TITLE: Bridging the gap of late-gestation nephrogenesis using a non-human primate model

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Key points:

- Single-cell RNA sequencing analysis of late-gestation primate nephron progenitor cells (NPCs) identifies rhesus-specific self-renewing NPCs that are not observed in mid-gestation humans.
- Differential expression and ligand-receptor interactions suggest a compositional shift in WNT signaling within the naive NPC population during LBN.
- *FZD7* localized to the connecting segment and *SEMA3D* was identified in the ureteric bud (UB) tips of late-gestation primates.

Abstract:

Background: Prematurity is associated with low nephron endowment and an increased risk of chronic kidney disease. Human nephrogenesis is complete at 34-36 weeks gestation, with 60% of nephrons forming during the third trimester through lateral branch nephrogenesis (LBN). We hypothesized that a differentiated but dividing population of nephron progenitor cells (NPCs) would contribute to the amplification of nephrons in late gestation.

Methods: Single-cell RNA-sequencing (scRNA-Seq) was performed on cortically-enriched fetal rhesus kidneys (n=9) from late second trimester and third trimester during LBN. This data was integrated with publicly available human scRNA-seq datasets from 8-18 weeks gestation kidneys (n=8) using state-of-the-art bioinformatics pipelines. Differentially expressed genes and ligand-receptor interactions were assessed and validated using RNAScope™ on human and rhesus archival tissue.

Results: scRNA-Seq of 64,782 rhesus cells revealed 37 transcriptionally distinct cell populations, including 7,879 rhesus NPCs. Pseudotime analyses identified a late gestation-specific lineage branch of differentiated NPC in rhesus that was not observed in mid-gestation humans. Differential expression analyses identified increased *SFRP1, FZD4*, and *TLE2* and decreased *FZD7*, *SHISA2*, *SHISA3*, and *TLE4* within the late-gestation rhesus NPC compared to mid-gestation human NPC and increased SEMA3D within the rhesus UB tip, suggesting a compositional shift in WNT and SEMA signaling components within the naive NPC population during LBN.

Conclusion: The rhesus macaque uniquely enables molecular studies of late-gestation primate nephrogenesis.

Our study suggests the hypothesis that a transitional state of self-renewing NPC supported by compositional shifts in key pathways may underlie the switch from branching phase nephrogenesis to lateral branch nephrogenesis and support ongoing nephron formation in late gestation.

INTRODUCTION

The period of new nephron formation, or nephrogenesis, in human kidney development typically extends through 34-36 weeks gestation (WG), with 60% of nephrons forming after 23 WG through a process termed lateral branch nephrogenesis (LBN).[1] Infants born prematurely must undergo LBN postnatally and are at the low end of nephron endowment and at high risk for chronic kidney disease (CKD) later in life.[2-10] Although preterm infants continue LBN postnatally, this process is associated with accelerated loss of SIX1 and RET expression in niche progenitors and earlier (by ~14 days) cessation of nephrogenesis.[11] In all mammals, nephrons are formed in a process associated with bifurcating branching of the ureteric bud (UB), which we will refer to as branching phase nephrogenesis (BpN) (**Fig 1A**). In mice, BpN is the only mode of nephron formation and persists until postnatal day 2 (P2), at which point the progenitor pool differentiates en masse to amplify the nephron/tip ratio 2-5-fold and nephrogenesis is completed.[12, 13] In contrast, human nephrogenesis transitions through three stages, from BpN (5-15 WG) to the short arcading phase (15-22 WG) **(Fig 1B)** to LBN (23-36 WG), where an elongating, non-branching UB tip induces nephrons, each directly connecting to the ureteric stalk after induction **(Fig 1C)**.[14-17] BpN generates 33,000 human nephrons through 15 cycles of bifurcative branching,[14] while arcading[15, 16, 18] and LBN[14-17] combine to create additional nephrons at each of the 33,000 terminal branch tips to bring the final nephron count between 200,000 and 2.7 million.[14, 19] While mouse models have been instrumental in elucidating the fundamental paradigms that control nephron progenitor cell (NPC) behavior and nephron formation, which appear to be highly conserved in the earliest stages of human kidney organogenesis,[20] they cannot provide insight into this critical LBN period of development that produces the majority of nephrons in humans and is aberrantly regulated in babies born preterm, affecting approximately 10% of all births or over 13 million infants born annually[21].

The rhesus macaque (*Macaca mulatta*) gestational period is 165 days, and that in like humans, can be divided into three trimesters,[22] with LBN present in late gestation.[17] Given these similarities, we established the rhesus as an LBN model and identified age-dependent changes within the late gestation nephron progenitor cells[17] compared to early/mid-gestation human nephrogenesis **(Fig 1)**[23-25] and the fully differentiated kidney. However, the mechanisms of LBN, including UB elongation, maintenance of NPCs, and nephron induction, remain uncharacterized at the molecular level. Specifically, it is unclear how self-renewing NPCs are maintained in a

niche, comprised of a single non-branching UB tip, despite a known change in transcriptional signature[17] and an increase in WNT signaling over time[26].

In this study, we used integrative single-cell transcriptomics to perform the first comprehensive and high-resolution comparison of nephrogenic niche progenitors during LBN and BpN to investigate how nephron amplification occurs independent of branching in the primate-specific phases of nephron production. Our data support that a transitional self-renewing NPC, which exhibits features classically associated with an early induced state, contribute to ongoing nephron formation but also can also be maintained within the niche through changes in the signaling milieu. Potential compositional changes in the WNT and SEMA3 signaling pathways between the NPC and UB may retain these cells that would otherwise differentiate at earlier stages of kidney development.

METHODS

Animals

All primate procedures were performed at the California National Primate Research Center at University of California Davis according to the Institutional Animal Care and Use Committee approved protocol #20330 awarded to Claire Chougnet. Fresh kidneys from control animals euthanized in accordance with the protocol were removed in necropsy and shipped overnight on ice in histidine-tryptophan-ketoglutarate (HTK) solution. Kidney tissue was either prepared for single cell RNA sequencing as described below or fixed in 4% PFA or NBF and embedded in paraffin.

Rhesus single-cell RNA sequencing analyses

For all rhesus samples, count matrices from raw FASTQ files were generated using cellranger (v6.0.1) and rhesus reference genome (Mmul assembly 10). To remove ambient mRNA, we applied the SoupX protocol on the count matrices with automatic calculation of the contamination fraction. Post ambient mRNA removal, we used DoubletFinder to remove doublet cells using the parameters recommended by the authors of DoubletFinder. Rhesus genes were translated to human orthologs (using hg19, Ensmbl release 75). The intersection of genes observed between the human scRNA-seq datasets (see the next section in Methods) and the human orthologs observed in rhesus scRNA-seq datasets were considered for all downstream analyses.

Sample preparation and sequencing, cell type annotation, and subclustering can be found in the supplemental methods

Human single-cell RNA sequencing

Count matrices of the human scRNA-seq datasets were downloaded from GSE114530, GSE112570, and GSE102596. We filtered the count matrices for only the cells that had a cell type annotation in the original publication. To keep cross-species comparisons and analyses consistent, we also filtered the count matrices to only those genes that were also seen in the human ortholog genes from the rhesus samples. Additional information can be found in the supplement methods

Cross-species analyses of the early transitional NPCs

Differential gene expression analysis

Differentially expressed genes of NPCs and UB C6 in rhesus as compared to those in the human dataset were determined using the software cellHarmony, which calls a moderated t-test. Log-normalized gene expression matrices from human and rhesus were provided as the input. Genes with fold change greater than 1.2 and FDR-corrected p-value less than 0.05 were considered differentially expressed.

Lineage trajectory inference, pseudotime analysis, and Cell-cell signaling interactions

Pseudotime analysis was done separately on the rhesus and human datasets using Monocle2.

Cell-cell signaling interactions were determined individually for the human and rhesus datasets using the R package CellChat (v2.1.2). Additional information can be found within the supplemental methods.

RESULTS

Lateral branch nephrogenesis in the human and rhesus is characterized by elongation of a single active ureteric tip that induces nephrons without branching.

