 4, P2–P4) treated with IWR-1 as mentioned above. Sections were co-stained with DAPI to visualize nuclei.

(C) Model system showing the role of Wnt in trophoblast organoid self-renewal and differentiation.  $\beta$ -cat,  $\beta$ -catenin; CTB, cytotrophoblast; EVT, extravillous trophoblast; STB, syncytiotrophoblasts; TCF, T cell factor.



Using a defined cocktail of growth factors and inhibitors, shown to promote organoid growth of various types of adult epithelial stem cells (Dutta et al., 2017; Kretzschmar and Clevers, 2016), we derived long-term expanding CTB-ORGs from sixth- to seventh-week placentae. Notably, organoids could not be established from tissues of the 10th to 12th weeks, suggesting that expandable TSCs might be absent at later stages of pregnancy. The proliferative 3D structures of early placenta express markers of human CTB stemness in the outer epithelial cell layers and spontaneously undergo cell fusion toward the center, thereby generating functionally active, CG $\beta$ -secreting STBs. Genome-wide mRNA expression profiling revealed great similarities of CTB-ORGs to freshly isolated vCTBs as well as to STBs generated by 2D *in vitro* differentiation. Proliferation progressively declined from outside to inside of CTB-ORGs. Hence, we speculate that, similar to the 2D primary cultures, the absence of proliferation in the inner part of CTB-ORGs could promote cell fusion.

Based on previously established criteria for human trophoblast origin (Hemberger et al., 2010; Lee et al., 2016; Li et al., 2014), CTB-ORGs were shown to express marker genes for CTB stemness and identity. Besides TP63, promoting vCTB proliferation (Li et al., 2014), ELF5 was continuously expressed upon passaging of CTB-ORGs. The *ELF5* gene promoter was found to be hypomethylated in CTB-ORGs, another indication for their trophoblast origin (Hemberger et al., 2010; Lee et al., 2016). AP-2 $\alpha$ , AP-2 $\gamma$ , and GATA3, restricted to trophoblasts in first-trimester placenta (Biadasiewicz et al., 2011; Paul et al., 2017), were also present in CTB-ORGs. GATA3 is also expressed in STBs of placental tissues (Paul et al., 2017), providing an explanation for the increasing GATA3 mRNA levels at long-term passaging of CTB-ORGs, which accompanies the rising CG $\beta$  levels in these cultures. TEAD4, an activator of Cdx2 expression and trophoctoderm (TE) development in mice (Nishioka et al., 2009; Yagi et al., 2007), was also detected in the majority of CTB nuclei of CTB-ORGs.

The key regulator of murine trophoblast development, Cdx2 (Strumpf et al., 2005), was shown to be weakly produced by a subset of human TE cells (Deglincerti et al., 2016). In early placenta it is absent from cell columns and appears in clusters of vCTBs, particularly in regions closer to the chorionic plate (Haider et al., 2016; Horii et al., 2016; Soncin et al., 2018). Herein, CDX2 was only detected in a small number of CTBs of organoids, and its mRNA was weakly expressed, as in the previously established human TSCs (Okoe et al., 2018). The heterogeneous distribution of the factor in the placenta could explain the low yield of CDX2<sup>+</sup> cells in CTB isolations, likely containing higher amounts of distal villi lacking CDX2 (Haider et al., 2016). Notably, its mRNA levels tended to decrease

at high passage numbers of CTB-ORGs, suggesting that the low-abundant CDX2<sup>+</sup> cell population eventually cannot be maintained long-term. CDX2<sup>+</sup> cells could represent another trophoblast subtype, for example residual TE stem cells, which require different conditions for continuous cell proliferation. However, cyclin A expression also declined at high passage numbers. Hence, a decline of transcription factors, associated with trophoblast stemness, by trend might also be explained by the diminishing proliferative capacity of CTB-ORGs. Loss of expandability of CTB-ORGs after P13 might indicate that the culture conditions for long-term self-renewal could be further improved. On the other hand, CTB-ORGs could not be established from placental tissues of the late first trimester. Hence, TSCs might have a limited lifespan *in vivo*, which could account for the loss of self-renewal upon long-term passaging of CTB-ORGs. Indeed, organoids derived from several other tissues also cease proliferation at higher passage numbers (Lee et al., 2018; Loomans et al., 2018). Nevertheless, our molecular analyses provide evidence for a self-renewing, Wnt-dependent vCTB precursor population in CTB-ORGs. These cells have the capacity to fuse, but also to form NOTCH1<sup>+</sup> EVT progenitors upon loss of Wnt stimulation, and could therefore be regarded as bipotential CTB stem cells.

