PNAS RESEARCH ARTICLE | AGRICULTURAL SCIENCES

Single-nuclei RNA sequencing (snRNA-seq) uncovers trophoblast cell types and lineages in the mature bovine placenta

Kimberly M. Davenport[a], M. Sofia Ortega[a], Hongyu Liu[a], Eleanore V. O'Neil[a], Andrew M. Kelleher[b], Wesley C. Warren[a,c], and Thomas E. Spencer[a,b,1]

Contributed by Thomas E. Spencer; received January 2, 2023; accepted February 14, 2023; reviewed by Lawrence P. Reynolds, Michael J. Soares, and Geetu Tuteja

Ruminants have a semi-invasive placenta, which possess highly vascularized placentomes formed by maternal endometrial caruncles and fetal placental cotyledons and required for fetal development to term. The synepitheliochorial placenta of cattle contains at least two trophoblast cell populations, including uninucleate (UNC) and binucleate (BNC) cells that are most abundant in the cotyledonary chorion of the placentomes. The interplacentomal placenta is more epitheliochorial in nature with the chorion developing specialized areolae over the openings of uterine glands. Of note, the cell types in the placenta and cellular and molecular mechanisms governing trophoblast differentiation and function are little understood in ruminants. To fill this knowledge gap, the cotyledonary and intercotyledonary areas of the mature day 195 bovine placenta were analyzed by single nuclei analysis. Single-nuclei RNA-seq analysis found substantial differences in cell type composition and transcriptional profiles between the two distinct regions of the placenta. Based on clustering and cell marker gene expression, five different trophoblast cell types were identified in the chorion, including proliferating and differentiating UNC and two different types of BNC in the cotyledon. Cell trajectory analyses provided a framework for understanding the differentiation of trophoblast UNC into BNC. The upstream transcription factor binding analysis of differentially expressed genes identified a candidate set of regulator factors and genes regulating trophoblast differentiation. This foundational information is useful to discover essential biological pathways underpinning the development and function of the bovine placenta.

cattle | placenta | single-nuclei RNA-seq | transcription factor | trophoblast

Significance

The placenta is essential for successful reproduction in mammalian species, and cattle have a unique synepitheliochorial type of placenta. Although the bovine placenta has been studied histologically and biochemically, the essential cellular and molecular factors and pathways governing trophoblast differentiation remain unclear. This study profiled the transcriptome of single cells in the mature bovine placenta and identified trophoblast cell types and candidate regulatory genes, factors and pathways involved in trophoblast cell fate and differentiation.

The efficiency of reproduction impacts the profitability and sustainability of both beef and dairy cattle operations (1–3). Of note, pregnancy loss is substantial in beef and dairy cattle, even in well-managed herds, and a major determinant of production efficiency (1, 4, 5). Pregnancy establishment and maintenance in cattle is influenced by numerous factors, including fertilization, embryonic cleavage and formation of a blastocyst, conceptus elongation and signaling for maternal recognition of pregnancy, maintenance of the corpus luteum and progesterone secretion, and development of a functional placenta (6–8). Pregnancy loss often occurs within the first 30 d after conception, with up to 50% of losses occurring within the first 7 d of pregnancy after fertilization (1, 4, 9). Up to an additional 20% of pregnancies are lost between days 30 and 60, which represents the pivotal period of growth and establishment of a functional placenta to support optimal embryo/fetal growth and development to term (1). A functional placenta is critical to support nutrient uptake and delivery for fetal growth, particularly during the second and third trimesters, and impacts offspring survivability and health into adulthood (1, 10–14). Moreover, the placenta produces unique hormones that impact fetal growth and maternal adaptations to pregnancy.

The mature bovine placenta has two unique anatomical regions. Placentomes are formed by interdigitation of chorionic villi of fetal placental cotyledons and maternal endometrial caruncles that vastly increase the surface area for maternofetal transport of nutrients that provide hematotrophic nutrition for fetal growth (14). The intercotyledonary areas of the placenta are between the placentomes and develop specialized areolae over the superficial openings of glands in the intercaruncular areas of the endometrium that provide histotrophic nutrition for fetal and placental growth (14, 15). Discrete cotyledons begin to form only after day 30, presumably over the caruncles of the endometrium (14, 16). Between days 30 and 60, the cotyledons and caruncles develop together into placentomes (14). During the ensuing 4 mo, the placentomes grow larger and vascularity increases to provide nutrients for subsequent exponential increases in fetal growth to term (17, 18). Of note, failure of placentome development is associated with pregnancy loss between

Author affiliations: [a]Division of Animal Sciences, University of Missouri, Columbia, MO 65211; [b]Department of Obstetrics, Gynecology, and Women's Health, University of Missouri, Columbia, MO 65211; and [c]Institute for Data Science and Informatics, University of Missouri, Columbia MO 65211

Author contributions: K.M.D., E.V.O., and T.E.S. designed research; K.M.D., M.S.O., E.V.O., A.M.K., and W.C.W. performed research; K.M.D., M.S.O., H.L., W.C.W., and T.E.S. analyzed data; and K.M.D. and T.E.S. wrote the paper.

Reviewers: L.P.R., North Dakota State University; M.J.S., University of Kansas Medical Center; and G.T., Iowa State University.

The authors declare no competing interest.

Copyright © 2023 the Author(s). Published by PNAS. This article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

[1]To whom correspondence may be addressed. Email: spencerte@missouri.edu.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2221526120/-/DCSupplemental.

Published March 13, 2023.

days 60 and 90 in cloned cattle pregnancies derived by nuclear transfer (19). Likewise, surgical ablation of caruncles in sheep decreases the number of placentomes formed and causes pregnancy loss or fetal growth retardation (20).

In cattle and other ruminants, placental development includes differentiation of mononucleate trophoblast cells into trophoblast binucleate cells (BNC) in the outer layer of the chorion (14, 21). By day 20 of pregnancy, BNC begin to appear, presumably arising from trophoblast uninucleate trophoblast cells (UNC) by mitotic polyploidization or endoreduplication (6, 14, 21, 22). The BNC are thought to differentiate from diploid UNC by cell division without cytokinesis, which produces a BNC with two 2N nuclei. This process occurs throughout pregnancy, predominantly in the cotyledons after day 30, and results in a constant 15 to 20% of the trophectoderm being BNC at various stages of maturation (23). The differentiation process produces BNC below the trophoblast tight junctions, without any desmosomal attachments to closely adjacent trophoblast UNC nor penetration of the basement membrane or basal lamina (24). Maturation is thought to involve mitotic polyploidization to produce two larger 4N nuclei (25, 26) coincident with a considerable increase in cell size and production of placenta-specific hormones. Fully differentiated and mature BNC serve as the basis for the formation of the unique synepitheliochorial form of placentation observed in cattle (14, 27). In the placentome, fully granulated BNC migrate through, while maintaining, tight junctions with other trophoblast cells and then are thought to fuse with a uterine luminal epithelium (LE) cell. This allows for the delivery of secretory granules containing unique placenta-specific hormones, including chorionic somatomammotropin hormone two (CSH2 also known as placental lactogen) and pregnancy-associated glycoproteins (PAGs) as well as exosomes, microvesicles, and other factors throughout gestation into the endometrial stroma and thus maternal circulation by exocytosis (14, 27). Of relevance, pregnancy loss after day 30 in beef and dairy cattle pregnancies is associated with lower circulating PAG concentrations after day 20, which could be attributed to compromised BNC differentiation and thus problems with placentation (28).

Although the morphology and histology of the bovine conceptus and placenta have been studied in detail, little is known about the cell and molecular biology of trophoblast cell differentiation. Previous work in the placenta of humans (29–31) demonstrated that single cell analysis can capture the multiple cell types that constitute the placenta. Herein, single-nuclei RNA-seq (snRNA-seq) of the mature bovine placenta was performed to determine cell types and their gene expression patterns, compositional changes, inferred trajectories, and regulatory factors in the cotyledonary and intercotyledonary regions. The mature bovine placenta (sourced at day 195 in this study) provides the opportunity to capture the greatest number of BNCs to characterize their transcriptome and model their formation, as BNCs are thought to continually form and eventually fuse with the maternal endometrial epithelium. This molecular knowledge serves as a framework and resource to advance understanding of cellular and molecular regulation of placenta development, function, and evolution in mammals.

Results

Single-Nuclei Analysis Identifies Distinct Cell Types in the Cotyledonary and Intercotyledonary Placenta. The reproductive tract of gestating cattle was obtained on day 195 of gestation. The uterus was isolated, and the intercotyledonary areas of the placenta were obtained. Placentomes were then removed, and the cotyledonary areas of the placenta separated from the endometrial caruncle by manual dissection. Nuclei were isolated from each region of the placenta, and snRNA-seq libraries prepared with the 10× Chromium system (*SI Appendix*, Table S1). Seurat was used to normalize expression profiles (32) and identified 13 distinct clusters in the cotyledonary and 11 in the intercotyledonary area, which were assigned to cell types based on the expression of previously reported marker genes (*Materials and Methods* and Datasets S1 and S2). The uniform manifold approximation and projection (UMAP) (33) was used to display these clusters in two dimensions (Fig. 1 *A* and *C*). With this approach, the local and global topological structure of the clusters is preserved, with subtypes of the major cell lineages being displayed proximal to each other.

