Single-cell transcriptomics reveal differences between chorionic and basal plate cytotrophoblasts and trophoblast stem cells

Robert Morey[1,2,3†], Francesca Soncin[1,2,3†], Sampada Kallol[1,2,3], Nirvay Sah[1,2,3], Zoe Manalo[1,2,3], Tony Bui[1,2,3], Jaroslav Slamecka[1,2,3], Virginia Chu Cheung[1,2,3], Don Pizzo[1], Daniela F. Requena[1,2,3], Ching-Wen Chang[1,2], Omar Farah[1,2], Ryan Kittle[1,2,3], Kelly Lam[1,2,3], Morgan Meads[1,2,3], Mariko Horii[1,2,3], Kathleen M. Fisch[3,4], Mana M. Parast[1,2,3*]

[1*]Department of Pathology, School of Medicine, University of California San Diego, La Jolla, 92093, CA, USA.
[2]Sanford Consortium for Regenerative Medicine, La Jolla, 92093, CA, USA.
[3]Center for Perinatal Discovery, University of California San Diego, La Jolla, 92093, CA, USA.
[4]Department of Obstetrics, Gynecology, and Reproductive Sciences, School of Medicine, University of California San Diego, La Jolla, 92093, CA, USA.

*Corresponding author(s). E-mail(s): mparast@health.ucsd.edu;
†These authors contributed equally to this work.

1 Supplementary Figures

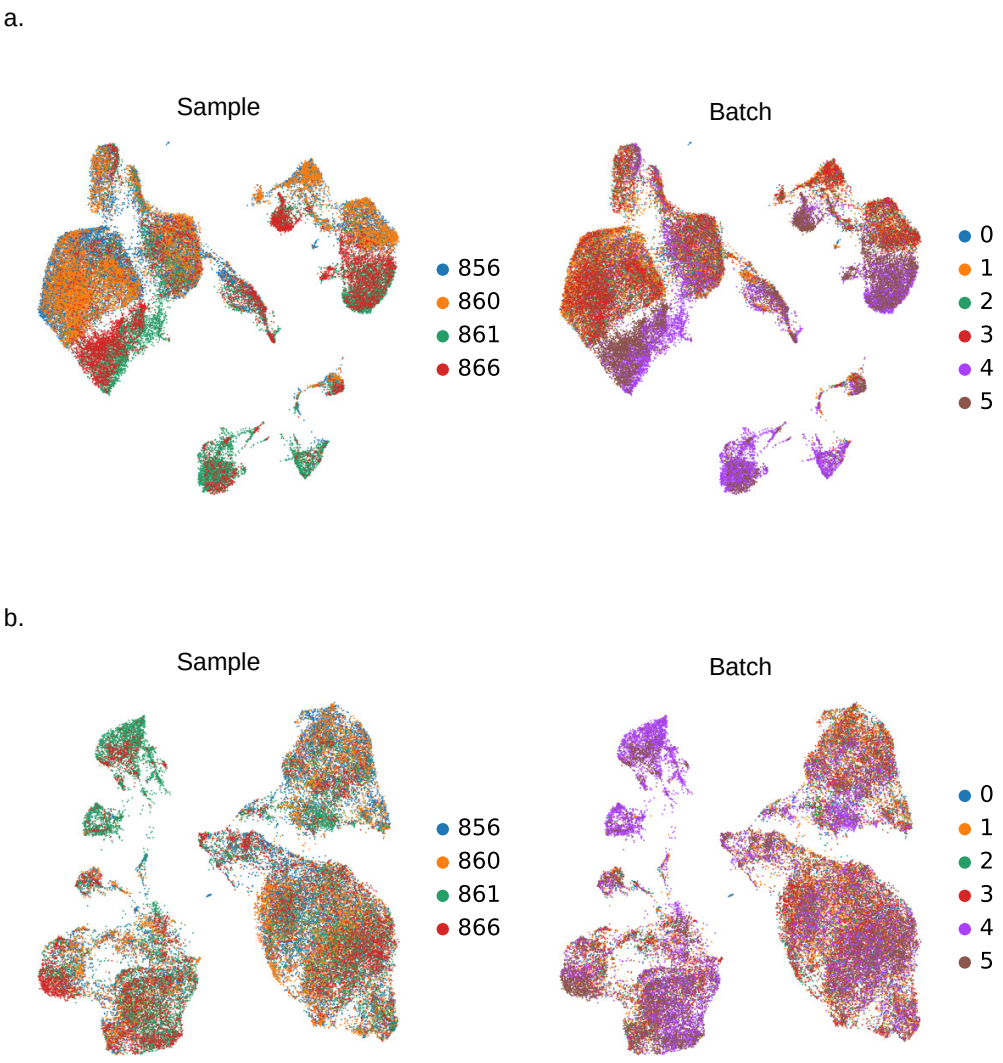

Fig. 1 First trimester placental single cell RNA-seq: Integration UMAPs. (**a**) UMAPs of placental samples before integration colored by the patient number (left) and batch number (right). (**b**) UMAPs of placental samples after integration colored by the patient number (left) and batch number (right).