Wnt signaling in adult stem cells and organoids controls self-renewal, lineage specification and differentiation (Frank et al., 2016; Kretzschmar and Clevers, 2017). Previous data suggested that it also plays a pivotal role in human placental development (Knöfler and Pollheimer, 2013; Sonderegger et al., 2010). Here, we show that the exogenous stimulation of Wnt signaling maintained growth of CTB-ORGs, coinciding with the expression of LGR5, nuclear  $\beta$ -catenin, and TCF-1. The latter, expressed in a subset of vCTBs, was shown to control self-renewal of different stem cell types (Huls et al., 2013; Yi et al., 2011). Since TCF-3 and -4 proteins were undetectable in proliferative CTBs of tissues and organoids, we speculate that TSC proliferation could be mainly triggered by nuclear  $\beta$ -catenin-TCF-1 complexes. Notably, self-renewal in CTB-ORGs was not maintained in the absence of the Wnt stimulators, suggesting that the endogenous, autocrine Wnt loops are not sufficient for long-term CTB proliferation, despite the expression of different Wnt ligands in vCTBs (Sonderegger et al., 2007). Withdrawal of R-spondin and CHIR99021 promoted a transient increase in cyclin A expression, trophoblast outgrowth, and development of proliferative NOTCH1<sup>+</sup> progenitors. The latter likely develop into HLA-G<sup>+</sup> EVTs, as has been recently shown in 2D cultures (Haider et al., 2016). At later stages the overall proliferative capacity and size of Wnt<sup>-</sup> CTB-ORGs organoid decreased, suggesting that TSCs could not be maintained under these conditions. Noteworthy, NOTCH1<sup>+</sup> progenitors of Wnt<sup>-</sup>



CTB-ORGs and cell columns *in vivo* lack nuclear  $\beta$ -catenin as well as expression of TCF-1, -3, and -4, indicating that canonical Wnt signaling could be dispensable for their growth. In contrast, all EVT nuclei *in situ* express TCF-3 and -4, as well as nuclear  $\beta$ -catenin in approximately 8% of cells (Pollheimer et al., 2006). During 3D differentiation of CTB-ORGs, some of the EVT nuclei accumulated nuclear  $\beta$ -catenin, whereas its membranous staining decreased. Developing EVTs of Wnt<sup>-</sup> organoids also induced nuclear TCF-3 and -4 in an autocrine fashion, as also previously noticed in primary CTBs differentiating on a 2D matrix (Meinhardt et al., 2014; Pollheimer et al., 2006). Since TCF-1 is absent from EVTs and TCF-3 is only weakly expressed, induction of TCF- $\beta$ -catenin activity could be mostly achieved by the increase of nuclear TCF-4 expression, accompanying the EMT-like process of these cells (Meinhardt et al., 2014). Along those lines, NOTCH1<sup>+</sup> progenitors accumulated, whereas EVTs poorly formed when CTB-ORGs were treated with IWR-1, 2 days after removal of R-spondin and CHIR99021. Thus, the data suggest that exogenous reinforcement of Wnt signaling could promote long-term expansion of TSCs in CTB-ORGs, whereas autocrine activation of the pathway likely controls EVT formation and the specific features of these cells, such as invasion and migration (Meinhardt et al., 2014; Pollheimer et al., 2006).