In the cotyledonary placenta, most cells were BNC (40.8%) or UNC (13.9%). Two different types of UNC trophoblast populations and three populations of BNC trophoblasts were identified (Fig. 1*A*). Other cell populations defined by the analysis included endothelial cells (3.1% of total), macrophages (3.0% of total), leukocytes (0.8% of total), neutrophils (0.4% of total), mesenchyme (0.2% of total), as well as endometrial luminal epithelium (LE; 0.4% of total). Two cell populations of unknown identity (together 37.4% of total) were also present (Fig. 1*A*). These unknown cell populations displayed marker genes related to mitochondrial function and were not able to be distinctly categorized. The architecture of different cell types in the chorionic villi of the cotyledon within the placentome is shown in Fig. 1*B*.

Nine trophoblast populations were present in the intercotyledonary placenta (Fig. 1*C*) with more UNC trophoblasts (37.0% of total cells) than BNC (12.8% of total cells). Other cell populations identified included mesenchymal cells (1.2% of total), endothelial cells (0.6% of total) as well as macrophages and leukocytes that comprised 0.4% and 0.2% of total cells, respectively. A small population of endometrial LE was also identified and represented 0.1% of the total cells. A population of cells with unknown identity were also identified that represented 47.5% of the total cells. The architecture of different cell types in the intercotyledonary placenta is shown in Fig. 1*D*.

Overall, gene expression of different cell types and trophoblast populations were similar between the cotyledonary and intercotyledonary areas of the placenta (Fig. 1 *E* and *F*). Marker genes that were used to identify BNC trophoblast populations included *CSH2* (placental lactogen), pregnancy-associated genes (*PAG10*, *PAG17*) and prolactin-related proteins (*PRP1*, *PRP2*) (14, 26), which clustered separately from UNC trophoblast populations that expressed *PAG2* and *PAG8*. Mesenchyme cell types were positive for *VIM* which is expressed solely in stromal-mesenchymal cells in the human placenta (34) as well as *IGF2*. Immune cell populations included macrophages expressing *C1QA* and *C1QB*, neutrophils expressing *BIRC5* and *CENPF* among other markers, and leukocytes expressing *BOLA*, *CD3E*, and *CD52*.

Identification of Trophoblast Cell Populations. The subpopulation of trophoblast cells in the two regions of the mature bovine placenta were then reclustered and further analyzed (Fig. 2). Six clusters of trophoblasts that included both UNC and BNC were identified in the cotyledon (Fig. 2*A*), with the majority BNC. The abundance of BNC was confirmed in the placentome chorioallantois by immunofluorescence using a rabbit anti-bovine PAG antibody that detects PAG4 and PAG6 (Fig. 2*B*). The UNC expressed primarily *PAG2* and *PAG8*, while BNC expressed primarily *PAG3*, *PAG16*, and *PAG17* along with *CSH2* (Fig. 2*C*). Of note, *PAG2* and *PAG8* are ancient PAGs expressed only in UNC, whereas the modern PAGs (*PAG3, PAG4, PAG16, PAG17*) are in BNC

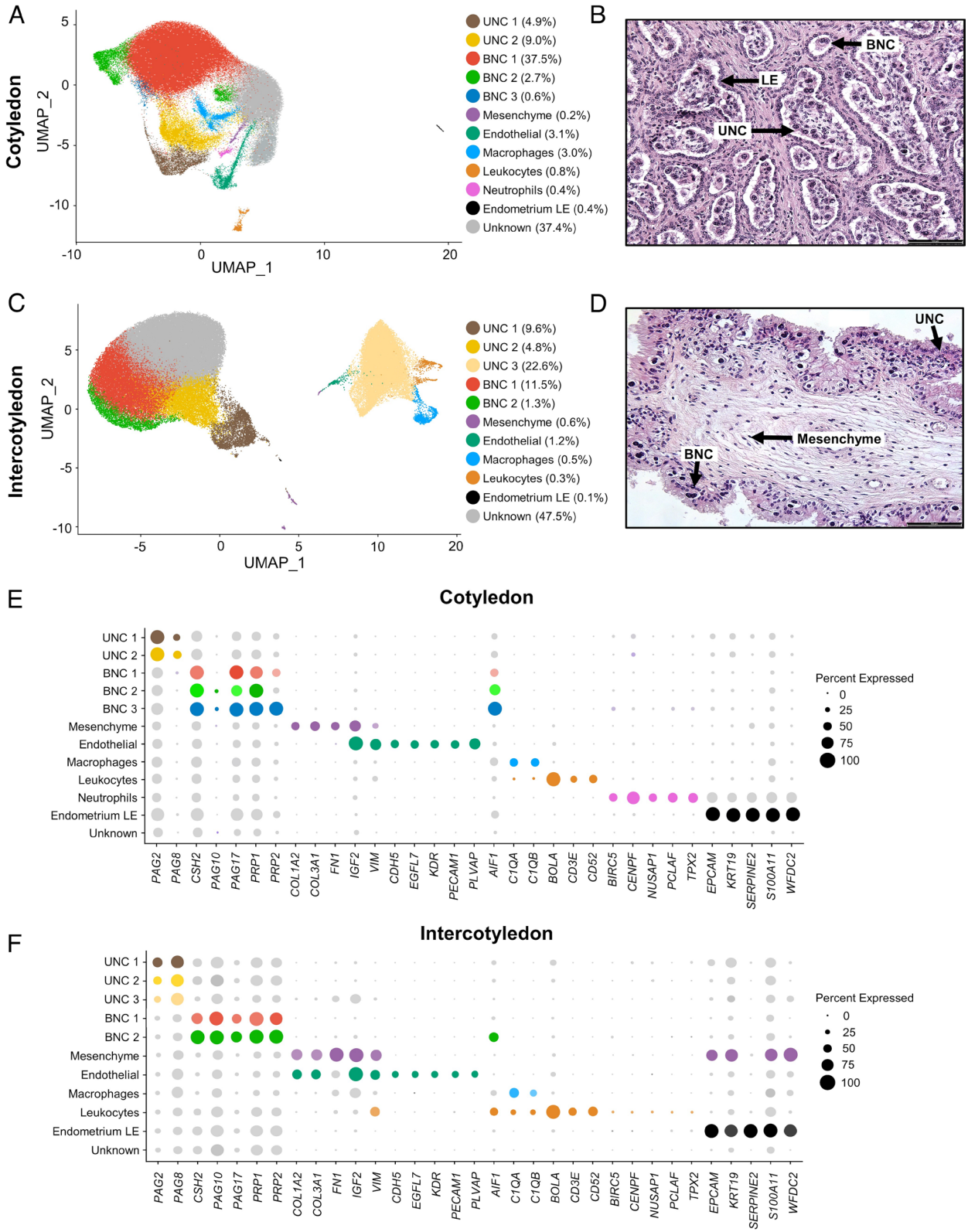

Fig. 1. Single-cell analysis of the mature day 195 bovine placenta. Uniform Manifold Approximation Plot (UMAP), where dots represent single cells and are colored by cell type for the cotyledonary (*A*) and intercotyledonary (*C*) areas of the placenta. Average proportions of cells in the cotyledonary areas of the placenta are provided for each cell type. Histological sections of the placentome (*B*) and intercotyledonary placenta (*D*). Dot plots of gene expression for markers of identified cell types in the cotyledonary (*E*) and intercotyledonary (*F*) areas of the placenta. BNC, binucleate trophoblast cell; LE, endometrial luminal epithelium; UNC, uninucleate trophoblast cell.

of the bovine placenta (35). The UNC also expressed trophoblast markers *PEG3* and *PEG10* along with transcription factors such as *FOXO4*, *GATA2*, *GATA3*, and *TCF7L2* (*SI Appendix*, Fig. S2). *GATA2* is conserved in trophoblast cells across mammals and regulates self-renewal of trophoblast stem and progenitor cells in the mouse placenta (36). The BNC expressed *PRP2*, *PRP3*, and *PRP4* (*SI Appendix*, Fig. S2). Two populations of BNC were identified based on *CSH2* and BNC-specific *PAG* marker gene

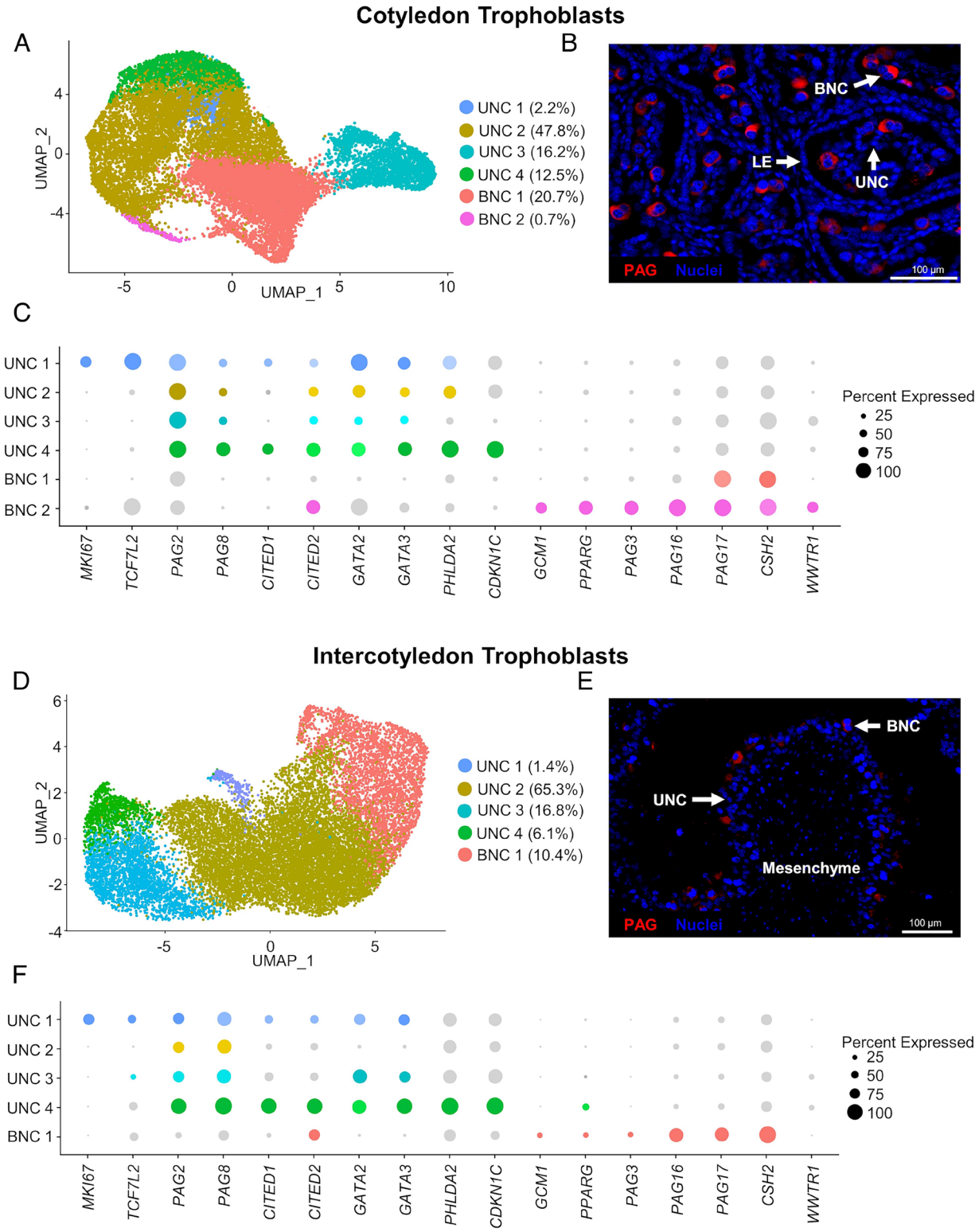

Fig. 2. Clustering and identification of trophoblast subpopulations present in the day 195 bovine placenta. (*A*) UMAP subclustering of trophoblast populations in the cotyledon. (*B*) The identification of BNC in the cotyledon. BNC were visualized by immunofluorescence analysis using a BNC-specific PAG antibody (red) staining. Nuclei (blue) were visualized by Hoechst nuclear staining. (*C*) Dot plots of select marker genes across trophoblast subpopulations in cotyledon. (*D*) UMAP sub-clustering of trophoblast populations in the intercotyledonary area of the placenta. (*E*) Immunoreactive BNC-specific PAGs in the intercotyledonary placenta. (*F*) Dot plots of select marker genes across trophoblast subpopulations in the intercotyledonary placenta. BNC, binucleate trophoblast cell; LE, luminal epithelium; UNC, uninucleate trophoblast cell.

expression. The cotyledonary BNC 2 population also expressed *WWTR1*, a Hippo signaling cofactor that optimizes trophoblast progenitor self-renewal and is essential for differentiation into the invasive extravillous trophoblast cell lineage in humans (37). The differential expression of *CITED2*, a gene expressed in numerous human and mouse placental cell types that plays a role in invasive extravillous trophoblast development in the rat and human (38), was found in two different BNC populations. Differentiating UNC were distinguished by expression of the cell cycle marker *CDKN1C*, an imprinted gene and potent growth regulator that

functions by inhibiting the activity of cyclin-dependent kinases to control cell proliferation and differentiation in the embryo and human placenta (39, 40). Proliferating UNC expressed proliferation markers *MKI67*, *MCM2*, and *PCNA*. A hallmark of fully differentiated BNC is the cessation of proliferation after endoreduplication (41).

The subpopulations of trophoblast present in the intercotyledonary placenta were like those identified in the cotyledon, except the intercotyledonary area did not display two distinct BNC populations (Fig. 2*D*). The expression of *PAGs* within UNC was like that of the cotyledon; however, *PAG12* was more abundant in UNC and *PAG10* was more abundant in the BNC of intercotyledonary placenta (*SI Appendix*, Fig. S2). Note the reduced abundance of BNC in the chorion of the intercotyledonary placenta assessed by immunofluorescence using a BNC-specific PAG antibody (which recognizes PAG4, PAG6) (Fig. 2*E*). Proliferating and differentiating UNC displayed similar expression profiles and characteristics across the two placenta locations (Fig. 2*F*).

Next, *CDKN1C* and *WWTR1* expression was interrogated at the mRNA and protein level in the day 195 bovine placenta (*SI Appendix*, Fig. S3) to visually identify distinct UNC and BNC populations in each region. *CDKN1C* expression was observed in several UNC populations (UNC 2, UNC 3, UNC 4). Immunoreactive CDKN1C protein was detected most abundantly in the cytoplasm of UNCs in the cotyledonary as well as intercotyledonary placenta, but not in the BNC. *WWTR1* mRNA was most abundant in one UNC cell type (UNC 3) and one BNC cell type in cotyledon (BNC 2) but was minimally expressed in trophoblasts of the intercotyledonary placenta. Similarly, immunoreactive WWTR1 protein was observed in the cytoplasm of a subset of UNC and BNC in the cotyledon and not detectable in trophoblasts of the intercotyledonary placenta.

Single-Cell Trajectory Analysis of Trophoblast Cells. Single-cell trajectory "pseudotime" analysis was conducted using Monocle3 (42) to better understand how trophoblast cells transition from one state to the next (Fig. 3). In both the cotyledonary (Fig. 3*A*) and intercotyledonary placenta (Fig. 3*B*), trophoblast cell trajectory was anchored in the proliferating UNC with two different transitions before a final transition to BNC. Of note, the trajectories became circular at the BNC trophoblast population, which indicates a terminal transition or differentiation event. Slingshot trajectory analysis (43) provided a similar cell differentiation path (*SI Appendix*, Fig. S4) which supports the predicted UNC to BNC differentiation trajectory identified with Monocle3.

A total of 7,835 and 8,495 differentially expressed genes (DEGs) were identified across trajectories in the pseudotime analysis (Datasets S3 and S4). The expression of 10 DEGs with known functions in mammalian trophoblast development or marker genes of mature BNC across pseudotime is provided in Fig. 3*C* for the cotyledonary and intercotyledonary placenta. Note the increased expression of *CSH2* and *PAG17* and decreased *PAG2* expression as the trophoblast cells transition into a terminally differentiated BNC population.

Identification of Candidate Transcription Factors Involved in Trophoblast Differentiation. Enrichment for transcription factor binding sites near DEGs across the UNC to BNC trajectory was investigated using ChIP-X Enrichment Analysis Version 3 and the Encyclopedia of DNA Elements (ENCODE) database (44). Twelve transcription factors with significantly enriched binding sites (5 kb upstream or downstream of the transcription start site) were expressed in at least one trophoblast cell population across both areas of the placenta (Fig. 4*A* and Table 1). Most of the 12 transcription factors were abundant in the proliferating UNC of the cotyledonary placenta or in the differentiating UNC of the intercotyledonary placenta (Fig. 4*A*). The transcription factors *CTCF*, *JUN*, *MYC*, *YY1*, *TCF7L2*, *BHLHE40*, *GATA2*, *TFAP2C*, *IRF3*, *TFAP2A*, *GATA3*, and *NR2F2* were abundant in the proliferating and differentiating UNC. The expression of these transcription factors was not detectable in the BNC of the placenta. In fact, very few transcription factors were expressed in BNC compared with UNC. The transcription factors expressed in BNC (*CITED2*, *GCM1*, *HAND1*, and *PPARG*) were present in the BNC 2 population (*SI Appendix*, Fig. S2).

Binding sites for the transcription factor CTCF had the greatest enrichment in DEGs across the UNC to BNC differentiation trajectory, followed by JUN, MYC, YY1 and TCF7L2, BHLHE40, GATA2, TFAP2C, IRF3, TFAP2A, and GATA3 and NR2F2 (Table 1). The binding sites of each transcription factor were significantly enriched the least for NR2F2 and GATA3 (9.5%) and most for CTCF (29.9%) of the total number of DEGs across pseudotime (Table 1). Genes with nearby transcription factor binding sites for BHLHE40 and GATA2 follow the expression pattern of the transcription factors, as they are most highly expressed in proliferating and differentiating UNC and decreased in BNC regardless of area of the placenta (Fig. 4*B*). The expression of *MYC*, *YY1*, *TCF7L2*, *JUN*, *CTCF*, *GATA3*, *IRF3*, *TFAP2C*, *TFAP2A,* and *NR2F2* transcription factors followed the same expression pattern as their associated genes with DNA binding sites, indicating their potential role in activating transcription of genes in the three types of UNC trophoblasts (*SI Appendix*, Figs. S5–S7).

Gene ontology analyses of genes with enriched transcription factor binding sites included biological processes related to regulation of translation, protein processing, and mRNA splicing (*SI Appendix*, Fig. S8). Previously described interactions between transcription factors were also queried in the STRING database (45) and all transcription factors had at least one known interaction with another transcription factor that were derived from curated databases and/or experimentally determined (*SI Appendix*, Fig. S9). The MYC and GATA3 transcription factors had the greatest number of connections (9 connections each) and were coexpressed with five and four other transcription factors, respectively. The transcription factors GATA2 and JUN were found to interact with 6, YY1 with 5, and NR2F2, TFAP2A, and CTCF with 4 other transcription factors. The mRNA abundance of transcription factors in this dataset (Fig. 4) indirectly supports evidence of coexpression from the STRING database.

Next, *BHLHE40* (abundant mRNA expression in the intercotyledonary placenta) and *GATA2* (abundant mRNA expression in the cotyledonary placenta) expression was interrogated at the mRNA and protein level in the day 195 bovine placenta (*SI Appendix*, Fig. S3). *BHLHE40* mRNA as well as BHLHE40 protein was observed predominantly in the cytoplasm of UNC trophoblasts. *GATA2* mRNA was most abundant in the proliferating UNC trophoblast and detected in other trophoblast cell types. GATA2 protein was detected most abundantly in the UNC of the placentome and intercotyledonary placenta.

Discussion

This study is the first to resolve specific cell type populations and their individual transcriptome profiles with snRNA-seq analyses to advance our understanding of the mature midgestation bovine placenta. These populations have been studied previously only by histology and immunohistochemistry techniques (14, 21–22, 23, 46). The snRNA-seq analysis identified major cell types in the two different areas of the chorion of the bovine placenta and

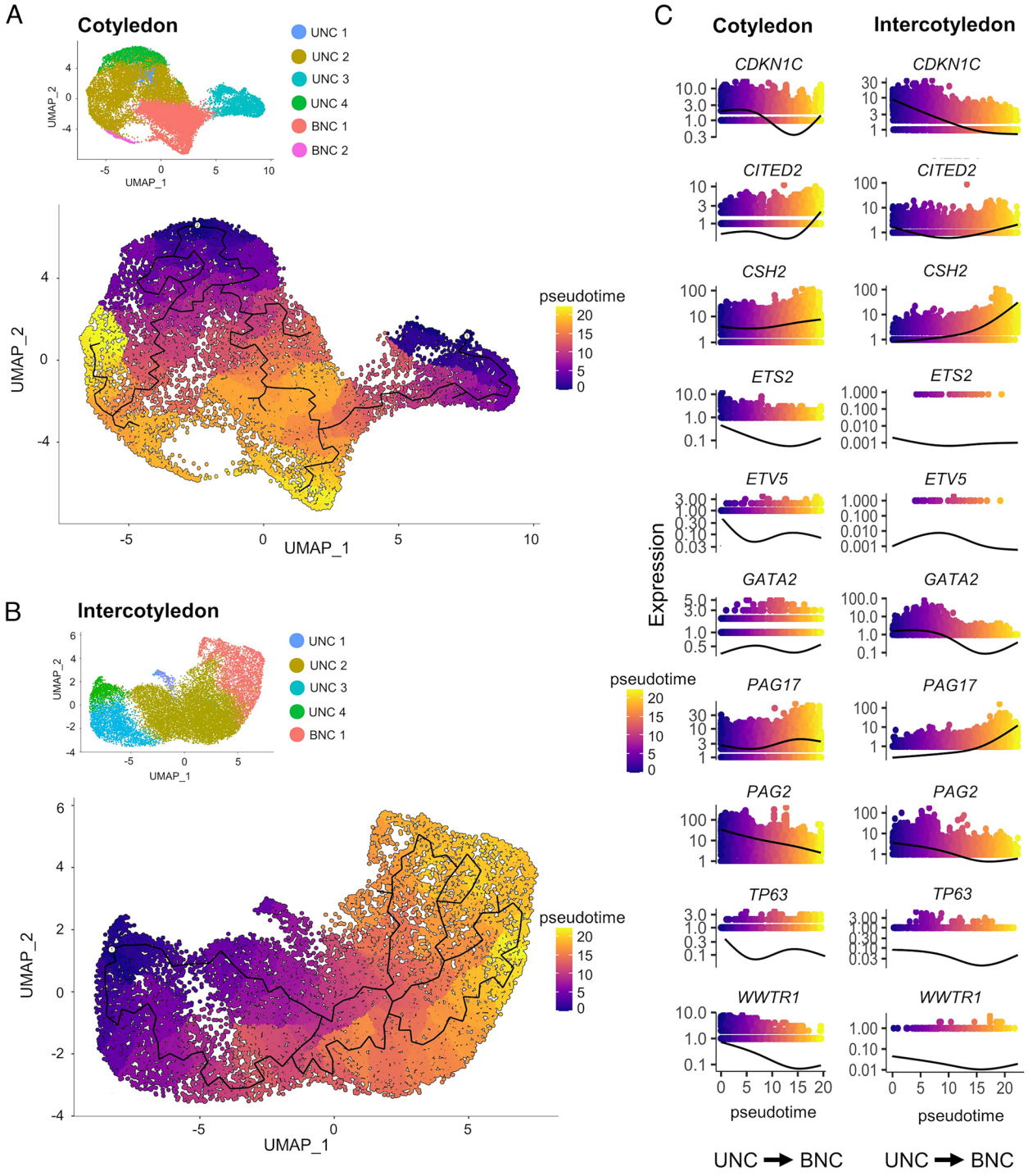

Fig. 3. Analyses of pseudotime trajectory of trophoblast differentiation in the (*A*) cotyledonary and (*B*) intercotyledonary placenta. (*C*) Differentially expressed genes across pseudotime for both areas of the bovine placenta.

differentiated the UNC from BNC trophoblasts. The BNC were determined by the expression of marker genes *CSH2* and binucleate-specific *PAGs*, such as *PAG17*, as those genes are not expressed in UNC and other cell populations (6, 14). The behavior of individual nuclei within a BNC, including those BNC that have fused with the maternal endometrial epithelium, was not able to be determined in this study and warrants further study. Of note, the UNC expressed genes identified in cytotrophoblasts from human and mouse placenta as well as human organoids (46–48).