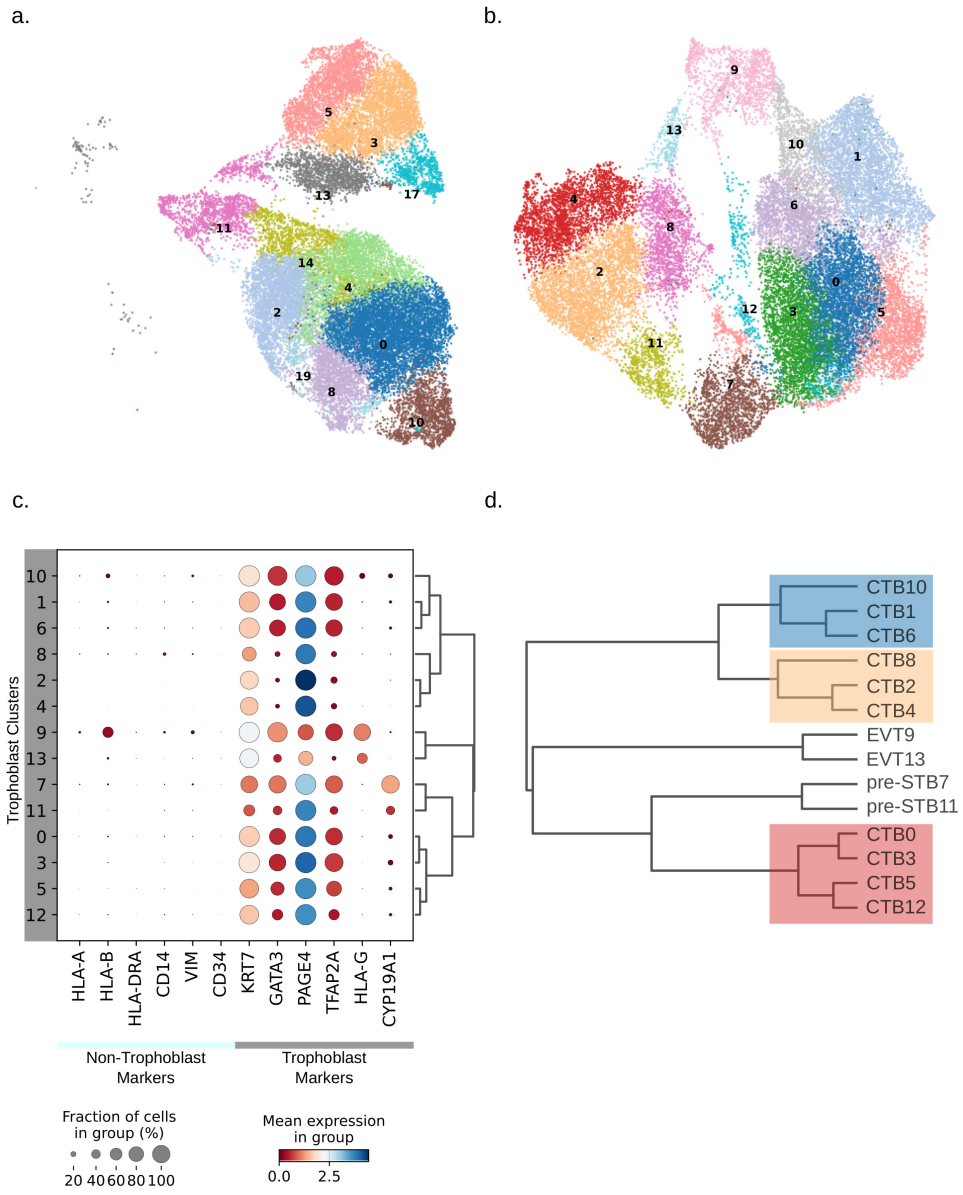

Fig. 2 First trimester placental single cell RNA-seq: Removal of non-trophoblast clusters and reclustering of trophoblast cells. (**a**) UMAP following the removal of clusters in **Fig. 1a** deemed to be non-trophoblast cells. (**b**) Reclustering of cells after removal of most of the non-trophoblast cells. (**c**) Dot plot and hierarchical clustering showing expression of trophoblast- and non-trophoblast-associated genes in reclustered UMAP shown in (b). (**d**) Dendrogram showing the transcriptional similarity between clusters in UMAP shown in (b), and annotated using dotplot shown in **Fig. 1c**, with clades colored by the cluster color of UMAP in **Fig. 1d** following the combining of clusters.

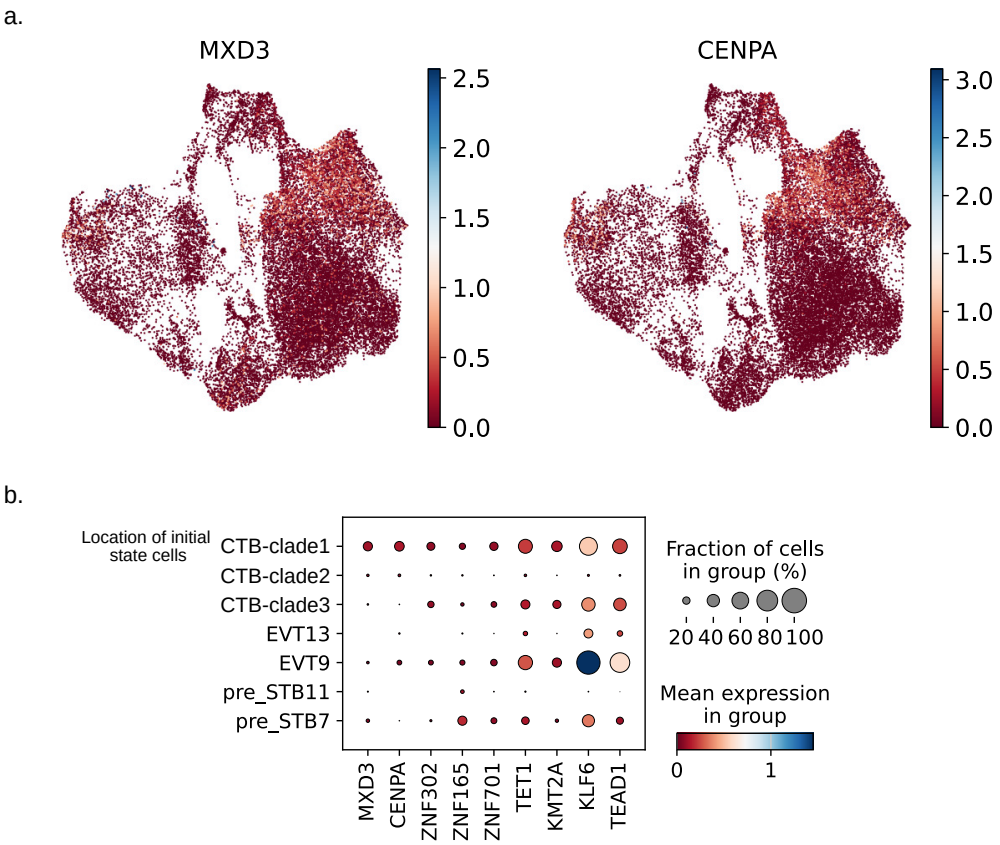

Fig. 3 Upregulated transcription factors in initial state cells. (**a**) UMAP showing the expression of the transcription factors MXD3 and CENPA that were differentially upregulated (adj. p-value < 0.05 and log fold change > 1) in the initial state cells (n=30), compared to the rest of the trophoblast cells in our integrated first trimester trophoblast dataset (**Table S10**), and were part of the CTB1 rank velocity genes (**Table S9**). (**b**) Dot plot showing the mean cluster expression and the fraction of cells in each cluster expressing differentially upregulated transcription factors (adj. p-value < 0.05 and log fold change > 1) in the initial state cells (n=30) compared to the rest of the trophoblast cells in our integrated first trimester trophoblast dataset (**Table S10**), and were part of the CTB1 rank velocity genes (**Table S9**).