In conclusion, the present study shows that EGF signaling, inhibition of TGF- $\beta$ , and reinforcement of the Wnt pathway is required for long-term expansion of CTB-ORGs. Removal of Wnt stimulators decreases vCTB self-renewal and induces development of proliferative EVT progenitors lacking nuclear  $\beta$ -catenin and TCFs. The NOTCH1<sup>+</sup> progenitors could give rise to adjacent EVTs, reactivating canonical Wnt- $\beta$ -catenin-TCF activity in an autocrine fashion. Hence, CTB-ORGs develop in a 3D arrangement, mimicking the sequential molecular steps of *in vivo* cell column formation and differentiation. Follow-up studies will be necessary to further optimize culture conditions for trophoblast self-renewal, specification, and differentiation in CTB-ORGs.

## EXPERIMENTAL PROCEDURES

### Tissue Collection

First-trimester placentae (sixth to seventh week of gestation) were obtained from elective pregnancy terminations. Utilization of tissues and all Experimental Procedures were approved by the Medical University of Vienna ethics boards (no. 084/2013) and required written informed consent.

### Trophoblast Organoid Formation and Cultivation

Villous CTBs were purified from 6th- to 7th-week placentae as mentioned in Supplemental Experimental Procedures. Cells were

re-suspended in ice-cold 0.5 mL basic trophoblast organoid medium (b-TOM) containing advanced DMEM/F12 (Invitrogen), 10 mM HEPES, 1 $\times$  B27 (Gibco), 1 $\times$  N2 (Gibco), and 2 mM glutamine (Gibco), centrifuged for 3 min at 1,000 rpm, and re-suspended in ice-cold advanced trophoblast organoid medium (a-TOM) consisting of b-TOM supplemented with 100 ng/mL R-spondin (PeproTech), 1  $\mu$ M A83-01 (R&D Systems), 100 ng/mL recombinant human epidermal growth factor (rhEGF, R&D Systems), 50 ng/mL recombinant murine hepatocyte growth factor (rmHGF, PeproTech), 2.5  $\mu$ M prostaglandin E2 (R&D Systems), 3  $\mu$ M CHIR99021 (Tocris), and 100 ng/mL Noggin (R&D Systems). EGF, Wnt stimulators (R-spondin, CHIR99021, and prostaglandin E2) and inhibition of TGF- $\beta$  signaling (A83-01) were critical for maintenance of organoids. Removal of Noggin or HGF, respectively, showed marginal effects on organoid growth, but were left in the cultures to eventually limit differentiation (Li and Parast, 2014) and promote trophoblast survival (Dash et al., 2005). Growth factor-reduced Matrigel (GFR-M, Corning) was added to reach a final concentration of 60%. Solution (40  $\mu$ L) containing 10<sup>5</sup> vCTBs was placed in the center of 24-well plates. After 2 min at 37°C, the plates were turned upside down to ensure equal spreading of the cells in the solidifying GFR-M-forming domes. After 15 min, the plates were turned again and the domes were carefully overlaid with 500  $\mu$ L prewarmed a-TOM. Organoids were allowed to form for 4–6 days at P0. Bright-field and phase contrast images of organoid cultures were taken using EVOS FL Cell Imaging System microscope (Life Technologies). To generate videos of growing organoids, bright-field images were taken every 90 min with the Lionheart automated live cell imager system (BioTek) and processed using Gen5 software (BioTek). Organoids could be formed from all placentae when using tissue of the sixth to seventh week of gestation. For additional description of methods see Supplemental Experimental Procedures.

## ACCESSION NUMBERS

RNA-seq data reported in this manuscript are accessible at GEO: GSE109976.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one video and can be found with this article online at <https://doi.org/10.1016/j.stemcr.2018.07.004>.

## AUTHOR CONTRIBUTIONS

S.H., P.L., and M.K. designed the research. S.H., G.M., V.K., M.G., and J.P. conducted the experiments. L.S. performed statistical analyses. U.K. and A.E. performed electron microscopy. S.M. provided organoid expertise. T.R.B. conducted bioinformatic analyses. J.P. designed graphics. C.F. contributed new reagents and placental material. P.L. and M.K. wrote the manuscript.

## ACKNOWLEDGMENTS

This study was supported by the Austrian Science Fund (grant P-28417-B30 to M.K. and grant P-30941-B30 to P.L.).



Received: May 4, 2018  
Revised: July 9, 2018  
Accepted: July 9, 2018  
Published: August 2, 2018

## REFERENCES

- Aplin, J.D. (2010). Developmental cell biology of human villous trophoblast: current research problems. *Int. J. Dev. Biol.* *54*, 323–329.
- Apps, R., Murphy, S.P., Fernando, R., Gardner, L., Ahad, T., and Moffett, A. (2009). Human leucocyte antigen (HLA) expression of primary trophoblast cells and placental cell lines, determined using single antigen beads to characterize allotype specificities of anti-HLA antibodies. *Immunology* *127*, 26–39.
- Baczyk, D., Dunk, C., Huppertz, B., Maxwell, C., Reister, F., Gianouliias, D., and Kingdom, J.C. (2006). Bi-potential behaviour of cytotrophoblasts in first trimester chorionic villi. *Placenta* *27*, 367–374.
- Biadasiewicz, K., Sonderegger, S., Haslinger, P., Haider, S., Saleh, L., Fiala, C., Pollheimer, J., and Knöfler, M. (2011). Transcription factor AP-2alpha promotes EGF-dependent invasion of human trophoblast. *Endocrinology* *152*, 1458–1469.
- Bilban, M., Tauber, S., Haslinger, P., Pollheimer, J., Saleh, L., Pehamberger, H., Wagner, O., and Knöfler, M. (2010). Trophoblast invasion: assessment of cellular models using gene expression signatures. *Placenta* *31*, 989–996.
- Boretto, M., Cox, B., Noben, M., Hendriks, N., Fassbender, A., Roose, H., Amant, F., Timmerman, D., Tomassetti, C., Vanhie, A., et al. (2017). Development of organoids from mouse and human endometrium showing endometrial epithelium physiology and long-term expandability. *Development* *144*, 1775–1786.
- Burton, G.J., Jauniaux, E., and Charnock-Jones, D.S. (2010). The influence of the intrauterine environment on human placental development. *Int. J. Dev. Biol.* *54*, 303–312.
- Chang, C.W., and Parast, M.M. (2017). Human trophoblast stem cells: real or not real? *Placenta* *60 (Suppl 1)*, S57–S60.
- Chen, B., Dodge, M.E., Tang, W., Lu, J., Ma, Z., Fan, C.W., Wei, S., Hao, W., Kilgore, J., Williams, N.S., et al. (2009). Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nat. Chem. Biol.* *5*, 100–107.
- Dash, P.R., Whitley, G.S., Ayling, L.J., Johnstone, A.P., and Cartwright, J.E. (2005). Trophoblast apoptosis is inhibited by hepatocyte growth factor through the Akt and beta-catenin mediated up-regulation of inducible nitric oxide synthase. *Cell Signal* *17*, 571–580.
- Deglincerti, A., Croft, G.F., Pietila, L.N., Zernicka-Goetz, M., Siggia, E.D., and Brivanlou, A.H. (2016). Self-organization of the in vitro attached human embryo. *Nature* *533*, 251–254.
- Dutta, D., Heo, I., and Clevers, H. (2017). Disease modeling in stem cell-derived 3D organoid systems. *Trends Mol. Med.* *23*, 393–410.
- Evain-Brion, D., and Malassine, A. (2003). Human placenta as an endocrine organ. *Growth Horm. IGF Res.* *13 (Suppl A)*, S34–S37.
- Fock, V., Plessl, K., Draxler, P., Otti, G.R., Fiala, C., Knöfler, M., and Pollheimer, J. (2015). Neuregulin-1-mediated ErbB2-ErbB3 signaling protects human trophoblasts against apoptosis to preserve differentiation. *J. Cell Sci.* *128*, 4306–4316.
- Frank, D.B., Peng, T., Zepp, J.A., Snitow, M., Vincent, T.L., Penkala, I.J., Cui, Z., Herriges, M.J., Morley, M.P., Zhou, S., et al. (2016). Emergence of a wave of Wnt signaling that regulates lung alveologenesis by controlling epithelial self-renewal and differentiation. *Cell Rep.* *17*, 2312–2325.
- Gamage, T.K., Chamley, L.W., and James, J.L. (2016). Stem cell insights into human trophoblast lineage differentiation. *Hum. Reprod. Update* *23*, 77–103.
- Genbacev, O., Donne, M., Kapidzic, M., Gormley, M., Lamb, J., Gilmore, J., Larocque, N., Goldfien, G., Zdravkovic, T., McMaster, M.T., et al. (2011). Establishment of human trophoblast progenitor cell lines from the chorion. *Stem Cells* *29*, 1427–1436.
- Haider, S., Meinhardt, G., Saleh, L., Fiala, C., Pollheimer, J., and Knöfler, M. (2016). Notch1 controls development of the extravillous trophoblast lineage in the human placenta. *Proc. Natl. Acad. Sci. USA* *113*, E7710–E7719.
- Hemberger, M., Udayashankar, R., Tesar, P., Moore, H., and Burton, G.J. (2010). ELF5-enforced transcriptional networks define an epigenetically regulated trophoblast stem cell compartment in the human placenta. *Hum. Mol. Genet.* *19*, 2456–2467.
- Horii, M., Li, Y., Wakeland, A.K., Pizzo, D.P., Nelson, K.K., Sabatini, K., Laurent, L.C., Liu, Y., and Parast, M.M. (2016). Human pluripotent stem cells as a model of trophoblast differentiation in both normal development and disease. *Proc. Natl. Acad. Sci. USA* *113*, E3882–E3891.
- Huls, G., van Es, J., Clevers, H., de Haan, G., and van Os, R. (2013). Loss of Tcf7 diminishes hematopoietic stem/progenitor cell function. *Leukemia* *27*, 1613–1614.
- Hustin, J., Jauniaux, E., and Schaaps, J.P. (1990). Histological study of the materno-embryonic interface in spontaneous abortion. *Placenta* *11*, 477–486.
- Jain, A., Ezashi, T., Roberts, R.M., and Tuteja, G. (2017). Deciphering transcriptional regulation in human embryonic stem cells specified towards a trophoblast fate. *Sci. Rep.* *7*, 17257.
- James, J.L., Stone, P.R., and Chamley, L.W. (2007). The isolation and characterization of a population of extravillous trophoblast progenitors from first trimester human placenta. *Hum. Reprod.* *22*, 2111–2119.
- Kessler, M., Hoffmann, K., Brinkmann, V., Thieck, O., Jackisch, S., Toelle, B., Berger, H., Mollenkopf, H.J., Mangler, M., Sehoul, J., et al. (2015). The Notch and Wnt pathways regulate stemness and differentiation in human fallopian tube organoids. *Nat. Commun.* *6*, 8989.
- Khong, T.Y., Liddell, H.S., and Robertson, W.B. (1987). Defective haemochorial placentation as a cause of miscarriage: a preliminary study. *Br. J. Obstet. Gynaecol.* *94*, 649–655.
- Knöfler, M., and Pollheimer, J. (2013). Human placental trophoblast invasion and differentiation: a particular focus on Wnt signaling. *Front. Genet.* *4*, 190.
- Kovats, S., Main, E.K., Librach, C., Stubblebine, M., Fisher, S.J., and DeMars, R. (1990). A class I antigen, HLA-G, expressed in human trophoblasts. *Science* *248*, 220–223.



- Kretzschmar, K., and Clevers, H. (2016). Organoids: modeling development and the stem cell niche in a dish. *Dev. Cell* 38, 590–600.
- Kretzschmar, K., and Clevers, H. (2017). Wnt/beta-catenin signaling in adult mammalian epithelial stem cells. *Dev. Biol.* 428, 273–282.
- Kunath, T., Yamanaka, Y., Detmar, J., MacPhee, D., Caniggia, I., Rossant, J., and Jurisicova, A. (2014). Developmental differences in the expression of FGF receptors between human and mouse embryos. *Placenta* 35, 1079–1088.
- Lee, C.Q., Gardner, L., Turco, M., Zhao, N., Murray, M.J., Coleman, N., Rossant, J., Hemberger, M., and Moffett, A. (2016). What is trophoblast? A combination of criteria define human first-trimester trophoblast. *Stem Cell Reports* 6, 257–272.
- Lee, J., Bscke, R., Tang, P.C., Hartman, B.H., Heller, S., and Koehler, K.R. (2018). Hair follicle development in mouse pluripotent stem cell-derived skin organoids. *Cell Rep.* 22, 242–254.
- Li, Y., Moretto-Zita, M., Leon-Garcia, S., and Parast, M.M. (2014). p63 inhibits extravillous trophoblast migration and maintains cells in a cytotrophoblast stem cell-like state. *Am. J. Pathol.* 184, 3332–3343.
- Li, Y., and Parast, M.M. (2014). BMP4 regulation of human trophoblast development. *Int. J. Dev. Biol.* 58, 239–246.
- Lim, K.H., Zhou, Y., Janatpour, M., McMaster, M., Bass, K., Chun, S.H., and Fisher, S.J. (1997). Human cytotrophoblast differentiation/invasion is abnormal in pre-eclampsia. *Am. J. Pathol.* 151, 1809–1818.
- Loomans, C.J.M., Williams Giuliani, N., Balak, J., Ringnalda, F., van Gurp, L., Huch, M., Boj, S.F., Sato, T., Kester, L., de Sousa Lopes, S.M.C., et al. (2018). Expansion of adult human pancreatic tissue yields organoids harboring progenitor cells with endocrine differentiation potential. *Stem Cell Reports* 10, 712–724.
- Meinhardt, G., Haider, S., Haslinger, P., Proestling, K., Fiala, C., Pollheimer, J., and Knöfler, M. (2014). Wnt-dependent T-cell factor-4 controls human extravillous trophoblast motility. *Endocrinology* 155, 1908–1920.
- Nishioka, N., Inoue, K., Adachi, K., Kiyonari, H., Ota, M., Ralston, A., Yabuta, N., Hirahara, S., Stephenson, R.O., Ogonuki, N., et al. (2009). The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. *Dev. Cell* 16, 398–410.
- Okabe, H., Toh, H., Sato, T., Hiura, H., Takahashi, S., Shirane, K., Kabayama, Y., Suyama, M., Sasaki, H., and Arima, T. (2018). Derivation of human trophoblast stem cells. *Cell Stem Cell* 22, 50–63.e6.
- Paul, S., Home, P., Bhattacharya, B., and Ray, S. (2017). GATA factors: master regulators of gene expression in trophoblast progenitors. *Placenta* 60 (Suppl 1), S61–S66.
- Pijnenborg, R., Anthony, J., Davey, D.A., Rees, A., Tiltman, A., Vercruyse, L., and van Assche, A. (1991). Placental bed spiral arteries in the hypertensive disorders of pregnancy. *Br. J. Obstet. Gynaecol.* 98, 648–655.
- Pijnenborg, R., Dixon, G., Robertson, W.B., and Brosens, I. (1980). Trophoblastic invasion of human decidua from 8 to 18 weeks of pregnancy. *Placenta* 1, 3–19.