We previously evaluated the morphology of third-trimester (~130 days gestation (dg)) rhesus kidney, which is equivalent to 32 weeks gestation in the human.[17] However, despite identifying changes within the nephrogenic niche, we were unable to identify signaling pathways in the original dataset due to the sparsity of NPCs nearing nephrogenesis cessation at ~130dg (316 NPCs). In this study, we obtained rhesus fetal kidney tissue from three donors at 104-108dg (~25-26 WG in the human or late second trimester). Tissue was cleared and 3D rendering

of the nephrogenic niche was assembled as previously described (**Fig 1D-G**).[17] Like the ~130dg rhesus samples, the new samples demonstrated rosette-like organization of the nephrogenic niche as well as LBN morphology, but with higher numbers of NPCs (**Fig 1D, E**). LBN can be identified by the SIX2+ NPC cap associated with a single UB tip, with CDH1+ lateral branches extending from the ureteric stalk below (**Fig 1G blue arrow**). However, our previous study identified that rare bifurcating branching events still occurred at late gestation but occurred prior to 130dg. In this earlier LBN period, we identified rare ongoing BpN (**Fig 1G yellow arrow**), highlighting that this gestational age is suitable for capturing the LBN transition.

To investigate how nephron induction occurs during LBN in the absence of UB branching, we performed morphologic characterization of both human (25-26 WG) and rhesus (104-108 dg) fetal kidneys (**Fig 2B-C**). Our findings showed that the process is stereotypic and highly conserved between the two species, leading us to develop the model summarized in Fig 2A. At the leading distal edge of the epithelium, RET expression across the bulbous UB tip is spread to cover all contact with the asymmetrically located SIX1+ cells. **(Fig 2A-C; 1**). RET also extended below the UB tip, where its protein expression pattern followed and mirrored locations associated with induction and formation of the pre-tubular aggregate and renal vesicle (**Fig 2A-C; 2-3**). The site of distal nephron fusion occurred at the RET+ UB tip (**Fig 2A-C; 4**). The UB tip then extended past this fusion site with the new nephron as it continued to grow as an unbranched elongating tubule, and it continuously maintained close contact with the undifferentiated NPCs (**Fig 2 A-C; 5**).

Single-cell RNA-sequencing reveals late-gestation specific progenitor cell populations undergoing lateral branch nephrogenesis

Overall, the morphologic pattern of nephron induction and formation during LBN appeared similar to that observed during early stages of BpN, with the major exception being that nephrogenesis was uncoupled from UB branching. We hypothesized that comprehensive molecular characterization of the niche progenitors through transcriptomic studies would uncover the mechanisms underlying this distinct behavior. We supplemented our scRNA-seq dataset of four fetal rhesus kidneys at ~130dg[17] with five additional rhesus samples, including two samples at 105-108dg, for a total of nine cortically-enriched fetal kidney samples from distinct donors (**Supplemental Table 1**). We identified 37 transcriptionally distinct cell clusters from 64,782 cells using our previous cluster annotations as reference (**Supplemental Fig 1A**, **Supplemental Table 2, 3**).[17] We then

integrated these data with available human fetal kidney datasets (GSE114530[23], GSE112570[24], and GSE102596[25]) from seven samples (representing BpN and arcading), consisting of 29,484 cells, using Harmony (**Fig 3A, Supplemental Fig 1B, Supplemental Table 4**).[27] Four clusters (c25, c26, c28, c29) were marked with definitive NPC markers such as *SIX1*, *SIX2*, *CITED1* and *MEOX1*, with cluster 25 containing the naive NPCs (in rhesus, n = 7879 cells; in human, n=9434 cells) (**Fig 3B**). UB markers identifying clusters 11 and 13 included *WNT11*, *WNT9B*, *GATA3*, *ADAMTS18*, and *ELF5* (**Supplemental Fig 1C**). To specifically interrogate and resolve the niche progenitor states, we subclustered the four NPC and two UB progenitor clusters using ICGS2, an unsupervised non-negative matrix factorization-based clustering pipeline, which yielded 8 subclusters of NPCs and 6 subclusters of UB progenitors (**Fig 3C**, **Supplemental Table 5, 6**). NPC C4 represented the most naive subcluster, with high expression of the naive NPC markers, including *SIX1, SIX2, MEOX1, EYA1, CITED1, ITGA8, and TMEM100*. Clusters C8 and C3 represented proliferating NPC (*TYMS*, *PCNA*, *TOP2A*, *CENPF*, *CENPE*, *CDC20*), while cluster C7 presented primed NPC (*DLK1, FAM132A, AKAP12, SNAI2*) and cluster C2 contained further differentiated NPC markers (*CYSTM1*, *FXYD6*, *SYTL2*) (**Fig 3D, Supplemental Table 7**).

To reduce the likelihood that comparative analyses between rhesus and human would be confounded by committed NPC, we filtered the NPCs to include those that expressed at least two of the NPC markers: *SIX1*, *SIX2*, and *ITGA8 (*referred as enriched NPC, or eNPC, hereon*).* In rhesus, 80% of these 2,719 eNPC came from the 105-108dg samples (**Supplemental Fig 2, Supplemental Table 5, 6**). *CITED1* was not chosen as a marker for filtering due to its relatively broad expression throughout the rhesus cells (**Fig 3B**). After filtering for eNPC, 64.5% (previously 31.5%) and 93.9% (previously 55.8%) of the cells were from NPC C4 in rhesus and human, respectively (**Supplemental Fig 2**). To investigate the characteristics of the removed human C4 cells, we performed differential gene expression of eNPCs compared to the removed cells. We noted increased expression of *SIX1*, *SIX2,* and *ITGA8* in the eNPCs, supporting this subset as the most naïve progenitors while maintaining a sufficient number of cells in C4. Cells removed demonstrated upregulation in genes related to heat shock responses (HSPA1A, HSPA1B, HSPB1, HSP90AA1, TCIM), potentially representing differences in dissociation protocols. (**Supplemental Table 7**). Interestingly, we noted three subclusters (C1, C5, C6) in the unfiltered dataset that were rhesus predominant (**Supplemental Fig 2A**), and they all largely remained after filtering, supporting their identification as late-gestation specific progenitors. The marker genes characterizing

these three clusters post-filtering include *SPC25* (C1), *UTRN*, *SFRP2*, *DUSP6*, *EMX2*, *DLL1*, *ANXA4*, *FN1*, *CYSTM1*, *FXYD5* (C5), and *MAL* and *FBLN2* (C6) (**Fig 3D, Supplemental Table 7**).

Late-gestation NPC show a distinct trajectory from mid-gestation human NPC

To orthogonally confirm that C4 cluster represented the most naive NPC state and to delineate the differentiation trajectories using an unsupervised approach, we used Monocle[29] to perform pseudotime analysis of the eNPCs at mid gestation (human) and late gestation (rhesus) (**Fig 4**). In the mid-gestation human (**Fig 4A**), the first and only branch splits the NPC C4 into more differentiated NPC C4 (left branch) and NPC C8 (right branch), a proliferative NPC cluster (**Supplemental Table 7**). NPC C2 and C7 overlap with the left branch, while C1 and C3, the two most differentiated NPC, overlap with the right branch. Branch points along the constructed cell trajectory mark a suggested split into two or more distinct cell fates. Despite having a comparable number of C4 NPCs in rhesus and human, the pseudotime analysis identified two branch points in late-gestation rhesus NPCs compared to only one branch in the mid-gestation NPC (**Fig 4B)**. In rhesus, the initial lineage branch point (Branch 1) creates two NPC populations: the right branch containing a subset of differentiated NPCs (NPC C4 and C8) found only in the late-gestation rhesus and the left “root” also containing C8 cells in addition to naïve progenitors (C4). Similar to branch 2 in the human dataset, the less differentiated left arm (Branch 2) either gives rise to NPC C7 (induced NPC), overlapping with NPC C4 and C8 (proliferating NPC), or to C1 and C3 (right arm post Branch 2), characterized by markers of cell cycle. Clusters C2, C5, and C6, found primarily in rhesus, represent a mixed population of both terminal branches, consisting of early differentiated NPC (C2), primed NPC (C6: *HEY1, PLOD2)*, markers of proximal (SFRP2, HES4, EMID1 for C5 and JAG1, CLDN1, GRIN2A for C6) and distal nephron (C5: SFRP2, HES4, EMID1)

To further understand this unexpected lineage bifurcation, we examined the transcriptional differences in the rhesus C4 cells using Monocle BEAM analysis and published literature.[24, 28, 30] Branch 1, found only in rhesus, distinguishes the differentiating eNPCs (left branch) vs self-renewing eNPCs (right branch). This rhesus-specific right branch of branch 1 includes NPC markers *EYA1*, *TMEM100, NRXN1, ITGA8*, and *RSPO3*, as well as what has previously been referred to as primed NPC[24], including *SFRP1*, *ROBO2*, and *TPM1*. However, this branch also contains markers of proliferation (*ATP1B1, CAMK2D*), proximal (*MYC*), medial-distal (*TRIB2*), and distal segment (*LTBP4, PCDH18, COL6A3*). Additionally, these progenitors include markers typically associated with

extracellular matrix/interstitial progenitors including *COL1A2*, *DCN*, *VCAN*, and basement membrane markers *COL14A1* and *HMCN1*. (**Fig 4C, Supplemental Table 8**). In the left (more differentiated) branch of branch 1, genes included primed NPC markers *HEY1, ID1, ID3*, and *ID4*, proliferation markers *TOPS2A, NUSAP1, MKI67, CDC20*, and early renal vesicle/broadly proximal markers *JAG1, HES4, SFRP2* and distal markers *CCND1* and *BMP7*. Interestingly, both sides expressed early proximal marker CDH6 and podocyte marker *ADAMTS9*. These data indicate that the left-sided population would be the one giving rise to more differentiated NPC populations and derivatives via established developmental paradigms.

Branch 2 of rhesus and the single branch point of human demonstrate further differentiation into primed NPC (left branch) and proliferating cells (right branch). There is down-regulation of *RSPO3* in both left and right branches, but more prominent on the right. The left branch shows increased expression of ID1 and ID3 (primed NPC), medial/distal markers (*VIM, IGF2,* and *LTBP1*), proximal tubule markers (*FTL, CD81)*, and extracellular matrix COL1A1, as well as markers *MEIS3, TXNIP, HSBP1,* and *TSC22D1* (**Supplemental Fig 3**). The right branch has high expression of proliferation markers including *TOP2A, MKI67, NUSAP1, CCNA2, and CDK1.* In the human, we see a similar separation of differentiated NPC on the left, dominated by C4 but also C7 and C2, and cell cycle on the right, composed mostly of C8 with some C3. These findings suggest a transitional state of NPC in late-gestation that does not exist in mid-gestation and may provide the source of self-renewing NPC in late-gestation despite their induced state.

Enriched NPCs present age-dependent differences in *WNT* signaling

To evaluate the age-dependent changes in NPCs, we first performed differential expression of the NPC subclusters in rhesus vs human (**Fig 5A, Supplemental Table 9**). Genes prominent in the WNT pathway were identified within the NPC, including Frizzled (FZD) receptors, WNT inhibitors, and Transducin-Like Enhancer of Split (TLE) co-repressors (**Fig 5B)**. Specifically, we identified increased *FZD4 (C7)* and *TLE2* and decreased *FZD7 (C3), SHISA2 (C4, C7), SHISA3 (C4), and TLE4 (C4, C7, C8)* **(Fig 5B**). We noted an increase in secreted Frizzled-related protein-1 (*SFRP1)* in the late-gestation rhesus relative to mid-gestation human, strongest within naïve NPC (C4) but present throughout the late-gestation NPC population, consistent with our findings of increased *SFRP1* within the self-renewing NPC branches of the Monocle pseudotime analyses. We also noted increased *SFRP2* in the more differentiated NPC clusters C2,3,5, and 6.

The mechanisms controlling nephrogenesis involve cross-talk of signaling pathways between the NPCs and the closely associated UB tips (**Fig 2**). Our initial subclustering (**Fig. 3C**) of UB cells (clusters c11 and c13) from the integrated dataset identified four stalk clusters (C1, C2, C3, C4) and two tip clusters (C5, C6), consisting of 3,507 total cells in rhesus and 391 cells[23] in human (**Fig 3C, Supplemental Table 10**). Marker genes of the UB tip of the late-gestation rhesus included *HIP1R, MYH10, STC1, AQP3 in C5* and *AKR1B1* and *EHF* in the UB C6, in addition to known UB markers *WNT9B*, *GATA3* and *GATA2* (**Fig 5C, Supplemental Figure 4A, Supplemental Table 11).** Over 95% of the UB cells from the human dataset were classified as UB C6, which limited the temporal comparative analyses to only UB C6 among the UB cells. Supporting a possible reduction in WNT response in the UB tip, we identified overall low expression of AXIN2 in the rhesus UB clusters (**Supplemental Figure 4A)**.

To further understand the signaling between eNPCs and UB, we interrogated the possible secretory receptor/ligand interactions between eNPC and UB in mid vs late gestation using CellChat.[31] Within the late-gestation rhesus, we identified distinct compositional changes within the WNT pathway (**Fig 5D-E, Supplemental Figure 4B, Supplemental Table 12**). While both human and rhesus exhibited WNT4 signaling to FZD4/6/8 from the more differentiated eNPC (C2), the rhesus NPC subcluster C5 demonstrated WNT signaling to UB tip and stalk through WNT5B ligand and FZD 4/6/8 interaction (**Fig 5D**). Additionally, WNT9B ligands within the UB tip C5 and C6 interacted with all of the NPC populations FZD2/3/4/6/8 and LRP5/6 receptors as expected (**Fig 5E**). Combined with the overall higher expression of the secreted antagonist SFRP1 in the naïve NPCs of rhesus, these data suggest an important role for differential modulation of WNT signaling activity as a mechanism governing LBN morphogenesis, consistent with the known role of the pathway as a pivotal regulator of kidney organogenesis.[26, 32-36] To further validate these findings, *in situ* hybridization was used to spatially localize the expression of differentially expressed components within the niche. We identified strong co-staining of *SFRP1* with *SIX1* in the cap NPC population (**Fig 6A**) in late-gestation rhesus and human, with extension surrounding the UB tip. This staining pattern confirmed our DEG analysis within the late-gestation rhesus and supports our pseudotime gene expression changes in the C4 rhesus progenitor population. *SFRP1* extended into the surrounding stroma in addition to the NPC. *SFRP2* was highly expressed in the early differentiated nephron structures, consistent with its high transcriptional level in the induced NPC *WNT4+*

population of cells.[26, 37] We next also noted a change in *FZD* composition within our DEG and highlighted in the cell chat analysis. Based on DEG, we anticipated high expression of *FZD4* and low expression of *FZD7* in late gestation compared to mid gestation. On tissue validation, we identified diffuse staining of FZD4 in both the UB and NPC (**Fig 6B**)*.* *FZD7*, while still present in the NPC, was most prominent in connecting segment of the distal nephron fusing to the UB tip (**Fig 6B**), which was not anticipated based on DEG. This unexpected localization was also seen at the protein level in the human archival sample, with increasing FZD7 protein expression at the distal fusion points with increasing gestational age, and present in examples for arcading (17wg) and LBN (27-30wg) (**Fig 6C**). Returning to the larger dataset, we identified increased *FZD7* in cluster 15 in late gestation. Although this cluster is labeled as Loop of Henle (with marker genes including *MAP1B*, *TFAP2B*, and *IRX2*), there is some overlap of these genes with collecting duct and urothelial expressions, including *FRAS1*, *TSPAN13*, *LLGL2*, *CPEP2* and *PTPN3*, suggesting broad expression of *FZD7* from Loop of Henle to collecting duct epithelium during the fusion of the nephron to the ureteric stalk.

Given that decreased *SHISA2* and *SHISA3* were identified on our differential expression analysis we next evaluated the expression of these two genes **(Supplemental Fig 4C)**. Despite the low expression in the DEG analysis, we identified strong *SHISA2* within the NPC and early differentiating structures at late gestation. Interestingly, *SHISA3*, while still present in the NPC, appeared most prominent in the surrounding stroma (**Supplemental Fig 4C**). *TLE2* and *TLE4* were also evaluated using RNAScope™. *TLE4* was present within the NPC of late-gestation rhesus and human, while no distinct pattern was visible in *TLE2* expression (**Supplemental Fig 4D**).

Enriched UB cells present age-dependent differences in *SEMA3* signaling

SEMA3D expression was significantly upregulated in UB C6 of late-gestation rhesus compared to mid-gestation human samples (FDR p-value < 0.05) (**Supplemental Table 11**). Using CellChat, we identified SEMA3A and SEMA3B signaling from the differentiated eNPC C2, C3, and C5 clusters communicating to the UB stalk and tip clusters via receptors NRP1/2:PLXN1/2 in the late gestation rhesus samples as described above. We next interrogated the possible secretory receptor/ligand interactions from the UB to eNPC in the late gestation rhesus which suggested reciprocal semaphorin signaling in the niche (**Fig 5E**). SEMA3B ligand signaling to the NRP1/2 and PLXN1/2 receptors was observed ubiquitously across eNPCs, with the strongest

interactions noted in eNPC clusters C5 and C6. In contrast, SEMA3D signaling to these receptors originated specifically from the UB tip clusters. As *SEMA3D* was a prominent marker of the rhesus UB tip in both differential expression and UB to NPC secretory communication, we validated this marker using RNAScope™. We identified *SEMA3D* within the UB tip of late-gestation human and rhesus (**Fig 6D)**. We also noted *SEMA3D* signal within the interstitium of the more mature deep cortex of the kidney.

DISCUSSION

Through our cross-time, cross-species investigation of the late-gestation rhesus macaque compared to mid-gestation human, we identified molecular differences in the nephron progenitor cells, including distinct NPC clusters in late gestation and distinct trajectories of the naïve NPC in late gestation. Our data suggests a transitional NPC state supported by a potential change in the signaling milieu between the NPC and UB within WNT and SEMA3 signaling pathways, allowing for this differentiated but self-renewing NPC to be maintained within the niche. While we acknowledge the limitations of RNA analyses, this hypothesis provides a potential mechanism for nephron amplification during LBN.

Distinct progenitor trajectories are seen within late-gestation rhesus

Distinct trajectory of the most naïve NPC in late gestation suggests there may be a population of late-gestation that does not exist mid-gestation NPC; a population of early differentiated NPC that contribute to self-renewal and remain in the niche. Rather than a single lineage branch dividing the self-renewing NPC from the differentiating NPC, the rhesus instead has two lineage branches, with a proliferating branch of NPC consisting of NPC C4 and C8. This right branch maintained strong cell cycle signals, markers of the naive NPC, as well as markers associated with the interstitial progenitors and early basement membrane markers, suggesting a shift in both location and early function despite their naive state that may be needed for LBN-nephrogenesis. This transitional proliferating population is distinct from those cells that go on to fully differentiate and become nephrons. The cells undergoing LBN may remain in a high cell cycle and early committed cell state (expressing early BM markers). Slowing of cell proliferation has been associated in the aging of the progenitor niche[38, 39]. However, changes in cell cycle expression at the RNA level are not the full story, as low translation in stem cell populations, independent of cell cycle, have been shown to maintain the progenitor state compared to high translation in differentiated cells.[40] Given that these populations of cells still maintain their progenitor signature,

they may represent the LBN and/or arcading phase of a self-renewing but early differentiated cell that can contribute to nephron formation in late gestation.

Changes in WNT signaling in late gestation may drive the transition from BpN to LBN

One of the potential pathways identified through this investigation included alterations within WNT signaling in late gestation compared to mid gestation. Jarmas et al found that the "tipping point" for nephron progenitor exit from the niche is controlled by the gradual increase in the translation of WNT agonists in individual nephron progenitor cells, enhancing the response to ureteric bud-derived WNT9b inputs.[26] It remains unknown how this shift in WNT responsiveness with age could impact ongoing nephrogenesis in a model with prolonged LBN prior to nephrogenesis cessation. SFRP1 is a secreted WNT antagonist that interacts directly with WNT ligand and can block its interaction with receptors.[41-44] Our data suggests that increased expression of SFRP1 within the naïve NPC may shield these nephron progenitor cells from the action of WNT agonists, as suggested by low expression of downstream target AXIN2, allowing for prolonged, ongoing nephrogenesis in late gestation without branching. Without early gestation rhesus samples for a within-species analyses this remains speculative. Future experiments including overexpression of *SFRP1* in a genetically tractable model of the NPC population may shed light on this question.

SHISA2 and *SHISA3* were downregulated in late gestation in the rhesus datasets, however our tissue validation still supports *SHISA2* was still present within the NPC in late gestation. SHISA2 has been shown to modulate both WNT and FGF signaling[45] and without an earlier time point we cannot conclude that there is decrease within the NPC at late gestation. Interestingly, SHISA3 had low presence in the NPC but had strong presence within the stroma. This supports our previous finding of interstitial progenitor markers within the LBN progenitor population. Similar to GNDF,[46] this may represent a shift in the spatial orientation of SHISA from the NPC to the stroma in late gestation, but its role in supporting LBN requires future investigation.

FZD7 identified the connecting segment/fusion of the distal nephron to the UB rather than the NPC population itself in late gestation. However, mid-gestation human data supports its expression throughout the NPC, as well as podocytes and loop of Henle, but only faintly expressed in the connecting tubule.[47] Our single cell data also supports expression within the Loop of Henle, despite tissue validated presence at the fusion of the developing nephron. FZD7 has also been identified as putative receptor for WNT6, which leads to de novo tubulogenesis in

kidney epithelial cells in culture,[48] but its role within the fusion of the nephron is not known. FZD7 can interact with LRP5/6 to transmit canonical Wnt signaling, as well as through ROR2 to transmit non-canonical Wnt signals.[49] WNT5A, with potential signaling highlighted within the rhesus-only clusters, may play a role in this distal fusion during LBN. WNT5A non-canonical signaling through ROR2 plays an important role in mediating cell proliferation and migration within the metanephric mesenchyme around the wolffian duct[50], further supporting this hypothesis.

The role of semaphorin 3D in UB elongation and induction of lateral branch nephrogenesis

Semaphorins are guidance proteins, mediating short and long-range repulsive and attractive guidance that influence organogenesis and disease.[51] Explant kidney cultures exposed to Sema3a demonstrate reduced ureteric branching, reduced GDNF expression, and reduced Ret phosphorylation. Conversely, Sema3a knockdown/knockout models resulted in increased bud branching.[52] Our study supports that *SEMA3D* is present within UB tips, as well as the surrounding interstitium, while our Cell chat data supports that *SEMA3B* signaling is prominent in the stalk. Our data suggests that increased *SEMA3D* signaling within the UB tip supports elongation rather than bifurcating branching, but future studies comparing the balance of *SEMA3B* to *SEMA3D* in the stalk vs tip require further investigation in early to late gestation. If early to mid-gestation tissue in the rhesus were available in the future, we would perform similar trajectory analyses of the UB overtime to better capture these changes.

The future of lateral branch nephrogenesis analyses

In summary, the gene expression approaches performed in this study help overcome current limitations in identifying pathways acting late in development, as the direct study of late-gestation human kidney development is not possible. The rhesus is the only resource that can be leveraged to define critical cellular and signaling changes that occur during human kidney development at single-cell resolution. Our study highlights a potential transitional, proliferating NPC that amplifies nephrons in late gestation LBN, which is the critical first step to maintaining these pathways otherwise impacted by preterm birth. However, the concepts proposed in this study remain hypothetical. Several limitations exist, resulting in unanswered questions. One of the greatest questions of this analysis is whether the identified changes within the late gestation NPC are time-dependent or species-dependent. As already discussed, no transcriptional data exists for the late-gestation human kidney, and

available human scRNA-seq datasets are limited to a developmental period before LBN begins. Additionally, human fetal archival material from autopsy can be challenging to obtain and can be subject to RNA degradation.[53, 54] Future work will seek to define signaling pathways in the rhesus at mid-gestation before LBN begins, with validation studies at both translational and protein levels to further test these hypotheses. Acquisition of these earlier gestation rhesus datasets will allow us to answer the many questions that remain when defining the unique maturational changes BpN to LBN transition and provide the high tissue samples for spatial validation of our computational findings.

DISCLOSURES

MPS receives research funding from Otsuka that is not relevant to this work

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AUTHOR CONTRIBUTIONS

R Kopan and MP Schuh conceptualized and planned the experiments, K Thakkar, R Kopan, and MP Schuh analyzed data. S Yarlagadda, L Alkhudairy, A Potter, and MP Schuh performed the experiments including single-cell dissociation. K Thakkar, N Salomonis, P Chaturvedi, and K Thorner performed bioinformatics analysis in collaboration with MP Schuh. K Thakkar and MP Schuh assembled figures. K Thakkar, KW McCracken, and MP Schuh wrote the manuscript. The final manuscript incorporated input from all authors.

DATA AVAILABILITY

The raw and processed files of scRNA-seq data from rhesus samples are deposited in GEO (Accession number: GSE305708). scRNA-seq data from human samples are available at GSE114530, GSE112570, and GSE102596. Code and scripts for reproducibility are available at https://github.com/kairaveet/integrative-nephrogenesis.

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FIGURES

Fig 1. Stages of nephrogenesis in human and comparative rhesus morphology. A) During branch phase nephrogenesis (BpN), nephrons are formed in a process associated with bifurcating branching of the ureteric bud (UB). **B)** 15 weeks gestation (WG), human nephrogenesis transitions to arcading phase followed by **C)** LBN at 23-36 WG, where an elongating, non-branching UB tip induces nephrons. Rhesus gestation in days included for reference. **D-F)** Early-LBN (108dg) rhesus displays a rosette-like organization of the NPC niche, similar to both 129dg rhesus and 32 wg human. with evidence of both LBN and rare BpN. G) the early-LBN rhesus (108dg) represents a critical transition period from BpN to LBN. Although lateral branches (blue arrow) are seen along the ureteric stalk, there is also evidence of recent BpN branching events (yellow arrow).