The knowledge of trophoblast subpopulations was improved after sub-clustering of the original trophoblast clusters found in cotyledonary and intercotyledonary areas of the placenta. The UNC expressed *GATA2*, a known transcription factor in UNC (38, 49–51). A subpopulation of UNC expressed proliferation marker *MKI67* and stem cell markers *TBX3* (52) and *RIF1* (53), and another subpopulation expressed *CDKN1C* that encodes a protein regulating the cell cycle and endoreduplication (54). The BNC are presumed to arise via endoreduplication that requires cessation of the cell cycle, and the UNC 4 trophoblasts expressing *CDKN1C* may be differentiating to BNC (54). Indeed, trophoblast cell trajectory analyses predicted the differentiation of UNC 4 into BNC in both the cotyledonary and intercotyledonary placenta. CDKN1C (p57Kip2)

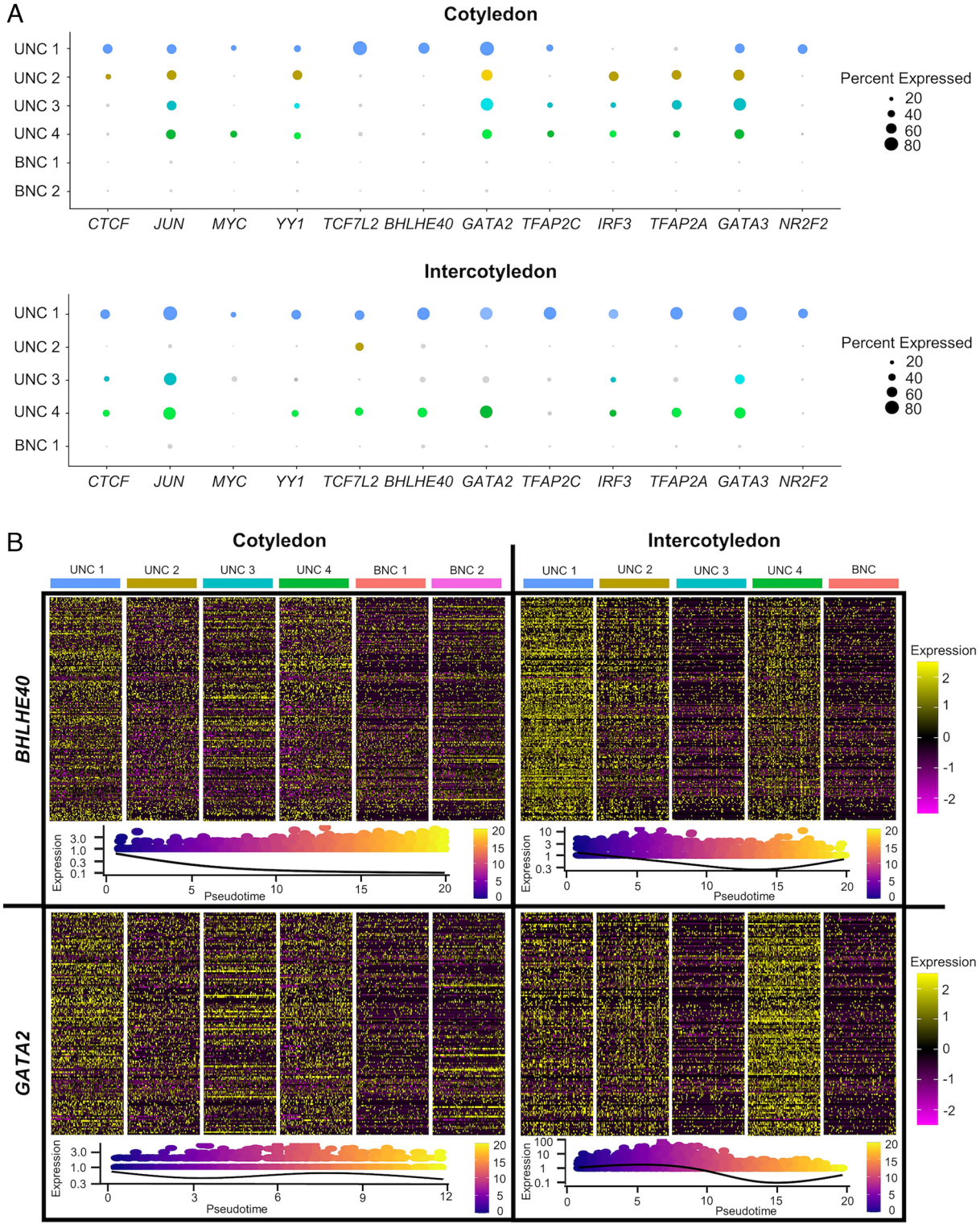

Fig. 4. Identification of candidate transcription factors regulating trophoblast trajectory. (*A*) Dot plots of gene expression for select transcription factors in each trophoblast cell type of the cotyledonary and intercotyledonary placenta. (*B*) Heatmap of expression of genes in trophoblast cell types with binding sites for BHLHE40 and GATA2 transcription factors in the bovine placenta based on pseudotime progression analysis.

inhibits the cyclin-dependent kinase CDK1 and is involved in the differentiation of trophoblast stem cells into trophoblast giant cells in the rodent placenta (54). The *CDKN1C* gene is imprinted (55, 56) and associated with large offspring syndrome in both cattle and humans (56–60). Although it is not known if *CDKN1C* is imprinted in the bovine placenta, parent-specific influences on gene expression in the mammalian placenta exist (61, 62).

The proportion of BNC in the cotyledonary and intercotyledonary placenta was comparable to reports from other studies that the cotyledonary chorion contains much more abundant numbers of BNC (21). The BNC are likely most abundant in the cotyledon, as the hormones they produce have greater access to the maternal circulation given that uterine blood flow is preferentially directed to the caruncles. A cluster of nuclei adjacent to BNCs in both

Table 1. Twelve significant (FDR < 0.01) transcription factors expressed in trophoblasts that have overrepresentation of gene targets differentially expressed across pseudotime, the number of genes and percent of total differentially expressed genes (DEGs), and binding motif

Transcription factor	Percent of total DEGs	No of genes	Odds ratio	FDR	Binding motif*
CTCF	30.0%	281	1.6	6.5E-10	
JUN	28.4%	266	1.7	1.4E-10	
MYC	26.5%	248	2.1	4.4E-19	
YY1	22.2%	208	2.2	5.4E-17	
TCF7L2	22.1%	207	2.2	1.6E-14	
BHLHE40	19.5%	183	2.1	2.7E-14	
GATA2	14.8%	139	1.8	7.7E-09	
TFAP2C	14.6%	137	1.8	3.3E-08	
IRF3	14.0%	131	1.9	1.1E-08	
TFAP2A	12.9%	121	1.6	2.2E-05	
GATA3	9.5%	89	2.2	5.7E-09	
NR2F2	9.5%	89	1.7	4.5E-05	

*Binding motifs procured from the HOMER transcription factor motif database.

tissues expressed *WWTR1*, a gene involved with the YAP pathway which regulates maintenance of cytotrophoblast-like fate and essentially blocking the transition to a syncytiotrophoblast in humans (37, 63, 64). Cell trajectory analysis of trophoblasts predicted a framework for UNC to BNC differentiation (Fig. 5). In this framework, the proliferating UNC 1 (marked by *MKI67* and *TCF7L2*) develop into a UNC 2–3 (marked by *PAG2* and *PAG8*). UNC 2–3 then form differentiating UNC (marked by *CDKN1C*) that could differentiate into two different types of BNC marked by up-regulation of BNC-specific genes (*CSH2, PAG3, PAG16, PAG17, PRP2, PRP3*) and loss of UNC-specific PAGs (*PAG2, PAG8*) (35). Two different BNC populations were observed in the cotyledonary but not intercotyledonary placenta. Given that the BNC 2 in the cotyledon was only 0.7% of total trophoblast cells, this could represent a true biological difference in the areas of the placenta or simply lower numbers of BNC in the intercotyledonary area that influenced cell type identification. The present study and trophoblast differentiation framework complement previous morphological and histological studies. Those studies identified intermediate developmental stages of UNC and BNC trophoblasts that differed in size, level of polyploidy, abundance of cytoplasmic granules, and location in the chorion epithelium (26, 65).

Many of the DEGs across the trajectory are associated with trophectoderm fate or placental function in other mammals, including *ETV5*, which prevents differentiation of trophoblast stem cells by regulating *FGF2* (66). The top 10 DEGs across pseudotime in both tissues included placental viral attachment factor *PROS1* (67), *RCAN1* which has been related to inflammation in the placenta (68), *SOD1* which is downregulated in preterm birth placentas in humans (69), placental iron uptake mediator gene *TFRC* (70), and *TP63* which inhibits differentiation of cytotrophoblasts in human trophoblast cells (71). In fact, all the "core" placental transcriptome genes (72) were expressed in the cotyledon (CPM > 10) as determined by bulk RNA-seq analysis of the chorion (Dataset S5). Seventeen (17) of those core genes were enriched in BNC, 14 in UNC, six in mesenchymal cells, five in endothelial cells, two in neutrophils, and one in leukocytes. Further, 85 genes were expressed in the mature bovine placenta of the 127 genes found to be significantly associated with a specific placentation shape (72) (Dataset S6). The mature bovine placenta also expressed 16 of the 22 genes used as markers for human cytotrophoblast, extravillous trophoblast, and syncytiotrophoblast in culture (73) (Dataset S6). Two genes (*CTNNB1* and *LRP5*) profiled in human cytotrophoblast were enriched in UNC stem cells (73). The genes *CD9* and *FN1* profiled in extravillous trophoblast were enriched in endothelial and mesenchyme clusters, respectively, while placental lactogen (*CSH2* in cattle) and *HSD3B1* profiled in syncytiotrophoblast were enriched in BNC and UNC trophoblast, respectively (73). This suggests that

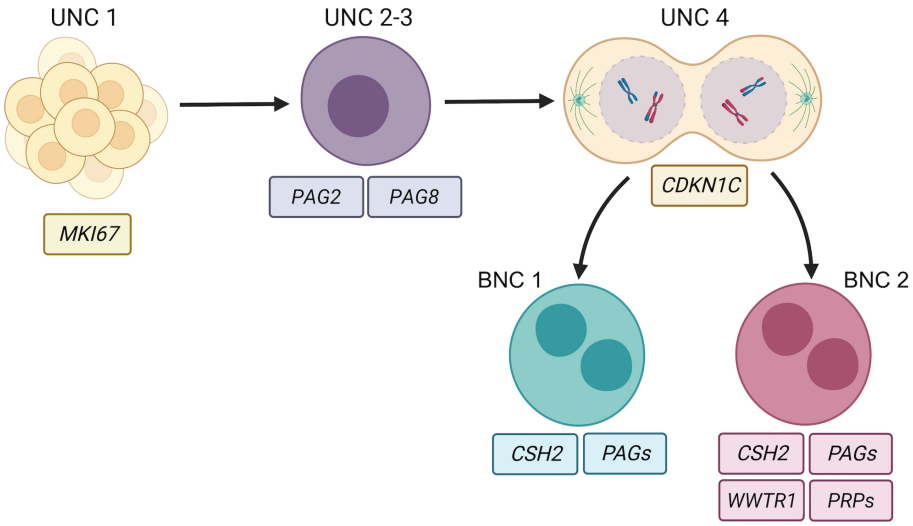

Fig. 5. Proposed trophoblast cell differentiation based on pseudotime analyses and marker gene analysis. Trophoblast lineage was predicted to begin with proliferating trophoblasts UNC. Differentiation of UNC into BNC involves the loss of UNC-specific PAGs and cell cycle inhibition during endoreduplication. Differentiating UNC can develop in two different BNC types that express BNC-specific genes.

some mechanisms relating trophoblast fate and differentiation in cattle are conserved with other mammalian species despite differences in placental structures (72).

The present study identified expressed transcription factors with binding sites near DEGs in the trophoblast differentiation trajectory that are involved with many essential and relevant cellular processes, including cell cycle progression and trophoblast development, in other species. The transcription factor with the most significant enrichment was MYC, which is involved with cell cycle progression, apoptosis, and cellular transformation in mammals (74). Six of the 10 transcription factors are directly linked to trophoblast function in mammals, including JUN which has been shown to be involved with trophoblast invasion (75), BHLHE40 which has been associated with pre-eclampsia (76), and GATA2 and GATA3 (36, 39, 51, 77), NR2F2 (78), and TFAP2A and TFAP2C (79–81) involved with trophoblast differentiation. Of note, GATA2, GATA3, TFAP2A, and TFAP2C have been shown to form a network of transcription factors to regulate early trophoblast progenitor specification by activating associated genes and inhibiting pluripotency genes such as OCT4 (39). Further, YY1 has been shown to promote trophoblast invasion by promoting *MMP2* expression, and mRNA expression of *YY1* is associated with recurrent miscarriage in humans (82). YY1 is also involved with DNA looping and facilitating enhancer-promoter interactions across the genome in a similar manner to CTCF (83). CTCF can act as both a transcription factor and insulator for topologically associated domains in chromatin looping (84, 85), regulates pregnancy specific glycoprotein (*PSG1*, *PSG2*, *PSG4*, *PSG5*, *PSG6*, *PSG8*, *PSG9*, and *PSG11*) expression in a human trophoblast cell line (86). Lastly, the transcription factor TCF7L2 has been shown to be involved with stem cell differentiation and epithelial to mesenchymal transition (87, 88), while IRF3 is an interferon regulatory factor expressed in the bovine placenta (89). The previously characterized involvement of these transcription factors in critical processes during mammalian placentation further supports that the mechanisms driving trophoblast fate may be conserved in cattle, despite very different placental structures (72).

The transcription factors and associated genes enriched in UNC that were minimally expressed or absent in BNC suggests that there could be regulatory mechanisms that are silencing gene expression during trophoblast differentiation. Further analysis of UNC 2–3 and BNC 1 trophoblast populations found 456 DEGs in the cotyledonary and 685 DEGs in the intercotyledonary placenta (Datasets S3 and S4). Using a database of human transcription factors (90), 20 transcription factors (*ASH1L, BBX, EPAS1, FOXO4, GATA2, GATA3, GPBP1, JUN, JUND, KLF6, NFE2L2, PEG3, RXRA, SON, TCF7L2, TFAP2A, TFDP1, YBX1, ZNF292, ZFHX3*) were more abundant in the UNC 2–3 trophoblasts but only one transcription factor (*NME2*) more abundant in BNC 1 trophoblasts. Similarly in the intercotyledonary placenta, 41 transcription factors (*ASH1L, BAZ2A, BBX, EPAS1, ETS2, ETV3, FOXO4, GATA2, GATA3, GPBP1, GTF2B, HAND1, HBP1, IRF3, KLF6, MGA, MLXIPL, MSX1, MYSM1, NCOA2, NFAT5, NFE2L1, NFE2L2, PEG3, PRDM2, RXRA, SAFB, SMAD4, SON, SPEN, TBX3, TERF2, TET2, TFAP2A, TFDP2, TRAFD1, ZFHX3, ZMAT1, ZNF131, ZNF292, ZZZ3*) were more abundant in the UNC 2–3 trophoblasts, but only three transcription factors more abundant in the BNC trophoblasts (*NME2, PPARG, YBX1*). Thus, the loss of specific transcription factors during differentiation of UNC into mature BNC appears to be a critical component of cell fate and lineage differentiation. Future studies should focus on discovering the genetic and epigenetic mechanisms underlying differentiation of proliferative UNC to nonproliferative BNC with particular attention to genes encoding transcription factors.

The cellular mechanism driving differentiation of UNC into BNC is posited to be endoreduplication or acytokinetic mitoses resulting in a polyploid cell (14). The underlying genetic process and impact of endoreduplication is essentially unknown for trophoblast differentiation in the ruminant placenta. Various physiologic states or pathologic conditions can induce cells to exit their mitotic cycle and differentiate into polyploid cells (91). In rodents, trophoblast giant cells are the first cell type to terminally differentiate during embryogenesis and arise from the trophectoderm layer in the blastocyst (54). The differentiation of placental trophoblast stem cells to trophoblast giant cells is caused by transition from a mitotic cell cycle to an endoreduplication that is triggered by CDKN1C (p57Kip2) inhibition of CDK1 activity, finally resulting in an increased amount of nuclear DNA (54). Induction of CDKN1A (p21CIP1) serves to prevent apoptosis in cells undergoing multiple rounds of endoreduplication (54). In the present study, CDKN1C was found to be most abundant in the UNC 4 population that is likely UNC trophoblasts differentiating into BNC. Future studies need to be focused on developing an in vitro culture system to derive

mature BNC from UNC to understand the critical cellular and molecular processes governing trophoblast differentiation in the bovine placenta (92). Further, studies of isolated UNC and BNC need to be conducted to understand the genetic and epigenetic regulation of endoreduplication-based differentiation and induction of genes expressed only in BNC. These studies are critically important, as trophoblast BNC in the chorion are essential for pregnancy establishment and formation of a placenta for maintenance of pregnancy and support of fetal growth to term in ruminants (19, 22).

Data, Materials, and Software Availability. Sequence data have been deposited in the Gene Expression Omnibus (GEO) with accession no. GSE214407 and are publicly available (93).

ACKNOWLEDGMENTS. We would like to thank the A.M.K. and T.E.S. laboratory for helpful discussions and the University of Missouri Genomics Technology Core for assistance with library preparation and sequencing. This work was supported by United States Department of Agriculture, National Institute of Food and Agriculture, and Agriculture and Food Research Initiative (USDA NIFA AFRI) Competitive Grant (2019-67015-28998).

1. M. C. Wiltbank *et al.*, Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. *Theriogenology* **86**, 239–253 (2016).
2. J. M. Sánchez *et al.*, Embryonic maternal interaction in cattle and its relationship with fertility. *Reprod. Domest. Anim.* **53**, 20–27 (2018).
3. M. F. Smith, R. D. Geisert, J. J. Parrish, Reproduction in domestic ruminants during the past 50 yr: Discovery to application. *J. Anim. Sci.* **96**, 2952–2970 (2018).
4. M. G. Diskin, D. G. Morris, Embryonic and early foetal losses in cattle and other ruminants. *Reprod. Domest. Anim.* **43**, 260–267 (2008).
5. M. G. Diskin, S. M. Waters, M. H. Parr, D. A. Kenny, Pregnancy losses in cattle: Potential for improvement. *Reprod. Fertil. Dev.* **28**, 83–93 (2016).
6. T. E. Spencer, P. R. Hansen, Implantation and establishment of pregnancy in ruminants. *Adv. Anat. Embryol. Cell Biol.* **216**, 105–135 (2015).
7. C. V. Barnwell *et al.*, Differences in mRNA populations of short and long bovine conceptuses on Day 15 of gestation. *Mol. Reprod. Dev.* **83**, 424–441 (2016).
8. J. G. N. Moraes *et al.*, Uterine influences on conceptus development in fertility-classified animals. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E1749–E1758 (2018).
9. R. Sartori, M. R. Bastos, M. C. Wiltbank, Factors affecting fertilisation and early embryo quality in single- and superovulated dairy cattle. *Reprod. Fertil. Dev.* **22**, 151–158 (2010).
10. R. M. Eley *et al.*, Development of the conceptus in the bovine. *J. Dairy Sci.* **61**, 467–473 (1978).
11. C. L. Ferrell, S. P. Ford, Blood flow steroid secretion and nutrient uptake of the gravid bovine uterus. *J. Anim. Sci.* **50**, 1113–1121 (1980).
12. G. J. King, Comparative placentation in ungulates. *J. Exp. Zool.* **266**, 588–602 (1993).
13. R. Leiser, C. Pfarrer, M. Abd-Elnaeim, V. Dantzer, Feto-maternal anchorage in epitheliochorial and endotheliochorial placental types studied by histology and microvascular corrosion casts. *Placenta* **9**, 21–39 (1998).
14. J. A. Green, R. D. Geisert, G. A. Johnson, T. E. Spencer, Implantation and placentation in ruminants. *Adv. Anat. Embryol. Cell Biol.* **234**, 129–154 (2021).
15. G. A. Johnson *et al.*, Cellular events during ovine implantation and impact for gestation. *Anim. Reprod.* **15**, 843–855 (2018).
16. A. C. Assis Neto *et al.*, Morpho-physical recording of bovine conceptus (Bos indicus) and placenta from days 20 to 70 of pregnancy. *Reprod. Domest Anim.* **45**, 760–772 (2010).
17. R. A. Laven, A. R. Peters, Bovine retained placenta: Aetiology, pathogenesis and economic loss. *Vet. Rec.* **139**, 465–471 (1996).
18. R. Leiser *et al.*, Fetal villosity and microvasculature of the bovine placentome in the second half of gestation. *J. Anat.* **191**, 517–527 (1997).
19. J. R. Hill *et al.*, Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. *Biol. Reprod.* **63**, 1787–1794 (2000).
20. D. J. Mellor, B. Mitchell, I. C. Matheson, Reductions in lamb weight caused by pre-mating caruncloctomy and mid-pregnancy placental ablation. *J. Comp. Pathol.* **87**, 623–633 (1977).
21. F. B. P. Wooding, The ruminant placental trophoblast binucleate cell: An evolutionary breakthrough. *Biol. Reprod.* **107**, 705–716 (2022).
22. J. D. Haeger, N. Hambruch, C. Pfarrer, Placental development and its control in cattle. *Bioscientifica Proc.* **8**, 12 (2019).
23. F. B. Wooding, Frequency and localization of binucleate cells in the placentomes of ruminants. *Placenta* **4**, 527–539 (1983).
24. J. Attiger, A. Boos, K. Klisch, Morphological characterization of basally located uninucleate trophoblast cells as precursors of bovine binucleate trophoblast giant cells. *Cells Tissues Organs.* **205**, 151–163 (2018).
25. K. Klisch, P. D. Thomsen, V. Dantzer, R. Leiser, Genome multiplication is a generalised phenomenon in placentomal and interplacentomal trophoblast giant cells in cattle. *Reprod. Fertil. Dev.* **16**, 301–306 (2004).
26. K. Klisch, C. Pfarrer, G. Schuler, B. Hoffman, R. Leiser, Tripolar acytokinetic mitosis and formation of feto-maternal syncytia in the bovine placentome: different modes of the generation of multinuclear cells. *Anat. Embryol. (Berl)* **200**, 229–237 (1999).
27. F. B. Wooding, D. C. Wathes, Binucleate cell migration in the bovine placentome. *J. Reprod. Fertil.* **59**, 425–430 (1980).
28. S. T. Reese *et al.*, Pregnancy associated glycoproteins (PAGs) and pregnancy loss in high vs sub fertility heifers. *Theriogenology* **1**, 7–12 (2019).
29. J. C. H. Tsang *et al.*, Integrative single-cell and cell-free plasma RNA transcriptomics elucidates placental cellular dynamics. *Proc. Natl. Acad. Sci. U.S.A.* **114**, E7786–E7795 (2017).
30. R. Vento-Tormo *et al.*, Single-cell reconstruction of the early maternal-fetal interface in humans. *Nature* **563**, 347–353 (2018).
31. R. Pique-Regi *et al.*, Single cell transcriptional signatures of the human placenta in term and preterm parturition. *Elife* **8**, e52004 (2019).
32. A. Butler, P. Hoffman, P. Smibert, E. Papalexi, R. Satija, Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat. Biotechnol.* **36**, 411–420 (2018).
33. L. McInnes, J. Healy, N. Saul, L. Großberger, UMAP: Uniform manifold approximation and projection. *JOSS* **3**, 861 (2018).
34. W. Abou-Kheir, A. Eid, R. El-Merahbi, R. Assaf, G. Daoud, A unique expression of keratin 14 in a subset of trophoblast cells. *PLoS One* **10**, e0139939 (2015).
35. M. Polei, J. Günther, D. Koczan, R. Fürbass, Trophoblast cell differentiation in the bovine placenta: Differentially expressed genes between uninucleate trophoblast cells and trophoblast giant cells are involved in the composition and remodeling of the extracellular matrix and O-glycan biosynthesis. *BMC Mol. Cell Biol.* **17**, 1 (2020).
36. S. Paul, P. Home, B. Bhattacharya, S. Ray, GATA factors: Master regulators of gene expression in trophoblast progenitors. *Placenta* **60**, S61–S66 (2017).
37. S. Ray *et al.*, Hippo signaling cofactor, WWTR1, at the crossroads of human trophoblast progenitor self-renewal and differentiation. *Proc. Natl. Acad. Sci. U.S.A.* **119**, e2204069119 (2022).
38. M. Kuna *et al.*, CITED2 is a conserved regulator of the uterine-placental interface. *Proc. Natl. Acad. Sci. U.S.A.* **120**, e2213622120 (2022).
39. C. Krendl *et al.*, GATA2/3-TFAP2A/C transcription factor network couples human pluripotent stem cell differentiation to trophectoderm with repression of pluripotency. *Proc. Natl. Acad. Sci. U.S.A.* **114**, E9579–E9588 (2017).
40. A. C. Saunders, B. McGonnigal, A. Uzun, J. Padbury, The developmental expression of the CDK inhibitor p57(kip2) (Cdkn1c) in the early mouse placenta. *Mol. Reprod. Dev.* **83**, 405–412 (2016).
41. F. B. Wooding, Current topic: The synepitheliochorial placenta of ruminants: Binucleate cell fusions and hormone production. *Placenta* **13**, 101–113 (1992).
42. C. Trapnell *et al.*, The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat. Biotechnol.* **32**, 381–386 (2014).
43. K. Street *et al.*, Slingshot: Cell lineage and pseudotime inference for single-cell transcriptomics. *BMC Genomics* **19**, 477 (2018).
44. A. B. Keenan *et al.*, ChEA3: Transcription factor enrichment analysis by orthogonal omics integration. *Nucleic Acids Res.* **47**, W212–W224 (2019).
45. D. Szklarczyk *et al.*, The STRING database in 2021: Customizable protein-protein networks, and functional characterization of user-uploaded gene/measurement sets. *Nucleic Acids Res.* **8**, D605–D612 (2021).
46. F. B. Wooding, G. Morgan, M. R. Brandon, S. Camous, Membrane dynamics during migration of placental cells through trophectodermal tight junctions in sheep and goats. *Cell Tissue Res.* **276**, 387–397 (1994).
47. F. B. Wooding, G. Morgan, C. L. Adam, Structure and function in the ruminant synepitheliochorial placenta: Central role of the trophoblast binucleate cell in deer. *Microsc. Res. Tech.* **38**, 88–99 (1997).
48. M. Y. Turco *et al.*, Trophoblast organoids as a model for maternal-fetal interactions during human placentation. *Nature* **564**, 263–267 (2018).
49. M. Knöfler *et al.*, Human placenta and trophoblast development: Key molecular mechanisms and model systems. *Cell Mol. Life Sci.* **76**, 3479–3496 (2019).
50. Q. Li, H. Wu, Y. Wang, H. Wang, Current understanding in deciphering trophoblast cell differentiation during human placentation. *Biol. Reprod.* **107**, 317–326 (2022).
51. H. Bai *et al.*, Regulation of trophoblast-specific factors by GATA2 and GATA3 in bovine trophoblast CT-1 cells. *J. Reprod. Dev.* **57**, 518–525 (2011).
52. T. Esmailpour, T. Huang, TBX3 promotes human embryonic stem cell proliferation and neuroepithelial differentiation in a differentiation stage-dependent manner. *Stem. Cells* **30**, 2152–2163 (2012).
53. Y. Mei, Y. B. Liu, S. Cao, Z. W. Tian, H. H. Zhou, RIF1 promotes tumor growth and cancer stem cell-like traits in NSCLC by protein phosphatase 1-mediated activation of Wnt/β-catenin signaling. *Cell Death Dis.* **9**, 942 (2018).
54. Z. Ullah, M. J. Kohn, R. Yagi, L. T. Vassilev, M. L. DePamphilis, Differentiation of trophoblast stem cells into giant cells is triggered by p57/Kip2 inhibition of CDK1 activity. *Genes. Dev.* **22**, 3024–3036 (2008).
55. L. Lefebvre, The placental imprintome and imprinted gene function in the trophoblast glycogen cell lineage. *Reprod. Biomed. Online* **25**, 44–57 (2012).
56. M. D. Simmers, K. M. Hudson, M. Baptissart, M. Cowley, Epigenetic control of the imprinted growth regulator *Cdkn1c* in cadmium-induced placental dysfunction. *Epigenetics*, 10.1080/15592294.2022.2088173 (2022).
57. S. J. Tunster, M. Van de Pette, R. M. John, Fetal overgrowth in the Cdkn1c mouse model of Beckwith-Wiedemann syndrome. *Dis. Model. Mech.* **4**, 814–821 (2011).
58. K. M. Robbins, Z. Chen, K. D. Wells, R. M. Rivera, Expression of KCNQ1OT1, CDKN1C, H19, and PLAGL1 and the methylation patterns at the KvDMR1 and H19/IGF2 imprinting control regions is conserved between human and bovine. *J. Biomed. Sci.* **19**, 95 (2012).
59. A. M. Driver, W. Huang, J. Kropp, F. Peñagaricano, H. Khatib, Knockdown of CDKN1C (p57(kip2)) and PHLDA2 results in developmental changes in bovine pre-implantation embryos. *PLoS One* **8**, e69490 (2013).
60. M. López-Abad, I. Iglesias-Platas, D. Monk, Epigenetic characterization of CDKN1C in placenta samples from non-syndromic intrauterine growth restriction. *Front. Genet.* **7**, 62 (2016).
61. D. Pilvar, M. Reiman, A. Pilvar, M. Laan, Parent-of-origin-specific allelic expression in the human placenta is limited to established imprinted loci and it is stably maintained across pregnancy. *Clin. Epigenet.* **11**, 94 (2019).
62. P. Dini *et al.*, Parental bias in expression and interaction of genes in the equine placenta. *Proc. Natl. Acad. Sci. U.S.A.* **118**, e2006474118 (2021).
63. Z. Huang *et al.*, The novel role of Hippo-YAP/TAZ in immunity at the mammalian maternal-fetal interface: Opportunities, challenges. *Biomed. Pharmacother.* **126**, 110061 (2020).
64. S. Saito *et al.*, Requirement for expression of WW domain containing transcription regulator 1 in bovine trophectoderm development. *Biochem. Biophys. Res. Commun.* **555**, 140–146 (2021).
65. K. Klisch *et al.*, DNA content and ploidy level of bovine placentomal trophoblast giant cells. *Placenta* **20**, 451–458 (1999).

66. K. Zhu *et al.*, Etv5 safeguards trophoblast stem cells differentiation from mouse EPSCs by regulating fibroblast growth factor receptor 2. *Mol. Biol. Rep.* **47**, 9259–9269 (2020).
67. O. Guzeloglu-Kayisli *et al.*, Zika virus-infected decidual cells elicit a gestational age-dependent innate immune response and exaggerate trophoblast Zika permissiveness: Implication for vertical transmission. *J. Immunol.* **205**, 3083–3094 (2020).
68. H. Y. Yuan *et al.*, MicroRNA-34a targets regulator of calcineurin 1 to modulate endothelial inflammation after fetal cardiac bypass in goat placenta. *Placenta* **51**, 49–56 (2017).
69. P. Lin *et al.*, Network analysis reveals important genes in human placenta. *J. Obstet. Gynaecol. Res.* **47**, 2607–2615 (2021).
70. C. Mandò *et al.*, Transferrin receptor gene and protein expression and localization in human IUGR and normal term placentas. *Placenta* **32**, 44–50 (2011).
71. Y. Li, M. Moretto-Zita, S. Leon-Garcia, M. M. Parast, p63 inhibits extravillous trophoblast migration and maintains cells in a cytotrophoblast stem cell-like state. *Am. J. Pathol.* **184**, 3332–3343 (2014).
72. D. L. Armstrong *et al.*, The core transcriptome of mammalian placentas and the divergence of expression with placental shape. *Placenta* **57**, 71–78 (2017).
73. H. Okae *et al.*, Derivation of human trophoblast stem cells. *Cell Stem. Cell* **22**, 50–63 (2018).
74. B. Rahat, A. Hamid, R. Ahmad Najar, R. Bagga, J. Kaur, Epigenetic mechanisms regulate placental c-myc and hTERT in normal and pathological pregnancies; c-myc as a novel fetal DNA epigenetic marker for pre-eclampsia. *Mol. Hum. Reprod.* **20**, 1026–1040 (2014).
75. B. Peng *et al.*, AP-1 transcription factors c-FOS and c-JUN mediate GnRH-induced cadherin-11 expression and trophoblast cell invasion. *Endocrinology* **156**, 2269–2277 (2015).
76. C. Mi *et al.*, BHLHE40 plays a pathological role in pre-eclampsia through upregulating SNX16 by transcriptional inhibition of miR-196a-5p. *Mol. Hum. Reprod.* **26**, 532–548 (2020).
77. A. Ralston *et al.*, Gata3 regulates trophoblast development downstream of Tead4 and in parallel to Cdx2. *Development* **137**, 395–403 (2010).
78. M. A. Hubert, S. L. Sherritt, C. J. Bachurski, S. Handwerger, Involvement of transcription factor NR2F2 in human trophoblast differentiation. *PLoS One* **5**, e9417 (2010).
79. P. Kuckenberg, C. Kubaczka, H. Schorle, The role of transcription factor Tcfap2c/TFAP2C in trophectoderm development. *Reprod. Biomed. Online* **25**, 12–20 (2012).
80. S. Kaiser *et al.*, Reduced gene dosage of Tfap2c Impairs trophoblast lineage differentiation and alters maternal blood spaces in the mouse placenta. *Biol. Reprod.* **93**, 31 (2015).
81. R. M. Karvas *et al.*, Stem-cell-derived trophoblast organoids model human placental development and susceptibility to emerging pathogens. *Cell Stem. Cell.* **29**, 810–825 (2022).
82. F. J. Tian *et al.*, The YY1/MMP2 axis promotes trophoblast invasion at the maternal-fetal interface. *J. Pathol.* **239**, 36–47 (2016).
83. A. S. Weintraub *et al.*, YY1 is a structural regulator of enhancer-promoter loops. *Cell* **171**, 1573–1588 (2017).
84. L. Braccioli, E. de Wit, CTCF: A Swiss-army knife for genome organization and transcription regulation. *Essays Biochem.* **63**, 157–165 (2019).
85. Q. Wu, P. Liu, L. Wang, Many facades of CTCF unified by its coding for three-dimensional genome architecture. *J. Genet. Genomics* **47**, 407–424 (2020).
86. D. S. Jeong, M. H. Kim, J. Y. Lee, Depletion of CTCF disrupts PSG gene expression in the human trophoblast cell line Swan 71. *FEBS Open Bio.* **11**, 804–812 (2021).
87. K. Hayashi, R. C. Burghardt, F. W. Bazer, T. E. Spencer, WNTs in the ovine uterus: Potential regulation of periimplantation ovine conceptus development. *Endocrinology* **148**, 3496–3506 (2007).
88. X. Zhang *et al.*, Wnt signaling promotes hindgut fate commitment through regulating multi-lineage genes during hESC differentiation. *Cell Signal.* **29**, 12–22 (2017).
89. T. Shirozu *et al.*, Expression dynamics of bovine MX genes in the endometrium and placenta during early to mid pregnancy. *J. Reprod. Dev.* **62**, 29–35 (2016).
90. S. A. Lambert *et al.*, The human transcription factors. *Cell* **8**, 650–665 (2018).
91. J. Zhang, Q. Qiao, H. Xu, R. Zhou, X. Liu, Human cell polyploidization: The good and the evil. *Semin. Cancer Biol.* **81**, 54–63 (2022).
92. V. V. Pillai *et al.*, RhoA/ROCK signaling antagonizes bovine trophoblast stem cell self-renewal and regulates preimplantation embryo size and differentiation. *Development* **149**, dev200115 (2022).
93. K. M. Davenport, T. E. Spencer, Single nuclei transcriptomics of the bovine placenta at mid gestation predicts transcription factors involved with trophoblast fate. Gene Expression Omnibus. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE214407. Deposited 28 September 2022.