- Pijnenborg, R., Vercruyse, L., and Hanssens, M. (2006). The uterine spiral arteries in human pregnancy: facts and controversies. *Placenta* 27, 939–958.
- Plessl, K., Haider, S., Fiala, C., Pollheimer, J., and Knöfler, M. (2015). Expression pattern and function of Notch2 in different subtypes of first trimester cytotrophoblast. *Placenta* 36, 365–371.
- Pollheimer, J., Loregger, T., Sonderegger, S., Saleh, L., Bauer, S., Bilban, M., Czerwenka, K., Husslein, P., and Knöfler, M. (2006). Activation of the canonical wntless/T-cell factor signaling pathway promotes invasive differentiation of human trophoblast. *Am. J. Pathol.* 168, 1134–1147.
- Roberts, R.M., and Fisher, S.J. (2011). Trophoblast stem cells. *Biol. Reprod.* 84, 412–421.
- Romero, R., Kusanovic, J.P., Chaiworapongsa, T., and Hassan, S.S. (2011). Placental bed disorders in preterm labor, preterm PROM, spontaneous abortion and abruptio placentae. *Best Pract. Res. Clin. Obstet. Gynaecol.* 25, 313–327.
- Sheppard, B.L., and Bonnar, J. (1976). The ultrastructure of the arterial supply of the human placenta in pregnancy complicated by fetal growth retardation. *Br. J. Obstet. Gynaecol.* 83, 948–959.
- Soncin, F., Khater, M., To, C., Pizzo, D., Farah, O., Wakeland, A., Rajan, K.A.N., Nelson, K.K., Chang, C.W., Moretto-Zita, M., et al. (2018). Comparative analysis of mouse and human placentae across gestation reveals species-specific regulators of placental development. *Development* 145. <https://doi.org/10.1242/dev.156273>.
- Sonderegger, S., Husslein, H., Leisser, C., and Knöfler, M. (2007). Complex expression pattern of Wnt ligands and frizzled receptors in human placenta and its trophoblast subtypes. *Placenta* 28 (Suppl A), S97–S102.
- Sonderegger, S., Pollheimer, J., and Knöfler, M. (2010). Wnt signaling in implantation, decidualisation and placental differentiation—review. *Placenta* 31, 839–847.
- Strumpf, D., Mao, C.A., Yamanaka, Y., Ralston, A., Chawengsakso-phak, K., Beck, F., and Rossant, J. (2005). Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development* 132, 2093–2102.
- Tanaka, S., Kunath, T., Hadjantonakis, A.K., Nagy, A., and Rossant, J. (1998). Promotion of trophoblast stem cell proliferation by FGF4. *Science* 282, 2072–2075.
- Turco, M.Y., Gardner, L., Hughes, J., Cindrova-Davies, T., Gomez, M.J., Farrell, L., Hollinshead, M., Marsh, S.G.E., Brosens, J.J., Critchley, H.O., et al. (2017). Long-term, hormone-responsive organoid cultures of human endometrium in a chemically defined medium. *Nat. Cell Biol.* 19, 568–577.
- Windsperger, K., Dekan, S., Pils, S., Gollet, C., Kunihs, V., Fiala, C., Kristiansen, G., Knöfler, M., and Pollheimer, J. (2017). Extravillous trophoblast invasion of venous as well as lymphatic vessels is altered in idiopathic, recurrent, spontaneous abortions. *Hum. Reprod.* 32, 1208–1217.
- Wu, Z., Zhang, W., Chen, G., Cheng, L., Liao, J., Jia, N., Gao, Y., Dai, H., Yuan, J., Cheng, L., et al. (2008). Combinatorial signals of activin/nodal and bone morphogenic protein regulate the early lineage segregation of human embryonic stem cells. *J. Biol. Chem.* 283, 24991–25002.



Yabe, S., Alexenko, A.P., Amita, M., Yang, Y., Schust, D.J., Sadovsky, Y., Ezashi, T., and Roberts, R.M. (2016). Comparison of syncytiotrophoblast generated from human embryonic stem cells and from term placentas. *Proc. Natl. Acad. Sci. USA* *113*, E2598–E2607.

Yagi, R., Kohn, M.J., Karavanova, I., Kaneko, K.J., Vullhorst, D., De-Pamphilis, M.L., and Buonanno, A. (2007). Transcription factor TEAD4 specifies the trophoblast lineage at the beginning of mammalian development. *Development* *134*, 3827–3836.

Yelavarthi, K.K., Fishback, J.L., and Hunt, J.S. (1991). Analysis of HLA-G mRNA in human placental and extraplacental membrane cells by in situ hybridization. *J. Immunol.* *146*, 2847–2854.

Yi, F., Pereira, L., Hoffman, J.A., Shy, B.R., Yuen, C.M., Liu, D.R., and Merrill, B.J. (2011). Opposing effects of Tcf3 and Tcf1 control Wnt stimulation of embryonic stem cell self-renewal. *Nat. Cell Biol.* *13*, 762–770.

Zdravkovic, T., Nazor, K.L., Larocque, N., Gormley, M., Donne, M., Hunkapillar, N., Giritharan, G., Bernstein, H.S., Wei, G., Hebrok, M., et al. (2015). Human stem cells from single blastomeres reveal pathways of embryonic or trophoblast fate specification. *Development* *142*, 4010–4025.

Zhou, Y., Damsky, C.H., and Fisher, S.J. (1997). Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome? *J. Clin. Invest.* *99*, 2152–2164.