Fig 2. Schematic of the lateral branch nephrogenesis: nephron induction without UB branching. A) the schematic representation of nephron induction and fusion through LBN with representative immunofluorescent images from **B)** human and **C)** rhesus in late gestation. There is broad RET (yellow) expression across the UB tip and in contact with nephron progenitor cells (NPC, green) (panel 1). RET expression is visualized along the side of the ureteric tip with contact to the formation of renal vesicles (panels 2-3). Distal CDH1+ (red) fusion of the nephron occurs near the base of the UB tip in close proximity to the RET+ cells, with NPC just above this fusion point (panel 4). The bulbous UB tip then outpouches or grows above this fusion point, with the NPC traveling with it (panel 5), leaving the recently formed “lateral branch” below.

Fig 3. Cross-species integrative single-cell transcriptomics characterizes heterogeneity and temporal differences in NPCs. A) Integrated UMAP of 94,266 cells from cortically-enriched fetal kidneys from 9 rhesus (late second and third trimesters) samples and 5 human (8-13 weeks old) samples. Cells are colored by cellHarmony cluster labels (left panel) and by species (right panel). **B)** UMAP from panel A colored by gene expression of key NPC progenitor marker genes. **C)** Integrated UMAP of NPC and UB cells from rhesus and human samples. Cells are colored by cell subclusters within NPCs and UB (left panel) and by species (right panel). **D)** Dot plot indicating average expression of major marker genes of NPC subclusters in human (top panel) and rhesus (bottom panel) datasets. Dots are sized by the percent of cells that expressed the marker

gene. **E)** UMAP from Panel C highlighting the enriched NPCs (eNPCs) defined by expression for: *SIX1*, *SIX2*, *ITGA8* (at least 2 of the three genes expressed). The highlighted cells are colored by species, similar to right panel C.

Fig 4. Developmental trajectory of NPCs. Lineage trajectory of NPCs constructed by Monocle2 for **A)** human and **B)** rhesus. Pseudotime increases from top to the bottom of the tree. To clearly illustrate where clusters are located, the lineage tree is reproduced to highlight each rhesus cell population. **C)** Heatmap of normalized gene expression showing branch-dependent genes (Monocle BEAM test; FDR < 10%) as rows along the two fates of Branch 1 in rhesus. Left and right segments show the gene expression change along the left and right branches, respectively, after Branch 1 in the Monocle tree in panel B. Columns are points in pseudotime, and the dividing middle segment of the heatmap indicates beginning of pseudotime. Selected genes are highlighted on the right. Rows are clustered using hierarchical clustering.

Fig 5. Age-dependent changes in WNT signaling. A) Heatmap indicating the log2 fold changes of differentially expressed genes (FDR p-value < 0.001) of each NPC subcluster in rhesus as compared to human cells. Top 150 differentially expressed genes are considered from each comparison. **B, C)** Dot plot indicating average expression of **B)** selected genes associated with WNT signaling in NPC subclusters in human (top panel) and rhesus (bottom panel) datasets, and **C)** marker genes of UB subclusters in rhesus. Dots are sized by the percent of cells that expressed the gene. **D, E)** Bubble plots showing the WNT-associated ligand-receptor pairs (rows) that contribute to secreted signaling **D)** from NPC to UB and **E)** UB to NPC in rhesus. Dots are colored by communication probability and sized by p-value.

Fig 6. RNAScope™ validation of WNT pathway and SEMA3D. A) Representative late-gestation rhesus and human kidney with co-localization of SFRP1 and SIX1, with additional SFRP1 expression within the surrounding stroma. SFRP2 was noted further away from the cortical edge, likely representing the WNT4+ population of NPC. **B)** FZD 4 and 7 expression in the late-gestation rhesus and human. While FZD4 was broadly expressed in the NPC and developing nephron, FZD7 localized to the connecting segment or the distal nephron and fusion to the UB. **C)** FZD7 protein expression in the developing human kidney. FZD7 is seen at the fusion of the nephron segments in examples of arcading (17 wg) and lateral branch nephrogenesis (27-30 wg).

D) Late-gestation rhesus and human demonstrate strong *SEMA3D* signal in the outer UB and UB tip, with additional SEMA3D staining identified within the maturing interstitium.

Figure 1

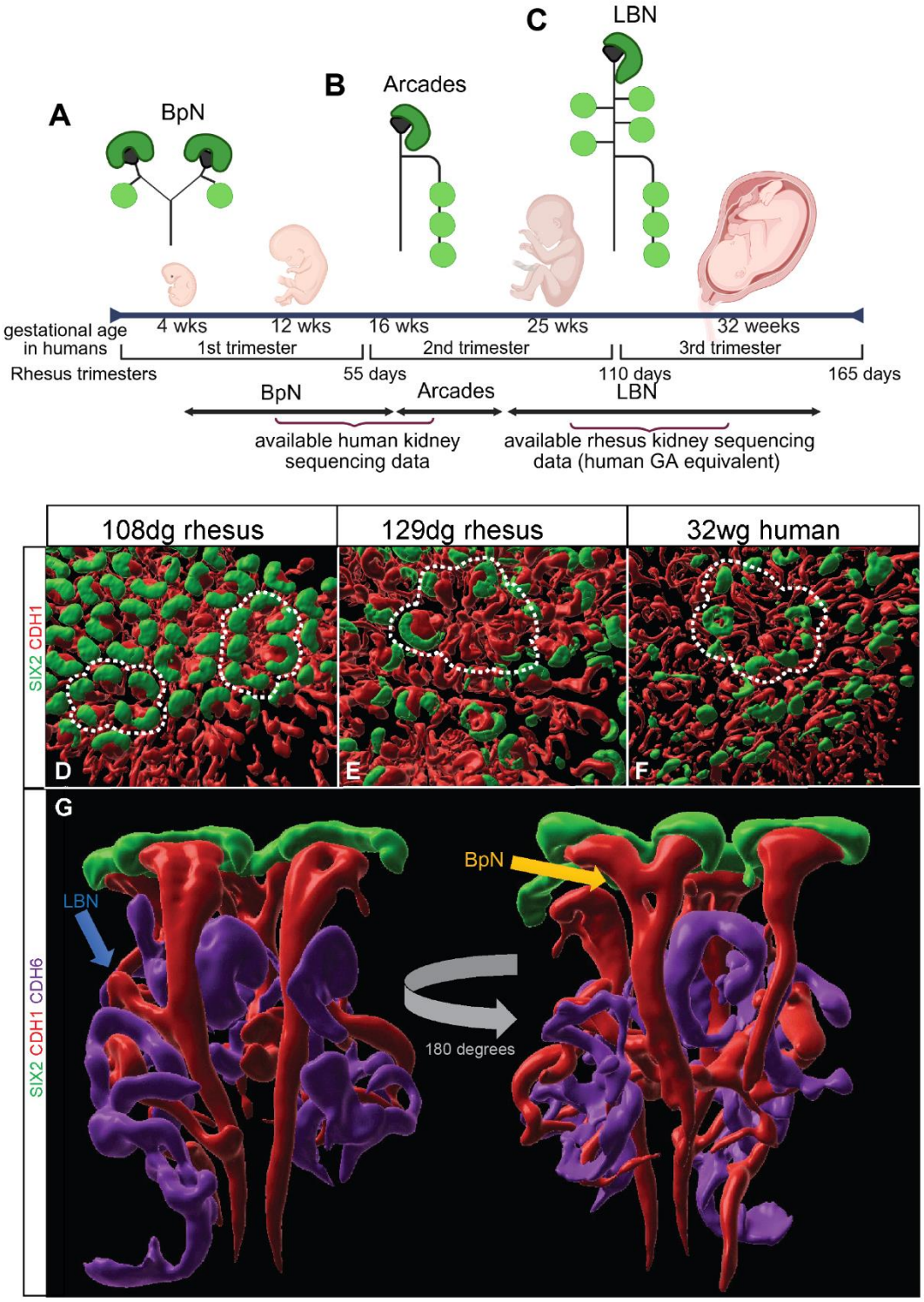

A
BpN
B
Arcades
C
LBN
gestational age in humans
4 wks
12 wks
16 wks
25 wks
32 weeks
1st trimester
2nd trimester
3rd trimester
Rhesus trimesters
55 days
110 days
165 days
BpN
Arcades
LBN
available human kidney sequencing data
available rhesus kidney sequencing data (human GA equivalent)
108dg rhesus
129dg rhesus
32wg human
SIX2 CDH1
D
E
F
G
SIX2 CDH1 CDH6
LBN
BpN
180 degrees

Figure 2

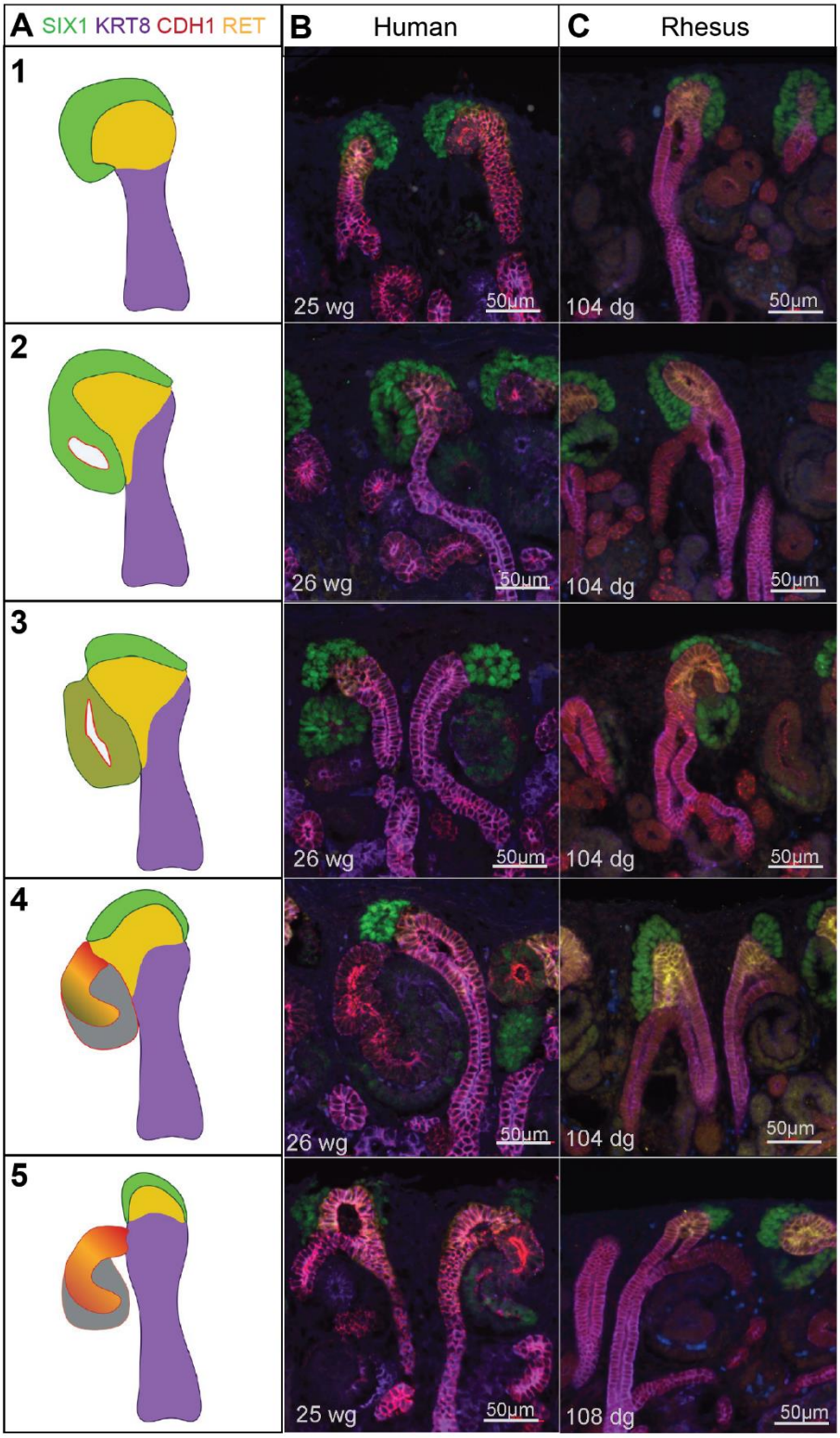

A SIX1 KRT8 CDH1 RET
B Human
C Rhesus
1
2
3
4
5
25 wg
50µm
104 dg
50µm
26 wg
50µm
104 dg
50µm
26 wg
50µm
104 dg
50µm
26 wg
50µm
104 dg
50µm
25 wg
50µm
108 dg
50µm

Figure 3

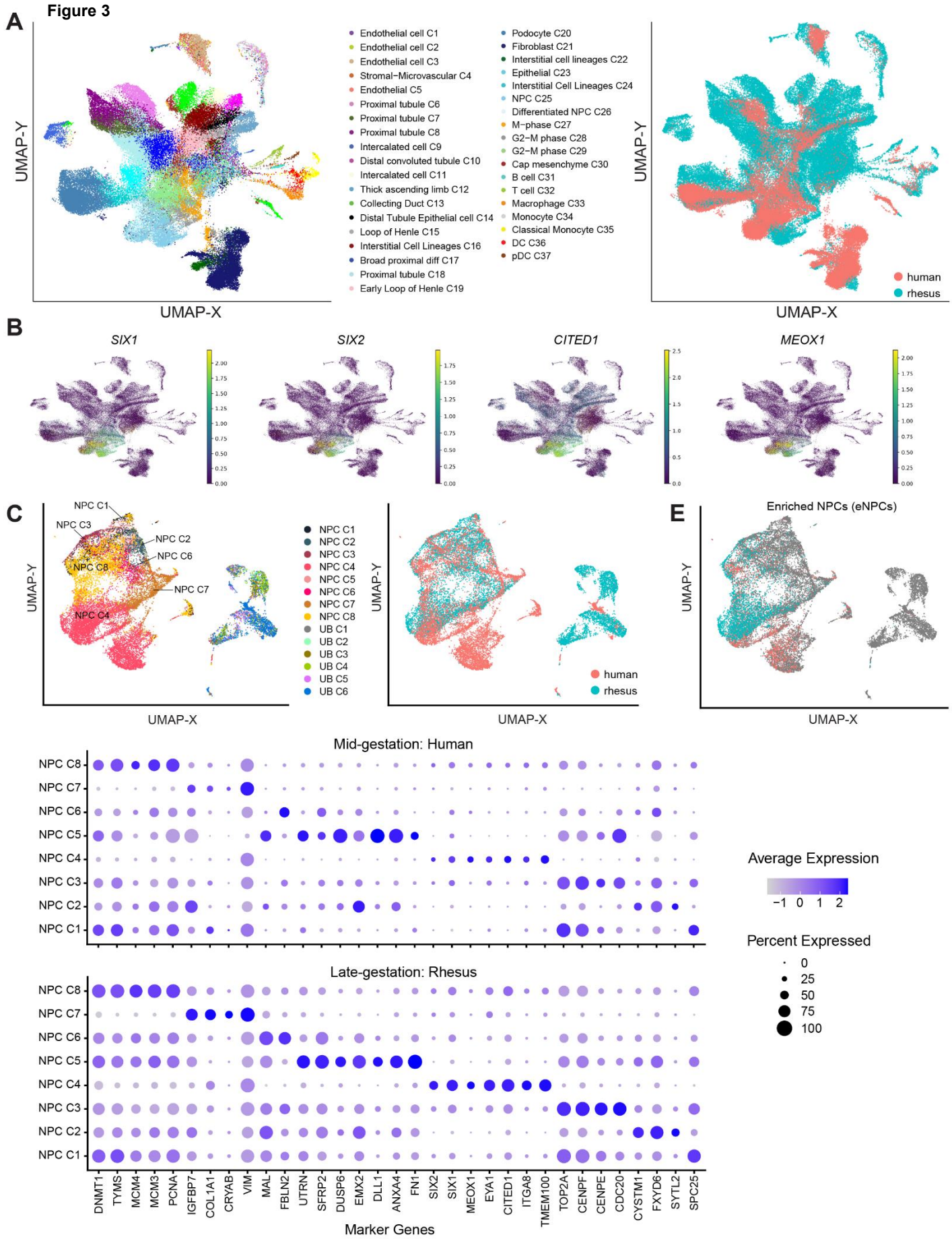

A
UMAP-Y
UMAP-X
Endothelial cell C1
Endothelial cell C2
Endothelial cell C3
Stromal−Microvascular C4
Endothelial C5
Proximal tubule C6
Proximal tubule C7
Proximal tubule C8
Intercalated cell C9
Distal convoluted tubule C10
Intercalated cell C11
Thick ascending limb C12
Collecting Duct C13
Distal Tubule Epithelial cell C14
Loop of Henle C15
Interstitial Cell Lineages C16
Broad proximal diff C17
Proximal tubule C18
Early Loop of Henle C19
Podocyte C20
Fibroblast C21
Interstitial cell lineages C22
Epithelial C23
Interstitial Cell Lineages C24
NPC C25
Differentiated NPC C26
M−phase C27
G2−M phase C28
G2−M phase C29
Cap mesenchyme C30
B cell C31
T cell C32
Macrophage C33
Monocyte C34
Classical Monocyte C35
DC C36
pDC C37
human
rhesus
B
SIX1
SIX2
CITED1
MEOX1
C
NPC C1
NPC C3
NPC C2
NPC C6
NPC C8
NPC C7
NPC C4
UB C1
UB C2
UB C3
UB C4
UB C5
UB C6
E
Enriched NPCs (eNPCs)
Mid-gestation: Human
Late-gestation: Rhesus
Average Expression
Percent Expressed
Marker Genes
DNMT1
TYMS
MCM4
MCM3
PCNA
IGFBP7
COL1A1
CRYAB
VIM
MAL
FBLN2
UTRN
SFRP2
DUSP6
EMX2
DLL1
ANXA4
FN1
SIX2
SIX1
MEOX1
EYA1
CITED1
ITGA8
TMEM100
TOP2A
CENPF
CENPE
CDC20
CYSTM1
FXYD6
SYTL2
SPC25

