Article

Generation of a Single-Cell RNAseq Atlas of Murine Salivary Gland Development

Murine salivary gland developmental scRNAseq atlas

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HIGHLIGHTS

Generated scRNAseq atlas of E12, E14, E16, P1, P30, and adult SMG

Smgc and *Bpifa2* define two proacinar populations in the developing SMG

Loss of *Ybx1*, *Eno1*, *Sox11*, and *Atf4* associated with acinar phenotype

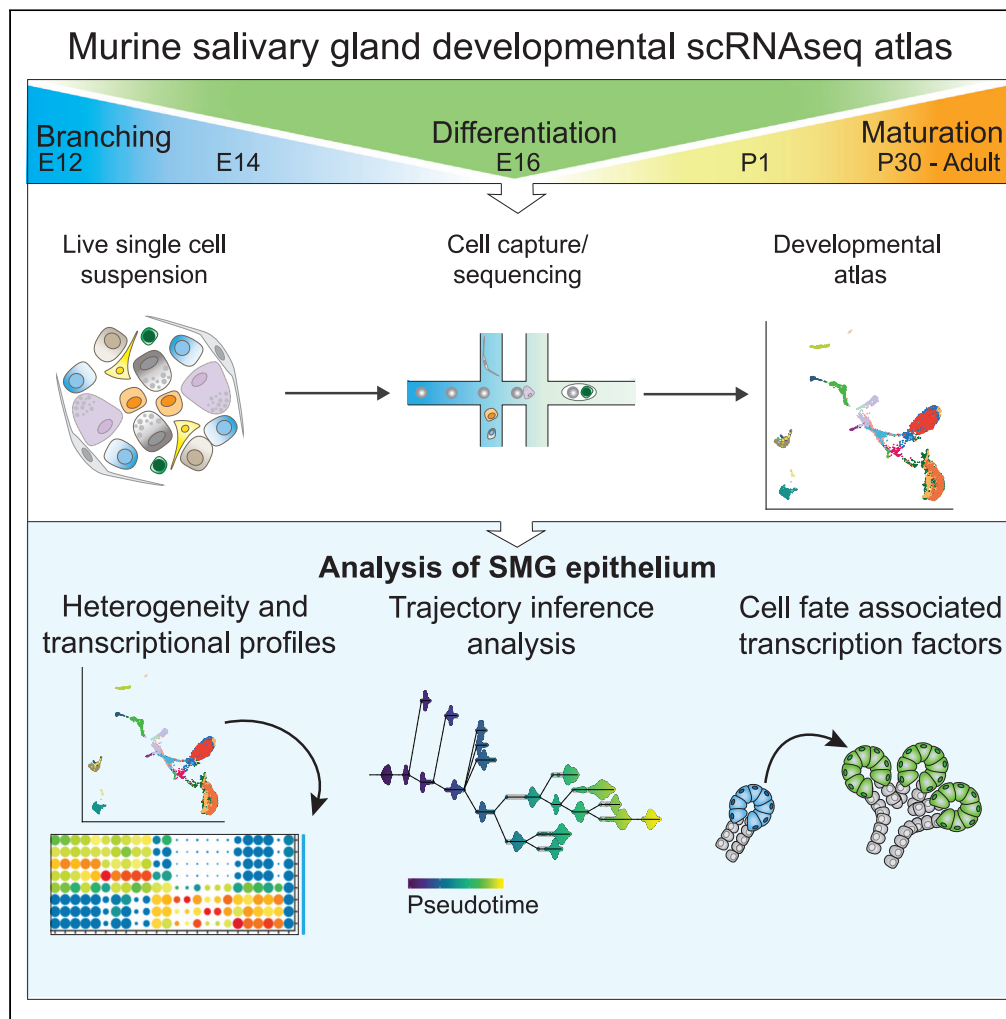*Gstt1* defines *Kit*-negative and sexually dimorphic intercalated duct cells

Article

Generation of a Single-Cell RNAseq Atlas of Murine Salivary Gland Development

Belinda R. Hauser,[1,3] Marit H. Aure,[1,3] Michael C. Kelly,[2] Genomics and Computational Biology Core, Matthew P. Hoffman,[1,*] and Alejandro M. Chibly[1,4,*]

SUMMARY

Understanding the dynamic transcriptional landscape throughout organ development will provide a template for regenerative therapies. Here, we generated a single-cell RNA sequencing atlas of murine submandibular glands identifying transcriptional profiles that revealed cellular heterogeneity during landmark developmental events: end bud formation, branching morphogenesis, cytodifferentiation, maturation, and homeostasis. Trajectory inference analysis suggests plasticity among acinar and duct populations. We identify transcription factors correlated with acinar differentiation including *Spdef, Etv1,* and *Xbp1,* and loss of *Ybx1, Eno1, Sox11,* and *Atf4.* Furthermore, we characterize two intercalated duct populations defined by either *Gfra3* and *Kit,* or *Gstt1.* This atlas can be used to investigate specific cell functions and comparative studies predicting common mechanisms involved in development of branching organs.

INTRODUCTION

In salivary glands and other developmentally similar branching organs like mammary gland, pancreas, kidney, lung, and lacrimal glands epithelial heterogeneity and cell lineages are not well understood. Developmentally, branching organs undergo a sequence of key events that are conserved across tissues, namely, initiation, tubulogenesis, branching morphogenesis, cytodifferentiation, and homeostasis (Goodwin and Nelson, 2020). These processes require coordinated regulation of proliferation, differentiation, and cell interactions with their microenvironment (Patel and Hoffman, 2014), which are also critical for regeneration. Proper characterization of the heterogeneous branching epithelium and identification of the molecular factors involved in the transition between development stages will inform therapeutic strategies aiming to regenerate injured organs.

Mouse submandibular gland (SMG) development begins at embryonic day (E) 11 with an epithelial placode forming within a condensed mesenchyme. Epithelial progenitors form an end bud at E12 that gives rise to all epithelial cells in the adult gland (Athwal, et al., 2019). Branching morphogenesis begins ~E13.5 and continues throughout fetal development and cell differentiation of proacinar and myoepithelial cells (MECs) begins ~E16. At postnatal day 1 (P1), functional acinar differentiation is required for saliva secretion and further acinar and ductal differentiation continues postnatally (Hauser and Hoffman, 2015; Patel and Hoffman, 2014; Tucker, 2007). The specific cell types and molecular factors required for the progression through these stages and those involved in lineage commitment and specification of terminally differentiated cells such as secretory acinar cells are not well known. This is of clinical relevance because acinar cells are lost or permanently damaged during autoimmune disease or as a consequence of cancer therapies (Jensen, et al., 2019) and little is known about the regulation of acinar development and regeneration.

Single-cell RNA sequencing (scRNAseq) has made it possible to discover new and rare cell types, explore cellular heterogeneity, and identify cell-fate-determining factors during developmental trajectories (Hedlund and Deng, 2018; Zappia, et al., 2018). Recently, scRNAseq analyses in branching organs including the kidney (Combes, et al., 2019), lung (Angelidis, et al., 2019; Reyfman, et al., 2019), pancreas (Qadir, et al., 2020; Byrnes, et al., 2018; Sznurkowska, et al., 2018), mammary gland (Bach, et al., 2017), and many others have helped characterize tissue-specific heterogeneity and cell lineages during specific developmental stages or physiological conditions (i.e., embryonic development, aging, or injury). scRNAseq offers a unique opportunity to investigate acinar and duct development in salivary glands to identify cells that

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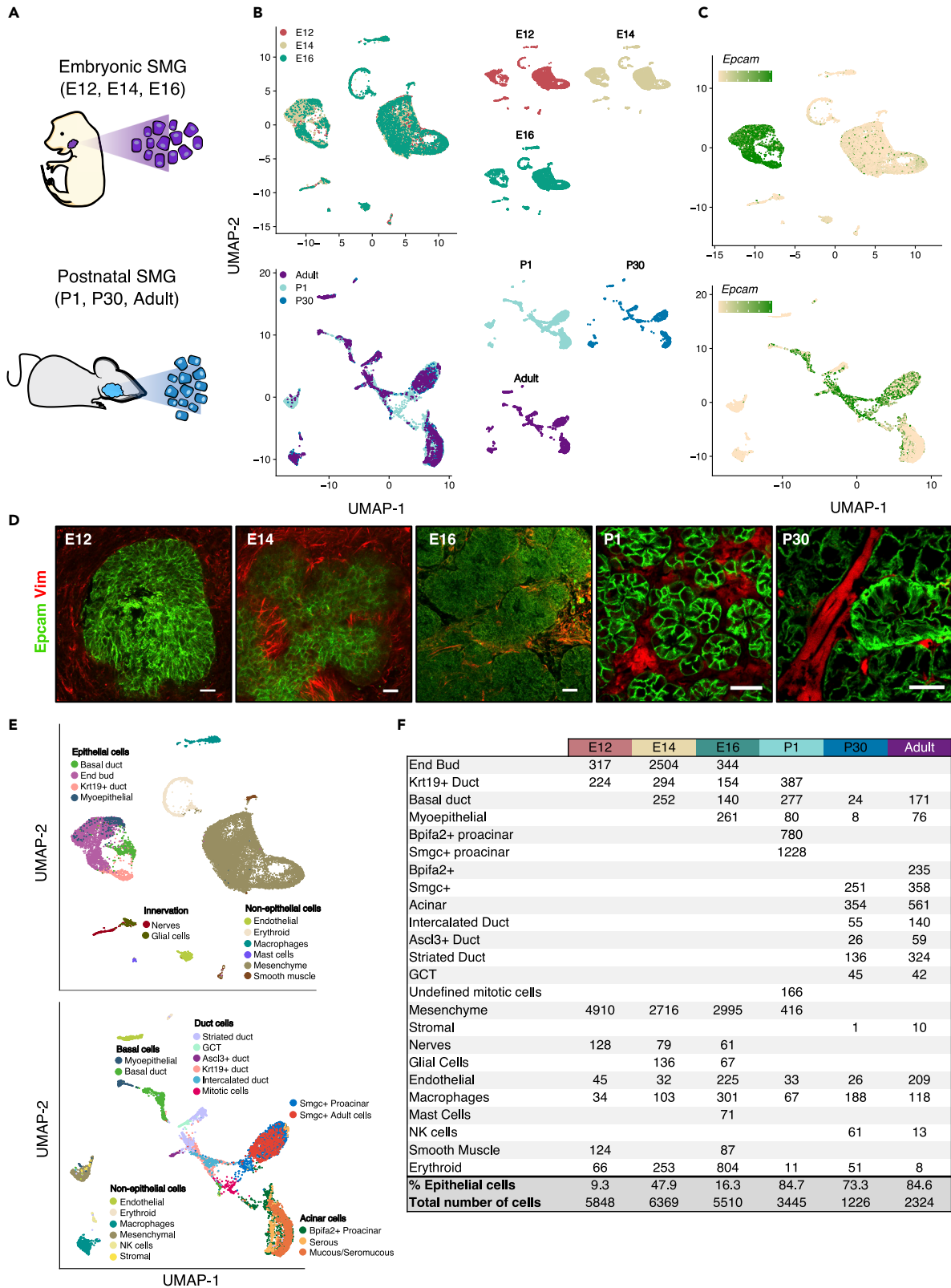
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https://doi.org/10.1016/j.isci.2020.101838

A

Embryonic SMG
(E12, E14, E16)

Postnatal SMG
(P1, P30, Adult)

B

C

D

E

F

	E12	E14	E16	P1	P30	Adult
End Bud	317	2504	344			
Krt19+ Duct	224	294	154	387		
Basal duct		252	140	277	24	171
Myoepithelial			261	80	8	76
Bpifa2+ proacinar				780		
Smgc+ proacinar				1228		
Bpifa2+						235
Smgc+					251	358
Acinar					354	561
Intercalated Duct					55	140
Ascl3+ Duct					26	59
Striated Duct					136	324
GCT					45	42
Undefined mitotic cells				166		
Mesenchyme	4910	2716	2995	416		
Stromal					1	10
Nerves	128	79	61			
Glial Cells		136	67			
Endothelial	45	32	225	33	26	209
Macrophages	34	103	301	67	188	118
Mast Cells			71			
NK cells					61	13
Smooth Muscle	124		87			
Erythroid	66	253	804	11	51	8
% Epithelial cells	9.3	47.9	16.3	84.7	73.3	84.6
Total number of cells	5848	6369	5510	3445	1226	2324

Figure 1. scRNAseq Analysis of Murine SMG Development

(A and B) Single-cell suspensions from embryonic and postnatal SMG were used to build scRNAseq libraries. Data integration of embryonic and postnatal stages is shown in separate UMAPs colored by developmental stage.

(C) UMAPs showing expression of *Epcam*.

(D) Immunostaining of Epcam (green) and Vimentin (red). Scale bars, 20 μm.

(E) Clusters were annotated based on expression of known markers.

(F) Table showing cell numbers within each population from each stage.

may be primed to produce acinar cells and define key transcription factors (TFs) involved in cell-fate decisions.

Here, we generate a scRNAseq resource of key stages of SMG development (E12, E14, E16, P1, P30, and adult). This atlas provides a transcriptional signature of cells at each developmental stage and highlights the epithelial heterogeneity of the gland. Furthermore, we show the potential of this resource through computational analysis to evaluate developmental trajectories, identify TFs potentially involved in acinar and duct differentiation, and characterize subpopulations of discrete cell types in anatomically defined compartments of the gland. This atlas can be coupled with other scRNAseq databases to predict cell functions and identify biologically conserved processes across multiple tissues. The database is a useful resource for the field to identify target genes and understand organogenesis in developmental and regenerative studies.

RESULTS

Generation of Transcriptional Atlas Capturing Landmark Events during SMG Development

We generated six individual scRNAseq libraries of murine SMG development using the 10X Genomics platform (Figures 1A, 1B, and S1). We chose embryonic stages corresponding to primary end bud formation (E12), branching morphogenesis (E14), and onset of cell differentiation (E16), as well postnatal days 1 and 30 (P1, P30) and adult glands (10 months). Unsupervised clustering and differential expression analyses were performed with the SEURAT package (Stuart, et al., 2019; Butler, et al., 2018) to identify cell populations. Embryonic epithelial populations were identified by expression of *Epcam* (all epithelium), *Krt14* and *Krt5* (basal duct), *Krt19* (duct), *Sox9* and *Aqp5* (end bud and proacinar), and *Acta2* (myoepithelial). Postnatal epithelium was annotated according to expression of known cell type-specific markers summarized in Figure S1. Non-epithelial clusters were identified by expression of *Pecam1* (endothelial), *Tubb3* (nerves), *Ncam1* (glial), *Alas2+* (erythroid), *Adgre1* (macrophages), *Kit* (mast cells), *Nkg7* and *Gzma* (natural killer cells), and *Acta2+Epcam-* (smooth muscle). A cluster of undefined mitotic epithelial cells was also observed at P1. Subsequent data integration was performed for embryonic and postnatal stages individually for visualization and to adjust for potential batch effects between samples (Figure 1B). The proportion of epithelial (*Epcam+*) and non-epithelial cells (*Vim+*) recovered was consistent with immunostaining for these proteins (Figures 1C and 1D), and as expected, the number of epithelial clusters increased throughout development reflecting epithelial specialization (Figures 1E and 1F). The transcriptional profiles for all identified cell populations are provided in Data S1. Using this resource, we aimed to (1) dissect the heterogeneity of the SMG epithelium and identify cell type-specific markers, (2) to identify putative cell-fate-determinant TFs involved in acinar and ductal cell specification and differentiation, and (3) to characterize epithelial cell subpopulations.

Transcriptional Heterogeneity Identified in Embryonic SMG Epithelium

To identify putative epithelial subpopulations, epithelial cells from each developmental stage were separated bioinformatically and re-clustered for downstream analysis maintaining their assigned cell annotations from Figure 1E. We anticipated unique heterogeneity across developmental stages; thus, individual analyses were performed for each stage. To choose an optimal resolution for unsupervised clustering, we tested 12 different resolution values and compared their performance using clustree package in R (not shown). We then chose a resolution that accurately discriminated cell types according to their previously assigned annotations.

Unsupervised clustering of E12, E14, and E16 epithelium resulted in seven to nine clusters (Figure 2A). Immunofluorescence images and UMAPs of *Krt5* and *Krt14* expression are shown to confirm that unsupervised clustering accurately represents the spatiotemporal expression of these progenitor markers throughout development (Figures 2B–2D). As expected, spatial separation between clusters was increased
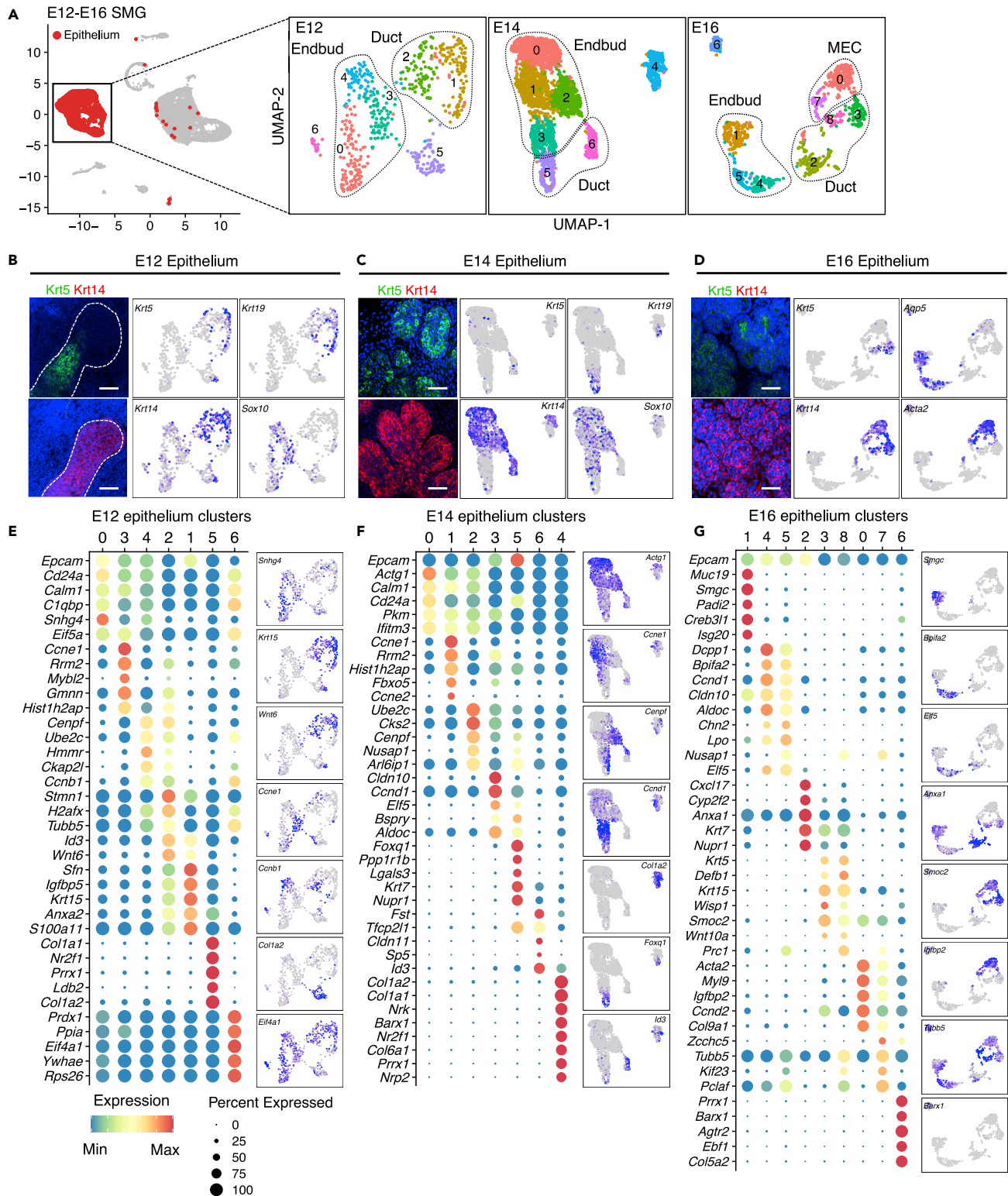
Figure 2. SEURAT Analysis of Embryonic SMG Epithelium

(A) From the embryonic integrated library, epithelial clusters from individual stages were separated and re-clustered with SEURAT. Individual UMAPs are shown, and labeled outlines indicate main cell types.

Figure 2. Continued

(B–D) Representative images of Krt5 (green) and Krt14 (red) whole-mount staining of embryonic SMG and complimentary UMAPs. Additional UMAPs are shown for *Sox10, Krt19, Aqp5,* and *Acta2.* Scale bars, 50 μm.

(E–G) Dot plots with scaled expression (color of the dot) and percentage of expression (size of the dot) of the top five genes for each cluster from Figure 2A. A representative gene from each group is shown in a UMAP.

at E16 likely due to ongoing cell specification, which was also evident by expression of the proacinar marker *Aqp5* in clusters 1, 4, and 5 (Figures 2A and 2D).

As the number of resulting clusters is dependent on the chosen resolution for analysis, it does not necessarily indicate the number of discrete subpopulations. Therefore, we also performed differential expression analysis of all clusters to cluster-specific gene expression. The top five expressed markers per cluster are shown in Figures 2E–2G, and their complete transcriptional profile is provided in Data S2. Observed epithelial heterogeneity at E12 was primarily due to the proliferative state of cells as shown by expression of cell cycle S phase (*Ung*) and G2/M phase (*Aurka, Mki67*) markers (Figures S2A and S2B). Proliferative end bud cells were found in cluster 2, whereas proliferative *Krt19*+ duct cells were in cluster 4. At E14, proliferative end bud cells were represented by cluster 2 but a discrete cluster of proliferative duct cells was not identified (Figures S2C and S2D). In addition, cluster 3 at E14 identified a subpopulation of end bud cells characterized by expression of *Cldn10*, which was distinct from end bud cells in clusters 0 and 1 characterized by *Actg1* expression (Figure 2F). These are likely to represent the inner and outer layers of the end bud, respectively (Figure S2G). At E16, proliferative end bud, basal duct, and MECs were found in clusters 5, 8, and 7, respectively (Figures 2G, S2E, and S2F). In addition, end bud cells were also divided in two major groups characterized by expression of *Muc19* and its splice isoform *Smgc* in cluster 1, and *Bpifa2* (parotid secretory protein or Psp) and *Dcpp1* in clusters 4 and 5 (Figure 2G). Although DEGs were enriched in a cluster or cell population, these cell-defining genes were often expressed elsewhere to lesser degrees.

At all stages, there were undefined cell clusters (clusters 5 at E12, 4 at E14, and 6 at E16) expressing mesenchymal markers *Col1a1, Col1a3a1,* as well as relatively low levels of *Krt14, Krt19, Sox10,* and *Epcam* (Figures 2E–2G and S2). In addition, cluster 6 at E12 is defined by high expression of genes such as *Prdx1, Ppia,* and *Eif4a1* that are broadly expressed within many cell clusters in both epithelial and non-epithelial cells (Figure 2E). As cell doublets were removed during analyses, these undefined clusters may represent either uncommitted cells, or cells that are undergoing epithelial to mesenchymal transition, or vice versa. They may be homologous to a *KRT17*+ matrix producing epithelial cell associated with pulmonary fibrosis (Habermann, et al., 2020). Further analysis of these cells is required to understand their identity.

scRNAseq of Postnatal SMG Reveals Two Related but Distinct Proacinar Populations

Clustering of epithelial cells from postnatal glands resulted in 10 epithelial clusters at P1 and P30 and 12 clusters in adult gland. Most of the previous annotations remained in a single cluster, but proacinar and acinar cells were represented by multiple discrete clusters at all postnatal stages, highlighting their heterogeneity (Figures 3A and 3B). The top five DEGs per cluster and representative UMAP of a gene from each cluster are shown (Figures 3C–3E). Duct populations at P1 were defined by high keratin expression and fell into two main groups: basal duct (BD) in cluster 4 (*Krt14*+ *Krt17*+) and differentiating ducts that express *Krt19* and *Krt7* in cluster 2 (Figure 3C). In the P30 and adult gland, well-separated duct clusters represented *Kit*+ intercalated duct (ID) cells, striated ducts (SD), *Ascl3*+ cells, and BD cells, all of which had unique transcriptional signatures (Figures 3C and 3D). Granular convoluted tubules (GCTs) were also in a discrete cluster at P30 (cluster 5) but were mixed with SD cells in the adult dataset (cluster 2), which was exclusively from female glands. MECs were in cluster 9 at P1 and cluster 8 in the adult gland (Figures 3C and 3E), and were defined by expression of *Acta2, Myh11,* and *Cnn1.* At P30, MECs clustered together with BD cells (Figures 3A and 3D), likely due to the low number of MECs recovered (Figure 1E). For further analyses, MECs at P30 were manually annotated based on expression of *Acta2* and *Cnn1.* Proliferation in P30 and adult gland was limited to a few cells in multiple clusters (Figures S3C–S3F). Last, few undefined cells that expressed the mesenchymal genes *Col1a1* and *Col3a1* were scattered across multiple clusters at all postnatal stages (Figure S3).

The mouse SMG is known to produce both serous and mucous secretions, and the greatest heterogeneity for identified cell types was observed within proacinar and acinar cells (Figures 3A and 3B). Proacinar cells at P1 were found across seven clusters that fell into three major groups based on top DEGs: *Smgc*+ proacinar (clusters 0, 3, and 6), *Bpifa2*+ proacinar (clusters 1, 7, and 8), and mitotic cells (cluster 5) (Figures 3A,
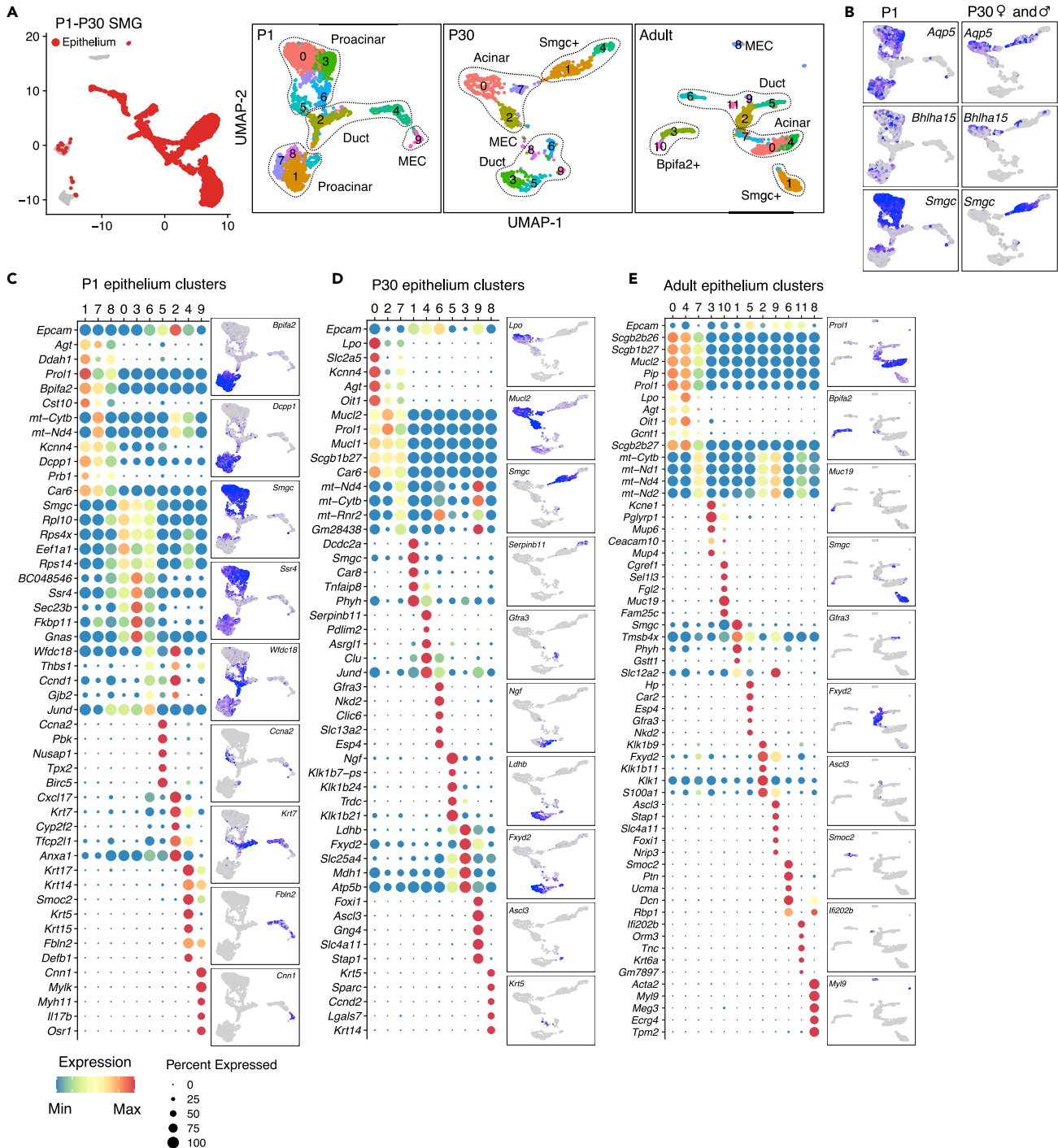
Figure 3. SEURAT Analysis of Postnatal SMG Epithelium

(A) From the postnatal integrated library, epithelial clusters from individual stages were separated and re-clustered with SEURAT. Individual UMAPs are shown, and labeled outlines indicate main cell types.

(B) UMAPs of expression of *Bhlha15*, *Aqp5*, and *Smgc* in P1 and P30 SMG.

(C–E) Dot plots with scaled expression (color of the dot) and percentage of expression (size of the dot) of the top five genes for each cluster from Figure 3A. A representative gene from each group is shown in a UMAP.
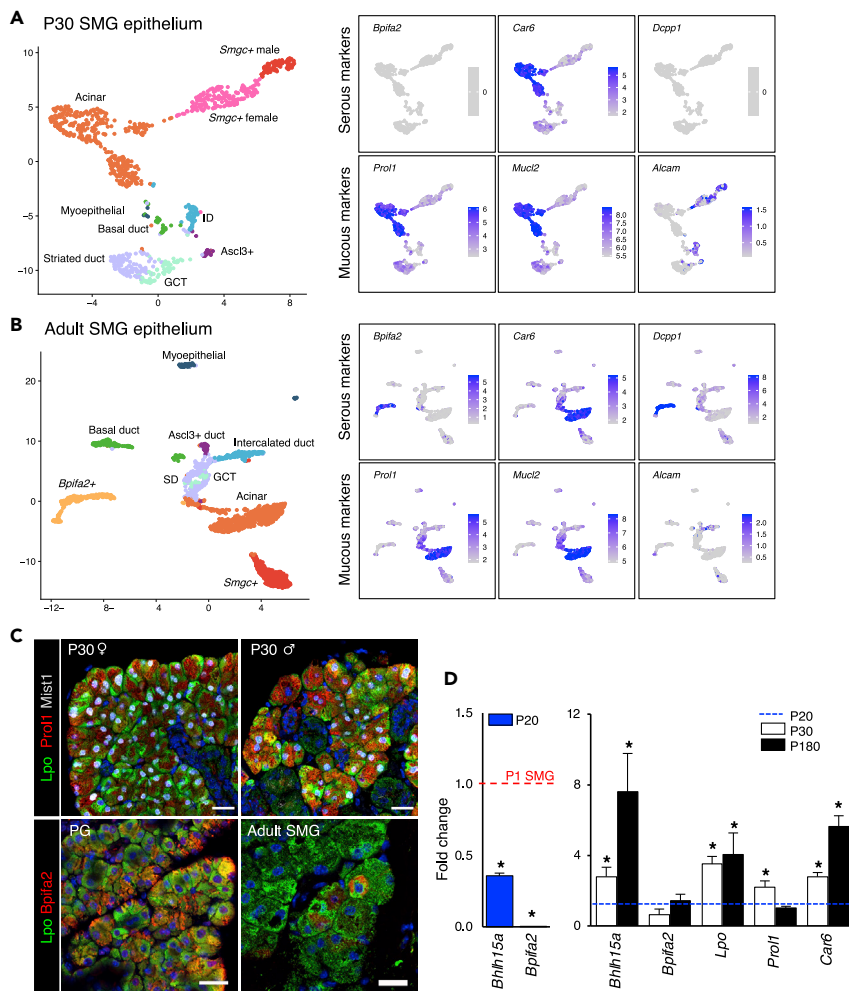
Figure 4. Expression of Serous and Mucous Genes in Acinar Cells

(A and B) Epithelium from P30 and adult SMG colored and annotated by cell type with UMAPs showing expression of serous and mucous markers alongside.

(C) Immunostaining of Lpo (green), Prol1 (red), and Mist1 (white) in P30 SMG (top panel) and Bpifa2 (Red) in adult PG and SMG (bottom panel).

(D) Left qPCR graph shows decreased expression of *Bhlha15* and *Bpifa2* in P20 SMG normalized to P1 SMG. Graph on the right shows qPCR of selected serous and mucous genes in P30 and P180 female SMG normalized to P20. Data are represented as mean ± SEM and asterisks denote statistical significance (p < 0.05) compared with baseline (n = 3, two-tailed t test).

3C, S3A, and S3B). Individual clusters within the first two groups shared similar transcriptional profiles with varying expression levels of their defining genes. Mature acinar cells defined by expression of *Aqp5* and *Bhlha15* (Mist1) were found in clusters 0, 2, and 7 in P30 (Figure 3D) and clusters 0 and 4 in adults (Figure 3E). They expressed varying levels of both serous and mucous secretory genes suggesting a seromucous phenotype (Nelson, et al., 2013; Kivela, et al., 1999; Mirels, et al., 1998). These included prolactin-induced protein (*Pip*), lactoperoxidase (*Lpo*), mucin-like 1 and 2 (*Mucl1, Mucl2*), proline-rich lacrimal 1 (*Prol1*, also known as *Muc10*), and carbonic anhydrase 6 (*Car6*) (Figures 3C, 3D, 4A, and 4C). Notably, *Bpifa2* was expressed in a discrete population (clusters 3 and 10, Figures 3A and 3E), which co-expressed *Dcpp* genes in adult SMG (Figure 4B). Immunostaining showed that Bpifa2 delineates serous cells in parotid gland and was detected in some Lpo+ acinar cells in adult SMG (Figure 4D). This population was not found in our P30 data and was not detected by immunostaining in P30 SMG from males and females (not shown); however, *Bpifa2* expression was detected by qPCR in postnatal glands (Figure 4D). Taken together, this suggests that *Bpifa2+* cells in adult SMG may represent an additional population of serous-like acinar cells.
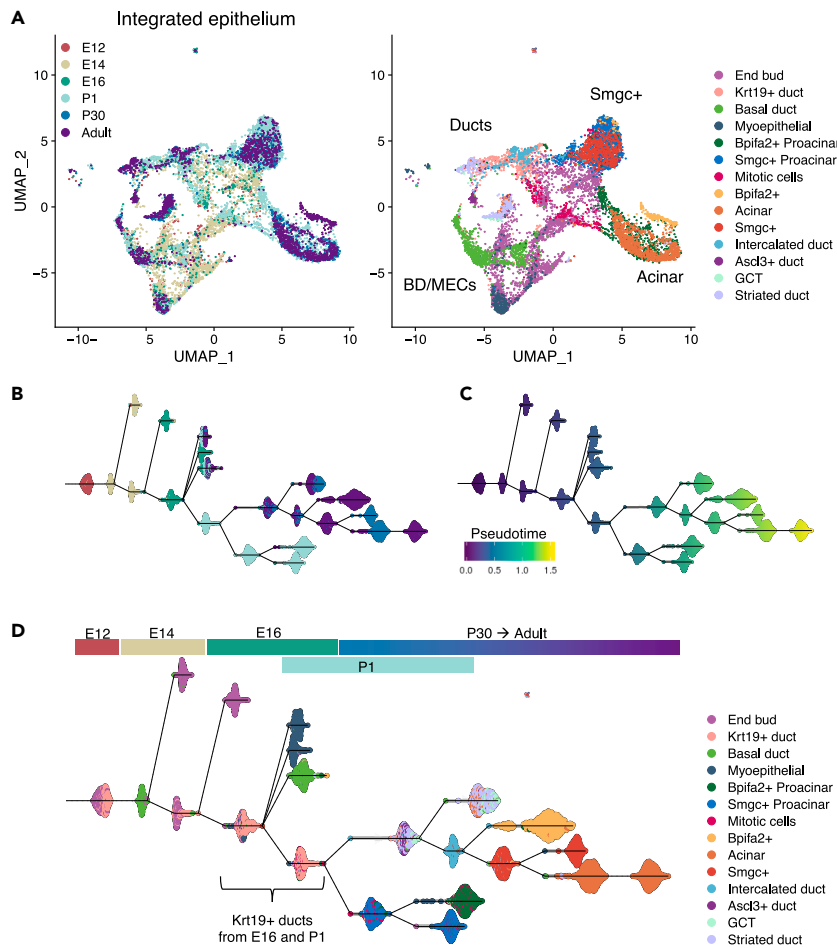
Figure 5. Trajectory Inference Analysis of SMG Epithelium

(A) Integrated and re-clustered epithelial cells from all stages shown in UMAP. Left panel is colored by stage and right panel by cell type.

(B and C) TI analysis using the PAGA-tree algorithm in Dynverse package. The determined pseudotemporal trajectory is colored by stage (B) and pseudotime score (C).

(D) Distribution of specific cell types along the trajectory is indicated by color and labels.

Thus, we refer to *Bpifa2*+ cells in adult gland as serous acinar cells and *Car6* *Prol1*+ *Lpo*+ cells as seromucous acinar cells.

Trajectory Inference Analysis Predicts Plasticity across Epithelial Populations

To further explore possible lineage relationships and developmental trajectories, we integrated epithelial cells from all stages (Figure 5A) maintaining cell type annotations. Four regions could be identified in the resulting integrated UMAP, which corresponded to Smgc+, acinar, basal, and specialized duct cells, respectively. Resolution between individual populations was greatly reduced, likely because of the influence of embryonic cell types, which were spread throughout the UMAP projection. Next, we performed trajectory inference (TI) analysis with Dynverse package in R (Saelens, et al., 2019), which aligns cells along an unbiased pseudotemporal trajectory called pseudotime based on their transcriptional similarities. Dynverse compares >50 TI methods and chooses the most appropriate one to evaluate individual cell transcriptomes in a given dataset to infer their order in pseudotime. Here, TI was performed using PAGA-tree algorithm (Wolf, et al., 2019). Although pseudotime is determined in an unbiased manner, we manually selected E12 epithelial cells as the origin of the trajectory because they are the most primitive population in our dataset.

The inferred pseudotime trajectory accurately represented the biological progression of developmental stages (Figures 5B–5D) with embryonic populations receiving the lowest pseudotime scores. However,

the resulting trajectory did not reflect the natural developmental progression of specific cell types according to reported lineage-tracing studies. For instance, lineage tracing of *Kit, Krt5,* and *Krt14* in the adult SMG shows that these populations are lineage-restricted during homeostasis (Kwak, et al., 2018; May, et al., 2018; Kwak, et al., 2016), whereas our predicted trajectory situated *Kit+* cells at a branching point preceding *Smgc+* and seromucous acinar cells (Figure S4). Nonetheless, plasticity of multiple salivary gland cell types including myoepithelial, *Kit+*, and *Krt5+* cells has been reported under severe damage (Ninche, et al., 2020). Thus, our TI analysis may be more informative of potential plasticity rather than lineage trajectories. Accordingly, primitive cell populations like end buds and *Krt19+* ducts were often found together in multiple branch points and closely related cell types remained in similar branches (Figure 5D). MECs and BD cells from E16 onward were in terminal branches with low pseudotime scores, likely because of their early specification. Similarly, a fraction of end bud cells from E14 and E16 were in terminal branches. This is not surprising as not every cell from a given developmental stage is expected to function as a progenitor or to maintain plasticity.

Notably, *Krt19+* duct and mitotic cells from E16 and P1 were localized at the branching point from which all other postnatal populations were derived, except for BD and MECs (Figure 5D). The most striking result was the separation between P1 proacinar cells and acinar populations, which were expected to appear in a continuous branch. Although it is possible that batch effect between developmental stages may influence these observations, this could also reflect the differences within the acinar compartment between neonatal and mature glands described in the previous section (Figure 4). The significance of our TI analysis to predict plasticity between populations will require further studies. Nonetheless, because of our consistent observations regarding complexity and heterogeneity in the acinar lineage, we next aimed to determine the TFs potentially involved in acinar cell development.

Identification of Transcription Factors Involved in Acinar Specification and Differentiation

To identify genes associated with acinar cell development, we performed Pearson correlation analysis across our integrated dataset (Figure 6A) to find genes correlated with expression of *Bhlha15*. *Bhlha15* is a well-known acinar gene that encodes the TF Mist1, which is regulated by *Xbp1* in acinar cells (Lo, et al., 2017; Hess et al., 2016; Huh, et al., 2010). Not surprisingly, the genes with the strongest correlations to *Bhlha15* were the acinar markers *Lpo* (0.38), *Aqp5* (0.37), and *Pip* (0.36) (Data S3). We specifically focused on TFs and found that *Creb3l1* (0.30), *Spdef* (0.27), *Etv1* (0.23), *Creb3l4* (0.20), and *Xbp1* (0.19) were the most positively correlated TFs, whereas *Eno1* (−0.34), *Ybx1* (−0.32), and *Sox11* (−0.32) were the most negatively correlated TFs (Data S3). The 10 strongest positive and negative correlated TFs with *Bhlha15* are shown in Figure 6B.

We hypothesized that TFs positively correlated with *Bhlha15* would become expressed after cytodifferentiation begins at E16 (Nelson, et al., 2013), and that in turn, negatively correlated genes would be downregulated. Indeed, when viewed as a heatmap along with *Bhlha15*, positively correlated genes were enriched after E16 (Figure 6C). To investigate if these TFs were involved in specific developmental transitions, we compared related populations from contiguous developmental stages. The top five enriched TFs (if present) for each transition are shown as violin plots, and the complete list is provided in Data S4. Not surprisingly, higher expression of positive *Bhlha15*-correlated genes (*Ehf, Spdef,* and *Xbp1*) occurred at E16 (Figure S5A), whereas negative *Bhlha15*-correlated genes (*Eno1, