

Comparative analysis of the syncytiotrophoblast in placenta tissue and trophoblast organoids using snRNA sequencing

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Madeline M Keenen, Liheng Yang, Huan Liang, Veronica J Farmer, Rizban E Worota, Rohit Singh, Amy S Gladfelter , Carolyn B Coyne 

Duke University School of Medicine, Department of Cell Biology, Durham, United States • Duke University School of Medicine, Department of Integrative Immunobiology, Durham, United States • Duke University School of Medicine, Department of Biostatistics and Bioinformatics, Durham, United States • Duke Human Vaccine Institute, Durham, United States

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eLife Assessment

This **important** study uses single-cell transcriptomics to analyze syncytiotrophoblasts in two trophoblast organoid models compared to primary placental tissue, providing **compelling** insights into syncytialization and highlighting the utility of organoid models in placental research. It also serves as an invaluable resource for the field.

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Abstract

The outer surface of chorionic villi in the human placenta consists of a single multinucleated cell called the syncytiotrophoblast (STB). The unique cellular ultrastructure of the STB presents challenges in deciphering its gene expression signature at the single-cell level, as the STB contains billions of nuclei in a single cell. There are many gaps in understanding the molecular mechanisms and developmental trajectories involved in STB formation and differentiation. To identify the underlying control of the STB, we performed comparative single nucleus (SN) and single cell (SC) RNA sequencing on placental tissue and tissue-derived trophoblast organoids (TOs). We found that SN RNA sequencing was essential to capture the STB population from both tissue and TOs. Differential gene expression and pseudotime analysis of TO-derived STB identified three distinct nuclear subtypes reminiscent of those recently identified *in vivo*. These included a juvenile nuclear population that exhibited both CTB and STB marker expression, a population enriched in genes involved in oxygen sensing, and finally a subtype enriched in transport and GTPase signaling molecules. Notably, suspension culture conditions of TOs that restore the native orientation of the STB (STB^{out}) showed elevated expression of canonical STB markers and pregnancy hormones, along with a greater proportion of the STB nucleus subtype specializing in transport and GTPase signaling, compared to those cultivated with an inverted STB polarity (STBⁱⁿ). Gene regulatory analysis identified novel markers of STB differentiation conserved in tissue and

TOs, including the chromatin remodeler RYBP, that exhibited STB-specific RNA and protein expression. CRISPR/Cas9 knockout of RYBP in STBⁱⁿ TOs did not impact cell-cell fusion; however, bulk RNA sequencing revealed downregulation of the pregnancy hormone CSH1 and upregulation of multiple genes associated with the oxygen-sensing STB nuclear subtype. Finally, we compared STB gene expression signatures amongst first trimester tissue, full-term tissue, and TOs, identifying many commonalities but also notable variability across each sample type. This indicates that STB gene expression is responsive to its environmental context. Our findings emphasize the utility of TOs to accurately model STB differentiation and the distinct nuclear subtypes observed *in vivo*, offering a versatile platform for unraveling the molecular mechanisms governing STB functions in placental biology and disease.

Introduction

During the course of human gestation, the developing fetus forms an entire external organ to support its growth—the placenta. While the fetal organs undergo development, the placenta assumes a multifaceted role, serving to facilitate molecular exchange, perform essential metabolic functions, produce hormones, prevent loss of immune tolerance, and act as a barrier against the vertical transmission of pathogens (Aye et al., 2022 [↗](#); Benirschke et al., 2012 [↗](#); Costa, 2016 [↗](#); Megli and Coyne, 2021 [↗](#)). The placenta's remarkable functional complexity is underscored by its distinctive cellular architecture. Its outer layer encompasses a giant single cell called the syncytiotrophoblast (STB), that contains billions of nuclei and envelops the chorionic villi (Barker et al., 1973 [↗](#); Burton and Jauniaux, 1995 [↗](#); Haeussner et al., 2014 [↗](#)). The STB is formed via cell-cell fusion of the underlying cytotrophoblast (CTB) cell population. CTBs reside on the basement membrane of chorionic villi to contribute new nuclei into the STB or lie at the interface between the placenta villi and the maternal decidua in multi-cell layered structures termed cell columns (CTB-CC) (**Figure 1A** [↗](#)) (Boyd and Hamilton, 1970 [↗](#)). CTBs closer to the maternal decidua are more differentiated than their counterparts lower in the column and eventually undergo epithelial to mesenchymal transition (EMT) to form fully differentiated extravillous trophoblast cells (EVT) that invade into the decidua (**Figure 1A** [↗](#)) (Arutyunyan et al., 2023 [↗](#); Turco and Moffett, 2019 [↗](#)). The molecular mechanisms and environmental cues that drive CTB differentiation into either STB or EVT lineages is an area of active research and defining these trajectories are essential to understand placenta development and pathogenesis.

Several groups have applied single-cell RNA sequencing (SC) of primary tissue throughout gestation, trophoblast organoids (TOs), and trophoblast stem cells (TSCs) to characterize EVT differentiation (Arutyunyan et al., 2023 [↗](#); Li et al., 2024 [↗](#); Liu et al., 2018 [↗](#); Marsh et al., 2022 [↗](#); Pique-Regi et al., 2019 [↗](#); Shannon et al., 2024 [↗](#); Suryawanshi et al., 2018 [↗](#); Vento-Tormo et al., 2018 [↗](#)). This has generated a lineage map of CTB-to-EVT differentiation with identification of its terminal states and the potential transcription factors involved. However, these datasets contain very few cells from the STB, limiting characterization of the CTB-to-STB differentiation process. This scarcity likely arises because the STB, being a large single cell, is excluded during the single-cell isolation and size filtration steps required for the 10x Genomics microfluidics pipeline. Therefore, approaches like single nucleus RNA sequencing (SN) may be essential to properly capture the heterogeneity of STB gene expression. In fact, a recent study performed SN on placenta tissue (six first trimester and six full-term tissues) and captured STB nuclei and defined their lineage trajectories at each gestational age (Wang et al., 2024 [↗](#)). Their analysis suggests the STB can bifurcate into at least two nuclear lineages post-fusion, associated with either hormone expression and GTPase signaling or with an oxygen response. This implies different functions of the STB may be attributed to distinct individual nuclei within the same giant cell. However, how nuclei with distinct gene expression arise and how they impact the function of the entire STB cell is not known. Dissecting nuclear heterogeneity in the STB will require a molecular biology and genetic toolkit that has been largely inaccessible for human pregnancy models.

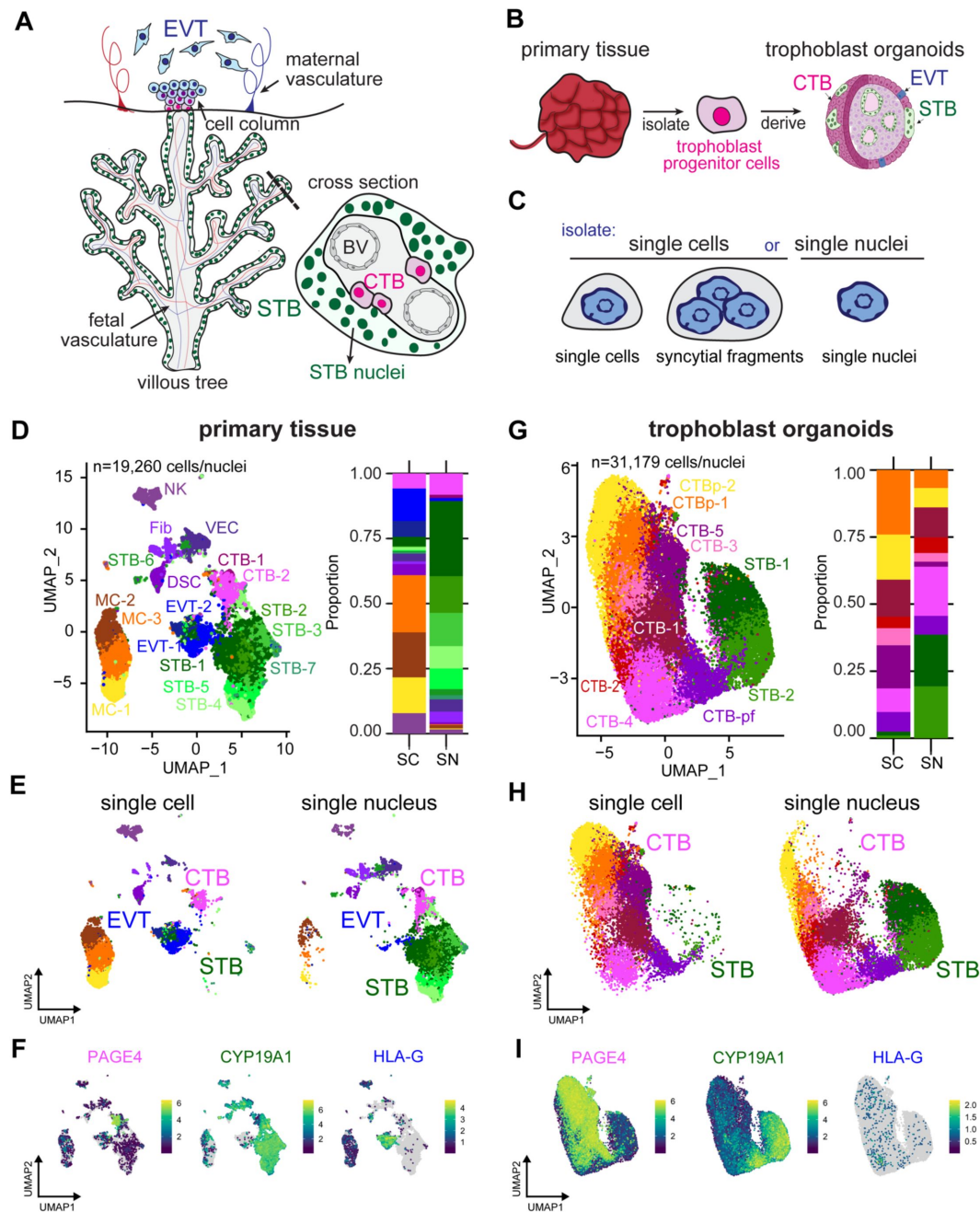


Figure 1

Single nucleus sequencing is essential to capture the STB lineage in full-term tissue and TOs.

A Cartoon of placenta villous tree and cross section, with the multinucleated STB highlighted in green, progenitor CTB cells in magenta, and extravillous cytotrophoblasts in blue. **B** Schematic of organoid generation via isolation of trophoblast progenitor cells from full-term placental tissue. **C** Schematic of experimental setup. Both tissue and TOs were processed into either single cells/syncytial fragments or single nuclei and sequenced. UMAP of integrated SC and SN datasets collected from primary tissue (**D**) and TOs (**G**). Cell/nucleus types are annotated as follows: cytotrophoblast (CTB), cytotrophoblast pre-fusion (CTB-pf), syncytiotrophoblast (STB), extravillous trophoblast (EVT), dendritic stem cell (DSC), vascular endothelial cell (VEC), fibroblasts (Fib), natural killer cell (NK), and macrophage (MC). Cell/nucleus types that contained multiple clusters are identified with -number after the name. **E** and **H** UMAP of the integrated SC and SN dataset separated into individual UMAPs by single cell or nucleus processing type for primary tissue (**E**) and TOs (**H**). **F** and **I** Expression of key trophoblast markers, including PAGE4 (CTB), CYP19A1 (STB), and HLA-G (EVT) in primary tissue (**F**) and TOs (**I**).

A major challenge in establishing genetically tractable and accessible models for the human placenta is the remarkable diversity of placental structures amongst mammals, and notably the variations in the tissue architecture and cell types seen even between humans and mice (Hemberger et al., 2020 [DOI](#); Wooding and Burton, 2008 [DOI](#)). In the last several years, trophoblast organoids (TOs) have emerged as powerful tools for studying trophoblast differentiation. CTB progenitor cells can be isolated from tissue throughout gestation, as CTB remain mitotic throughout pregnancy (Haider et al., 2018 [DOI](#); Mayhew, 2014 [DOI](#); Turco et al., 2019 [DOI](#); Yang et al., 2022 [DOI](#)). They can subsequently be maintained in a proliferative state capable of trophoblast differentiation by using a growth factor cocktail and cultivation in extracellular matrix (Okoe et al., 2018 [DOI](#); Turco et al., 2019 [DOI](#); Yang et al., 2022 [DOI](#)), and spontaneously fuse to form STB (Haider et al., 2018 [DOI](#); Li et al., 2023 [DOI](#); Turco et al., 2019 [DOI](#); Yang et al., 2022 [DOI](#)). In standard culture conditions, TOs exhibit an inverted architecture compared to placental villi *in vivo*, with an outward facing proliferative CTB layer and a largely inward facing STB (STBⁱⁿ) (Haider et al., 2018 [DOI](#); Turco et al., 2019 [DOI](#); Yang et al., 2022 [DOI](#)). Our recent work has established a method to reverse the cellular polarity of TOs to their native orientation, resulting in organoids containing very large (>50 nuclei) STB on the outermost layer and mononuclear CTBs positioned in the center (STB^{out}), and exhibit increased secretion of the STB-associated hormone human chorionic gonadotropin (hCG) (Yang et al., 2023 [DOI](#)). Recapitulating the native orientation of outward facing STB is essential to model key aspects of STB function *in vitro*, such as molecular transport and its role as a barrier to infection and immune cells. However, how changes in TO orientation affect the functional differentiation of STB remains an important and unexplored area of investigation. While the majority of CTBs in TOs differentiate into the STB, a small proportion can spontaneously differentiate into HLA- G⁺ EVT. This EVT percentage can be increased through a three-step treatment involving Neuregulin-1 (NRG1) (EVT^{enrich}) (Haider et al., 2018 [DOI](#); Turco et al., 2019 [DOI](#); Yang et al., 2022 [DOI](#)). Consequently, TOs offer the ability to induce differentiation along both STB and EVT lineages and have rapidly become a powerful and accessible tool to investigate trophoblast biology.

As TOs become increasingly prevalent as a research model, it is crucial to assess their resemblance to *in vivo* trophoblast cell types. Further, the gene expression signature of the STB and any heterogeneity that exists amongst nuclei remains enigmatic due to its unique multinucleated ultrastructure, both *in vitro* and *in vivo*. In this study, we performed comparative SC and SN on primary full-term placenta tissue and TOs. We found that SN is essential to capture STB gene expression both in tissue and TOs, while SC enriches for mitotic cells, maternal immune cells, and EVT cells in tissue. Differential gene expression and pseudotime analysis of distinct STB nuclei in TOs identified three distinct subtypes reminiscent of those recently identified *in vivo*: a juvenile population that exhibits both CTB and STB expression, an FLT1-expressing population enriched in genes involved in oxygen sensing, and a subtype enriched in expression of molecular transport and GTPase signaling molecules. While STBⁱⁿ and STB^{out} conditions maintain a similar proportion of CTB cell nuclei, STB^{out} TOs exhibited a higher proportion of the transport and GTPase STB-3 nuclear subtype while STBⁱⁿ exhibited a higher proportion of the oxygen sensing STB-2 subtype. Pseudotime and gene regulatory network analysis of RNA velocity identified genes linked to STB differentiation, including the chromatin effector RYBP which is enriched in STB^{out} TOs. To validate this analysis, we utilized CRISPR/Cas9 to knock-out RYBP in TOs. We found that deletion of RYBP in STBⁱⁿ TOs did not affect cell-cell fusion or STB formation, but bulk RNA sequencing demonstrated that RYBP KO resulted in a significant decrease in the expression of the pregnancy hormone CSH1 and an increase in expression of key genes that define the oxygen sensing STB-2 nuclear subtype. Finally, STB gene expression was compared between TOs and primary tissue at different stages of gestation. The CTBs were remarkably similar across all conditions, indicating that CTBs isolated from term placenta that are used for TO generation are comparable to those isolated at earlier stages of pregnancy. The STB displayed both commonalities and notable variability across the sample types. Together, this work demonstrates the capacity of TOs to mirror STB differentiation

and the nuclear subtypes seen *in vivo*, providing an accessible platform to dissect the key molecular pathways underlying placenta function, distinct STB subtypes, and trophoblast-related pregnancy disorders.

Results

Single nucleus sequencing is necessary to capture the gene expression signature of STBs in placental tissue and TOs

In this study, we set out to compare the transcriptional profile of full-term placental tissue composed of placental villi and decidua (**Figure 1A**, **1B**) to STBⁱⁿ TOs previously derived from placental tissue (**Figure 1B**). To assess whether SC or SN sequencing could effectively capture trophoblast cell populations, we processed matched samples into single cells/syncytial fragments or single nuclei (**Figure 1C**, **Materials and Methods**). The SC and SN datasets generated from primary placental tissue (**Figure 1D-F**) and TOs (**Figure 1G-I**) were integrated, and a graph-based clustering approach used to identify clusters (Butler et al., 2018; Hao et al., 2021; Satija et al., 2015; Stuart et al., 2019). Each dataset was visualized with a UMAP plot (**Figure 1D** and **1G**) and established gene expression markers were used to determine the cell or nuclear identity of each cluster (**Figure 1F**, **1I**, **Supplementary Figure 1.1C and 1.1F**, and **Supplemental Table 1-2**) (Arutyunyan et al., 2023; Derisoud et al., 2024; Vento-Tormo et al., 2018).

To determine how each sequencing technique affects the detection of trophoblast cell types, we first defined the cell/nucleus types in the primary tissue dataset. We identified two CTB clusters, seven STB clusters, and two EVT clusters, each expressing their respective markers (e.g., PAGE4, CYP19A1, and HLA-G) (**Figure 1D**, **1F**, **Supplemental Figure 1.1C**). The clusters for each cell type are labeled with a randomly ordered number. There was a similar distribution of these subtypes across all three donor tissues (**Supplemental Figure 1.1A-B**). As in previous single cell approaches, our SC tissue dataset captured only a small fraction of the STB cell type (6% of total cells are STB). In contrast, the predominant population in the SN preparation was STB nuclei, accounting for 76% of the total nuclei count, (**Figure 1D**–**1E** and **Supplemental Figure 1.1A-B**) and consistent with their relative prevalence *in vivo* (Mayhew, 2014; Mayhew and Simpson, 1994; Simpson et al., 1992). Specifically, the SN dataset on primary tissue had an 8:1 ratio of STB to CTB, resembling stereological estimates of placental trophoblast compositions at full-term (9:1) indicating that enzymatic digestion of nuclei is capturing a representative population of these trophoblast nucleus types. (**Figure 1D**). While the STB subtype is better captured by SN sequencing, SC sequencing exhibited significant enrichment in both EVT (18% of total in SC vs. 1.3% in SN) and macrophages (53% of total in SC vs. 1.9% in SN) (**Figure 1D**–**1E** and **Supplemental Figure 1.1A-B**). As a control for SC/SN dataset integration, cell/nucleus types were identified within the individual SC and SN datasets and proportions mirrored the integrated SC/SN dataset (**Supplemental Figure 1.2 and 1.3**). Thus differences between tissue processing for either SC or SN impact the cell types that can be recovered and subsequently sequenced and SN is essential for the analysis of the STB.

We next defined the nucleus types represented in the STBⁱⁿ TO dataset, in which we identified two proliferating CTB clusters (CTB-p), five non-proliferative CTB clusters (CTB-1-5), one pre-fusion CTB cluster with high expression of endogenous retroviral fusion genes (CTB-pf), and two STB clusters (STB 1-2) (**Figure 1G**–**1I** and **Supplemental Figure 1.1F**). The clusters for each cell type are labeled with a randomly assigned number and color, and therefore cluster numbers and color for each cell type cannot be directly compared between the tissue and TO individual datasets until they are integrated in **Figure 6**. Like placenta tissue, the SC dataset captured only a small number of the total STB present in TOs, with only 2.4% of the total cell population attributed to the STB (**Figure 1G** and **Supplemental Figure 1.1E**). In contrast, the STB accounted for 38% of the

total nuclear numbers captured by SN sequencing (**Figure 1G** and **Supplemental Figure 1.1E**). The differences in non-proliferative CTB populations (CTB 1-5) between SC and SN sequencing were less pronounced, with 57% of the total cell population from SC sequencing attributed to these cells and 56% of nuclei in the SN dataset (**Figure 1G** and **Supplemental Figure 1.1E**). Similarly, both SC and SN captured nearly identical numbers of CTB-pf cells (7%). However, the number of CTB-p were only 16% of the total population in SN despite accounting for 40% in SC, consistent with the challenge to isolate nuclei from mitotic cells for SN sequencing due to the breakdown of the nuclear envelope during mitosis (**Figure 1G**). Collectively, this comparison underscores the necessity of SN sequencing to representatively capture the gene expression of the STB population in both primary placental tissue and TOs. However, other cell types including mitotic CTB populations (TOs), EVT (tissue), and macrophages (tissue) are enriched in SC and demonstrate a combined SC/SN approach is necessary to capture every cell type in the human placenta or TOs.

Defining trophoblast lineage composition in distinct TO culture conditions

The STB has been historically understudied due to the challenges of its multinucleate architecture. Therefore, we next investigated the impact of TO culture conditions on trophoblast lineage composition using only SN sequencing because it more accurately captured the STB population. To do this, we used TOs isolated from three unique placentas and cultured them each in three distinct culture conditions—standard Matrigel conditions to generate STBⁱⁿ TOs, in suspension to generate STB^{out} TOs, and with NRG1 to enrich for EVT cells (EVT^{enrich}) (**Figure 2A-C**, **Materials and Methods**, **Supplemental Table 3-4**). Each organoid condition was processed into suspensions of nuclei, SN sequenced in parallel, and data from each biological replicate integrated, clustered, and plotted on a UMAP. (**Figure 2A-C**, **Supplementary Figure 2.1A**, **Supplemental Table 1-2**). The clusters for each cell type are labeled with numbers assigned in a random order.

We first assessed how CTB subtypes varied amongst the culturing methods that generated different organoid organizations. We found five populations of CTBs that were identified using well-established markers, such as CDH1 and TENM3, and accounted for 53% of the total population in STBⁱⁿ, 38% in STB^{out}, and 57% in EVT^{enrich} (**Figure 2A-C**, **Supplementary Figure 2.1B**). These CTBs could be delineated into multiple subtypes expressing proliferative markers (KI67 and PCNA in CTB-p), CTB cell column (CTB-CC) markers (LPCAT1, NOTCH1, and ITGA2), or pre-fusion intermediate markers (retroviral fusion protein ERVFRD-1 and GREM2 in CTB-pf) (**Supplementary Figure 2.1B**). STBⁱⁿ and STB^{out} TOs both contained two proliferative CTB populations (CTBp-1 and CTBp-2) that together accounted for 18% of the total nuclear population of each dataset (**Figure 2A-B**). In contrast, there was a single proliferative population in EVT^{enrich} that accounted for 10% of the population, was closer to EVT than CTB on the UMAP, and had downregulated CTB markers and upregulated EVT markers including HLA-G and MMP2. This suggests that TO-derived differentiated EVTs undergo mitosis, as observed *in vivo* (**Figure 2C**, **Supplementary Figure 2.1B, D**) (Arutyunyan et al., 2023). In the STBⁱⁿ condition, CTB-2 expressed canonical CTB-CC markers and accounted for 17% of the total nuclear population (**Supplementary Figure 2.1B-C**). The STB^{out} condition predominantly consisted of a single non-proliferative CTB population (CTB-1). A subset of this cluster expressed the CTB-CC marker ITGA2 suggesting this CC population was still present but not identified as a separate cluster (**Supplementary Figure 2.1B-C**). In contrast, three of the five CTB populations in EVT^{enrich} TOs expressed the CTB-CC markers ITGB6 and LPCAT1 (**Supplementary Figure 2.1B-C**), consistent with the function of CTB-CCs to differentiate into EVT (Turco and Moffett, 2019). Finally, CTB-pf accounted for 4% of the total population in STB^{out}/EVT^{enrich} TOs and 6% of STBⁱⁿ, suggesting culture conditions did not dramatically change the proportion of this intermediate cell type (**Figure 2A-C**, **Supplementary Figure 2.1B**). Thus, while most CTB identities are present across conditions, there are notable differences in the proportion of different CTB subtypes depending on culture conditions.

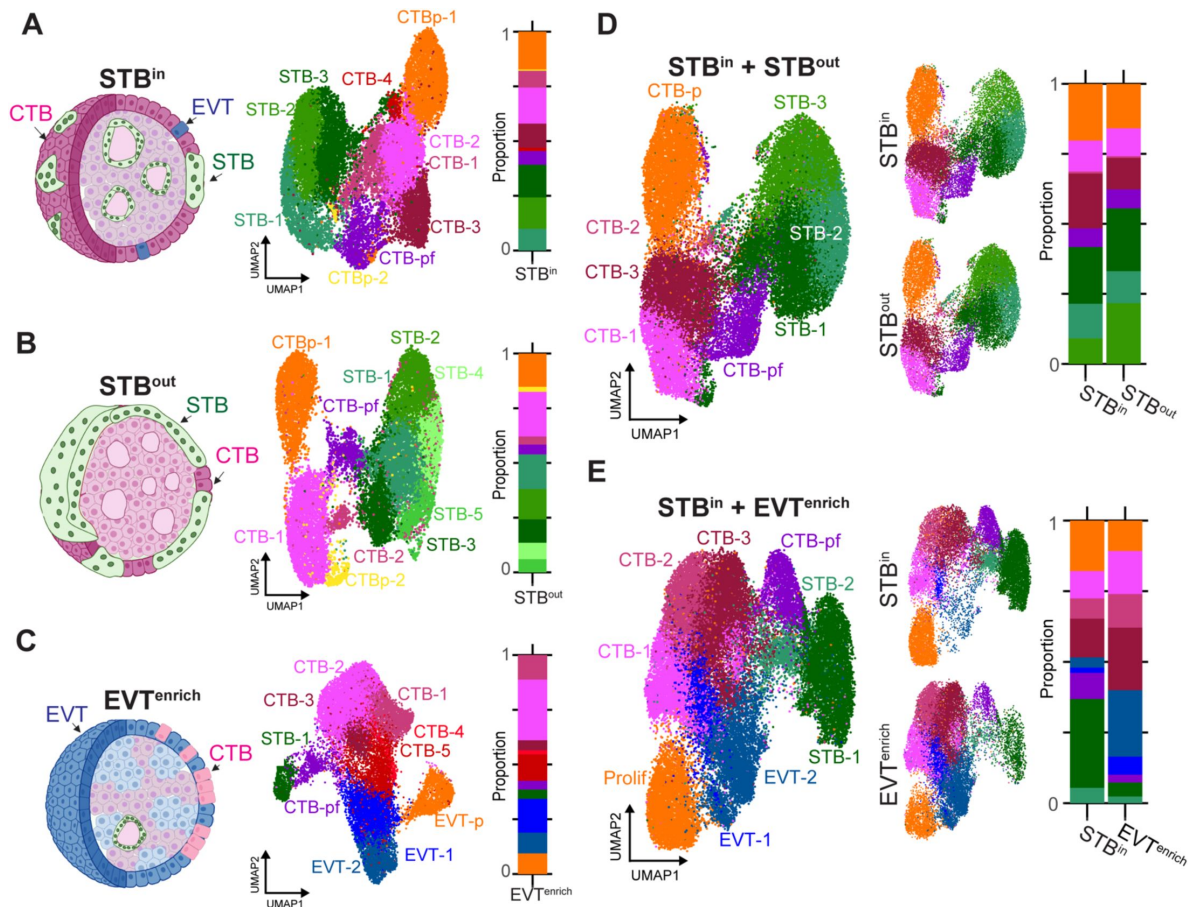


Figure 2

Comparison of TO gene expression in different culture conditions by SN RNA sequencing.

TOs were grown with the STB facing inward (STBⁱⁿ), outward (STB^{out}), or induced to differentiate into EVT (EVT^{enrich}). Schematic of TO nucleus type composition (left), UMAP of the individual SN datasets (middle), and barplot showing the proportions of each nucleus type (right) are shown for STBⁱⁿ (A), STB^{out} (B), or EVT^{enrich} (C). UMAP of the integrated datasets (left), integrated dataset split by culture condition (middle), and barplot showing the proportions of each nucleus type (right) are shown for the STBⁱⁿ+STB^{out} integrated dataset (D) or STBⁱⁿ+EVT^{enrich} dataset (E). Prolif in E refers to proliferative subtype encapsulating both EVT-p and CTB-p.

We next sought to determine how culture conditions influenced the differentiation of CTBs into either the STB or EVTs. In each culture condition STB nuclei were present, accounting for 39% (STBⁱⁿ), 53% (STB^{out}), and 4% (EVT^{enrich}) of the total population (**Figure 2A-C**). As anticipated, few to no EVTs were present in STBⁱⁿ and STB^{out} TOs (**Figure 2A-B**) (Turco et al., 2019; Yang et al., 2023, 2022). In contrast, EVTs accounted for 24% of the total population in EVT^{enrich} TOs and separated into two clusters (EVT-1, EVT-2) (**Figure 2C**). Each had an increased expression of the mature EVT markers HLA-G, MMP2, and DIO2 (**Supplemental Figure 2.1B**). Together, this demonstrates that STB^{out} culture conditions promotes further STB differentiation while EVT^{enrich} conditions promotes EVT differentiation.

To directly compare the relative populations and gene expression between TO culture conditions, we integrated the STBⁱⁿ dataset with either STB^{out} or EVT^{enrich} TOs (**Figure 2D-E**). Each nucleus type retained the expression of canonical markers (**Supplemental Figure 2.2A-B**), but EVT^{enrich} exhibited higher basal expression of the EVT markers HLA-G and DIO2 (**Supplemental Figure 2.2C**). The nucleus types in the STBⁱⁿ+STB^{out} dataset showed significant overlap on the UMAP, indicating relatively consistent gene expression across culture conditions (**Figure 2D**). However, the proportions were not identical for each nucleus type and reflected the differences observed in the individual datasets. Of note, there was a higher percentage of CTB-3 that expressed CTB-CC markers in STBⁱⁿ (20% of STBⁱⁿ and 11% of STB^{out}) and a decrease in the STB-3 population (9% of STBⁱⁿ and 20% of STB^{out}) (**Figure 2D**, **Supplemental Figure 2.2A**) suggesting that STB^{out} culture conditions promotes STB differentiation and prevents CTB-CC differentiation down the EVT lineage. Despite CTB-pf accounting for 7% of each dataset in STBⁱⁿ and STB^{out}, the STB:CTB ratio was nearly halved in STBⁱⁿ compared to STB^{out} (1.3:1 in STBⁱⁿ versus 2.5:1 in STB^{out}). This indicates a higher proportion of nuclei in STB^{out} TOs have undergone cell-cell fusion to become STB. Similarly, STB^{out} TOs expressed higher levels of key STB markers and hormones, indicating culture in suspension promotes enhanced STB differentiation (**Supplementary Figure 2.2D and Supplementary Figure 3.1D**). These results are consistent with our previous report demonstrating an increase in the number of STB nuclei and enhanced expression of hCG- genes in the STB^{out} condition (Yang et al., 2023). In contrast, the STBⁱⁿ+EVT^{enrich} merged dataset exhibited significantly less overlap on the UMAP and amongst nucleus type proportions (**Figure 2E**). In particular, STBⁱⁿ TOs had a dramatic increase in STB nucleus types (37% of STBⁱⁿ and 7% of EVT^{enrich}) while EVT^{enrich} exhibited an increase in EVT types (5% of STBⁱⁿ and 30% of EVT^{enrich}) (**Figure 2E**, **Supplemental Figure 2.2B**), validating EVT^{enrich} conditions promote CTB differentiation into EVT instead of STB.

Lastly, we compared STB^{out} TOs with a publicly available SN dataset from trophoblast stem cell (TSC) derived STB to identify differences in STB populations that might exist between these models (Wang et al., 2024). We integrated the STB^{out} TO dataset with the TSC dataset, performed clustering, and visualized the results on a UMAP. Both TO and TSC models showed a nearly equivalent composition of trophoblast nucleus types, including CTB-p, CTB-pf, EVTs, and two distinct STB populations (STB-1 and STB-2) (**Supplemental Figure 2.3A-C**). Despite similarities in nuclear proportions, pseudobulk differential expression analyses revealed significant differences in the transcriptional profiles of CTB-pf and STB populations derived from TOs and TSCs. TSC-derived CTB-pf and STB clusters highly expressed the EVT marker HLA-G, which was absent in TO-derived CTB-pf and STBs (**Supplemental Figure 2.3D**). Additionally, TO-derived CTB-pf and STBs showed significantly higher expression of STB-associated hormones and other factors, including PSGs, CGBs, CSH1, HOPX, and KISS1 (**Supplemental Figure 2.3D-E**) (Costa, 2016). Therefore, while the two models exhibit a similar proportion of nucleus types, there are notable gene expression differences within each.

Together, these data highlight that culture conditions not only influence the composition of trophoblast nucleus types but also drive their differentiation into distinct lineages, consistent with previous reports and underscoring the critical role of the environmental cues present in each

culture condition in shaping trophoblast identity (Arutyunyan et al., 2023 [DOI](#); Turco and Moffett, 2019 [DOI](#); Yang et al., 2023 [DOI](#), 2022 [DOI](#)).

Comparative transcriptional profiling reveals three distinct

STB subtypes with varied proportions in STBⁱⁿ and STB^{out} TOs

Given the increased proportion of STB nuclei in STB^{out} TOs and enhanced expression of typical STB markers in STB^{out} TOs, we next sought to identify the genes that define each STB subtype. To do this, we utilized the merged STBⁱⁿ+STB^{out} dataset that contained three STB subpopulations. This dataset exhibited differential enrichment of each STB subpopulation within the two culture conditions (**Figure 3A** [DOI](#)). We first analyzed the genes enriched in each STB subtype and identified hundreds of genes whose expression was conserved in the STB subtypes of both STBⁱⁿ and STB^{out} TOs (**Figure 3B** [DOI](#), **Supplemental Figure 3.1A**, **Supplemental Table 8**). STB-1 is closest to CTB-pf on the UMAP and expressed many genes previously associated with CTBs, including the transcription factors TEAD1 and TP63 (**Figure 2D** [DOI](#), **3B**) (Li et al., 2014 [DOI](#); Mizutani et al., 2022 [DOI](#)). In fact, when the top genes in STB-1 were plotted as a dotplot for every nucleus type in the dataset these genes were most enriched in the CTB subtypes (**Supplemental Figure 3.1B**). The gene ontology (GO) terms associated with STB-1-enriched genes are involved in RNA splicing, stem cell maintenance, and RAS signaling (**Figure 3C** [DOI](#), **Supplemental Figure 3.1C**). Candidate genes from each of these GO terms demonstrated expression predominantly in CTBs, intermediate expression in STB-1, and lower expression in the remaining two STB populations (**Supplemental Figure 3.1C**). STB-2 expressed a unique subset of genes that included the VEGF receptor FLT1, the TGFβ family member INHBA, and the insulin regulator PAPP2, associating this subtype with ER stress and oxygen sensing (**Figure 3B-C** [DOI](#)) (Barrios et al., 2021 [DOI](#); Li et al., 2022 [DOI](#); Sasagawa et al., 2021 [DOI](#), 2018 [DOI](#)). Female pregnancy was identified as an enriched GO term in STB-2 (**Figure 3C-D** [DOI](#)) and many of the genes found in this term are linked to angiogenesis. This included 8 pregnancy specific glycoprotein (PSG) paralogs that are pro-angiogenic, VEGFA, and the growth factor Angiopoietin-2 that facilitates vascular development in specific contexts (Akwii et al 2019, Moore et al 2021 [DOI](#)) (**Supplemental Table 9**). Interestingly, STB-2 was more abundant in STBⁱⁿ than STB^{out} (**Figure 3A** [DOI](#)). We validated this finding with RNA fluorescence in-situ hybridization (FISH) and found an increase in the expression of the STB-2 marker PAPP2 in STBⁱⁿ TOs compared to STB^{out} (**Figure 3D and F** [DOI](#) and **Supplemental Figure 3.3A**). Finally, the top genes in STB-3 included the sodium/bicarbonate transporter SLC4A4, the matrix metalloprotease ADAMTS6, the collagen receptor ITGA1, and protein kinase C epsilon PRKCE (**Figure 3B** [DOI](#)) (Cain et al., 2022 [DOI](#), 2016 [DOI](#); Zeltz and Gullberg, 2016 [DOI](#)). GO terms demonstrated that the STB-3 cluster was enriched for processes involved in GTPase signaling, vascular transport, and actin organization (**Figure 3C** [DOI](#), **Supplemental Figure 3.1C**). Both the vascular transport and transport across the blood brain barrier GO terms were enriched for molecules involved in the transport of small molecules, amino acids, and fatty acids (**Supplemental Table 9**). In contrast to STB-1 and STB-2, the percentage of STB-3 nuclei were doubled from 20% in STBⁱⁿ to 40% in STB^{out}. We validated this finding via RNA-FISH using the STB-3 marker ADAMTS6 and observed increased RNA expression in STB^{out} compared to STBⁱⁿ TOs (**Figure 3E and G** [DOI](#) and **Supplemental Figure 3.3B**). Interestingly, most pregnancy hormones were expressed at similar levels in the STB-2 and STB-3 subtypes but exhibited increased expression in STB^{out} compared to STBⁱⁿ (**Supplemental Figure 3.2C-D**). In summary, STB-1 exhibited intermediate gene expression between CTB and STB, STB-2 expressed vascular signaling/oxygen sensing factors, while STB-3 was enriched in transport/GTPase signaling functions. Of note, STB-1 and STB-2 nuclear subtypes were enriched in STBⁱⁿ TOs while STB-3 subtype was doubled in STB^{out} TOs, indicating STB nuclear subtype proportions are sensitive to the culture conditions TOs are grown in (**Figure 3A** [DOI](#)).

Given the different proportions of STB subtypes between STBⁱⁿ and STB^{out} TOs, we next sought to determine if gene expression changed within each STB subtype as a function of culture condition. Therefore, we performed pseudobulk differential expression analysis using DESeq2 to compare gene expression of each STB subtype between STBⁱⁿ and STB^{out} TOs and determined GO terms

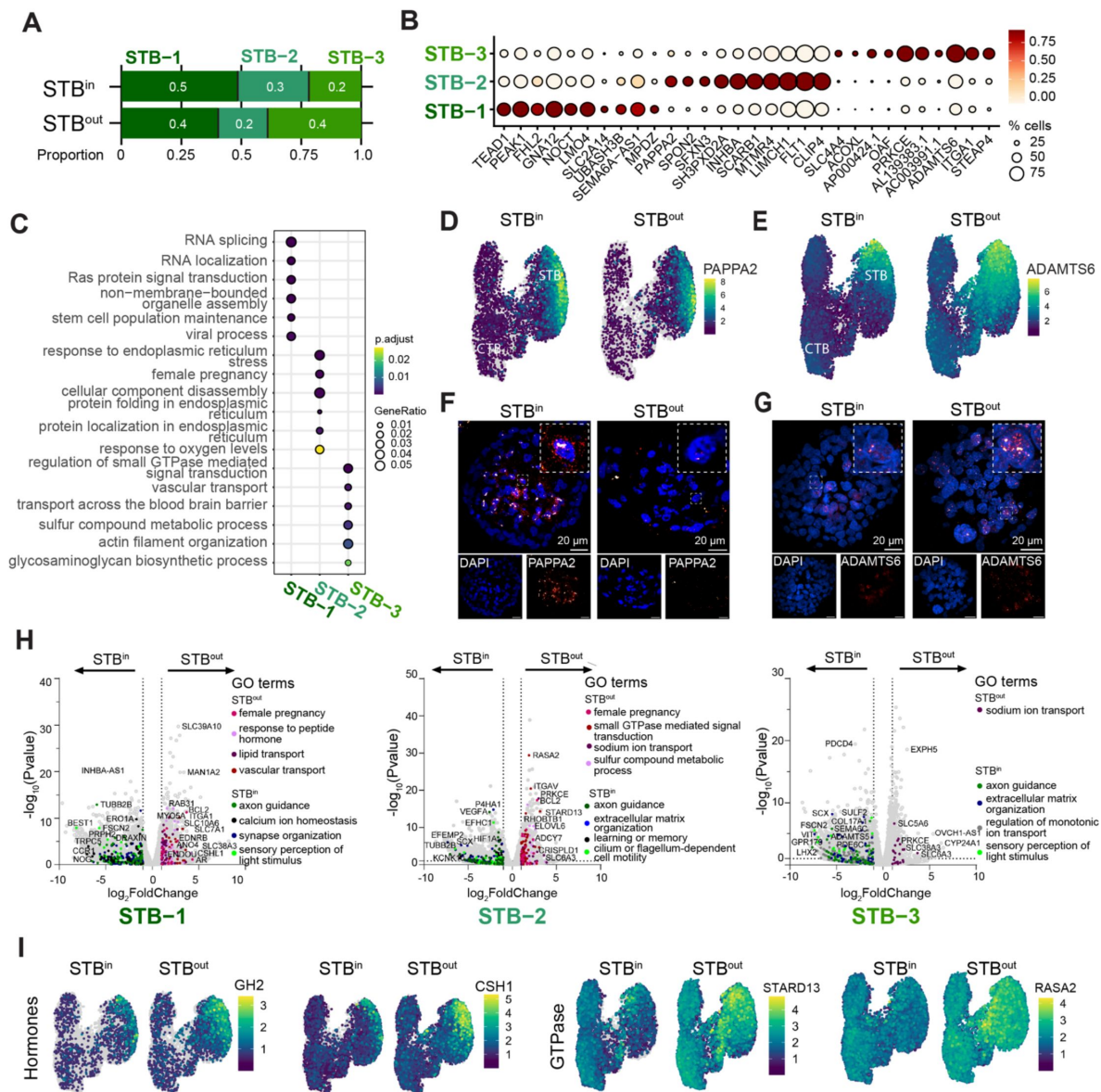


Figure 3

STB subtype analysis and expression differences in STBⁱⁿ vs STB^{out} TOs.