Figure 4

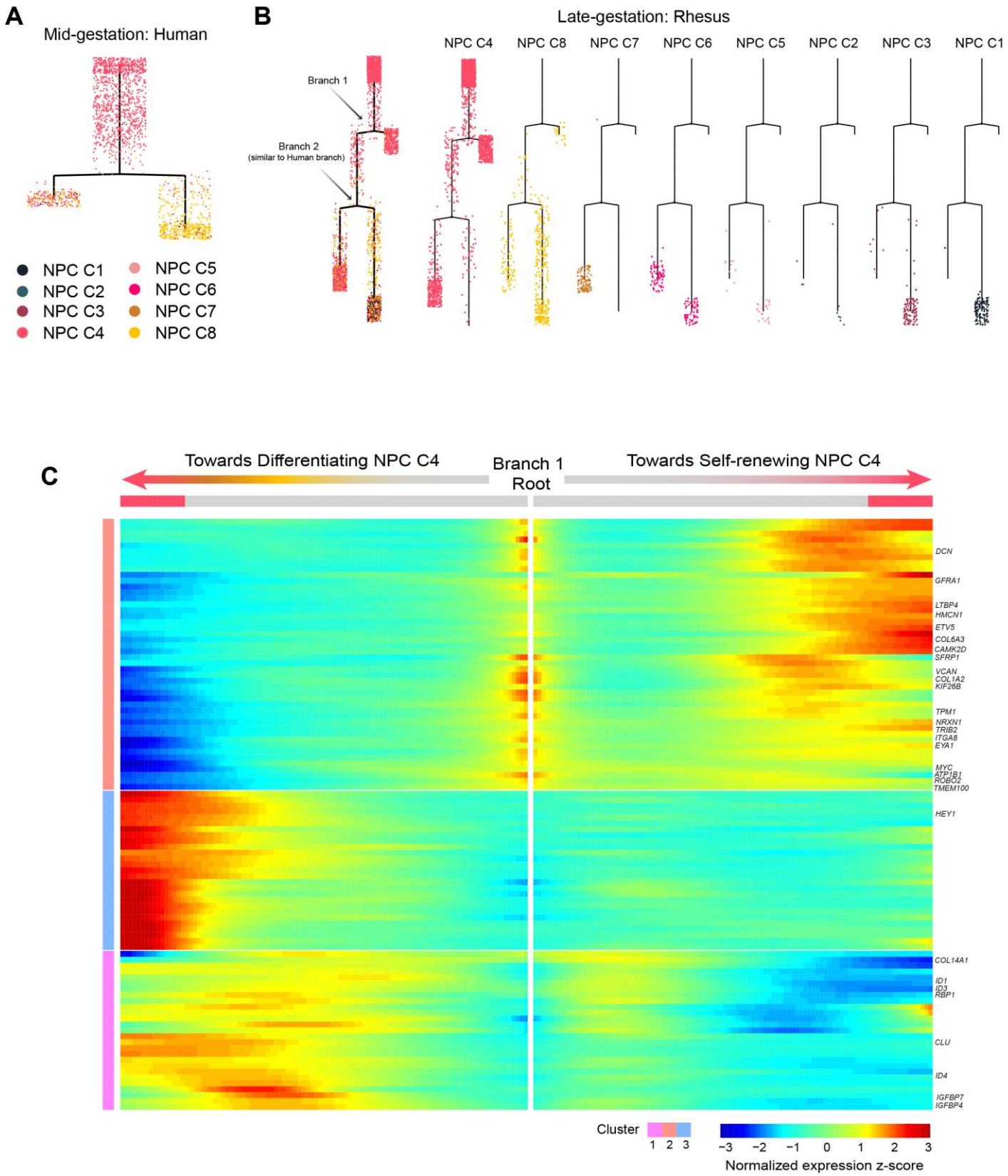

A
Mid-gestation: Human
NPC C1
NPC C2
NPC C3
NPC C4
NPC C5
NPC C6
NPC C7
NPC C8
B
Late-gestation: Rhesus
NPC C4
NPC C8
NPC C7
NPC C6
NPC C5
NPC C2
NPC C3
NPC C1
Branch 1
Branch 2
(similar to Human branch)
C
Towards Differentiating NPC C4
Branch 1
Root
Towards Self-renewing NPC C4
DCN
GFRA1
LTBP4
HMCN1
ETV5
COL6A3
CAMK2D
SFRP1
VCAN
COL1A2
KIF26B
TPM1
NRXN1
TRIB2
ITGA8
EYA1
MYC
ATP1B1
ROBO2
TMEM100
HEY1
COL14A1
ID1
ID3
RBP1
CLU
ID4
IGFBP7
IGFBP4
Cluster
1 2 3
−3 −2 −1 0 1 2 3
Normalized expression z-score

Figure 5

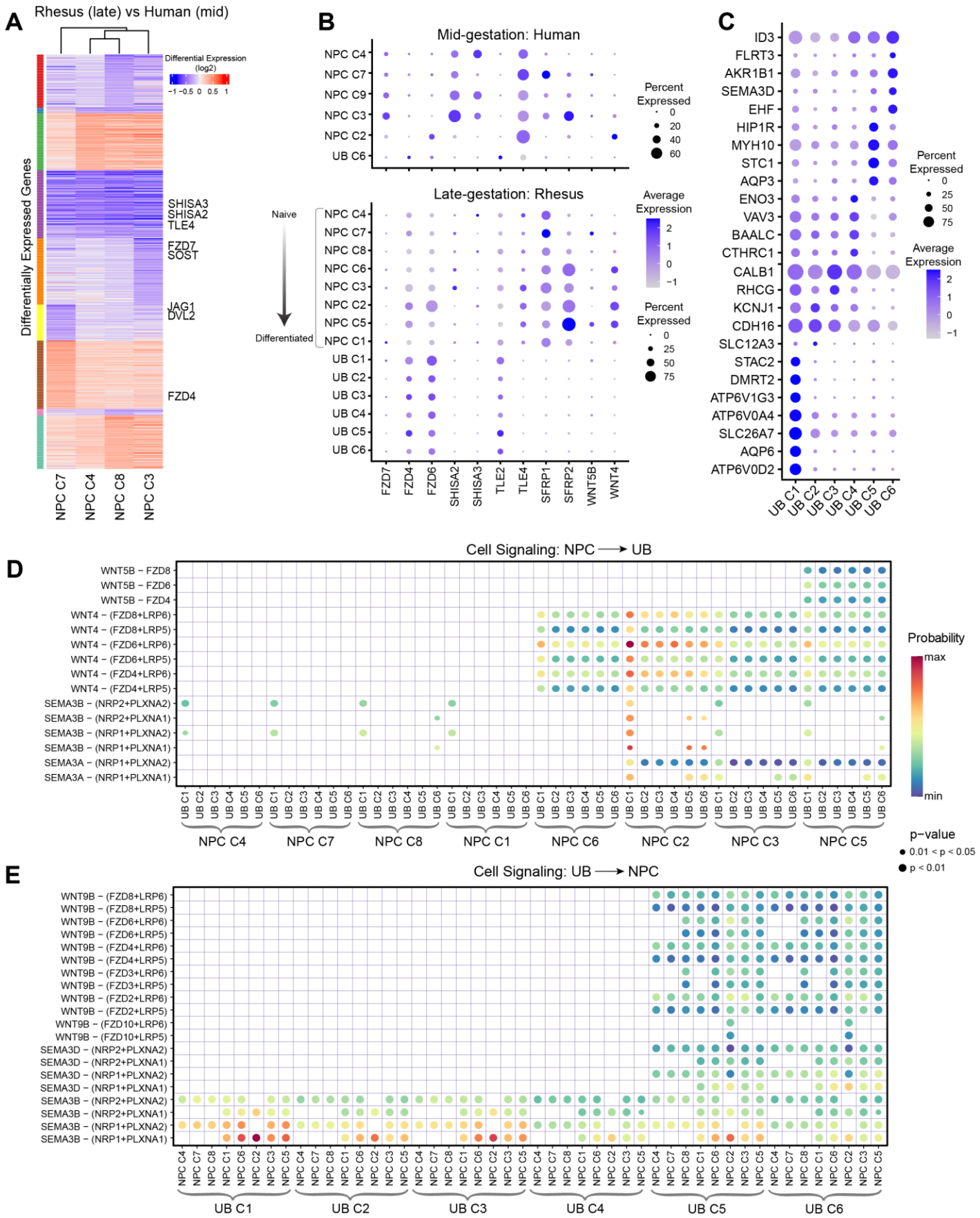
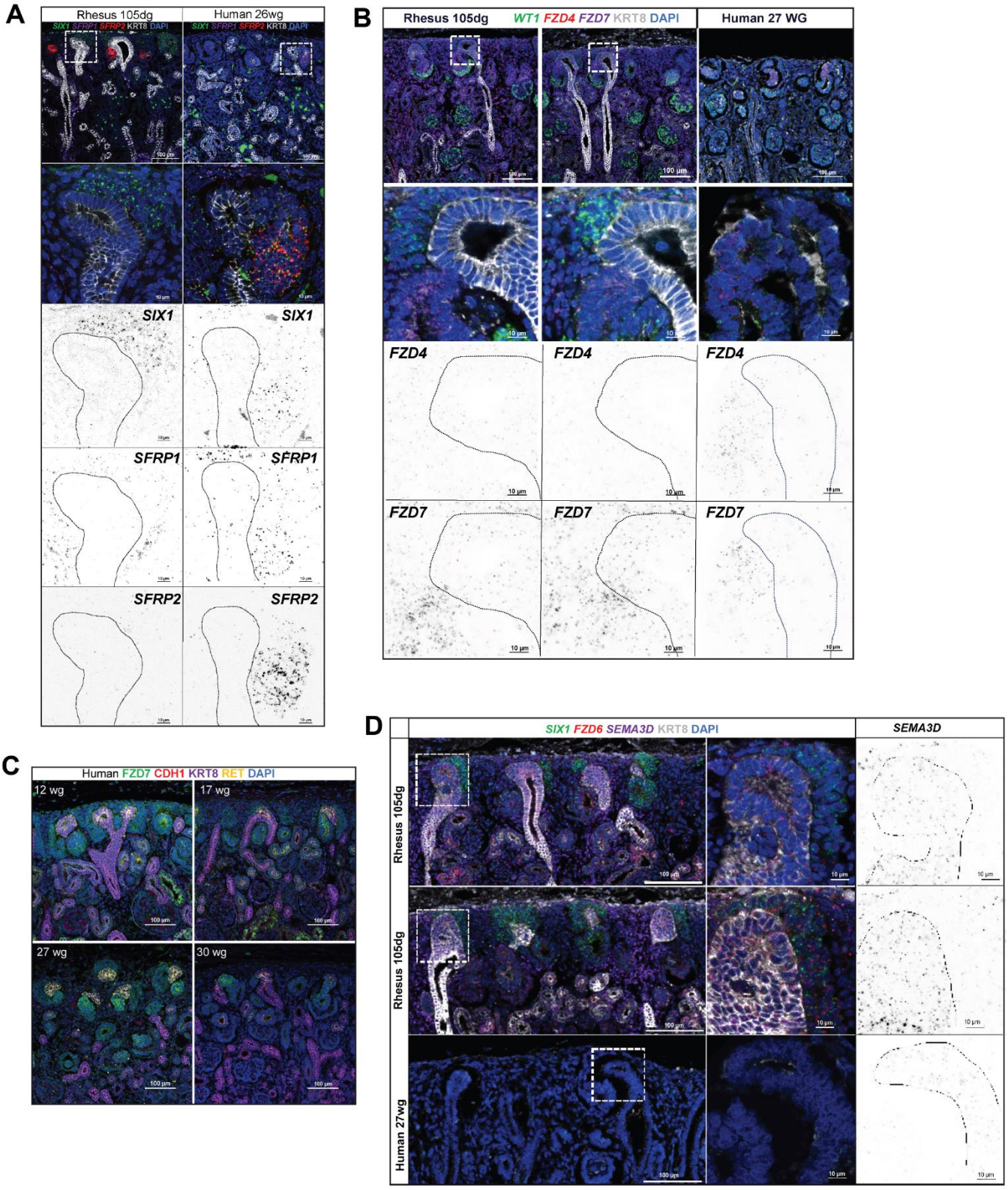

A
Rhesus (late) vs Human (mid)
Differential Expression (log2)
−1 −0.5 0 0.5 1
Differentially Expressed Genes
SHISA3
SHISA2
TLE4
FZD7
SOST
JAG1
DVL2
FZD4
NPC C7
NPC C4
NPC C8
NPC C3
B
Mid-gestation: Human
Late-gestation: Rhesus
Naive
Differentiated
Percent Expressed
Average Expression
FZD7
FZD4
FZD6
SHISA2
SHISA3
TLE2
TLE4
SFRP1
SFRP2
WNT5B
WNT4
C
ID3
FLRT3
AKR1B1
SEMA3D
EHF
HIP1R
MYH10
STC1
AQP3
ENO3
VAV3
BAALC
CTHRC1
CALB1
RHCG
KCNJ1
CDH16
SLC12A3
STAC2
DMRT2
ATP6V1G3
ATP6V0A4
SLC26A7
AQP6
ATP6V0D2
UB C1
UB C2
UB C3
UB C4
UB C5
UB C6
D
Cell Signaling: NPC → UB
WNT5B − FZD8
WNT5B − FZD6
WNT5B − FZD4
WNT4 − (FZD8+LRP6)
WNT4 − (FZD8+LRP5)
WNT4 − (FZD6+LRP6)
WNT4 − (FZD6+LRP5)
WNT4 − (FZD4+LRP6)
WNT4 − (FZD4+LRP5)
SEMA3B − (NRP2+PLXNA2)
SEMA3B − (NRP2+PLXNA1)
SEMA3B − (NRP1+PLXNA2)
SEMA3B − (NRP1+PLXNA1)
SEMA3A − (NRP1+PLXNA2)
SEMA3A − (NRP1+PLXNA1)
NPC C4
NPC C7
NPC C8
NPC C1
NPC C6
NPC C2
NPC C3
NPC C5
Probability
max
min
p−value
0.01 < p < 0.05
p < 0.01
E
Cell Signaling: UB → NPC
WNT9B − (FZD8+LRP6)
WNT9B − (FZD8+LRP5)
WNT9B − (FZD6+LRP6)
WNT9B − (FZD6+LRP5)
WNT9B − (FZD4+LRP6)
WNT9B − (FZD4+LRP5)
WNT9B − (FZD3+LRP6)
WNT9B − (FZD3+LRP5)
WNT9B − (FZD2+LRP6)
WNT9B − (FZD2+LRP5)
WNT9B − (FZD10+LRP6)
WNT9B − (FZD10+LRP5)
SEMA3D − (NRP2+PLXNA2)
SEMA3D − (NRP2+PLXNA1)
SEMA3D − (NRP1+PLXNA2)
SEMA3D − (NRP1+PLXNA1)
SEMA3B − (NRP2+PLXNA2)
SEMA3B − (NRP2+PLXNA1)
SEMA3B − (NRP1+PLXNA2)
SEMA3B − (NRP1+PLXNA1)
UB C1
UB C2
UB C3
UB C4
UB C5
UB C6

Figure 6


A
Rhesus 105dg
Human 26wg
SIX1 SFRP1 SFRP2 KRT8 DAPI
SIX1
SFRP1
SFRP2
B
Rhesus 105dg
WT1 FZD4 FZD7 KRT8 DAPI
Human 27 WG
FZD4
FZD7
C
Human FZD7 CDH1 KRT8 RET DAPI
12 wg
17 wg
27 wg
30 wg
D
SIX1 FZD6 SEMA3D KRT8 DAPI
SEMA3D
Rhesus 105dg
Rhesus 105dg
Human 27wg