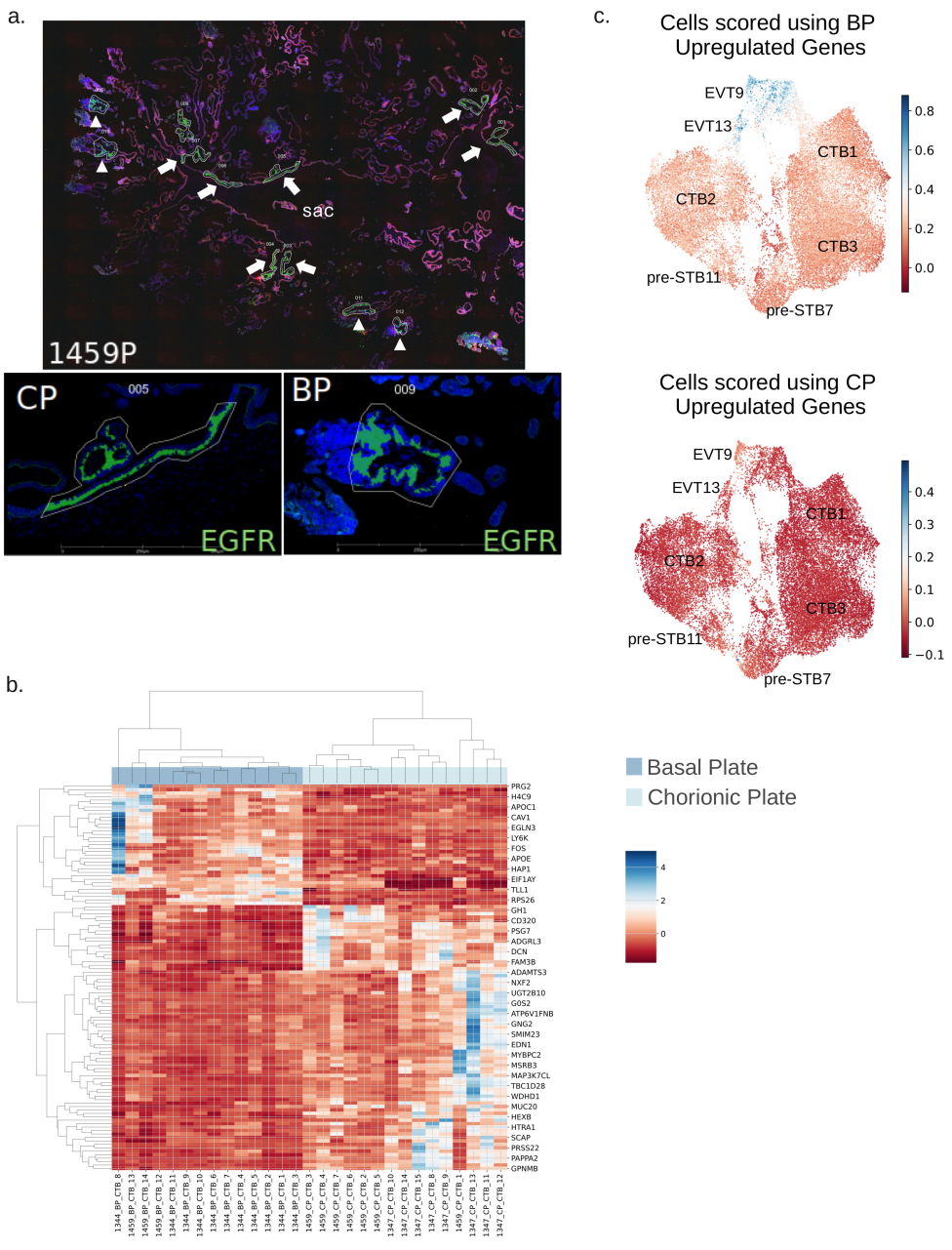

Fig. 4 Spatial transcriptomic profiling of basal and chorionic plate CTB. (**a**) Representative scan of formalin-fixed paraffin-embedded placental tissue (placenta # 1459P) used for GeoMx-based digital spatial profiling, showing tissue stained for EGFR to identify vCTB, with selection of Regions of Interest (ROI's) near the basal (BP, arrowheads) and chorionic plate (CP, arrows) based on spatial relation to the gestational sac. (**b**) Heatmap displaying the expression of genes that were determined to be differentially expressed (adj. P-value < 0.05 and log fold change > 1) between BP and CP CTB in 29 ROI's, located within three different placentae, using the GeoMx whole transcription atlas (WTA) panel. (**c**) UMAPs of early and late first trimester integrated scRNA-seq data, scored using the genes upregulated in the basal plate (top) or the chorionic plate (bottom), based on the GeoMx WTA data.

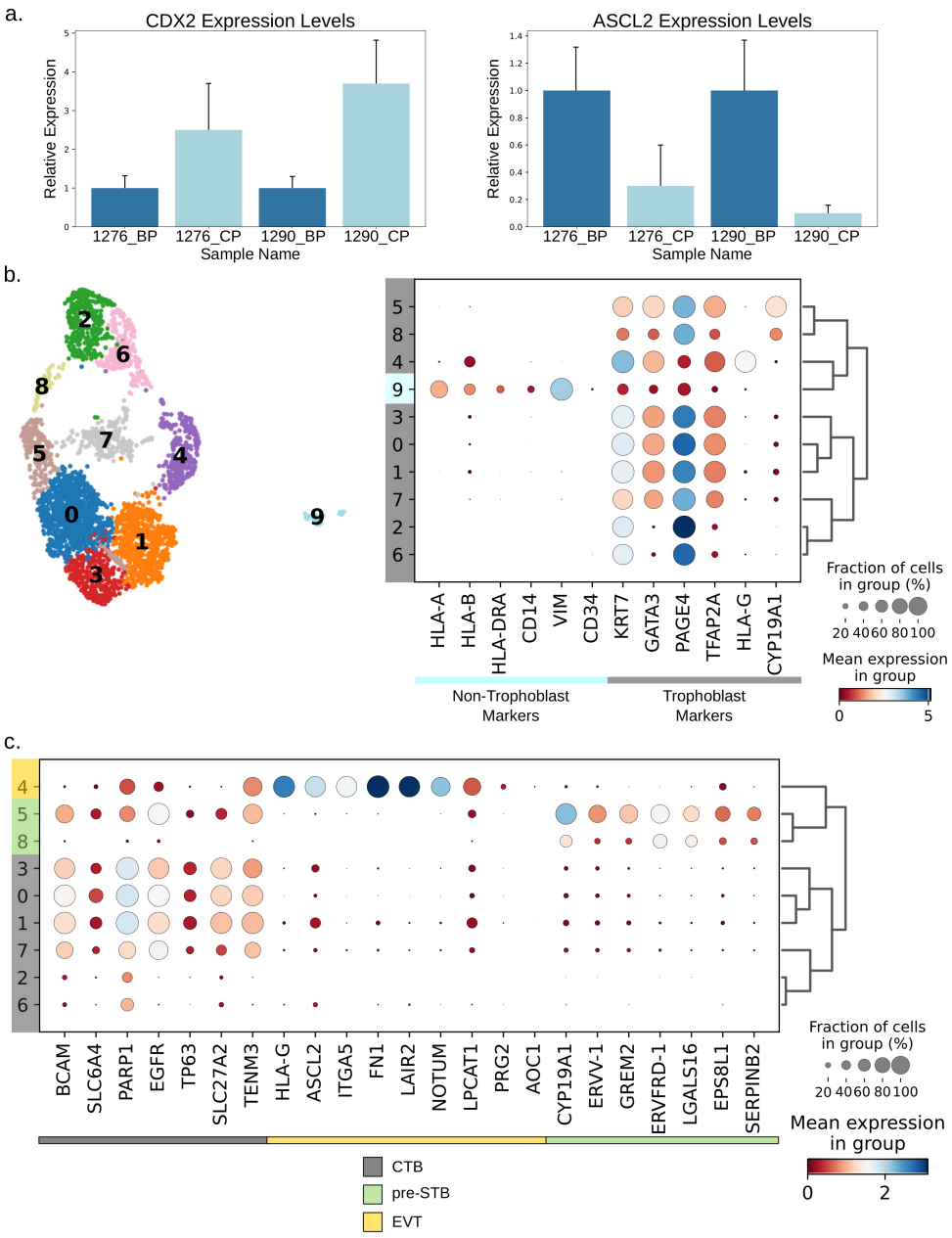

Fig. 5 Single cell RNA-seq of basal and chorionic plate CTB. (**a**) Q-PCR showing the relative expression of the chorionic plate marker CDX2 and the basal plate marker ASCL2 in cells separated into basal plate (BP, dark blue) and chorionic plate (CP, light blue) fractions. Single-cell RNA-seq was then subsequently performed on these fractions. (**b**) UMAP showing the clustering of the basal and chorionic plate scRNA-seq dataset (left) and dot plot showing the mean expression and fraction of cells expressing various trophoblast (grey) and non-trophoblast (light blue) marker genes (right). (**c**) Dot plot and hierarchical clustering of cell clusters in the BP and CP dataset, showing the mean expression and fraction of cells expressing trophoblast cell type-specific markers (CTB in grey, EVT in yellow, and pre-STB in green).

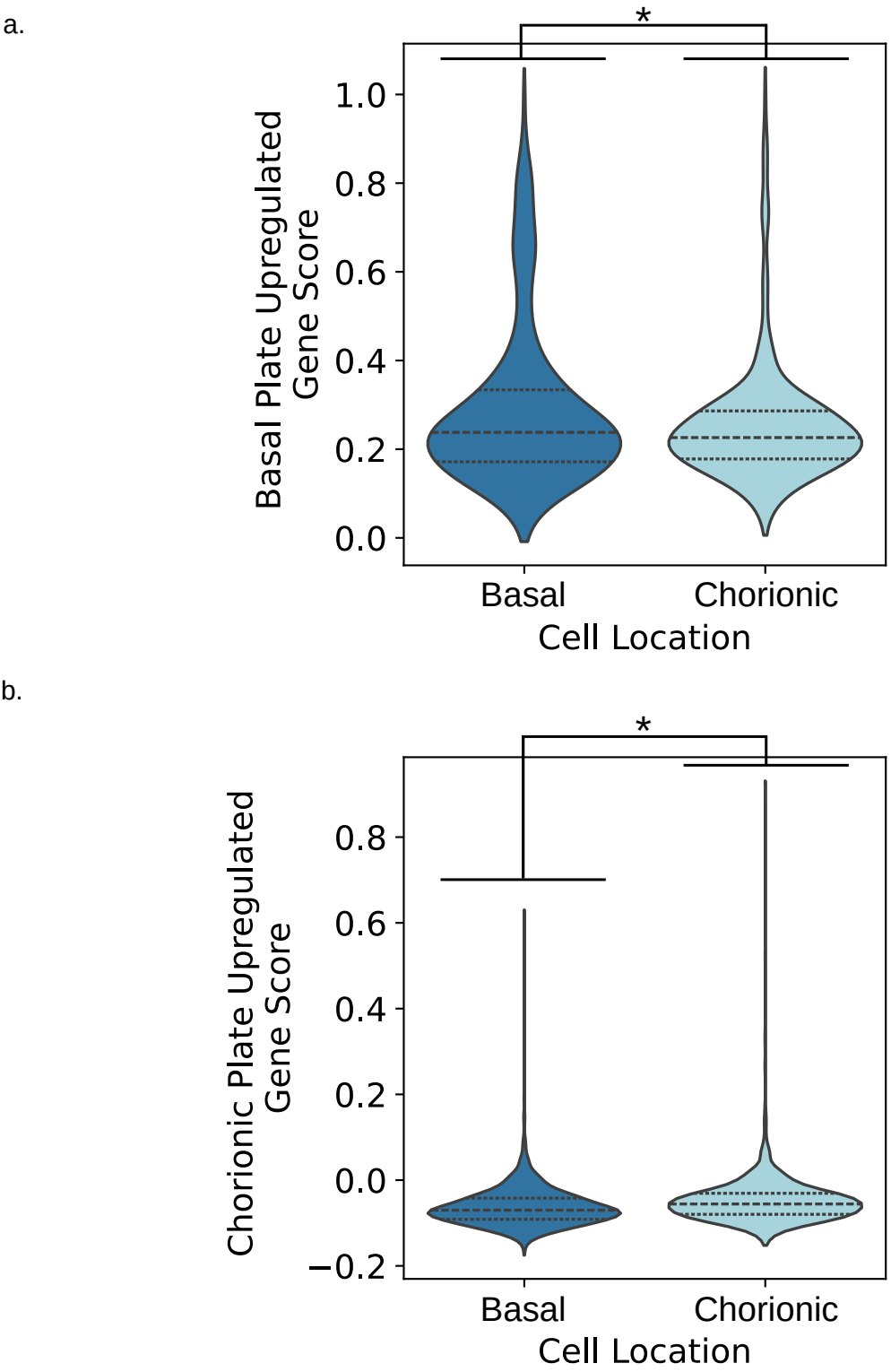

Fig. 6 Basal and chorionic plate cell scoring using spatial transcriptomics data. (**a**) Violin plot showing the basal and chorionic plate cells, scored using the genes found to be differentially upregulated in the basal plate regions of interest (ROIs) compared to the chorionic plate ROIs from the spatial transcriptomic dataset. (**b**) Violin plot showing the basal plate and chorionic plate cells scored using the genes found to be differentially upregulated in the chorionic plate ROIs compared to the basal plate ROIs from the spatial transcriptomic dataset. Dashed lines indicate quartiles and * indicates Wilcoxon rank-sum p-value < 0.001.

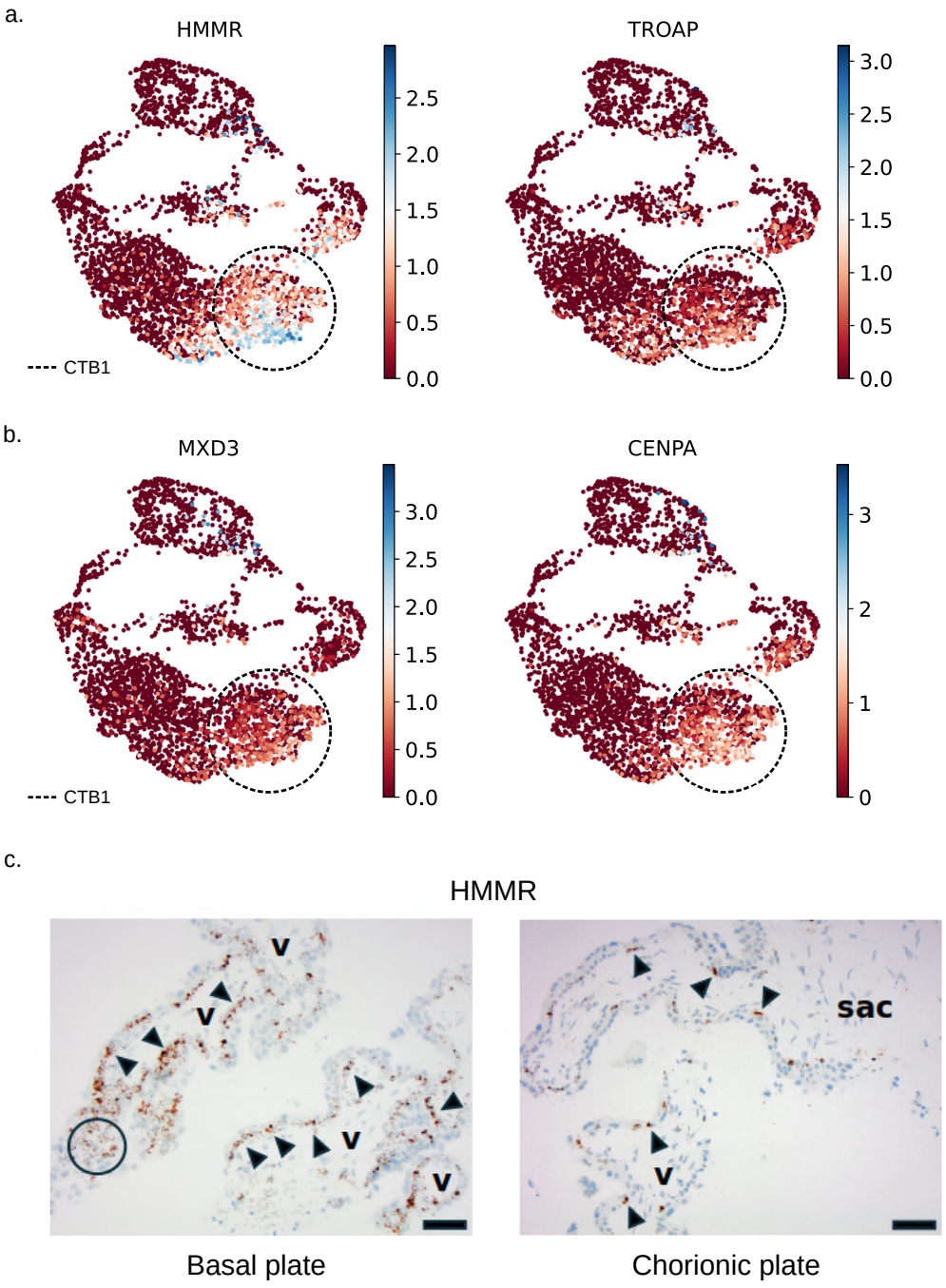

Fig. 7 Expression of initial state marker genes in the basal and chorionic plate. (**a**) UMAPs showing the expression of initial state marker genes *HMMR* and *TROAP* in basal and chorionic plate scRNA-seq dataset. Cluster CTB1 is marked by a dotted circle. (**b**) UMAPs showing the expression of initial state transcription factors *MXD3* and *CENPA* in basal and chorionic plate scRNA-seq dataset. Cluster CTB1 is marked by a dotted circle. (**c**) *In-situ* hybridization of the initial state marker, HMMR, in a 6-week placenta (same one shown in **Fig. 2d**), comparing expression at/near basal vs. chorionic plate. Villous CTB in chorionic villi ("v") are marked by arrowheads. The gestational sac is labeled "sac," while the proximal column trophoblast are marked by a circle. Scale bar=125 μm.

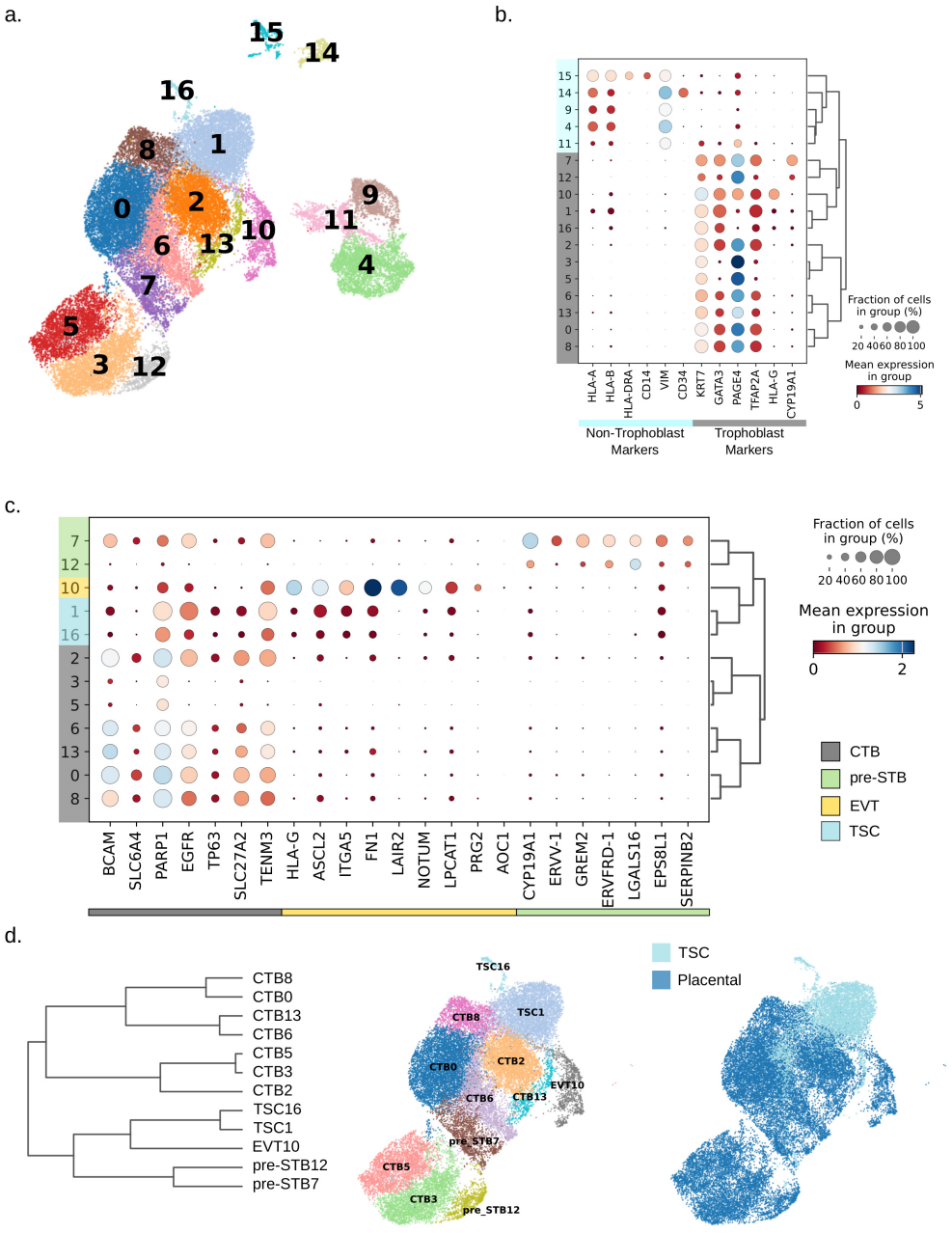

Fig. 8 Early first trimester and trophoblast stem cell (TSC) scRNA-seq dataset, following integration, clustering, and annotation. (**a**) UMAP of all cells from early first trimester placenta and TSC, following quality control filtering, integration, and clustering. (**b**) Dot plot and hierarchical clustering of cell clusters using trophoblast and non-trophoblast specific gene expression. (**c**) Dot plot and hierarchical clustering of cell clusters using cell type specific gene expression. (**d**) Hierarchical clustering dendrogram showing the transcriptomic similarity (Pearson correlation) between cell clusters (left), UMAP of clusters annotated based on cell type specific gene expression shown in part (c) (middle) and UMAP showing the location of TSC (light blue) vs. first trimester placental cells (dark blue) (right).

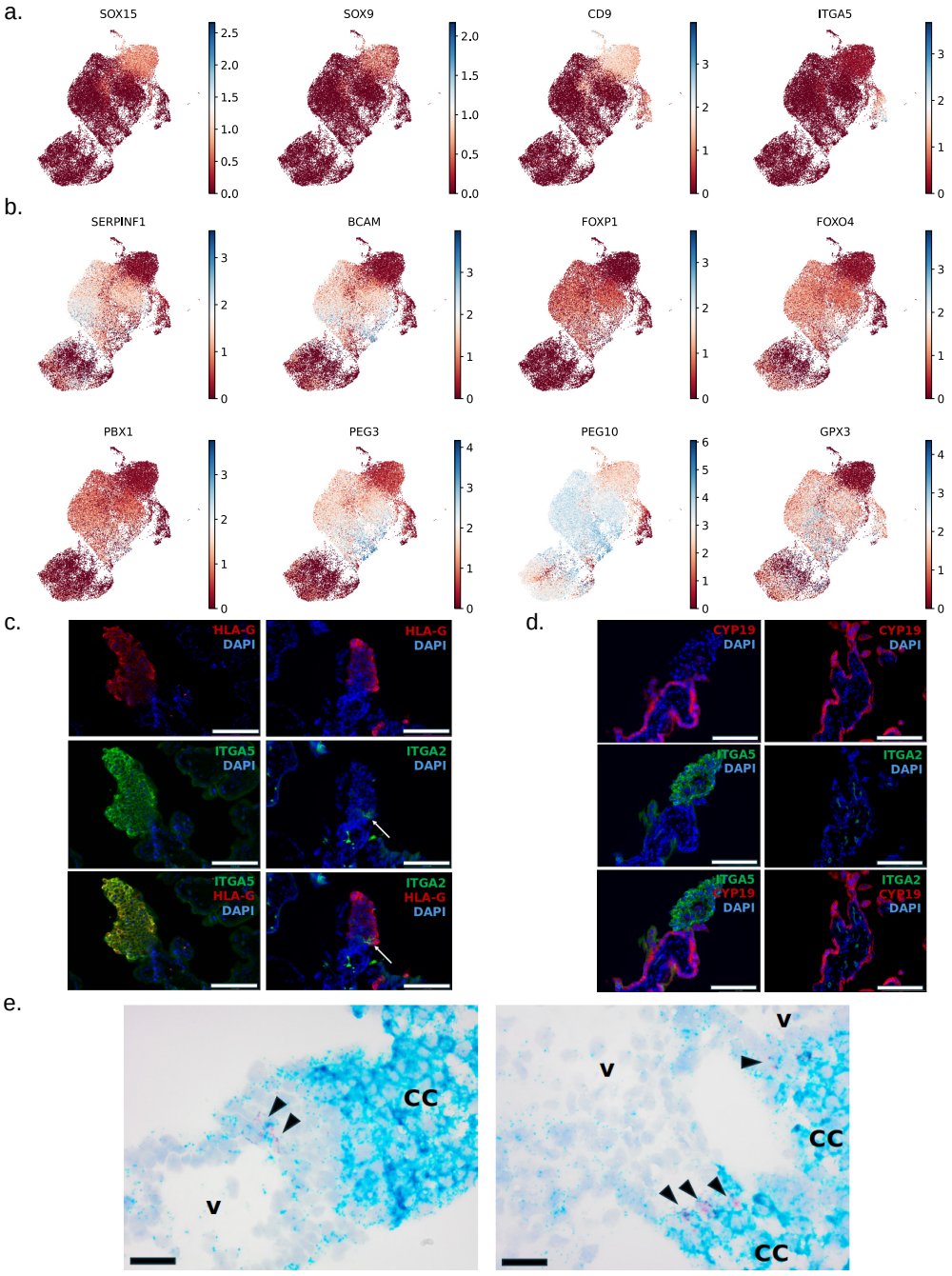

Fig. 9 Differences in gene expression between TSC and initial state CTB. (**a**) UMAPs showing the expression of genes upregulated in TSCs compared to initial state CTB. (**b**) UMAPs showing the expression of genes upregulated in initial state CTB compared to TSCs. (**c-d**) Immunohistochemistry of first trimester placenta with antibodies against ITGA5 and ITGA2, co-stained either with HLA-G (**c**) or CYP19A1 (**d**). ITGA5 is only expressed in column trophoblast, while ITGA2 is expressed in rare proximal column trophoblast (arrow in panel C), but also in fetal endothelial cells (panel D). Scale bar=156 μm. (**e**) *In-situ* hybridization of first trimester placenta with probes against *ITGA5* (teal) and *ITGA2* (magenta). Rare dual-positive cells are noted in the proximal columns (arrowheads). V=villous core; CC=cell column. Scale bar=62.5 μm.

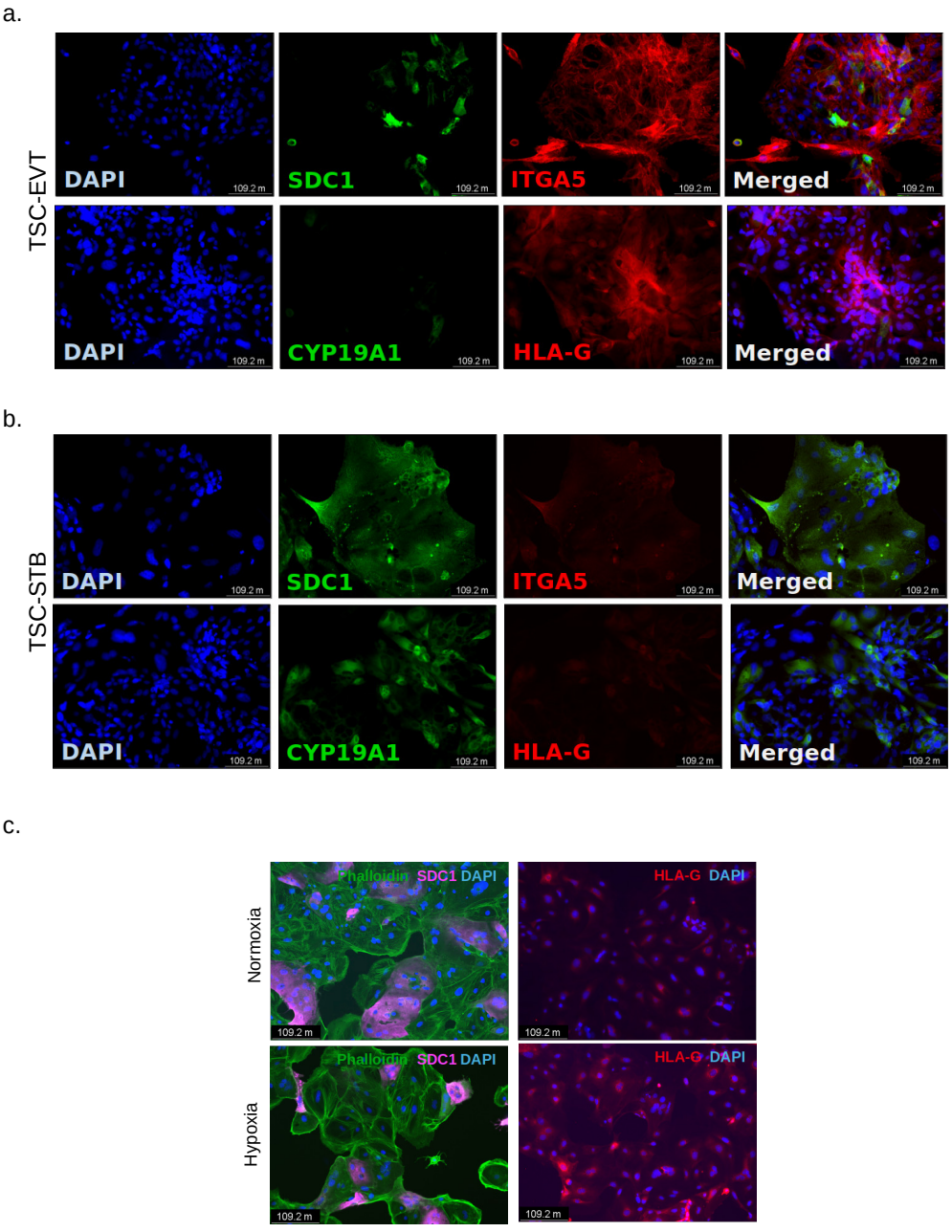

Fig. 10 Directed and spontaneous differentiation of TSC. (**a**) Immunohistochemistry of STB markers (SDC1 and CYP19A1), pcEVT marker (ITGA5), and EVT marker (HLA-G) following directed differentiation of TSCs to EVT. (**b**) Immunohistochemistry of STB markers (SDC1 and CYP19A1), pcEVT marker (ITGA5), and EVT marker (HLA-G) following directed differentiation of TSC to STB. (**c**) Immunohistochemistry of STB marker (SDC1) and EVT marker (HLA-G) following spontaneous differentiation of TSC (cultured in DMEM-F12+FBS in either normoxia/21% oxygen or hypoxia/2% oxygen). By morphology and staining, the TSCs formed large SDC1^{+} multinucleated cells in normoxia, but preferentially formed HLA-G^{+} mononuclear cells in hypoxia.

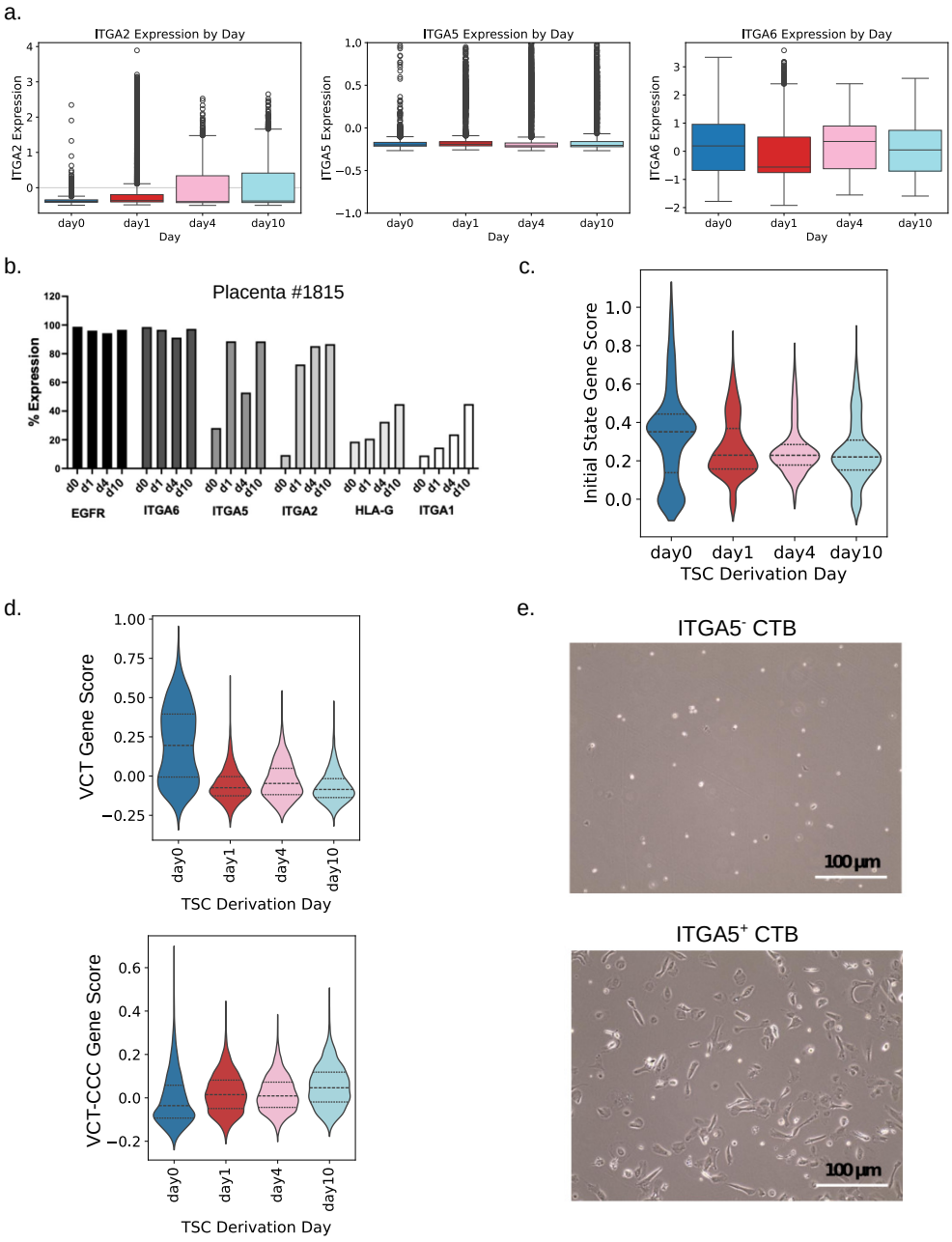

Fig. 11 Derivation of TSC from early first trimester CTB shows gradual progression toward a pcEVT phenotype. (**a**) Boxplots showing expression of *ITGA2, ITGA5*, and *ITGA6*, based on scRNA-seq of CTB (isolated from placenta #1815), cultured in TSC derivation media. (**b**) Percent of early gestation first trimester CTB expressing markers of CTB (EGFR, ITGA6), pcEVT (ITGA5, ITGA2), pan-EVT (HLA-G) and mature EVT (ITGA1), by flow cytometry, at 4 timepoints during TSC derivation, in placenta #1815. (**c**) Violin plot showing initial state scoring of cells grouped by day of derivation, in placenta #1815. Scoring was done using the significantly upregulated genes in initial state cells in first trimester placenta (**Fig. 2b, Table S10**). Dashed lines represent quartiles. (**d**) Violin plots grouped by day of derivation, scoring cells from placenta #1815, based on the top 50 uniquely expressed genes as outlined by Arutyunyan et al.[1], for the cell types villous cytotrophoblast (VCT) and cytotrophoblast cell columns derived from VCT (VCT-CCC). Dashed lines represent quartiles. (**e**) Isolated CTB and MACS-sorted for ITGA5 cells plated in 2D. Pictures show that only ITGA5^{+} cells adhered to the tested substrates (fibronectin), in both oxygen tensions tested (21% or 2%, 21% shown), in TSC media, while very few ITGA5^{-} cells did the same.

2 Supplementary Tables

Table S1: Sample and sequencing metrics.
Table S2: Top 200 marker genes in each cluster in the first trimester placenta samples.
Table S3: Marker genes after removal of non-trophoblast clusters, reclustering, and annotation of clusters.
Table S4: Cell numbers of each cluster by gestational age for UMAP shown in **Fig. S2d**.
Table S5: Top 200 marker genes in each cluster of the merged primary placenta dataset shown in **Fig. 1d**.
Table S6: Genes upregulated (adj. p-value < 0.05 and log fold change > 1) in CTB3 (primarily composed of late first trimester CTB) vs CTB1 and CTB2 (primarily composed of early first trimester CTB) (Top) and genes upregulated (> 1 Log2 fold change and adj. p-value < 0.05) in CTB1 and CTB2 (primarily composed of early first trimester CTB) vs CTB3 (primarily composed of late first trimester CTB) (Bottom).
Table S7: Genes upregulated (> 1.5 Log2 fold change and adj. p-value < 0.05) in CTB1 vs CTB2 (both primarily composed of early first trimester CTB).
Table S8: Genes upregulated (> 1.5 Log2 fold change and adj. p-value < 0.05) in CTB2 vs CTB3 (both primarily composed of early first trimester CTB).
Table S9: Genes that best explain the RNA velocity vector field in **Fig. 2a**. These genes, called rank velocity genes, may play a role in driving the differentiation of CTB. Rank velocity genes with a minimum r^2 value of 0.5 and a minimum correlation coefficient between spliced and unspliced genes of 0.3 by cluster.
Table S10: Genes upregulated (adj. p-value < 0.05 and log fold change > 1) in initial state cells (n=30) vs all other cells in the early and late first trimester integrated placental dataset.
Table S11: Genes upregulated (adj. p-value < 0.05 and log fold change > 1) in either the basal plate ROIs or chorionic plate ROIs in GeoMx spatial WTA dataset.
Table S12: Top 200 marker genes of all clusters after integration of early first trimester trophoblast and TSC single cell data (**Fig. 4a**).
Table S13: Genes upregulated (adj. p-value ¡ 0.05 and log fold change ¿ 1) in initial state cells (n=30) vs TSC, in the early first trimester and TSC integrated dataset (**Fig. 4a**).

References

[1] Arutyunyan, A. *et al.* Spatial multiomics map of trophoblast development in early pregnancy. *Nature* **616**, 143–151 (2023).