A Proportion of each STB subtype in the integrated STBⁱⁿ+STB^{out} dataset. **B** Top differentially expressed genes (DEGs) of each STB subtype is shown as a dot plot. Mean expression is shown as a color scale and percent of nuclei expressing each gene is demonstrated by size of the dot. **C** Top Biological Process GO terms associated with DEGs in each STB subtype was determined with clusterProfiler. **D** Feature Plots of PAPP2 in STBⁱⁿ and STB^{out} TOs. **E** Feature Plots of ADAMTS6 in STBⁱⁿ and STB^{out} TOs. **F** RNA FISH of PAPP2 in STBⁱⁿ and STB^{out} TOs. **G** RNA FISH of ADAMTS6 in STBⁱⁿ and STB^{out} TOs. **H** DESeq2 was used to find DEGs between STBⁱⁿ and STB^{out} datasets in STB-1, STB-2, and STB-3 clusters and plotted as a volcano plot. GO terms associated with DEGs in either STBⁱⁿ or STB^{out} were found with clusterProfiler and colored on the UMAP with select representative genes highlighted with text. **I** Feature Plot of representative target genes from E.

associated with the differentially expressed genes (DEGs) (**Figure 3H** and **Supplemental Table 10**) (Love et al., 2014). STB-1 and -2 of STB^{out} TOs were enriched for genes involved in pregnancy including genes in the human placenta lactogen family (CSH1 and CSHL1), growth hormone 2 (GH2), the STB specific gene ENDOU (Haider et al., 2018), and the androgen receptor (AR) (**Figure 3H-I**). In addition, STB in STB^{out} TOs exhibited increased expression of genes involved in GTPase signaling, including the RhoA GAPs STARD13 and GRAF3 (ARHGAP42) (Bai et al., 2013; Ching et al., 2003) (**Figure 3H-I**). All three STB subtypes in STB^{out} TOs exhibited an enrichment in transport- associated proteins, consistent with the STB being proximal to media (**Figure 3H**, **Supplemental Figure 3.1E**). In contrast, STBⁱⁿ TOs were enriched in extracellular matrix organization genes but their expression was not specific to STB subtypes (**Figure 3H** and **Supplementary Figure 3.1F**). The STB-2 subtype in STBⁱⁿ TOs had increased expression of the hypoxia associated genes LIMD1, HILPDA, and HIF1A as well as the angiogenesis-associated proteins VEGFA and FLT1 (**Figure 3H**, **Supplemental Figure 3.1G**) (Foxler et al., 2018; Rodriguez and Kersten, 2020; Shibuya, 2011), consistent with the increased proportion of the oxygen sensing STB-2 subtype in STBⁱⁿ. Together, these results demonstrate that there are at least three subpopulations of STB in trophoblast organoids, which differ both in their relative proportions and transcriptional signature between culture conditions.

Pseudotime and gene network analysis reveals gene regulators in STB^{out} TOs, including the chromatin remodeler RYBP

To assess whether distinct nuclear subtypes represented a STB differentiation trajectory in TOs, we next performed pseudotime gene expression inference using the integrated STBⁱⁿ and STB^{out} datasets described above (**Figure 2D**). We utilized the Slingshot algorithm to establish the global lineage structure for each dataset using undefined starting and ending clusters (Street et al., 2018). Following this, we depicted the pseudotime progression on the UMAP plot for the STB lineage (**Figure 4A**). This visualization revealed a continuous trajectory starting from CTB-p subtype, traversing through CTBs to CTB-pf, and progressing through STB-1 and STB-2 before culminating in the STB-3 subtype (**Figure 4A**). To find genes associated with pseudotime, we performed an association test with tradeSeq and found that STB differentiation was marked by an increase in well-known STB marker genes, including ADAM12, PLAC4, and PSG6 (**Figure 4B**) (Aghababaei et al., 2015; Chen et al., 2022; Moore et al., 2021; Tuohey et al., 2013). Next, we conducted a comparative pseudotime expression analysis between STBⁱⁿ and STB^{out} conditions. Our findings revealed enrichment of several STB- associated genes in STB^{out}, such as the secreted metalloproteinase protein ADAM12, the progesterone synthesis enzyme CYP11A1, and the proteoglycan synthesis gene MAN1A2 (**Figure 4C**) (Aghababaei et al., 2015; Zhu et al., 2023). Interestingly, the chromatin remodeler RYBP and transcriptional activator AFF1 were two genes most significantly associated with the CTB to STB pseudotime trajectory and enhanced in STB^{out} TOs (**Figure 4B-C**), suggesting they may play roles in initiating transcription of genes involved in STB^{out} nuclear differentiation.

To obtain a global view of candidate transcription factors and chromatin remodelers involved in STB differentiation in tissue, STBⁱⁿ, and STB^{out} TOs we next turned to a gene network analysis approach. We isolated the subset of nucleus types involved in STB differentiation (CTB, CTB-pf, and STBs) from each dataset and performed RNA velocity using spliced/unspliced matrices and plotted trajectories from each on a UMAP integrated for replicates of each sample type. This analysis creates a trajectory like slingshot but instead of accounting for pseudotime with bulk RNA expression, it leverages transcript splicing dynamics (Manno et al., 2018). The predominance of STB nuclei in the SN full-term tissue dataset precluded the ability to attain sufficient CTB-pf nuclei, but the velocity map demonstrated CTB differentiating into two STB lineages (**Supplemental Figure 4.1, full-term**). Given the decreased STB:CTB ratios present in organoids, we were able to capture high numbers of CTB-pf nuclei and observed that this population is a precursor to the STB, as anticipated (**Supplemental Figure 4.1, STBⁱⁿ and STB^{out}**). We then employed Velorama to infer gene regulation in these cells. Velorama is an RNA Velocity-based causal inference method that

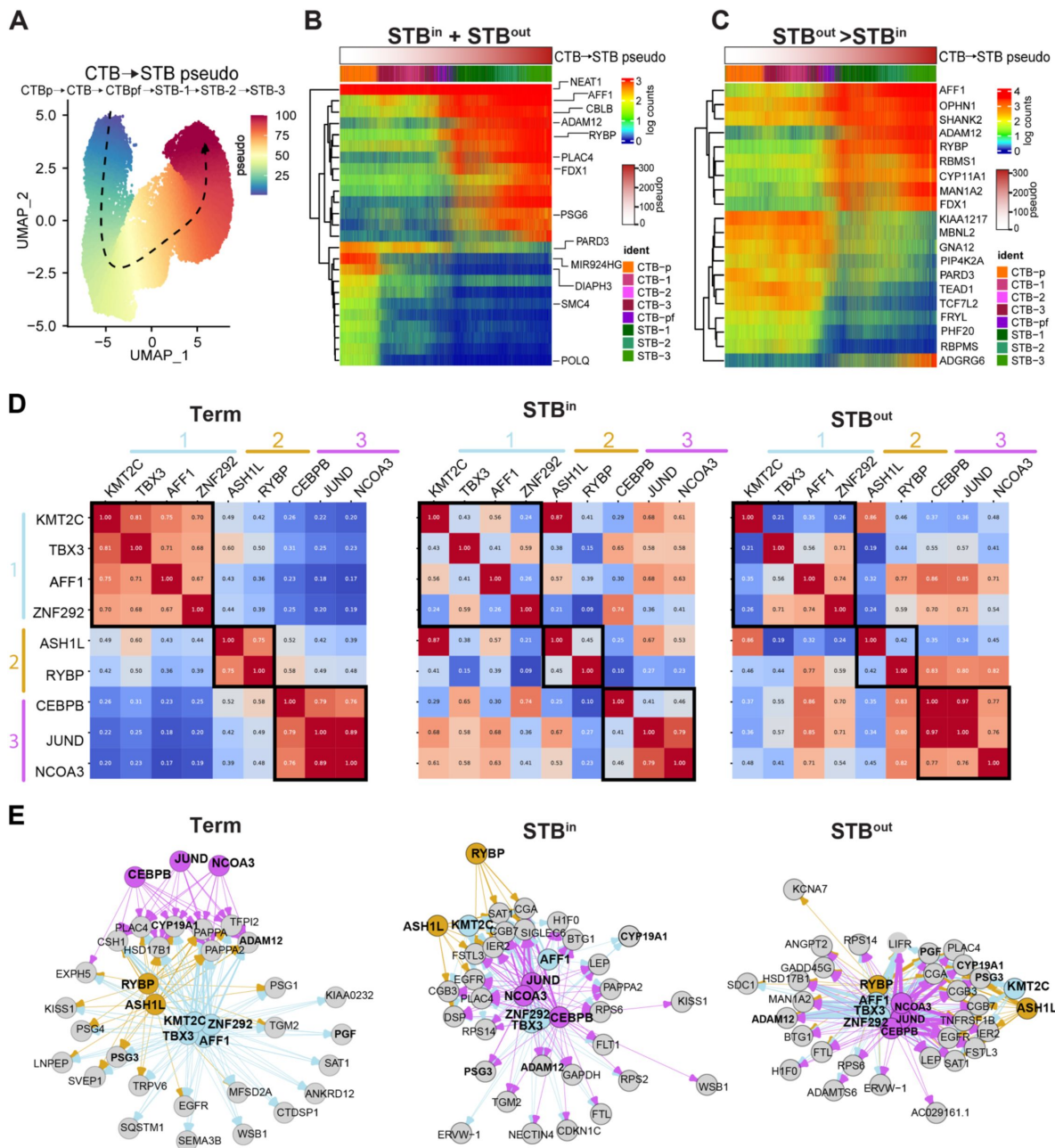


Figure 4

Trajectory and gene regulatory network analysis reveals transcription factors and chromatin remodelers associated with STB differentiation.

A Pseudotime was performed with Slingshot and assigned pseudotime value for each nucleus type is overlaid as a color scale on the UMAP from time 0 (blue) to time 100 (red). Arrow demonstrates directionality of the trajectory. Top genes associated with pseudotime (**B**) or enriched in STB^{out} compared to STBⁱⁿ (**C**) are plotted on a barplot with nuclei ordered with the pseudotime seen in **A**. Logcounts and pseudotime are demonstrated as a color scale bar and the identity of each nucleus is labeled with a color as indicated in the legend. **D** TF/CR and TG pairs were identified via Velorama and plotted as a similarity matrix to show the overlap in TGs for each TF/CR. Each module discussed in the text is labeled as 1, 2, or 3 and colored with blue, orange, or pink respectively. **E** Network graphs of TF/CRs and their respective TGs were plotted with igraph. Each TF/CR and respective arrows were false colored as per the module labels in **D**. Grey circles represent the target genes. Target genes that are shared among all three sample types are bolded. The width of the arrow from each TF/CR to each TG represents the interaction strength score determined with Velorama and the color of the arrow represents the module from which the interaction arises.

accounts for the multi-trajectory development of cellular state. It infers temporal causality on a directed acyclic graph where each node is a cell, and the edges indicate the velocity-implied direction of differentiation. Velorama derives gene regulatory networks by training a neural network to predict target gene expression profiles given regulator genes, such as transcription factors (TFs), and outputs an interaction score for every TF and target gene (TG) combination (Singh et al., 2024). Given the identification of the chromatin remodeler RYBP in the Slingshot analysis, we expanded Velorama to include other known chromatin remodelers (CRs), as they also have the potential to regulate gene expression levels.

To dissect the functional interactions between TF/CRs, we first compared the similarity of TGs interacting with each TF/CR and plotted this as a heatmap with a score of 1 indicating all TGs are shared and a score of 0 indicating no overlap (heatmap demonstrates high overlap in red and low overlap in blue, **Figure 4D**). In full-term tissue, these TF/CR fell into three modules that shared most target genes: 1- KMT2C/TBX3/AFF1/ZNF292 (blue), 2-ASH1L/RYPB (orange), and 3-CEBPB/JUND/NCOA3 (pink) (**Figure 4D**). In contrast to full-term tissue, most TGs in STBⁱⁿ organoids were associated with AFF1/JUND/NCOA3/ZNF292/TBX3/CEBPB in no module order (**Figure 4D**). Finally, STB^{out} appeared to have a similar distribution of TF/CRs as STBⁱⁿ, apart from an increased prevalence of RYPB association with target genes in STB^{out} (**Figure 4D**).

We next evaluated the specific TGs in each condition to predict the possible functions of the TF/CR and TG pairs. In full-term tissue, many STB-specific target genes were found to be associated using RNA velocity including CYP19A1, MFSD2A, ADAM12, PSGs, human placenta lactogen (CSH1), and the placenta specific insulin regulator PAPPA enriched in STB of full-term tissue (Wang et al., 2024). Module 3 was only associated with a handful of genes at full-term and with low interaction scores (interaction score demonstrated by arrow width). In contrast, Module 3 had a much stronger prevalence in both the number of target genes and interaction scores in both STBⁱⁿ and STB^{out} organoids with some similar genes (conserved genes bolded, ADAM12, CYP19A1, PGF, PSG3) and some unique to organoids, particularly of note being hCG genes (CGA, CGB4, CGB7) (**Figure 4E**), whose expression is known to be decreased in the STB of full-term tissue (Rull and Laan, 2005). STBⁱⁿ was the only condition to show a link between the TF/CRs and the VEGF receptor FLT1, consistent with the enrichment of hypoxic/angiogenic associated genes in the STB-2 subtype of STBⁱⁿ compared to STB^{out} (**Figure 3H, 4D**). Importantly, this analysis independently identified both RYPB and AFF1 as transcription effectors involved in the expression of STB TGs, consistent with our pseudotime analysis (**Figure 4A-C**). AFF1 was associated with most TGs in all three sample types (**Figure 4E**). In contrast, while RYPB was associated with most STB marker genes in full-term tissue and STB^{out}, it was associated with only a few TGs in STBⁱⁿ (**Figure 4E**). In fact, RYPB was associated with genes that were enriched in STB^{out} organoids in the Slingshot analysis (ADAM12 and MAN1A2) but not in STBⁱⁿ TOs (**Figure 4B-E**), supporting the hypothesis that RYPB could be a modulator of a specific lineage of STB nuclear subtype differentiation. In summary, this gene network analysis suggests that many TF/CR and TG interactions are shared amongst tissue and TOs. However, many interactions in TOs also change as a function of culture condition. While module 3 is most linked to TGs expressed in TOs, RYPB is linked specifically to TGs expressed in tissue and STB^{out} TOs, but not STBⁱⁿ TOs.

RYBP is a STB-specific nuclear marker involved in silencing gene expression characteristic of the STB- 2 nuclear subtype

To validate the analyses described above, we selected RYPB as a model target gene for its potential role in STB nuclear subtype differentiation and based on the availability of well-validated antibody reagents. RYPB contains chromatin remodeling activity as part of the PRC1 histone ubiquitination complex and can both increase and decrease the transcription of target genes (Rose et al., 2016; Silva et al., 2018). The expression dynamics of RYPB was directly correlated with the differentiation trajectory from CTB to STB, with upregulated expression beginning in CTB-pf (**Figure 4B**). We first visualized RYPB in full-term tissue sections, using E-cadherin as a marker

of CTB and Cytokeratin-7 as a marker of CTB and STB cells. STB can be clearly designated as regions expressing Cytokeratin-7 but not expressing E-Cadherin, as validated with the STB-specific marker ENDOU (**Supplementary Figure 5.1**). We found that RYBP exhibited STB-specific localization in term placenta tissue (**Figure 5A**). To confirm this result in TOs, we visualized RYBP at the protein level in each sample type with immunofluorescence (IF) using E-cadherin as a CTB marker and CGBs as STB specific markers (**Figure 5B-C**). RYBP exhibited STB specific expression in both STBⁱⁿ and STB^{out} TOs. Together this suggests that RYBP is a novel marker of STB nuclei in tissue and TOs. We hypothesized that RYBP could play a role in guiding STB nuclei toward a specific nuclear subtype during differentiation. To test this, we performed gene editing in TOs with CRISPR/Cas9 to delete RYBP and confirmed this knock-out by sequencing and IF (**Figure 5D** and **Supplemental Figure 5.2A**). In parallel, we generated TOs with AFF1 deletion, as it was also associated with the STB differentiation trajectory and confirmed the knockout by sequencing (**Figure 4** and **Supplemental Figure 5.2B**). However, despite testing several antibodies, none were suitable for IF localization of AFF1. We generated three homozygous knockout clones of each line (RYBP^{-/-} or AFF1^{-/-}) and compared their morphologies to three control TO lines that contained the Cas9 plasmid but a scrambled gRNA (WT). No difference in the overall morphology of TOs was found, and size and number of nuclei within the STB remained constant (**Figure 5D**). To determine if gene expression differences were found in the TOs lacking RYBP or AFF1, we performed bulk RNA sequencing of each RYBP^{-/-}, AFF1^{-/-} or WT lines in STBⁱⁿ TOs. We found that deletion of either RYBP or AFF1 resulted in significant increased expression of genes associated with the STB-2 oxygen sensing subtype, including FLT1, PAPP2, SPON2, SFXN3, and many of the PSG hormones involved in angiogenesis (**Figure 5E**, **Supplemental Figure 5.3**, **Supplemental Table 11**). RYBP^{-/-} TOs upregulated transcripts that were not expressed in WT TOs or AFF1^{-/-} TOs (**Figure 5E** and **Supplemental Figure 5.3**). RYBP is a component of a non-canonical Polycomb complex that transcriptionally silences genes (Rose et al., 2016). In addition to the upregulation of the STB-2 genes, its knockout led to the upregulation of many genes not typically expressed in TOs including COL18A1, SEMA5A, CARD11, and MSX1, likely due to the loss of this silencing function (**Figure 5E**). In contrast, the human placenta lactogen gene (CSH1) was significantly upregulated in WT TOs compared to RYBP^{-/-} and AFF1^{-/-} TOs, suggesting deletion of both RYBP and AFF1 affect the expression of a hormone increased in STB^{out} TOs (**Figure 5E**, **Supplemental Figure 5.3**, and **Supplemental Table 11**). However, no further STB-3 subtype genes were found to be upregulated compared to control in the STBⁱⁿ TOs tested. Together, these results validate the predictions from the trajectory analyses described above and suggest that AFF1 and RYBP act to silence the STB-2 nuclear subtype and activate human placenta lactogen in TOs.

Comparison of STB differentiation in TOs to first trimester and full-term placental tissue

Having identified several differences in STB differentiation between STBⁱⁿ and STB^{out} TOs, we next sought to identify how these differences relate to placenta tissue across gestation. We used a publicly available first trimester SN sequencing dataset and integrated this data with the full-term tissue and TOs described above (**Supplemental Figure 6.1A**). Because tissue-derived datasets contain a large proportion of non-trophoblast nucleus types, we subset the integrated dataset to contain only trophoblasts in the STB lineage, which included both proliferating CTBs (CTB-p), CTBs (CTB-1 and CTB-2), pre-fusion CTBs (CTB-pf), and five distinct STB populations (**Figure 6A**, **Figure 6B**, **Supplemental Figure 6.1A-D**). The proportion of STB:CTB was higher in full-term tissue than in first trimester tissue (7:1 at full-term vs 1.5:1 in first trimester, combined dataset), as anticipated given the higher proportion of CTBs in first trimester (**Figure 6B**) (Benirschke et al., 2012; Mayhew, 2014). Of note, the STB:CTB ratios in TOs more closely resembled first trimester than term tissue in both culture conditions (1:1 for STB^{out} and 0.6:1 for STBⁱⁿ) (**Figure 6B**). The tissue and TO samples exhibited dramatically different proportions of each CTB (CTB-1-2) subtype and each STB (STB1-5) subtype, which indicates sample to sample heterogeneity (**Figure 6A-B**, **Supplemental Figure 6.1A-C**). Therefore, we merged the two CTB and five STB populations

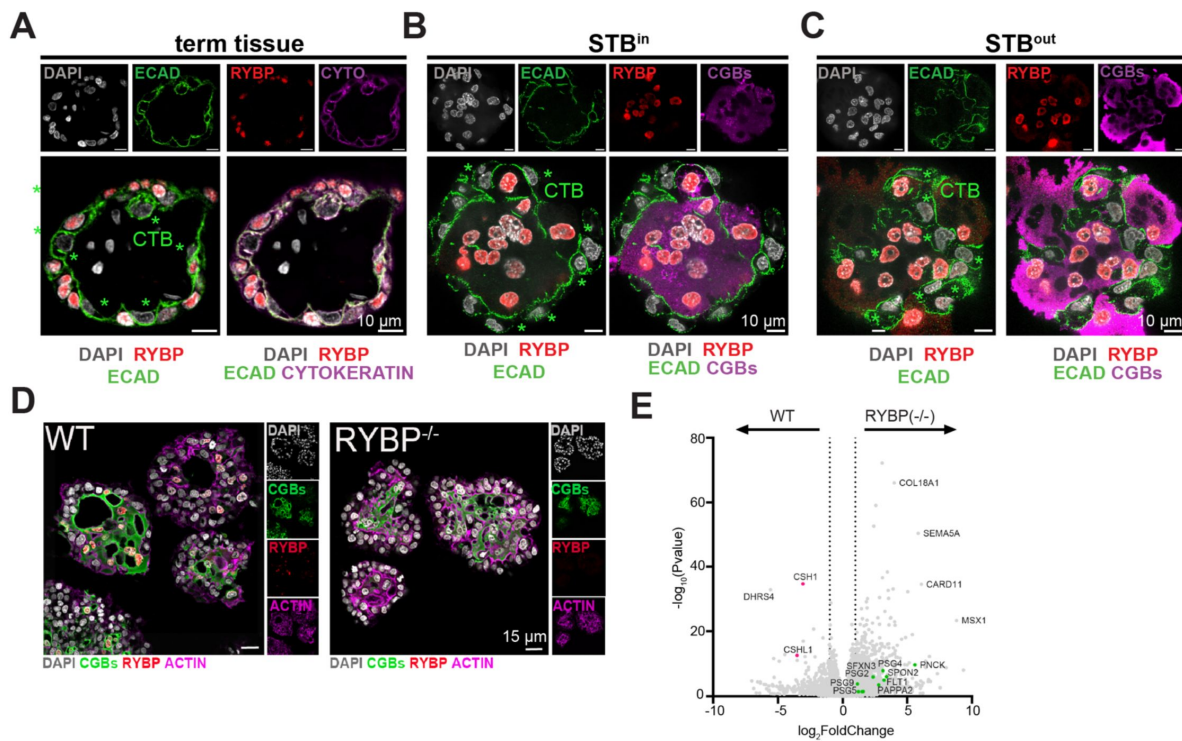


Figure 5

RYBP is a marker of STB and its deletion downregulates STB-2 marker genes.

A Immunofluorescence of full-term tissue was performed with E-cadherin (ECAD), cytokeratin (CYTO), and RYBP and stained with DAPI to label nuclei. Immunofluorescence of STBⁱⁿ TOs (**B**) or STB^{out} TOs (**C**) was performed with chorionic gonadotropin (CGBs) to label STB, RYBP, and E-cadherin (ECAD) to mark cell boundaries. TOs were further stained with DAPI to label nuclei. Green stars represent CTB cells, as marked by E-cadherin, and are negative for RYBP. **D** Immunofluorescence of WT or RYBP^{-/-} STBⁱⁿ TOs performed with RYBP and CGBs and stained with DAPI and Actin. **E** Bulk RNA sequencing of WT or RYBP^{-/-} STBⁱⁿ TOs was performed and analyzed with DESeq2 to determine differential gene expression. Dotted line represents a log2 fold change greater than 1. Genes enriched in the STB-2 subtype are labeled in green.

together to globally detect the conserved and differentially expressed genes among the STB or CTB populations (**Figure 6C**). We first analyzed the expression of canonical marker genes for each nucleus type (for example TP63/TENM3 in CTB and CYP19A1 /TFAP2A in the STB) and found that each sample expressed these markers (**Figure 6D**), suggesting general conservation across both tissue types and TO conditions. To dissect what was different amongst the samples, we next analyzed the enriched genes in each nucleus type and identified GO terms associated with these genes for each sample type (**Figure 6E** and **Supplemental Figure 6.1E** and **Supplemental Table 12 and 13**). GO terms associated with the CTB populations were remarkably conserved amongst all sample types (**Supplemental Figure 6E**). In contrast, STB exhibited greater diversity of functions between sample types (**Figure 6E**). While GO terms involved in ER to Golgi transport and hormone production were conserved in all STB samples, many processes were found only within a subset of sample types (**Figure 6E**). For example, genes with ER stress, ER associated degradation, and macroautophagy GO terms were expressed in TOs but not in tissue. In contrast, GTPase signaling and vesicle organization were present in first trimester, term, and STB^{out} TOs, but not in STBⁱⁿ (**Figure 6E**). Together, these findings suggest that the STB exhibits greater heterogeneity in function across sample types than do CTBs.

To determine what genes were significantly different in each sample, we performed pseudobulk analyses using DESeq2, plotted genes on a Volcano plot, and colored genes significantly associated with different GO terms (**Figure 6F** and **Supplemental Table 14**). We performed this analysis comparing STB in either first trimester tissue to TOs (STB^{out} or STBⁱⁿ) or full-term tissue to TOs (STB^{out} or STBⁱⁿ) (**Figure 6F**). Many GO terms were enriched across all comparisons. This included an increase in extracellular matrix, cell substrate adhesion, and connective tissue development genes within the tissue datasets, with a particular enrichment of collagen genes and matrix metalloproteases (ADAM and MMP family members) (**Figure 6F**) (Qu and Khalil, 2022). In contrast, the STB present in organoids exhibited an enrichment of genes involved in cytoplasmic translation/ ribosome biogenesis as well as oxidative phosphorylation/ DNA damage (**Figure 6F**). In addition, genes associated with cytokine production and the immune response based on GO terms were present in full-term tissue but not first trimester tissue or TOs (**Figure 6F**). Therefore, while STB of TOs and tissue share many conserved marker genes, a subset of STB gene expression is differentially regulated between tissue and TOs.

We next evaluated the expression of genes known to change throughout gestation in each sample type. For example, many hormones are differentially released throughout pregnancy with first trimester tissue exhibiting high hCG production (CGB genes) and full-term tissue releasing more human placenta lactogen (CSH genes) (Costa, 2016; Rull and Laan, 2005; SAMAN et al., 1966). Consistent with these gestational hormone trends, STB from first trimester expressed hCG genes while there was no expression in full-term tissue (**Figure 6G**, **Supplemental Figure 6.1F**). Further, full-term tissue exhibited an increase in CSH1 expression compared to first trimester (**Figure 6G**, **Supplemental Figure 6.1F**). STBⁱⁿ and STB^{out} TOs expressed both hormones in the STB suggesting organoids can produce hormones differentially expressed throughout gestation (**Figure 6G**, **Supplemental Figure 6.1F**). STB^{out} TOs exhibited an increase in expression of both hormones compared to STBⁱⁿ TOs (**Figure 6G**, **Supplemental Figure 6.1F**). Consistent with previous reports, there was an increase in the expression of FLT1 and decrease in PAPP in the STB of first trimester compared to full-term tissue, with TOs resembling first trimester expression (**Supplemental Figure 6.1G-H**) (Wang et al., 2024). Together, these results demonstrate that while the STB of each sample expresses key STB marker genes, both gestational age and trophoblast organoid culture conditions can modify the gene expression patterns of the STB.

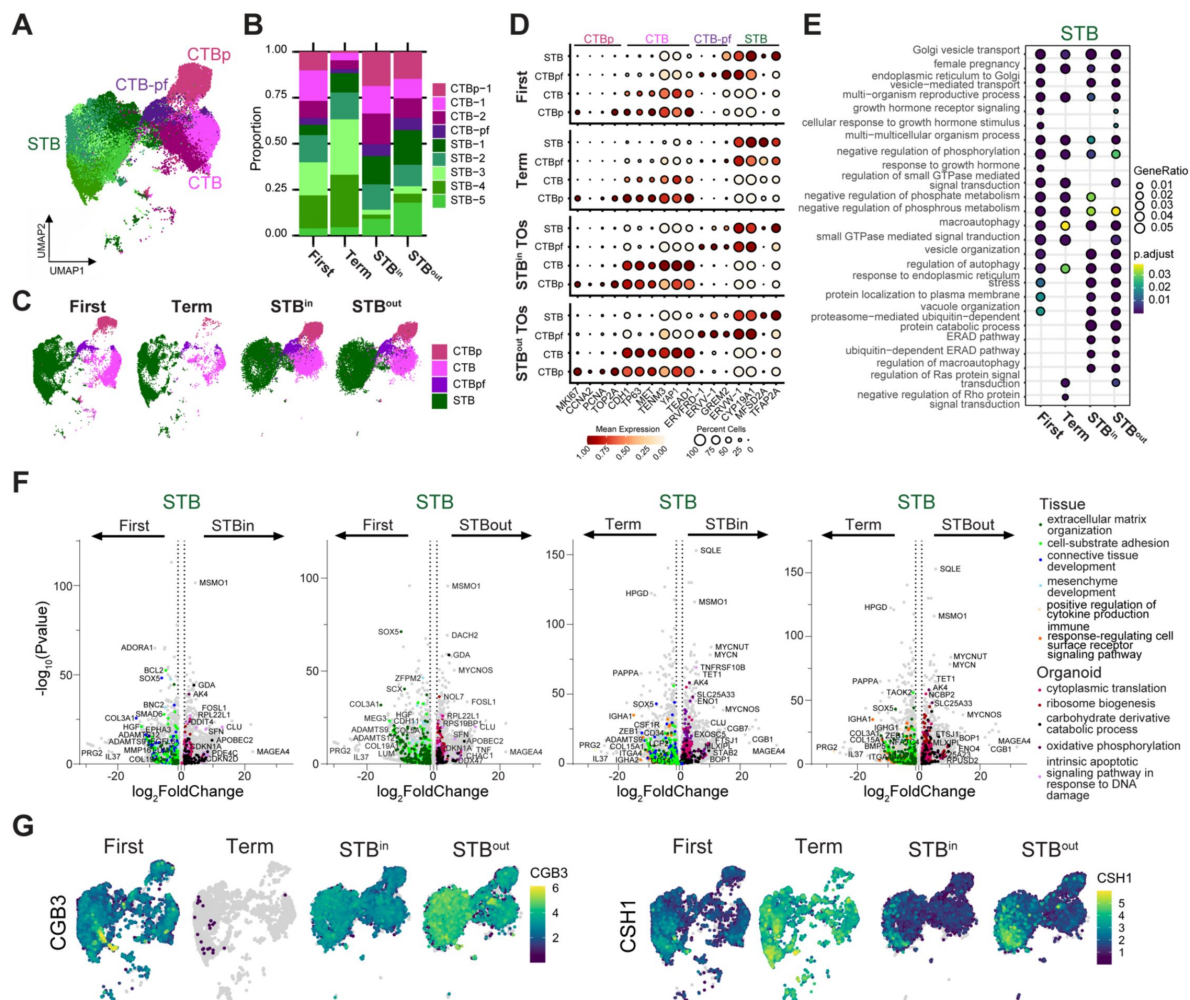


Figure 6

Similarities and differences between STB in first trimester and full-term tissue and TOs.

A UMAP of the integrated SN dataset including first trimester, full-term tissue, STBⁱⁿ TOs, and STB^{out} TOs. **B** Relative proportion of each nucleus type for each sample in the integrated dataset in **A**. **C** UMAP of the integrated dataset split by sample type. The five STB subtypes were merged into a single STB subtype while the two non-proliferative CTB subtypes were merged. **D** Dot plot of key trophoblast marker genes in the STB lineage with heatmap representing mean gene expression and size of the dot representing the percent of nuclei expressing each marker. The labels on the top of the graph refer to the established marker genes used to identify the identity of each cluster. **E** The top DEG in the STB of each sample type were analyzed for biological process GO term enrichment with clusterProfiler and plotted as a dotplot. **F** DESeq2 was used to find DEGs between different sample types for the merged STB cluster. GO terms for DEGs enriched in each sample type were found with ClusterProfiler and colored on the UMAP with select representative genes highlighted in text. **G** Feature plots demonstrating RNA expression of selected genes.

Discussion

A critical barrier to understanding human gestation has been the limited number of accessible models for the placenta. In this study, we conducted comparative SC and SN RNA sequencing on full-term placenta tissue and TOs. Our findings demonstrate that SN sequencing is crucial for capturing the STB lineage due to its distinct syncytial structure. In contrast, SC sequencing enriched for mitotic cell populations in TOs and EVT in tissue, suggesting the isolation approach chosen depends on your trophoblast cell type of interest. We characterized the nucleus types in each TO model and utilized DEG and pseudotime analyses to define three STB subtypes present in both STBⁱⁿ and STB^{out} TOs, albeit at different ratios. These include a juvenile population that exhibited intermediate CTB and STB expression (STB-1), an FLT1+ population enriched in genes involved in oxygen sensing and the stress response (STB-2), and a final subtype enriched in transport and GTPase signaling molecules (STB-3). We identified the chromatin remodeler RYBP as a gene linked to STB differentiation. We validated that RYBP exhibits STB specific expression in TOs and tissue via immunofluorescence and utilized CRISPR to knock out RYBP in TOs. Deletion of RYBP in TOs upregulated genes that define the STB-2 subtype and downregulated the placenta hormone human placenta lactogen. Finally, we compared STB gene expression between placental tissues from first trimester and full-term to TOs. This showed that although standard STB differentiation markers are maintained in all, there is substantial heterogeneity in STB gene expression between the different sample types. Together, these results draw important implications for our understanding of STB nuclear differentiation and show how TOs can serve as relevant STB models.

STB^{out} TOs grown in suspension maintain native polarity and exhibit an increase in syncytia size, with >50 nuclei/syncytia in STB^{out} compared to ~10 nuclei/syncytia under standard STBⁱⁿ culture conditions (Yang et al., 2023 [\[1\]](#)). However, it was unknown whether CTB grown via this method maintained their proliferative capacity. Here we show that the proportion of mitotic cells remains constant between TOs grown in STBⁱⁿ and STB^{out} conditions, suggesting culture in suspension is not a terminal, post-mitotic state. In fact, the proportion of STB nuclei increased in the STB^{out} condition was concurrent with a decrease in the CTB-CC population. *In vivo*, CTB-CC cells sit adjacent to the villous trees and maternal uterus and are thought to progressively differentiate into EVT cells that then can invade into the uterus (Arutyunyan et al., 2023 [\[2\]](#); Boyd and Hamilton, 1970 [\[3\]](#); Turco and Moffett, 2019 [\[4\]](#)). Therefore, the increase of STB in the STB^{out} condition could be caused by promotion of STB lineage and an inhibition of differentiation down a CTB-CC or EVT lineage.

The enrichment of STB nuclei in SN sequencing allowed us to identify and define three populations of STB in the STBⁱⁿ and STB^{out} TO conditions. STB-1 represented a juvenile population undergoing a transition from CTB to STB gene expression. These nuclei may have recently incorporated into the syncytia and are actively undergoing differentiation at the time of sequencing. STB-2 expressed the VEGF receptor FLT1 and is enriched in genes involved in responding to oxygen levels and ER stress, suggesting STB-2 might play a role in responding to low oxygen levels. Finally, STB-3 exhibits an increase in GTPase signaling molecules and transporter proteins. Importantly, culture conditions changed the relative enrichment of these subtypes. Whereas STBⁱⁿ TOs were enriched for the juvenile STB-1 and oxygen sensing STB-2 populations, STB^{out} TOs exhibited an increase in the transport/GTPase STB-3 subtype. We hypothesize the STB-3 subtype is a terminal differentiation state as it appears at the end of the slingshot pseudotime trajectory. In fact, in addition to exhibiting a higher proportion of the STB-3 subtype, STB^{out} TOs express higher concentrations of key STB pregnancy hormones in all nuclear subtypes suggesting culturing in suspension may promote terminal STB differentiation. An alternative hypothesis, however, is that nuclei in STBⁱⁿ and STB^{out} TOs exhibit distinct lineages trajectories. Testing these hypotheses will require future experiments tracing nuclear subtypes through time.

Why might changing culture conditions influence the distribution and gene expression of STB nuclear subtypes? We hypothesize growth in either extracellular matrix (STBⁱⁿ) or suspension (STB^{out}) impacts both the cell orientation and environmental cues each cell type is exposed to like oxygen concentrations, cell and membrane tension, and media flow dynamics. Future studies dissecting each environmental cue and the differentiation of STB nuclei through time and space will help elucidate the molecular mechanism driving each STB subtype. Of note, these three STB subtypes are remarkably like those recently defined in first trimester and full-term tissue: a juvenile population, a FLT1 expressing population enriched in genes involved in oxygen sensing, and a PAPP-A positive population that expresses GTPase signaling molecules and hormones (Wang et al., 2024 [\[link\]](#)). The distribution of STB subtypes defined in Wang et al (2024) [\[link\]](#) also changed throughout gestation with the oxygen sensing STB nuclear subtype increased in the first trimester and the GTPase/hormone subtype increased at term and implies the environmental cues the STB experiences throughout gestation may affect STB subtype distribution *in vivo* similar to what is seen in TOs. This resemblance suggests TOs can recapitulate similar nucleus subtypes as those seen *in vivo*, indicating their strength as an experimental model and highlights the importance of dissecting the environmental cues that contribute to each STB subtype *in vitro*.

How might these distinct nuclear transcriptional identities affect the function of the STB? Despite the STB being one large cytoplasm where molecules can freely mix by diffusion, it has long been suggested to contain distinct cytoplasmic zones that specialize in different functions (Benirschke et al., 2012 [\[link\]](#); Burgos and Rodríguez, 1966 [\[link\]](#); Burton, 1990 [\[link\]](#)). For example, STB cytoplasmic regions adjacent to the fetal vasculature dramatically thin presumably to facilitate diffusional exchange of gas and nutrients and express angiogenic promoting proteins. In contrast, regions where hormones are produced have dense packing of membranous organelles and express protein trafficking molecules (Baczyk et al., 2004 [\[link\]](#); Beck et al., 1986 [\[link\]](#); Burton and Jauniaux, 1995 [\[link\]](#); Clark et al., 1998 [\[link\]](#); Hempstock et al., 2003 [\[link\]](#); Jauniaux et al., 2003 [\[link\]](#); Khaliq et al., 2009 [\[link\]](#); Morrish and Marusyk, 1997 [\[link\]](#); Mouzon, 1997 [\[link\]](#); Sharkey et al., 1993 [\[link\]](#)). One potential mechanism for creating these distinct cytoplasmic zones is nuclear specialization, whereby individual nucleus identities might be spatially localized to different cytoplasmic regions, as has been demonstrated for nuclei in syncytial muscle fibers (Kim et al., 2020 [\[link\]](#), Petrany et al., 2020). Different nuclear identities likely arise from a combination of differentiation pathways and environmental cues. The generation of an organoid cell culture model that recapitulates the STB nuclear identities seen *in vivo* is a critical advancement towards deciphering the mechanisms that allow the giant STB cell to effectively carry out its many essential functions.

To dissect the transcription factors that drive STB differentiation we applied gene regulatory analysis and identified multiple TF/CR modules potentially involved in differentiation of STB in full-term tissue and TOs. Interestingly, the module most associated with STBⁱⁿ included the TF CEBPB, which is important for placenta development in mice and was recently found to be involved in STB differentiation in first trimester but not in full-term tissue (Bégay et al., 2004 [\[link\]](#); Wang et al., 2024 [\[link\]](#)). Consistent with these results, we found that the TF CEBPB was only associated with a few genes in our independent full-term tissue dataset, suggesting that STB of TOs might employ transcriptional programs characteristic of the first trimester. The final module includes the CR RYBP, which is involved in a non-canonical form of the PRC1 polycomb complex that ubiquitinylates histones and can modulate gene expression (Rose et al., 2016 [\[link\]](#); Silva et al., 2018 [\[link\]](#)). We demonstrate that RYBP exhibits STB-specific expression in TOs and tissue. We predicted that RYBP might mediate the differentiation of STB nuclei into specific nuclear subtypes. To test this idea, we deleted RYBP with CRISPR/Cas9 in TOs and observed an increase in many STB-2 nuclear subtype marker genes and a significant decrease in the human placenta lactogen gene. This suggests that RYBP acts to downregulate the STB-2 nuclear subtype in STBⁱⁿ TOs. Future work delving into the exact distribution of STB nuclear subtypes in STBⁱⁿ and STB^{out} TOs will enable us to dissect if RYBP also plays a role in activating the STB-3 subtype, or simply inhibiting the STB-2

subtype. Of note, RYBP deletion in mice is embryonic lethal due in part to a failure to form trophoctoderm and subsequent invasion defects (Pirity et al., 2005 [↗](#)), consistent with a possible role of RYBP on STB differentiation in human placenta.

While the global subtypes found in first trimester, full-term tissue, STBⁱⁿ TOs, and STB^{out} TOs were similar, many genes were differentially expressed in each STB population. One significant difference between the STB of full-term tissue and TOs was in hormone expression. It has long been appreciated that the STB differentially expresses hormones as a function of gestational age (Costa, 2016 [↗](#); Kumar and Magon, 2012 [↗](#)), but it was not known 1- how TOs mirror this expression and 2- how isolation of TOs from different stages of tissue gestation affected expression. We found that while the TOs used in this study were derived from full-term CTBs, the STB associated with these organoids express hormone transcripts classically associated with early gestation. This suggests that the cues that restrict these hormones to different gestational stages *in vivo* are not intrinsic to the isolated CTBs and can be studied in full-term derived TOs. Future work adding maternal cues to the TO system will help define how STB hormone levels are mechanistically modulated.

In conclusion, our study elucidates STB nucleus subtypes in TOs, tracks their proportions across culture conditions, and compares gene expression to STB *in vivo*. The fluctuating proportions of STB subtypes across

TO culture conditions imply that environmental cues can direct individual nuclei in the same cell into different identities. These findings underscore the power of TOs as an experimental model for studying the STB.

Methods

Tissue Processing for SC and SN sequencing

Placenta tissue was collected from patients undergoing scheduled C-sections at UNC Health consented under IRB 21-2055. Patient information, sequencing data, and tissue samples from these experiments was later transferred to Duke under the IRB Pro00113088. Immediately after placenta delivery a cotyledon from the center of the placenta was dissected. Samples from both decidua and villous tissue were snap frozen in liquid nitrogen for future processing within ten minutes of placenta delivery to minimize STB degradation. Additional samples were fixed in 10% buffered formalin for subsequent tissue paraffin embedding and slicing. The remaining villous/decidua tissue from the dissected cotyledon was then immediately processed into single cells, as described below. A list of the tissues used in this study is available in **Supplementary Table 1**.

SC processing

The cotyledon (decidua + villous, chorion removed) was chopped into fine pieces (<1mm) with a scalpel and washed with 1x PBS in cheese cloth until flow through was clear of blood. Tissue was placed into Trypsin media (1X PBS without calcium or magnesium, 0.2% Trypsin (Thermo 15090046), 0.53M EDTA) and incubated at 37°C for ten minutes in a shaking water bath. After incubation trypsin was inactivated with 100mLs of Wash Media (DMEM F12 media + 20% FBS). Supernatant was passed through a sterile cheese cloth, spun down, and resuspended to Resuspension Media (DMEM F12 media + 10% FBS). Remaining tissue was then placed into 25mL collagenase buffer (1mg/mL collagenase V (Sigma C9263) in Wash Media) and shaken at 37°C for ten minutes. Supernatant was passed through cheese cloth, spun down, and resuspended in Resuspension Media. Cell pellets from both digestion steps were combined and pelleted, resuspension media removed, and resuspended in 10mLs RBC lysis buffer (Thermo 00-4333-57)

and incubated at RT for 10min. Cells were passed through a 100µm filter, spun down, and passed through a Milltenyi Debris Removal Solution (130-109-398) gradient as per the manufacturer's instructions. The final pellet was then resuspended in 1X PBS + 0.04% BSA (Sigma A1595).

SN processing

Single nuclei were isolated with the 10X Chromium Nuclear Isolation Kit (CG000505) as per the User Guide with the following changes. 50mgs of frozen tissue was resuspended in lysis buffer on ice, dounced to homogenize, and incubated for a total of only 7 minutes from the resuspension step to centrifugation. In addition, while the initial centrifugation step in lysis buffer was performed at 500xg for 5 min to minimize time spent in lysis buffer, subsequent wash spins were done at 500xg for 10min to minimize loss. The final pellet was then resuspended in 1X PBS + 0.04% BSA (Sigma A1595) +10X Genomics supplied RNase inhibitor.

Organoid Culture

STBⁱⁿ, STB^{out}, and EVT^{enrich} TOs were derived, propagated, and differentiated as described previously (Yang et al., 2022 [eLife](#) and 2023). Briefly, STBⁱⁿ TOs were derived and maintained by sequential digestion of term placental chorionic villi with 0.2% trypsin-250 (Alfa Aesar, J63993), 0.02% EDTA (Sigma-Aldrich, E9884), and 1.0 mg/mL collagenase V (STEMCELL Technologies, 100-0681), followed by further mechanical disruption by pipetting. Pooled digests were washed with Advanced DMEM/F12 medium (Gibco 12634-010) and pelleted by centrifugation, then resuspended in ice-cold Matrigel (Corning 356231). Matrigel “domes” (40 µl/well) were plated into 24-well tissue culture plates (Corning 3526) and overlaid with 500 µL prewarmed term trophoblast organoid medium (tTOM) (**Supplementary Table 3**). Cultures were maintained in 37°C humidified incubator with 5% CO₂. Medium was renewed every 2-3 days. To generate STB^{out} TOs, mature STBⁱⁿ TOs were released from Matrigel domes with cell recovery solution (Corning, 354253) on ice for 30-60min, pelleted, washed one time with cold basal media (Advanced DMEM/F12 + 1% P/S + 1% L-glutamine + 1% HEPES), and then resuspended in pre-warmed tTOM supplemented with 5 µM Y-27632, and transferred into an ultra-low attachment 24-well plate (Corning 3473) for suspension culture at 37°C and 5% CO₂ for 48 hours. To generate EVT^{enrich} TOs, established STBⁱⁿ TOs were passaged into new Matrigel “domes” as described above and previously (Yang et al., 2022 [eLife](#)), and maintained in tTOM for ~ 5 days prior to switching to EVT differentiation media 1 (EVT m1 recipe: **Supplementary Table 4**) for 9 days culture, then replaced with EVT m2 with the same recipe as EVT m1, but lacking NRG1 for a further 3-4 days. A list of the TOs lines used in this study is available in **Supplementary Table 1**.

CRISPR/Cas9-mediated genes editing in TOs

To generate *RYBP* and *AFF1* knock out TOs line, two sets of sgRNA pairs for each gene with the target sites close to the 5 prime of genes ORF were selected from a pre-designed sgRNA database established by Synthego (<https://design.synthego.com/#/>), then each sgRNA pair (dual-sgRNAs) were cloned into individual cassettes on Lentiviral transfer plasmid with Cas9 ORF (Dual-gRNA lentivirus CRISPR vector). CRISPR lentivirus was produced in 293T cells by the transient transfection of the combination of above genes specific transfer plasmids, psPAX2 (packaging plasmids) and pMD2.G (envelop plasmids) at the ratio 2:1:1 following the standard lentivirus packaging protocols described previously ¹. Using the prepared lentivirus, we transduced the fully dissociated TO single-cell suspension overnight in an ultra-low attachment culture vessel. The cells were then replated into fresh Matrigel domes for further culture and recovered for recovery 2-4 days prior to puromycin selection (2 µg/ml) for ~4 days. Single organoid unit picking and dissociation for clonal expansion were performed after an additional two weeks of growth. Genomic DNA was purified from each individual single- organoid clone for sequencing validation. High-fidelity PCR was conducted on the predicted target region of the gRNAs using the primers listed in Supplemental Table 6. The purified PCR products were then subjected to Sanger sequencing and analyzed by comparing them to sequences from scramble gRNA-mediated single-

organoid clones. Immunofluorescent staining was performed to further validate knockout single-organoid clones. The sequences of the gRNAs used in this study can be found in Supplemental Table 5.

Organoid Processing for SC and SN sequencing

STBⁱⁿ TOs were processed into both single cells and nuclei for SC/SN sequencing while STB^{out} and EVT^{enrich} TOs were processed into single nuclei for SN sequencing via the following protocols.

SC processing

STBⁱⁿ TOs were dissociated by scraping Matrigel domes into 1 mL of pre-warmed TrypLE Express (Invitrogen, 12605036) and incubating at 37°C for 12 min, swirling the tube every 2-3 min. Dissociated organoids were pelleted at 1250 rpm for 3 min and re-suspended in 200 µL DMEM containing 10% FBS. Resuspended organoids were subjected to vigorous manual disruption using a single channel p200 pipette (Ranin, 17008652) for 3 min followed by the addition of 800 µL of DMEM containing 10% FBS. The disrupted suspension was then passed over a 40 µm filter cell strainer (Corning, 352098). Flow through was then centrifuged at 1250 rpm for 5min and the pellet resuspended in 250 µL of 1x PBS for a final volume of ~300 µL and cell counts of ~1 x 10⁶ cells/mL.

SN processing

TOs from each condition (STBⁱⁿ, STB^{out}, EVT^{enrich}) were harvested by scraping with a wide bore pipette, centrifuged to pellet (600xg for 6min), resuspended in 100µLs TrypLE (Thermo Fisher, 12605010), and incubated at 37°C for 10min. After incubation each sample was pipetted 100x with a P200 pipette to dissociate cells and placed on ice. Single nuclei were then isolated with the 10x Chromium Nuclear Isolation Kit (CG000505) as per the User Guide with the following changes. 500µLs of Lysis buffer was added to the TO/TrypLE solution and transferred to a 2mL Kimble Dounce (Millipore Sigma, D8938) on ice, dounced 10x, and subsequently incubated on ice for a total of 10 minutes. Remaining steps were performed as suggested, except for final wash step spins were performed at 500xg for 10min to minimize nuclei loss. The final pellet was then resuspended in 10mLs of 1X PBS + 0.04% BSA (Sigma A1595) +10X Genomiocs supplied RNase inhibitor, nuclei counted, and 10,000 nuclei run in each well of a chromium controller.

10X Genomics library generation, sequencing, and data analysis

SC suspensions were stained with Trypan Blue and counted to obtain live/dead cell ratios while SN were stained with Ethidium Homodimer-1 (Thermo E1169) and counted with a hemocytometer on a fluorescent microscope. 10,000 SC/SN of each sample type were loaded into individual chip wells and run on a 10x chromium controller with the Chromium Single Cell 3' Reagent Reagent Kit v3.1 (Dual Index) following the manufacturers protocol. Tissue libraries were then sequenced on an Illumina NovaSeq S2 at a targeted sequencing depth of 100,000 reads/cell or nucleus while organoid libraries were sequenced on an Illumina NovaSeq S4 at a targeted sequencing depth of 74,000 reads/cell or nucleus. Cell Ranger was then used to align reads to the human genome (GRCh38) and create a counts matrix.

SC and SN Data Analysis

Post-processing, quality control, and read alignment to the hg38 human reference genome were performed using 10x CellRanger package (v6.1.2, 10x Genomics). Gene expression matrices generated by the 10x CellRanger aggregate option were analyzed using Seurat (version 4.0) in R²⁻⁵. For SC, cells with at least 200 and no more than 10,000 unique expressed genes were included in downstream analysis, and cells with more than 25% mitochondrial reads were excluded from analysis. For SN, nuclei with at least 800 and no more than 10,000 unique features and less than 60,000 counts were included in downstream analysis, and nuclei with more than 7.5%

mitochondrial reads and 3% ribosomal reads were excluded from analysis. To eliminate batch effects, datasets from unique donors were normalized using the *sctransform* function (version 2) and integrated using the *FindIntegrationAnchors()* in Seurat (version 4.0) in R⁵. Variables regressed included nFeatures, nCounts, percent mitochondria and ribosomes, and X- and Y-linked genes to avoid sex-associated differences. Dimensional reduction was performed using the *RunPCA()* function to obtain the first 40 principal components, which was determined using *ElbowPlots()* across the first 50 dimensions. To identify clusters, Louvain clustering (Seurat *FindClusters()* function) was performed, and optimal resolution was determined using the *clustree()* function⁶ on a range of resolutions between 0.2-1.0, with 0.6 selected for TOs and 0.3 selected for tissue. To identify clusters enriched in combined files of TOs and tissue, dataset integration was performed using Harmony and Louvain clustering (Seurat) performed at a 0.3 resolution, as optimized using *clustree()*⁶. Differential expression analysis between clusters was performed using the Wilcoxon rank sum test (Seurat) using *FindAllMarkers()*, with genes with a log2 fold change threshold set to 0.25 and FDR- adjusted p-value < 0.05 considered significant. Pseudobulk differential expression analysis was performed using DESeq2 (Love et al.). GO term enrichment was performed with clusterProfiler using *compareCluster*.

Pseudotime

The *slingshot* package (version 2.6.0) in R was used to determine differentiation trajectories from clusters identified in Seurat with unbiased starting and ending roots⁷. The raw counts and above-generated slingshot object were used to run *evaluateK()* with the total number of knots ranging from 3 to 9. The optimal number of knots was determined to be 5. The *fitGAM()* function using *tradeSeq* (1.5.10) was run with this resulting value and gene expression along lineages identified using the *associationTest()* function⁸. Heatmaps of expression changes across lineages were generated using *ComplexHeatmap()* on log transformed counts and rasterized using the *ImageMagick* “Bessel” filter⁹. The *plotGenePseudotime()* function was used to visualize raw count gene expression in individual cells across lineages from the *slingshot* object.

RNA Velocity and Velorama

RNA velocity was performed via the python based program scVelo on snRNAseq data from both placenta tissues of three patients and organoids from three patient-derived TO cell lines (Bergen et al., 2020¹⁰). The organoids were further divided into STBⁱⁿ and STB^{out} subcategories, resulting in nine total datasets in our analysis. First, we generated UMAPs for each data source (full-term tissue, STBⁱⁿ, and STB^{out}) with corresponding trajectory vectors. For the TOs we included only the CTB, CTB-pf, and STB nucleus types in the analysis. For each data source, the sample datasets were integrated with Scanorama to eliminate dataset-specific batch effects for transcript counts as well as spliced and unspliced transcripts (Hie et al., 2018¹¹). The samples were then merged and UMAPs with trajectory vectors were created using the scVelo library. Then, we employed Geosketch to downsample the cell population represented in the UMAP while preserving transcriptomic heterogeneity (Hie et al., 2019¹²). This was done to enhance the clarity and distinction of cells on the UMAP. Second, we inferred gene regulatory networks with Velorama (Singh et al., 2024¹³). We compiled lists of human transcription factors (TFs) and genes coding for chromatin remodelers (CRs) from sources like <https://www.factorbook.org/tf/human>¹⁴ to use as regulatory genes, along with a selection of highly variable genes and genes of interest from the tissue and organoid datasets to use as our target genes. Velorama was then used to infer the gene regulatory networks under default settings and produced a total of nine regulatory-target gene interaction matrices for tissues and organoids raw data sets. Each interaction matrix provides scores that highlight the strength of the relationship between specific regulatory-target gene pairs. We then ranked the regulatory-target gene pairs by their interaction strength and identified top TFs in each sample, after filtering TFs by number of target genes among the top 500 pairs. Heatmaps were then created based on the overlap of target genes with a score of 1 representing

total overlap and a score of 0 indicating no overlap. Finally, top target genes in each cluster were found via sorting by interaction score and plotted as a network analysis with the R package igraph, with the width of the arrow representing the Velorama interaction strength score.

RNA extraction and bulk RNA-seq analysis

Total RNA from TOs was purified with the Sigma GenElute Universal total RNA purification kit (Sigma-Aldrich, RNB100) following manufacturer's instruction. Purified Total RNA concentration and quality was determined by Thermo scientific Nanodrop one. All RNA samples submitted for bulk RNA-seq were further run for QC evaluation for their RQN (RNA quality number, 10 for all samples) prior to library preparation by the Duke Sequencing and Genomic Technologies (SGT) using KAPA HyperPrep kit (Roche). Sequencing was performed on the NextSeq 1000 XLEAP using P2 flow cell. The reads were aligned to the human reference genome (GRCh38) using QIAGEN CLC Genomics (v20). Differential expression analysis was performed using the DESeq2 package in R with significance cutoff as 0.05 and fold change cutoff at $\log_2 \pm 2^{10}$. Files associated with bulk RNA-seq studies have been deposited into Sequence Read Archive (GSE288650). Volcano plots were generated using the EnhancedVolcano package in R¹¹ or in Graphpad Prism version 9.

Immunofluorescence in placenta tissue

FFPE tissue sections derived from the same patients sequenced in **Figure 1** [\[link\]](#) were removed from paraffin and rehydrated via an iteration through the following 3min wash steps (Xylene, Xylene, 1:1 Xylene:100% EtOH, 100% EtOH, 100% EtOH, 95% EtOH, 70% EtOH, 50% EtOH). Slides were then placed under running tap water for 5 minutes to re-hydrate then transferred to sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH6) and placed into boiling water for 20min for antigen revival. Slides were then placed under running tap water for 10minutes then transferred into PBS. To perform IF, tissue was permeabilized with 0.5% Triton X- 100 in 1X PBS for 20min at RT then washed 2x in 1X PBS. Red blood cell autofluorescence was quenched with TrueBlack Lipofuscin Autofluorescence Quencher (Biotium #2300) as per the manufacturer's instructions then washed 2x in 1X PBS. Tissue slides were then blocked in blocking buffer (1% BSA in 1X PBS) for 1 hour at RT. Primary antibodies were then incubated overnight at 4°C in 0.1% BSA in 1x PBS in the following dilutions: 1:1000 RYBP (Millipore Sigma HPA053357), 1:500 Cytokeratin-7 (Millipore Sigma MABT1490), and 1:200 E- cadherin (BD Biosciences 610181). Slides were rinsed 2x in 1X PBS, and incubated in secondary antibodies for 1 hour at 37°C (Invitrogen A-21247, A-11011, A-11001). Slides were rinsed 2x in 1X PBS, incubated in 1 µg/ml Hoechst in 1X PBS for ten minutes at RT, and finally washed 1x in 1X PBS. PBS was then removed and slides mounted with ProLong Diamond Antifade (Thermo P36965) and dried overnight prior to imaging. Slides were imaged on a Nikon Ti-E stand equipped with a Yokogawa CSU-W1 spinning disk confocal unit with a 40x Nikon Silicone objective and illuminated with 405/488/565/646 laser sources.

Cryosectioning and immunofluorescence staining of TOs

TOs were collected in microcentrifuges tubes pre-coated with regular FBS, then rinsed once with 1X PBS prior to fixing in 4% PFA at RT for 2 h. Pelleted TOs were washed twice with 1X PBS and resuspended in 1X PBS with 0.5 mL 20% (wt/vol) sucrose solution per sample, then transferred to 4°C overnight to allow all the organoid units to pellet into the bottom of sucrose solution. On the following day, 7.5% gelatin (wt/vol)/10% (wt/vol) sucrose embedding solution was pre-warmed at 37°C for 30 min, then organoid units were isolated from sucrose solution into small size molds (7×7×5 mm) and polymerize with embedding solution at 4 °C for 20 min before transferring into -80 °C for at least 3 hrs prior to cryosectioning.

To section above prepared organoid frozen blocks, the blocks were transferred from -80°C into the cryosection machine (Leica CM1950 Cryostat) at -20°C to equilibrate for 15 min, then 10 µm thickness sections cut. The cryosections were incubated at RT for 15 min before incubation in permeabilization buffer (0.5% <vol/vol> Triton X-100 in 1X PBS with or without 5% <wt/vol> goat

serum) for 45 min at RT. Cryosections were washed and blocked in 5%(v/v) goat serum or BSA/0.1%(v/v) Tween-20 in 1X PBS for 15 min at RT and then incubated with rabbit anti-human RYBP polyclonal antibody (Sigma, HPA053357), mouse anti-human hCG- β antibody (abcam, ab9582), rat anti-human E-cadherin/cadherin (Thermo Fisher 14-3249-82) diluted in above-described blocking solution at 4°C overnight. The following day cryosections were washed with 1X PBS and then incubated for 1 h at RT with Multi-rAb CoraLite Plus 488 Goat anti-mouse (Proteintech, RGAM002), Multi-rAb CoraLite Plus 594 Goat anti-rabbit (Proteintech, RGAR004), and either Donkey Anti-Rat IgG H&L (Alexa Fluor® 647) preadsorbed (Abcam ab150155) recombinant secondary antibodies or Alexa Fluor 647–conjugated phalloidin (Invitrogen, A22287). Cryosections were washed again with 1X PBS and mounted in Vectashield (Vector Laboratories, H-1200) containing 4',6-diamidino-2-phenylindole (DAPI). Images were captured using a Olympus Fluoview FV3000 inverted confocal microscope or a Nikon-Yokogawa CSU-W1 spinning disk confocal and contrast-adjusted in Photoshop or Fiji.

Cryosectioning and RNA fluorescence in situ hybridization (FISH) of TOs

TOs were collected in microcentrifuge tubes pre-coated with 1 mg/mL BSA, rinsed once with 1X PBS, and fixed in 4% PFA at RT for 30 minutes. After fixation, TOs were pelleted, washed twice with 1X PBS, and embedded in OCT within a cryomold. The embedded samples were then flash-frozen in 2-methylbutane and stored at -80°C. Cryosections (10 μ m thick) were cut using a cryostat and transferred onto microscope slides (Thermo Fisher 12-550-15). To remove OCT, sections were washed three times with 1X PBS, followed by permeabilization in 0.5% Triton X-100 in 1X PBS for 45 minutes at RT. After two additional PBS washes, sections were incubated for 30 minutes in 30% formamide wash buffer (30% vol/vol formamide in 2X saline-sodium citrate (SCC) buffer). For primary probe hybridization, TO sections were incubated overnight at 37°C in 30% hybridization buffer (30% (vol/vol) formamide, 1 mg/mL yeast tRNA, and 10% (wt/vol) dextran sulfate in 2X SCC buffer) with 1 μ M primary FISH probes targeting either PAPP2 or ADAMTS6 (**Supplemental Table 7**). The next day, sections were washed twice with 30% formamide wash buffer at 47°C for 30 minutes, followed by a 5-minute incubation in 10% formamide wash buffer (10% vol/vol formamide in 2X SCC buffer). Next, TO sections were incubated for 1 hour at 37°C with 5 nM of each secondary probe (bit1_5pCy5, bit11_5pCy5, bit12_5pCy5) in 10% hybridization buffer (**Supplemental Table 7**). Following secondary probe hybridization, sections were washed three times in 10% formamide wash buffer for 5 minutes each at room temperature. During the second wash, nuclei were counterstained with 1 μ g/mL DAPI or Hoechst diluted in 10% hybridization buffer. Finally, TO sections were mounted in Prolong Diamond Antifade (Thermo Fisher P36970) and imaged using a Nikon Ti-E stand equipped with a Yokogawa CSU-W1 spinning disk confocal unit. Imaging was performed with a 100 $\times$ Nikon silicone objective under 405/646 laser illumination.

Data Availability

The datasets analyzed in this paper can be accessed on GEO with the accession number GSE288650. In addition, the processed datasets can be interactively visualized online at <https://gladfelterlab.shinyapps.io/PlacentaRNAsequencing/>.

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Additional files

SupplementalTable_Figure3B [↗](#)

SupplementalTable_Figure3C [↗](#)

SupplementalTable_Figure3H [↗](#)

SupplementalTable_Figure5E [↗](#)

SupplementalTable_Figure6A [↗](#)

SupplementalTable_Figure6E [↗](#)

SupplementalTable_Figure6F [↗](#)

SupplementalTable7 [↗](#)

Supplemental Figures [↗](#)

References

- Aghababaei M., Hogg K., Perdu S., Robinson W.P., Beristain A.G (2015) **ADAM12-directed ectodomain shedding of E-cadherin potentiates trophoblast fusion** *Cell Death Differ* **22**:1970–1984 <https://doi.org/10.1038/cdd.2015.44>
- Arutyunyan A., Roberts K., Troulé K., Wong F.C.K., Sheridan M.A., Kats I., Garcia-Alonso L., Velten B., Hoo R., Ruiz-Morales E.R., Sancho-Serra C., Shilts J., Handfield L.-F., Marconato L., Tuck E., Gardner L., Mazzeo C.I., Li Q., Kelava I., Wright G.J., Prigmore E., Teichmann S.A., Bayraktar O.A., Moffett A., Stegle O., Turco M.Y., Vento-Tormo R (2023) **Spatial multiomics map of trophoblast development in early pregnancy** *Nature* **616**:143–151 <https://doi.org/10.1038/s41586-023-05869-0>
- Aye I.L.M.H., Aiken C.E., Charnock-Jones D.S., Smith G.C.S (2022) **Placental energy metabolism in health and disease—significance of development and implications for preeclampsia** *Am. J. Obstet. Gynecol* **226**:S928–S944 <https://doi.org/10.1016/j.ajog.2020.11.005>
- Baczyk D., Satkunaratnam A., Nait-Oumesmar B., Huppertz B., Cross J.C., Kingdom J.C.P (2004) **Complex Patterns of GCM1 mRNA and Protein in Villous and Extravillous Trophoblast Cells of the Human Placenta** *Placenta* **25**:553–559 <https://doi.org/10.1016/j.placenta.2003.12.004>
- Bai X., Lenhart K.C., Bird K.E., Suen A.A., Rojas M., Kakoki M., Li F., Smithies O., Mack C.P., Taylor J.M (2013) **The smooth muscle-selective RhoGAP GRAF3 is a critical regulator of vascular tone and hypertension** *Nat. Commun* **4**:2910–2910 <https://doi.org/10.1038/ncomms3910>
- Barker S.B., Cumming G., Horsfield K (1973) **Quantitative morphometry of the branching structure of trees** *J. Theor. Biol* **40**:33–43 [https://doi.org/10.1016/0022-5193\(73\)90163-x](https://doi.org/10.1016/0022-5193(73)90163-x)
- Barrios V., Chowen J.A., Martín-Rivada Á., Guerra-Cantera S., Pozo J., Yakar S., Rosenfeld R.G., Pérez-Jurado L.A., Suárez J., Argente J (2021) **Pregnancy-Associated Plasma Protein (PAPP)-A2 in Physiology and Disease** *Cells* **10**:3576 <https://doi.org/10.3390/cells10123576>
- Beck T., Schweikhart G., Stolz E (1986) **Immunohistochemical location of HPL, SP1 and β -HCG in normal placentas of varying gestational age** *Arch Gynecol* **239**:63–74 <https://doi.org/10.1007/bf02133965>
- Bégay V., Smink J., Leutz A (2004) **Essential Requirement of CCAAT/Enhancer Binding Proteins in Embryogenesis** *Mol. Cell. Biol* **24**:9744–9751 <https://doi.org/10.1128/mcb.24.22.9744-9751.2004>
- Benirschke K., Burton G.J., Baergen R.N (2012) **Multiple Pregnancies** In: *Pathology of the Human Placenta* pp. 761–880 https://doi.org/10.1007/978-3-642-23941-0_25
- Bergen V., Lange M., Peidli S., Wolf F.A., Theis F.J (2020) **Generalizing RNA velocity to transient cell states through dynamical modeling** *Nat. Biotechnol* **38**:1408–1414 <https://doi.org/10.1038/s41587-020-0591-3>
- Boyd J.D., Hamilton W.J (1970) **The Human Placenta** <https://doi.org/10.1007/978-1-349-02807-8>

Burgos M.H., Rodríguez E.M (1966) **Specialized zones in the trophoblast of the human term placenta** *Am J Obstet Gynecol* **96**:342–356 [https://doi.org/10.1016/0002-9378\(66\)90237-7](https://doi.org/10.1016/0002-9378(66)90237-7)

Burton G.J (1990) **On the varied appearances of the human placental villous surface visualised by scanning electron microscopy** *Scanning Microscopy* **4**:501–7

Burton G.J., Jauniaux E (1995) **Sonographic, stereological and Doppler flow velocimetric assessments of placental maturity** *Bjog Int J Obstetrics Gynaecol* **102**:818–825 <https://doi.org/10.1111/j.1471-0528.1995.tb10849.x>

Butler A., Hoffman P., Smibert P., Papalexi E., Satija R (2018) **Integrating single-cell transcriptomic data across different conditions, technologies, and species** *Nat. Biotechnol* **36**:411–420 <https://doi.org/10.1038/nbt.4096>

Cain S.A., Mularczyk E.J., Singh M., Massam-Wu T., Kielty C.M (2016) **ADAMTS-10 and -6 differentially regulate cell-cell junctions and focal adhesions** *Sci. Rep* **6**:35956 <https://doi.org/10.1038/srep35956>

Cain S.A., Woods S., Singh M., Kimber S.J., Baldock C (2022) **ADAMTS6 cleaves the large latent TGFβ complex and increases the mechanotension of cells to activate TGFβ** *Matrix Biol* **114**:18–34 <https://doi.org/10.1016/j.matbio.2022.11.001>

Chen Y., Siriwardena D., Penfold C., Pavlinek A., Boroviak T.E (2022) **An integrated atlas of human placental development delineates essential regulators of trophoblast stem cells** *Dev. (Camb., Engl.)* **149**:dev200171 <https://doi.org/10.1242/dev.200171>

Ching Y.-P., Wong C.-M., Chan S.-F., Leung T.H.-Y., Ng D.C.-H., Jin D.-Y., Ng I.O. (2003) **Deleted in Liver Cancer (DLC) 2 Encodes a RhoGAP Protein with Growth Suppressor Function and Is Underexpressed in Hepatocellular Carcinoma*** *J. Biol. Chem* **278**:10824–10830 <https://doi.org/10.1074/jbc.m208310200>

Clark D., Smith S., Licence D., Evans A., Charnock-Jones D (1998) **Comparison of expression patterns for placenta growth factor, vascular endothelial growth factor (VEGF), VEGF-B and VEGF-C in the human placenta throughout gestation** *J Endocrinol* **159**:459–467 <https://doi.org/10.1677/joe.0.1590459>

Costa M.A (2016) **The endocrine function of human placenta: an overview** *Reprod Biomed Online* **32**:14–43 <https://doi.org/10.1016/j.rbmo.2015.10.005>

Derisoud E., Jiang H., Zhao A., Chavatte-Palmer P., Deng Q (2024) **Revealing the molecular landscape of human placenta: a systematic review and meta-analysis of single-cell RNA sequencing studies** *Hum. Reprod. Updat. dmae* **006** <https://doi.org/10.1093/humupd/dmae006>

Foxler D.E., Bridge K.S., Foster J.G., Grevitt P., Curry S., Shah K.M., Davidson K.M., Nagano A., Gadaleta E., Rhys H.I., Kennedy P.T., Hermida M.A., Chang T., Shaw P.E., Reynolds L.E., McKay T.R., Wang H., Ribeiro P.S., Plevin M.J., Lagos D., Lemoine N.R., Rajan P., Graham T.A., Chelala C., Hodivala-Dilke K.M., Spendlove I., Sharp T.V (2018) **A HIF-LIMD1 negative feedback mechanism mitigates the pro-tumorigenic effects of hypoxia** *EMBO Mol. Med* **10**:e8304 <https://doi.org/10.15252/emmm.201708304>

Haeussner E., Buehlmeier A., Schmitz C., Koch F.E. von, Frank H.-G. (2014) **Novel 3D Microscopic Analysis of Human Placental Villous Trees Reveals Unexpected Significance of Branching Angles** *Sci. Rep* **4**:6192 <https://doi.org/10.1038/srep06192>

- Haider S., Meinhardt G., Saleh L., Kunihs V., Gamperl M., Kaindl U., Ellinger A., Burkard T.R., Fiala C., Pollheimer J., Mendjan S., Latos P.A., Knöfler M (2018) **Self-Renewing Trophoblast Organoids Recapitulate the Developmental Program of the Early Human Placenta** *Stem Cell Rep* **11**:537–551 <https://doi.org/10.1016/j.stemcr.2018.07.004>
- Hao Y., Hao S., Andersen-Nissen E., Mauck W.M., Zheng S., Butler A., Lee M.J., Wilk A.J., Darby C., Zager M., Hoffman P., Stoeckius M., Papalexi E., Mimitou E.P., Jain J., Srivastava A., Stuart T., Fleming L.M., Yeung B., Rogers A.J., McElrath J.M., Blish C.A., Gottardo R., Smibert P., Satija R (2021) **Integrated analysis of multimodal single-cell data** *Cell* **184**:3573–3587 <https://doi.org/10.1016/j.cell.2021.04.048>
- Hatterschide J., Natale C.A., Ridky T.W., White E.A (2023) **Monitoring cell fate in 3D organotypic human squamous epithelial cultures** *STAR Protoc* **4**:102101 <https://doi.org/10.1016/j.xpro.2023.102101>
- Hemberger M., Hanna C.W., Dean W (2020) **Mechanisms of early placental development in mouse and humans** *Nat. Rev. Genet* **21**:27–43 <https://doi.org/10.1038/s41576-019-0169-4>
- Hempstock J., Bao Y.-P., Bar-Issac M., Segaren N., Watson A.L., Charnock-Jones D.S., Jauniaux E., Burton G.J (2003) **Intralobular Differences in Antioxidant Enzyme Expression and Activity Reflect the Pattern of Maternal Arterial Bloodflow Within the Human Placenta** *Placenta* **24**:517–523 <https://doi.org/10.1053/plac.2002.0955>
- Hie B., Bryson B., Berger B (2018) **Efficient integration of heterogeneous single-cell transcriptomes using Scanorama** *Nat. Biotechnol* **37**:685–691 <https://doi.org/10.1038/s41587-019-0113-3>
- Hie B., Cho H., DeMeo B., Bryson B., Berger B (2019) **Geometric Sketching Compactly Summarizes the Single-Cell Transcriptomic Landscape** *Cell Syst* **8**:483–493 <https://doi.org/10.1016/j.cels.2019.05.003>
- ImageMagick (2023) **ImageMagick Studio LLC** <https://imagemagick.org>
- Jauniaux E., Hempstock J., Greenwold N., Burton G.J (2003) **Trophoblastic Oxidative Stress in Relation to Temporal and Regional Differences in Maternal Placental Blood Flow in Normal and Abnormal Early Pregnancies** *Am J Pathology* **162**:115–125 [https://doi.org/10.1016/s0002-9440\(10\)63803-5](https://doi.org/10.1016/s0002-9440(10)63803-5)
- Khaliq A., Li X.F., Shams M., Sisi P., Acevedo C.A., Whittle M.J., Weigh H., Ahmed A. (2009) **Localisation of Placenta Growth Factor (PlGF) in Human Term Placenta** *Growth Factors* **13**:243–250 <https://doi.org/10.3109/08977199609003225>
- Kim M., Franke V., Brandt B., Lowenstein E.D., Schöwel V., Spuler S., Akalin A., Birchmeier C (2020) **Single-nucleus transcriptomics reveals functional compartmentalization in syncytial skeletal muscle cells** *Nat Commun* **11**:6375 <https://doi.org/10.1038/s41467-020-20064-9>
- Kumar P., Magon N (2012) **Hormones in pregnancy** *Niger. Méd. J. : J. Niger. Méd. Assoc* **53**:179–183 <https://doi.org/10.4103/0300-1652.107549>
- Li F., Long Y., Yu X., Tong Y., Gong L (2022) **Different Immunoregulation Roles of Activin A Compared With TGF-β** *Front. Immunol* **13**:921366 <https://doi.org/10.3389/fimmu.2022.921366>

- Li Q., Sharkey A., Sheridan M., Magistrati E., Arutyunyan A., Huhn O., Sancho-Serra C., Anderson H., McGovern N., Esposito L., Fernando R., Gardner L., Vento-Tormo R., Turco M.Y., Moffett A (2024) **Human uterine natural killer cells regulate differentiation of extravillous trophoblast early in pregnancy** *Cell Stem Cell* **31**:181–195 <https://doi.org/10.1016/j.stem.2023.12.013>
- Li X., Li Z.-H., Wang Y.-X., Liu T.-H (2023) **A comprehensive review of human trophoblast fusion models: recent developments and challenges** *Cell Death Discov* **9**:372 <https://doi.org/10.1038/s41420-023-01670-0>
- Li Y., Moretto-Zita M., Leon-Garcia S., 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 <https://doi.org/10.1016/j.ajpath.2014.08.006>
- Liu Y., Fan X., Wang R., Lu X., Dang Y.-L., Wang Huiying, Lin H.-Y., Zhu C., Ge H., Cross J.C., Wang Hongmei (2018) **Single-cell RNA-seq reveals the diversity of trophoblast subtypes and patterns of differentiation in the human placenta** *Cell Res* **28**:819–832 <https://doi.org/10.1038/s41422-018-0066-y>
- Love M.I., Huber W., Anders S (2014) **Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2** *Genome Biol* **15**:550 <https://doi.org/10.1186/s13059-014-0550-8>
- Manno G.L., Soldatov R., Zeisel A., Braun E., Hochgerner H., Petukhov V., Lidschreiber K., Kastri M.E., Lönnerberg P., Furlan A., Fan J., Borm L.E., Liu Z., Bruggen D. van, Guo J., He X., Barker R., Sundström E., Castelo-Branco G., Cramer P., Adameyko I., Linnarsson S., Kharchenko P.V. (2018) **RNA velocity of single cells** *Nature* **560**:494–498 <https://doi.org/10.1038/s41586-018-0414-6>
- Marsh B., Zhou Y., Kapidzic M., Fisher S., Billewicz R (2022) **Regionally distinct trophoblast regulate barrier function and invasion in the human placenta** *eLife* **11**:e78829 <https://doi.org/10.7554/elife.78829>
- Mayhew T.M (2014) **Turnover of human villous trophoblast in normal pregnancy: What do we know and what do we need to know?** *Placenta* **35**:229–240 <https://doi.org/10.1016/j.placenta.2014.01.011>
- Mayhew T.M., Simpson R.A (1994) **Quantitative evidence for the spatial dispersal of trophoblast nuclei in human placental villi during gestation** *Placenta* **15**:837–44 [https://doi.org/10.1016/s0143-4004\(05\)80185-7](https://doi.org/10.1016/s0143-4004(05)80185-7)
- Megli C.J., Coyne C.B (2021) **Infections at the maternal-fetal interface: an overview of pathogenesis and defence** *Nat Rev Microbiol* :1–16 <https://doi.org/10.1038/s41579-021-00610-y>
- Mizutani T., Orisaka M., Miyazaki Y., Morichika R., Uesaka M., Miyamoto K., Yoshida Y (2022) **Inhibition of YAP/TAZ-TEAD activity induces cytotrophoblast differentiation into syncytiotrophoblast in human trophoblast** *Mol. Hum. Reprod* **28**:gaac032 <https://doi.org/10.1093/molehr/gaac032>
- Moore T., Williams J.M., Becerra-Rodriguez M.A., Dunne M., Kammerer R., Dveksler G. (2021) **Pregnancy-specific glycoproteins: evolution, expression, functions and disease associations** *Reproduction* **163**:R11–R23 <https://doi.org/10.1530/rep-21-0390>

- Morrish D.W., Marusyk H (1997) **Localization of human chorionic gonadotropin and placental lactogen by immunogold labeling for electron microscopy: Technique and limitations** *Microsc Res Techniq* **38**:176–187 [https://doi.org/10.1002/\(sici\)1097-0029\(19970701/15\)38:1/2<176::aid-jemt18>3.0.co;2-m](https://doi.org/10.1002/(sici)1097-0029(19970701/15)38:1/2<176::aid-jemt18>3.0.co;2-m)
- Mouzon S.H (1997) **The GLUT3 Glucose Transporter Isoform Is Differentially Expressed within Human Placental Cell Types** *J Clin Endocr Metab* **82**:2689–2694 <https://doi.org/10.1210/jc.82.8.2689>
- Okae H., Toh H., Sato T., Hiura H., Takahashi S., Shirane K., Kabayama Y., Suyama M., Sasaki H., Arima T (2018) **Derivation of Human Trophoblast Stem Cells** *Cell Stem Cell* **22**:50–63 <https://doi.org/10.1016/j.stem.2017.11.004>
- Pique-Regi R., Romero R., Tarca A.L., Sandler E.D., Xu Y., Garcia-Flores V., Leng Y., Luca F., Hassan S.S., Gomez-Lopez N (2019) **Single cell transcriptional signatures of the human placenta in term and preterm parturition** *eLife* **8**:e52004 <https://doi.org/10.7554/elife.52004>
- Pirity M.K., Locker J., Schreiber-Agus N (2005) **Rybp/DEDAF Is Required for Early Postimplantation and for Central Nervous System Development** *Mol. Cell. Biol* **25**:7193–7202 <https://doi.org/10.1128/mcb.25.16.7193-7202.2005>
- Qu H., Khalil R.A (2022) **Role of ADAM and ADAMTS disintegrin and metalloproteinases in normal pregnancy and preeclampsia** *Biochem. Pharmacol* **206**:115266 <https://doi.org/10.1016/j.bcp.2022.115266>
- Rodriguez M.A. de la R., Kersten S. (2020) **Regulation of lipid droplet homeostasis by hypoxia inducible lipid droplet associated HILPDA** *Biochim. Biophys. Acta (BBA) - Mol. Cell Biol. Lipids* **1865**:158738 <https://doi.org/10.1016/j.bbalip.2020.158738>
- Rose N.R., King H.W., Blackledge N.P., Fursova N.A., Ember K.J., Fischer R., Kessler B.M., Klose R.J (2016) **RYBP stimulates PRC1 to shape chromatin-based communication between Polycomb repressive complexes** *eLife* **5**:e18591 <https://doi.org/10.7554/elife.18591>
- Rull K., Laan M. (2005) **Expression of β -subunit of HCG genes during normal and failed pregnancy** *Hum. Reprod* **20**:3360–3368 <https://doi.org/10.1093/humrep/dei261>
- Samaan N., Yen S.C.C., Friesen H., Pearson O.H. (1966) **Serum Placental Lactogen Levels During Pregnancy and in Trophoblastic Disease** *J. Clin. Endocrinol. Metab* **26**:1303–1308 <https://doi.org/10.1210/jcem-26-12-1303>
- Sasagawa T., Nagamatsu T., Morita K., Mimura N., Iriyama T., Fujii T., Shibuya M (2018) **HIF-2 α , but not HIF-1 α , mediates hypoxia-induced up-regulation of Flt-1 gene expression in placental trophoblasts** *Sci. Rep* **8**:17375 <https://doi.org/10.1038/s41598-018-35745-1>
- Sasagawa T., Nagamatsu T., Yanagisawa M., Fujii T., Shibuya M (2021) **Hypoxia-inducible factor-1 β is essential for upregulation of the hypoxia-induced FLT1 gene in placental trophoblasts** *Mol. Hum. Reprod* **27**:gaab065 <https://doi.org/10.1093/molehr/gaab065>
- Satija R., Farrell J.A., Gennert D., Schier A.F., Regev A (2015) **Spatial reconstruction of single-cell gene expression data** *Nat. Biotechnol* **33**:495–502 <https://doi.org/10.1038/nbt.3192>
- Shannon M.J., McNeill G.L., Koksai B., Baltayeva J., Wächter J., Castellana B., Peñaherrera M.S., Robinson W.P., Leung P.C.K., Beristain A.G (2024) **Single-cell assessment of primary and**

stem cell- derived human trophoblast organoids as placenta-modeling platforms *Dev. Cell* **59**:776–792 <https://doi.org/10.1016/j.devcel.2024.01.023>

Sharkey A.M., Charnock-Jones D.S., Boocock C.A., Brown K.D., Smith S.K (1993) **Expression of mRNA for vascular endothelial growth factor in human placenta** *Reproduction* **99**:609–615 <https://doi.org/10.1530/jrf.0.0990609>

Shibuya M (2011) **Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis** *Genes Cancer* **2**:1097–1105 <https://doi.org/10.1177/1947601911423031>

Silva C.J.S. da, Simón R., Busturia A (2018) **Epigenetic and non-epigenetic functions of the RYBP protein in development and disease** *Mech. Ageing Dev* **174**:111–120 <https://doi.org/10.1016/j.mad.2018.03.011>

Simpson R.A., Mayhew T.M., Barnes P.R (1992) **From 13 weeks to term, the trophoblast of human placenta grows by the continuous recruitment of new proliferative units: A study of nuclear number using the disector** *Placenta* **13**:501–512 [https://doi.org/10.1016/0143-4004\(92\)90055-x](https://doi.org/10.1016/0143-4004(92)90055-x)

Singh R., Wu A.P., Mudide A., Berger B (2024) **Causal gene regulatory analysis with RNA velocity reveals an interplay between slow and fast transcription factors** *Cell Syst* **15**:462–474 <https://doi.org/10.1016/j.cels.2024.04.005>

Street K., Risso D., Fletcher R.B., Das D., Ngai J., Yosef N., Purdom E., Dudoit S (2018) **Slingshot: cell lineage and pseudotime inference for single-cell transcriptomics** *BMC Genom* **19**:477 <https://doi.org/10.1186/s12864-018-4772-0>

Stuart T., Butler A., Hoffman P., Hafemeister C., Papalexi E., Mauck W.M., Hao Y., Stoeckius M., Smibert P., Satija R (2019) **Comprehensive Integration of Single-Cell Data** *Cell* **177**:1888–1902 <https://doi.org/10.1016/j.cell.2019.05.031>

Suryawanshi H., Morozov P., Straus A., Sahasrabudhe N., Max K.E.A., Garzia A., Kustagi M., Tuschl T., Williams Z (2018) **A single-cell survey of the human first-trimester placenta and decidua** *Sci Adv* **4**:eaau4788 <https://doi.org/10.1126/sciadv.aau4788>

Tuohey L., Macintire K., Ye L., Palmer K., Skubisz M., Tong S., Kaitu'u-Lino T.J (2013) **PLAC4 is upregulated in severe early onset preeclampsia and upregulated with syncytialisation but not hypoxia** *Placenta* **34**:256–260 <https://doi.org/10.1016/j.placenta.2012.12.009>

Turco M.Y., Gardner L., Kay R.G., Hamilton R.S., Prater M., Hollinshead M., McWhinnie A., Esposito L., Fernando R., Skelton H., Reimann F., Gribble F., Sharkey A., Marsh S.G.E., O'Rahilly S., Hemberger M., Burton G.J., Moffett A (2019) **Trophoblast organoids as a model for maternal-fetal interactions during human placental development** *Nature* **564**:263–267 <https://doi.org/10.1038/s41586-018-0753-3>

Turco M.Y., Moffett A (2019) **Development of the human placenta** *Development* **146**:dev163428 <https://doi.org/10.1242/dev.163428>

Van den Berge K., de Bezieux H Roux, Street K., Saelens W., Cannoodt R., Saeys Y., Dudoit S., Clement L (2020) **Trajectory-based differential expression analysis for single-cell sequencing data** *Nat Commun* **11**:1201 <https://doi.org/10.1038/s41467-020-14766-3>

- Vento-Tormo R., Efremova M., Botting R.A., Turco M.Y., Vento-Tormo M., Meyer K.B., Park J.-E., Stephenson E., Polański K., Goncalves A., Gardner L., Holmqvist S., Henriksson J., Zou A., Sharkey A.M., Millar B., Innes B., Wood L., Wilbrey-Clark A., Payne R.P., Ivarsson M.A., Lisgo S., Filby A., Rowitch D.H., Bulmer J.N., Wright G.J., Stubbington M.J.T., Haniffa M., Moffett A., Teichmann S.A (2018) **Single-cell reconstruction of the early maternal-fetal interface in humans** *Nature* **563**:347–353 <https://doi.org/10.1038/s41586-018-0698-6>
- Wang M., Liu Y., Sun R., Liu F., Li J., Yan L., Zhang J., Xie X., Li D., Wang Y., Li S., Zhu X., Li R., Lu F., Xiao Z., Wang H (2024) **Single-nucleus multi-omic profiling of human placental syncytiotrophoblasts identifies cellular trajectories during pregnancy** *Nat. Genet* **56**:294–305 <https://doi.org/10.1038/s41588-023-01647-w>
- Wooding P., Burton G (2008) **Comparative Placentation, Structures Functions and Evolution** <https://doi.org/10.1007/978-3-540-78797-6>
- Yang L., Liang P., Yang H., Coyne C.B (2023) **Trophoblast organoids with physiological polarity model placental structure and function** *J. Cell Sci* **137**:jcs261528 <https://doi.org/10.1242/jcs.261528>
- Yang L., Semmes E.C., Ovies C., Megli C., Permar S., Gilner J.B., Coyne C.B (2022) **Innate immune signaling in trophoblast and decidua organoids defines differential antiviral defenses at the maternal-fetal interface** *eLife* **11**:e79794 <https://doi.org/10.7554/elife.79794>
- Zappia L., Oshlack A (2018) **Clustering trees: a visualization for evaluating clusterings at multiple resolutions** *Gigascience* **7** <https://doi.org/10.1093/gigascience/giy083>
- Zeltz C., Gullberg D (2016) **The integrin–collagen connection – a glue for tissue repair?** *J. Cell Sci* **129**:1284–1284 <https://doi.org/10.1242/jcs.188672>
- Zhu Y.-N., Pan F., Gan X.-W., Liu Y., Wang W.-S., Sun K (2023) **The Role of DNMT1 and C/EBPα in the Regulation of CYP11A1 Expression During Syncytialization of Human Placental Trophoblasts** *Endocrinology* **165**:bqad195 <https://doi.org/10.1210/endocr/bqad195>

Author information

Madeline M Keenen

Duke University School of Medicine, Department of Cell Biology, Durham, United States

Liheng Yang

Duke University School of Medicine, Department of Integrative Immunobiology, Durham, United States

ORCID iD: [0000-0001-6842-086X](https://orcid.org/0000-0001-6842-086X)

Huan Liang

Duke University School of Medicine, Department of Cell Biology, Durham, United States, Duke University School of Medicine, Department of Biostatistics and Bioinformatics, Durham, United States

Veronica J Farmer

Duke University School of Medicine, Department of Cell Biology, Durham, United States

Rizban E Worota

Duke University School of Medicine, Department of Integrative Immunobiology, Durham, United States

Rohit Singh

Duke University School of Medicine, Department of Cell Biology, Durham, United States, Duke University School of Medicine, Department of Biostatistics and Bioinformatics, Durham, United States

Amy S Gladfelter

Duke University School of Medicine, Department of Cell Biology, Durham, United States

For correspondence: amy.gladfelter@duke.edu

Carolyn B Coyne

Duke University School of Medicine, Department of Integrative Immunobiology, Durham, United States, Duke Human Vaccine Institute, Durham, United States

ORCID iD: [0000-0002-1884-6309](https://orcid.org/0000-0002-1884-6309)

For correspondence: carolyn.coyne@duke.edu

Editors

Reviewing Editor

Han Zhu

University of Colorado Anschutz Medical Campus, Aurora, United States of America

Senior Editor

Adèle Marston

University of Edinburgh, Edinburgh, United Kingdom

Reviewer #1 (Public review):

Summary:

This study provides an in-depth analysis of syncytiotrophoblast (STB) gene expression at the single-nucleus (SN) and single-cell (SC) levels, using both primary human placental tissues and two trophoblast organoid (TO) models. The authors compare the older TO model, where STB forms internally (STBin), with a newer model where STB forms externally (STBout). Through a series of comparative analyses, the study highlights the necessity of using both SN and SC techniques to fully understand placental biology. The findings demonstrate that the STBout model shows more differentiated STBs with higher expression of canonical markers and hormones compared to STBin. Additionally, the study identifies both conserved and distinct gene expression profiles between the TO models and human placenta, offering valuable insights for researchers using TOs to study STB and CTB differentiation.

Strengths:

The study offers a comprehensive SC- and SN-based characterization of trophoblast organoid models, providing a thorough validation of these models against human placental tissues. By comparing the older STBin and newer STBout models, the authors effectively demonstrate the improvements in the latter, particularly in the differentiation and gene expression profiles of STBs. This work serves as a critical resource for researchers, offering a clear

delineation of the similarities and differences between TO-derived and primary STBs. The use of multiple advanced techniques, such as high-resolution sequencing and trajectory analysis, further enhances the study's contribution to the field.

Weaknesses were addressed during the revision.

The authors effectively addressed my critiques in the rebuttal letter and made corresponding changes in the manuscript. Specifically, they: 1) emphasized the importance of TO orientation in influencing STB nuclear subtype differentiation by adding text to the introduction; 2) clarified the differences in cluster numbers and names between primary tissue and TO data, explaining that each dataset was analyzed independently with separate clustering algorithms and adding clarifying text to the results section; 3) included additional rationale for using SN over SC sequencing, particularly for studying the multinucleated STB; 4) acknowledged that their original evidence was insufficient to definitively determine STBout nuclei differentiation status and removed language suggesting STB-3 as a terminally differentiated subtype, presenting alternative hypotheses in the discussion; and 5) incorporated new figures and clarifications, including RNA-FISH experiments, to validate subtype-specific marker gene expression. Overall, the authors' revisions strengthened the manuscript and aligned well with my critiques.

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<https://doi.org/10.7554/eLife.101170.2.sa2>

Reviewer #3 (Public review):

In this report, Keenen et al. present a thoroughly characterized platform for identifying potential molecular mechanisms regulating syncytiotrophoblast cell functions in placental biology. Application of single cell assessments to identify developmental trajectories of this lineage have been challenging due to the complex, multinucleated structure of the syncytium. The authors provide a comprehensive comparative assessment of term placental tissue and three independent trophoblast organoid models. They use single cell and single nucleus RNA sequencing followed by differential gene expression and pseudotime analyses to identify subpopulations and differentiation trajectories. They further compare the datasets generated in this study to publicly available datasets from first trimester placental tissue. The work is timely as optimization of trophoblast organoids is an evolving topic in placental research. And careful characterization of in vitro models has been noted as essential for model selection and result interpretation in the field.

The study elucidates syncytiotrophoblast nucleus subtypes and proportions in three different organoid models and compares subtypes and gene expression signatures to placental tissues. This work advances the field by demonstrating the utility of different trophoblast organoids to model syncytiotrophoblast differentiation. The in-depth characterization of cell types comprising the different organoid models and how they compare to placental tissue will help to inform model selection for future experimentation in the field. Defining cell composition and cell differentiation trajectories will also aid in data interpretation for data generated by these tissue and model sources. Overall, the conclusions presented in the manuscript are well supported by the data. The figures, as presented, are informative and striking.

The authors present outstanding progress toward their overall aim of identifying, "the underlying control of the syncytiotrophoblast". They identify the chromatin remodeler, RYBP, as well as other regulatory networks that they propose are critical to syncytiotrophoblast development.

The initial study was limited in fully addressing the aim, however, as functional evidence for the contributions of the factors/pathways to syncytiotrophoblast cell development was absent. In a revised version of the manuscript, the authors report the first application of CRISPR-mediated gene silencing in a TO model. They use CRISPR-Cas9-mediated gene targeting to generate RYBP and AFF1 knockout models. Deletion of either RYBP or AFF1 increased STB-2 marker gene expression, as determined using bulk RNA-seq. Future experimentation will assess the distribution of STB nuclear subtypes in the RYBP and AFF1 knockout models and explore the essentiality of RYBP, AFF1, and other identified factors to syncytiotrophoblast development and function.

Localization and validation of the identified factors within tissue and at the protein level will also provide further contextual evidence to address the hypotheses generated. In a revised version of the manuscript, the authors localize STB markers PAPP2 and ADAMTS6 in TOs using RNA-FISH. Future work will aim to further validate the markers and hypotheses generated from this study.

Author response:

The following is the authors' response to the original reviews

We thank the public reviewers and editors for their insightful comments on the manuscript. We have made the following changes to address their concerns and think the resulting manuscript is stronger as a result. Specifically, we have 1) added RNA FISH data of specific STB-2 and STB-3 RNA markers to confirm their distribution changes between STBⁱⁿ and STB^{out} TOs, 2) removed language throughout the text that refer to STB-3 as a terminally differentiated nuclear subtype, and 3) generated CRISPR-mediated knock-outs of two genes identified by network analysis and validated their role in mediating STB nuclear subtype gene expression.

Reviewer #1 (Public review):

Strengths:

The study offers a comprehensive SC- and SN-based characterization of trophoblast organoid models, providing a thorough validation of these models against human placental tissues. By comparing the older STBⁱⁿ and newer STB^{out} models, the authors effectively demonstrate the improvements in the latter, particularly in the differentiation and gene expression profiles of STBs. This work serves as a critical resource for researchers, offering a clear delineation of the similarities and differences between TO-derived and primary STBs. The use of multiple advanced techniques, such as high-resolution sequencing and trajectory analysis, further enhances the study's contribution to the field.

Thank you for your thoughtful review—we appreciate your recognition of our efforts to comprehensively validate trophoblast organoid models and highlight key advancements in STB differentiation and gene expression.

Weaknesses:

While the study is robust, some areas could benefit from further clarification.

(1) The importance of the TO model's orientation and its impact on outcomes could be emphasized more in the introduction.

We agree that TO orientation may significantly influence STB nuclear subtype differentiation. As the STB is critical for both barrier formation and molecular transport *in vivo*, lack of exposure to the surrounding media in STBⁱⁿ TOs *in vitro* could compromise these functions and the associated environmental cues that influence STB nuclear differentiation. We have added text to the introduction to highlight this point (lines 117-120).

(2) The differences in cluster numbers/names between primary tissue and TO data need a clearer explanation, and consistent annotation could aid in comparison.

Thank you for highlighting that the comparisons and cluster annotations need clarification. In Figure 1, we did not aim to directly compare CTB and STB nuclear subtypes between TOs and tissue. Each dataset was analyzed independently, with clusters determined separately and with different resolutions decided via a clustering algorithm (Zappia and Oshlack, 2018). For example, for the STB, this approach identified seven subtypes in tissue but only two in TOs, making direct comparison challenging. To address this challenge, we integrated the SN

datasets from TOs and tissue in Figure 6. This integration allowed us to directly compare gene expression between the sample types and examine the proportions within each STB subtype. Similarly, in Figure 2, direct comparison of individual CTB or STB clusters across the separate datasets is challenging (Figures 2A-C) due to differences in clustering. To overcome this, we integrated the datasets to compare cluster gene expression and relative proportions (Figures 2D-E). Nonetheless, to address the reviewers concern we have added text to the results section to clarify that subclusters of CTB and STB between datasets should not be directly compared until the datasets are integrated in Figure 2D-E and Figure 6 (lines 166-167).

(3) The rationale for using SN sequencing over SC sequencing for TO evaluations should be clarified, especially regarding the potential underrepresentation of certain trophoblast subsets.

This is an important point as the challenges of studying a giant syncytial cell are often underappreciated by researchers that study mononucleated cells. We have added text to the introduction to clarify why traditional single cell RNA sequencing techniques were inadequate to collect and characterize the STB (lines 91-93).

(4) Additionally, more evidence could be provided to support the claims about STB differentiation in the STB^{out} model and to determine whether its differentiation trajectory is unique or simply more advanced than in STBⁱⁿ.

Our original conclusion that STB^{out} nuclei are more terminally differentiated than STBⁱⁿ was based on two observations: (1) STB^{out} TOs exhibit increased expression of STB-specific pregnancy hormones and many classic STB marker genes and (2) STB^{out} nuclei show an enrichment of the STB-3 nuclear subtype, which appears at the end of the slingshot pseudotime trajectory. However, upon consideration of the reviewer comments, we agree that this evidence is not sufficient to definitively distinguish if STB^{out} nuclei are more advanced or follow a unique differentiation trajectory dependent on new environmental cues. Pseudotime analyses provided only a predictive framework for lineage tracing, and these predictions must be experimentally validated. Real-time tracking of STB nuclear subtypes in TOs would require a suite of genetic tools beyond the scope of this study. Therefore, to address the reviewers' concerns we have removed language suggesting that STB-3 is a terminally differentiated subtype or that STB^{out} nuclei are more differentiated than STBⁱⁿ nuclei throughout the text until the discussion. Therein we present both our original hypothesis (that STB nuclei are further differentiated in STB^{out}) and alternative explanations like changing trajectories due to local environmental cues (lines 619-625).

Reviewer #2 (Public review):

Strengths:

(1) The use of SN and SC RNA sequencing provides a detailed analysis of STB formation and differentiation.

(2) The identification of distinct STB subtypes and novel gene markers such as RYBP offers new insights into STB development.

Thank you for highlighting these strengths—we appreciate your recognition of our use of SN and SC RNA sequencing to analyze STB differentiation and the discovery of distinct STB subtypes and novel gene markers like RYBP.

Weaknesses:

(1) Inconsistencies in data presentation.

We address the individual comments of reviewer 2 later in this response.

(2) Questionable interpretation of lncRNA signals: The use of long non-coding RNA (lncRNA) signals as cell type-specific markers may represent sequencing noise rather than true markers.

We appreciate the reviewer's attention to detail in noticing the lncRNA signature seen in many STB nuclear subtypes. However, we disagree that these molecules simply represent sequencing noise. In fact, many studies have rigorously demonstrated that lncRNAs have both cell and tissue specific gene expression (e.g., Zhao et al 2022, Isakova et al 2021, Zheng et al 2020). Further, they have been shown to be useful markers of unique cell types during development (e.g., Morales-Vicente et al 2022, Zhou et al 2019, Kim et al 2015) and can enhance clustering interpretability in breast cancer (Malagoli et al 2024). Many lncRNAs have also been demonstrated to play a functional role in the human placenta, including H19, MEG3, and MEG8 (Adu-Gyamfi et al 2023) and differences are even seen in nuclear subtypes in trophoblast stem cells (Khan et al 2021). Therefore, we prefer to keep these lncRNA signatures included and let future researchers test their functional role.

To improve the study's validity and significance, it is crucial to address the inconsistencies and to provide additional evidence for the claims. Supplementing with immunofluorescence staining for validating the distribution of STBⁱⁿ, STB^{out}, and EVT^{enrich} in the organoid models is recommended to strengthen the results and conclusions.

Each general trophoblast cell type (CTB, STB, EVT) has been visualized by immunofluorescence by the Coyne laboratory in their initial papers characterizing the STBⁱⁿ, STB^{out}, and EVT^{enrich} models (Yang et al, 2022 and 2023). We agree that it is important to validate the STB nuclear subtypes found in our genomic study. However, one challenge in studying a syncytia is that immunofluorescence may not be a definitive method when the nuclei share a common cytoplasm. This is because protein products from mRNAs transcribed in one nucleus are translated in the cytoplasm and could diffuse beyond sites of transcription. Therefore, RNA fluorescence in situ hybridization (RNA-FISH) is instead needed. While a systematic characterization of the spatial distribution of the many marker genes found each subtype is outside the scope of this study, we include RNA-FISH of one STB-2 marker (PAPPA2) and one STB-3 marker (ADAMTS6) in Figure 3F-G and Supplemental Figure 3.3. This demonstrates there is an increase in STB-2 marker gene expression in STBⁱⁿ TOs and an increase in STB-3 marker gene expression in STB^{out} TOs.

Reviewer #3 (Public review):

The authors present outstanding progress toward their aim of identifying, "the underlying control of the syncytiotrophoblast". They identify the chromatin remodeler, RYBP, as well as other regulatory networks that they propose are critical to syncytiotrophoblast development. This study is limited in fully addressing the aim, however, as functional evidence for the contributions of the factors/pathways to syncytiotrophoblast cell development is needed. Future experimentation testing the hypotheses generated by this work will define the essentiality of the identified factors to syncytiotrophoblast development and function.

We thank the reviewer for their thoughtful assessment, constructive feedback, and encouraging comments. We acknowledge that the initial manuscript primarily presented analyses suggesting correlations between RYBP and other factors identified in the gene network analysis and STB function. Understanding how gene networks in the STB are formed

and regulated is a long-term goal that will require many experiments with collaborative efforts across multiple research groups.

Nonetheless, to address this concern we have knocked out two key genes, RYBP and AFF1, in TOs using CRISPR-Cas9-mediated gene targeting. Bulk RNA sequencing of STBⁱⁿ TOs from both wild-type (WT) and knockout strains revealed that deletion of either gene caused a statistically significant decrease in the expression of the pregnancy hormone *human placental lactogen* and an increase in the expression of several genes characteristic of the oxygen-sensing STB-2 subtype, including *FLT-1*, *PAPPA2*, *SPON2*, and *SFXN3*. These findings demonstrate that knocking out RYBP or AFF1 results in an increase in STB-2 marker gene expression and therefore play a role in inhibiting their expression in WT TOs (Figure 5D-E and supplemental Figure 5.2). We also note that this is the first application of CRISPR-mediated gene silencing in a TO model.

Future work will visualize the distribution of STB nuclear subtypes in these mutants and explore the mechanistic role of RYBP and AFF1 in STB nuclear subtype formation and maintenance. However, these investigations fall outside the scope of the current study.

Localization and validation of the identified factors within tissue and at the protein level will also provide further contextual evidence to address the hypotheses generated.

We agree that visualizing STB nuclear subtype distribution is essential for testing the many hypotheses generated by our analysis. To address this, we have included RNA-FISH experiments for two STB subtype markers (*PAPPA2* for STB-2 and *ADAMTS6* for STB-3) in TOs. These experiments reveal an increase in *PAPPA2* expression in STBⁱⁿ TOs and an increase in *ADAMTS6* expression in STB^{out} TOs (Figure 3F-G and Supplemental Figure 3.3). Genomic studies serve as powerful hypothesis generators, and we look forward to future work—both our own and that of other researchers—to validate the markers and hypotheses presented from our analysis.

Recommendations for the authors:

Reviewing Editor Comments:

We strongly encourage the authors to further strengthen the study by addressing all reviewers' comments and recommendations, with particular attention to the following key aspects:

(1) Clarifying the uniqueness of the STB differentiation trajectory between STBⁱⁿ and STB^{out}, and determining whether STB^{out} represents a more advanced stage of differentiation compared to STBⁱⁿ. It is also important to specify which developmental stage of placental villi the STB^{out} and STBⁱⁿ are simulating.

We have revised the manuscript to remove definitive language claiming that STB-3 represents a terminally differentiated subtype or that STB^{out} nuclei are more differentiated than STBⁱⁿ nuclei. Instead, we now present our hypothesis and alternative explanations in the discussion (lines 619-625), and emphasize the need for experimental validation of pseudotime predictions to test these hypotheses.

(2) Utilizing immunofluorescence to validate the distribution of cell types in the organoid models.

The Coyne lab has previously performed immunofluorescence of CTB and STB markers in STBⁱⁿ and STB^{out} TOs (Yang et al 2023). The syncytial nature of STBs complicates immunofluorescence-based validation of the STB nuclear subtypes due translating proteins all sharing a single common cytoplasm and therefore being able to diffuse and mix. Instead,

we performed RNA-FISH for two STB subtype markers (PAPPA2, STB-2 and ADAMTS6, STB-3), which showed subtype-specific nuclear enrichment in STBⁱⁿ and STB^{out} TOs, respectively (Figure 3F-G and Supplemental Figure 3.3).

(3) Addressing concerns regarding the use of lncRNA as cell marker genes. Employing canonical markers alongside critical TFs involved in differentiation pathways to perform a more robust cell-type analysis and validation is recommended.

As discussed in detail above, we maintain that lncRNAs are valuable markers, supported by their demonstrated roles in cell and tissue specificity and placental function. These signatures provide important insights and hypotheses for future research, and we have clarified this rationale in the revised manuscript.

Reviewer #1 (Recommendations for the authors):

(1) The authors have presented an extensive SC- and SN-based characterization of their improved trophoblast TO model, including a comparison to human placental tissues and the previous TO iteration. In this way, the authors' work represents an invaluable resource for investigators by providing thorough validation of the TO model and a clear description of the similarities and differences between primary and TO-derived STBs. I would suggest that the authors reshape the study to further highlight and emphasize this aspect of the study.

We thank the reviewer for their thoughtful recommendation and agree that our datasets will serve as an invaluable resource for comparing in vitro models to in vivo gene expression. However, extensive validation is required to make definitive conclusions about the extent to which these systems mirror one another and where they diverge. For this reason, in this manuscript, we have focused on characterizing STB subtypes to provide a foundational understanding of the model and this poorly characterized subtype.

(2) Introduction, Paragraph 3: What is the importance of orientation for the trophoblast TO model? The authors may consider removing some of the less important methodologic details from this paragraph and including more emphasis on why their TO model is an improvement.

Text has been added to this paragraph to highlight the importance of outward facing STB orientation, which is essential to mirror the STB's transport function *in vitro* (lines 118-120).

(3) Results, Figure 1: In addition to the primary placental tissue plots showing all cell populations, it may be useful to have side-by-side versions of similar plots showing only the trophoblast subsets, so that the primary and TO data could be more easily compared visually.

This has been implemented and added to the Supplemental Figure 1.4.

(4) Results, Figure 1: In simple terms, what is the reason for ending up with different cluster numbers/names from the primary tissue and TO? Would it be possible to apply the same annotation to each (at least for trophoblast types) and thus allow direct comparison between the two?

As described above, each dataset was separately analyzed and clusters determined with an algorithm to determine the optimal clustering resolution. Therefore, the number of clusters between each dataset cannot be directly compared until the SN TO and tissue datasets are

integrated together in Figure 6. We have added text to the manuscript to make it clear that they should not be compared except for in bulk number until this point (230-232).

(5) Results, Figure 2: For subsequent evaluation of different in vitro TO conditions, did the authors use only SN sequencing because they wanted to focus on STB? Based on Figure 1, it seems some CTB subsets would be underrepresented if using only SN. Given that the authors look at both STB and CTB in their different TOs, is this an issue?

The CTB clusters that showed the greatest divergence between SC and SN datasets were those associated with mitosis and the cell cycle, likely due to nuclear envelope breakdown interfering with capture by the 10x microfluidics pipeline. While cytoplasmic gene expression provides valuable insights into CTB function, our manuscript focuses on the STB starting from Figure 2. Since the STB is captured exclusively by the SN dataset, we concentrated on this approach to streamline our analysis.

(6) Results, Figure 3: What do the authors consider to be the primary contributing factors for why the STB subsets display differential gene expression between STBⁱⁿ and STB^{out}? Is this due primarily to the cultural conditions and/or a result of the differing spatial arrangement with CTBs?

This is an intriguing question that is challenging to disentangle because the culture conditions are integral to flipping the orientation. The two primary factors that differ between STBⁱⁿ and STB^{out} TOs are the presence of extracellular matrix in STBⁱⁿ and direct exposure to the surrounding media in STB^{out}. We believe these environmental cues play a significant role in shaping the gene expression of STB subsets. Fully disentangling this relationship would require a method to alter the TO orientation without changing the culture conditions. While this is an exciting direction for future research, it falls outside the scope of the present study.

(7) Results, Figure 4: The authors' analysis indicates that the STB nuclei from the STB^{out} TO are likely "more differentiated" than those in STBⁱⁿ TO. Could the authors provide some qualitative or quantitative support for this? Is the STB^{out} differentiated phenotype closer to what would be observed in a fully formed placenta?

As discussed earlier, we agree with the reviewers that this claim should be removed from the text outside of the discussion.

(8) Results, Figure 5: Based on the trajectory analysis, do the authors consider that the STB from STB^{out} TO are simply further along the differentiation pathway compared to those from STBⁱⁿ TO, or do the STB from STB^{out} TO follow a differentiation pathway that is intrinsically distinct from STBⁱⁿ TO?

We think the idea of an intrinsically distinct pathway is a fascinating alternative hypothesis and have added it into the discussion. We do not find the pseudotime currently allows us to answer this question without additional experiments, so we have removed claims that the STB^{out} STB nuclei are further along the differentiation pathway.

(9) Results, Figure 6: A notable difference between the STB^{out} TO and the term tissue is that the CTB subsets are much more prevalent. Is this simply a scale difference, i.e. due to the size of the human placenta compared to the limited STB nuclei available in the STB^{out} TO? Or are there other contributing factors?

The proportion of CTB to STB nuclei in our term tissue (9:1) aligns with expectations based on stereological estimates. We believe the relatively low number of CTB nuclei in our dataset is

due to the need for a larger sample size to capture more of this less abundant cell type. Since the primary focus of this paper is on STB, and we analyzed over 4,000 STB nuclei, we do not view this as a limitation. However, future studies utilizing SN to investigate term tissue should account for the abundance of STB nuclei and plan their sampling carefully to ensure sufficient representation of CTB nuclei if this is a desired focus.

Reviewer #2 (Recommendations for the authors):

(1) The color annotations for cell types in Figure 2 are inconsistent between the different panels, and the term "Prolif" in Figure 2E is not explained by the authors.

We chose colors to enhance visibility on the UMAP. We do not wish readers to make direct comparisons between the different CTB or STB subtypes of the sample types until the datasets are integrated in Figure 2D. This is because an algorithm for the clustering resolution has been chosen independently for each dataset. Cluster proportions are better compared in the integrated datasets in Figure 2D. We have added text to the results section to make this clear to the reader (lines 166-167).

(2) In Figure 3 and Supplementary Figures 1.3, the authors frequently present long non-coding RNA (lncRNA) signals as cell type-specific markers in the bubble plots. These signals are likely sequencing noise and may not accurately represent true markers for those cell types. It is recommended to revise this interpretation.

As referenced above, there are many examples of lncRNAs that have biological and pathological significance in the placenta (H19, Meg3, Meg8) and lncRNAs often have cell type specific expression that can enhance clustering. We prefer to keep these signatures included and let future researchers determine their biological significance.

(3) In Figure 3C, the authors performed pathway enrichment analysis on the STB subtypes after integrating STB_in and STB_out organoids. The enrichment of the "transport across the blood-brain barrier" pathway in the STB-3 subtype does not align with the current understanding of STB cell function. Please provide corresponding supporting evidence. Additionally, please verify whether the other functional pathways represent functions specific to the STB subtypes.

Interestingly, many of the genes categorized under "transport across the blood-brain barrier" are transporters shared with "vascular transport." These include genes involved in the transport of amino acids (SLC7A1, SLC38A1, SLC38A3, SLC7A8), molecules essential for lipid metabolism (SLC27A4, SLC44A1), and small molecule exchange (SLC4A4, SLC5A6). Given that the vasculature, the STB, and the blood-brain barrier all perform critical barrier functions, it is unsurprising that molecules associated with these GO terms are enriched in the STB-3 subtype, which expresses numerous transporter proteins. Since the transport of materials across the STB is a well-established function, we have not included additional supporting evidence but have clarified the genes associated with this GO term in the text (lines 392-394 and supplemental Table 9).

(4) The pseudotime heatmap in Figure 4B is not properly arranged and is inconsistent with the differentiation relationships shown in Figure 4A. It is recommended to revise this.

We are uncertain which aspect of the heatmap in Figure 4A is perceived as inconsistent with Figure 4B. One distinction is that pseudotime in Figure 4A is normalized from 0 to 100 to fit the blue-to-yellow-to-red color scale, whereas in Figure 4B, the color scale is not normalized and the color bar ranging from white to red. This difference reflects our intent to simplify

Figure 4B-C, as the abundance of color between cell types and gene expression changes required a streamlined representation to ensure the figure remained clear and easy to interpret. This is classically done in the field and consistent with the default code in the slingshot package.

(5) In Figures 4C and 4D, although RYBP is highly expressed in STB, it is difficult to support the conclusion that RYBP shows the most significant expression changes. It is recommended to provide additional evidence.

The claim that RYBP exhibits the most significant expression changes was based on p-value ordering of genes associated with pseudotime via the associationTest function in slingshot and not with immunofluorescence data. The text has been revised to make this distinction clear (lines 390-393).

(6) In Figure 4E, staining for CTB marker genes is missing, and in Figure 4F, CYTO is difficult to use as a classical STB marker. It is recommended to use the CGBs antibody from Figure 4E as a STB marker for staining to provide evidence.

We have revised the Figure 5B-C to use e-Cadherin as a CTB marker gene in TOs and CGB antibody as a marker of STB.

In tissue, however, obtaining a good STB marker that does not overlap with the RYBP antibody (rabbit) in term tissue is difficult as the STB downregulates hCG expression closer to term to initiate contractions. SDC1 is often used but only labels the plasma membrane so does not help in distinguishing the STB cytoplasm. We have added an image of cytokeratin, e-Cadherin, and the STB marker ENDOU to validate that our current approach with e-Cadherin and cytokeratin allows us to accurately distinguish between CTB and STB cells.

(7) The velocity results in Figure 5A do not align with the differentiation relationships between cells and contradict the pseudotime results presented in Figure 4 by the authors.

The reviewer raises an interesting observation regarding the velocity map in Figure 5A, which appears to show a bifurcation into two STB subtypes. This observation aligns with similar findings reported in tissue by our colleagues (Wang et al., 2024). However, given the low number of CTB cells in our tissue dataset, we were cautious about making definitive conclusions about pseudotime without a larger sample size. Notably, the RNA velocity map closely resembles the pseudotime trajectory in TOs, with CTB transitioning into the CTB-pf subtype and subsequently into the STB. One potential explanation for discrepancies between tissue and TOs is the difference in nuclear age: nuclei in tissue can be up to nine months old, whereas those in TOs are only hours or days old. It is possible that the lineage in TOs could bifurcate if cultured for longer than 48 hours, but our current dataset captures only the early stages of the STB differentiation process. While exploring these hypotheses is fascinating, they are beyond the scope of this current study.

Reviewer #3 (Recommendations for the authors):

Amazing work - I greatly enjoyed reading the manuscript. Here are a few questions and suggestions for consideration:

Evidence presented throughout the results sections hints that the organoids may represent an earlier stage of placental development compared to the term. Increased hCG gene expression is observed, but as noted expression is decreased in term STB. STB:CTB ratios are also higher at term compared to the first trimester, etc. It was difficult to conclude definitively based on how data is presented in Fig 6 and discussed. Maybe there is no clear answer. Perhaps the altered cell type ratios in the organoid models (e.g.,

few STB in EVT enrich conditions) impact recapitulation of the in vivo local microenvironment signaling. As such, can the authors speculate on whether cell ratios could be strategically leveraged to model different gestational time points?

Along these same lines, syncytiotrophoblast in early implantation (before proper villi development) is often described as invasive and later at the tertiary villi stage defined by hormone production, barrier function, and nutrient/gas exchange. Do the authors think the different STB subtypes captured in the organoid models represent different stages/functions of syncytiotrophoblast in placental development?

Minor Comments

(1) Please clarify what the third number represents in the STB:CTB ratio (e.g., 1:3:1 and 2:5:1). EVT?

The first number is a decimal point and not a colon (ie 1.3 and 2.5). Therefore these numbers are to be read as the STB:CTB ratio is 1.3 to 1 or 2.5 to 1.

(2) Could consider co-localizing RYBP in term tissue with a syncytio-specific marker like CGB used for organoids (Fig 4F).

We addressed this concern in comment 6 to reviewer 2.

(3) Recommend defining colors-which colors represent which module in Figure 5C in the legend and main body text. I see the labels surrounding the heatmap in 5B, but defining colors in text (e.g. cyan, magenta, etc.) would be helpful. Do the gray circles represent targets that don't belong to a specific module? Are the bolded factor names based on a certain statistical cutoff/defining criteria or were they manually selected?

The text of both the results and figure legends has been revised to clarify these points.

(4) Data Availability: It would be helpful to provide supplemental table files for analyses (e.g., 5C to list the overlapping relationships in TGs for each TF/CR (5C) and 3E/6F to list DEG genes in comparisons).

Supplemental files for each analysis have been added (Supplemental Table 8-14). In addition, the raw and processed data is available on GEO and we have created an interactive Shiny App so people without coding experience can interact with each dataset (lines 917-919).

(5) "...and found that each sample expressed these markers (Figure 6D), suggesting..." Consider clarifying "these".

Text has been added to refer to a few of these marker genes within the text (line 540).

Citations

(1) Zappia L, Oshlack A. Clustering trees: a visualization for evaluating clusterings at multiple resolutions. *GigaScience*. 2018;7(7):giy083. PMID: PMC6057528

(2) Zhou J, Xu J, Zhang L, Liu S, Ma Y, Wen X, Hao J, Li Z, Ni Y, Li X, Zhou F, Li Q, Wang F, Wang X, Si Y, Zhang P, Liu C, Bartolomei M, Tang F, Liu B, Yu J, Lan Y. Combined Single-Cell Profiling of lncRNAs and Functional Screening Reveals that H19 Is Pivotal for Embryonic Hematopoietic Stem Cell Development. *Cell Stem Cell*. 2019;24(2):285-298.e5. PMID: 30639035

(3) Malagoli G, Valle F, Barillot E, Caselle M, Martignetti L. Identification of Interpretable Clusters and Associated Signatures in Breast Cancer Single-Cell Data: A Topic Modeling

Approach. *Cancers*. 2024;16(7):1350. PMID: PMC11011054

(4) Adu-Gyamfi EA, Cheeran EA, Salamah J, Enabulele DB, Tahir A, Lee BK. Long non-coding RNAs: a summary of their roles in placenta development and pathology†. *Biol Reprod*. 2023;110(3):431–449. PMID: 38134961

(5) Zheng M, Hu Y, Gou R, Nie X, Li X, Liu J, Lin B. Identification three LncRNA prognostic signature of ovarian cancer based on genome-wide copy number variation. *Biomed Pharmacother*. 2020;124:109810. PMID: 32000042

(6) Khan T, Seetharam AS, Zhou J, Bivens NJ, Schust DJ, Ezashi T, Tuteja G, Roberts RM. Single Nucleus RNA Sequence (snRNAseq) Analysis of the Spectrum of Trophoblast Lineages Generated From Human Pluripotent Stem Cells in vitro. *Front Cell Dev Biol*. 2021;9:695248. PMID: PMC8334858

(7) Isakova A, Neff N, Quake SR. Single-cell quantification of a broad RNA spectrum reveals unique noncoding patterns associated with cell types and states. *Proc Natl Acad Sci United States Am*. 2021;118(51):e2113568118. PMID: PMC8713755

(8) Morales-Vicente DA, Zhao L, Silveira GO, Tahira AC, Amaral MS, Collins JJ, Verjovski-Almeida S. Singlecell RNA-seq analyses show that long non-coding RNAs are conspicuously expressed in *Schistosoma mansoni* gamete and tegument progenitor cell populations. *Front Genet*. 2022;13:924877. PMID: PMC9531161

(9) Kim DH, Marinov GK, Pepke S, Singer ZS, He P, Williams B, Schroth GP, Elowitz MB, Wold BJ. Single-Cell

Transcriptome Analysis Reveals Dynamic Changes in lncRNA Expression during Reprogramming. *Cell Stem Cell*. 2015;16(1):88–101. PMID: PMC4291542

(10) Yang L, Liang P, Yang H, Coyne CB. Trophoblast organoids with physiological polarity model placental structure and function. *bioRxiv*. 2023;2023.01.12.523752. PMID: PMC9882188

<https://doi.org/10.7554/eLife.101170.2.sa0>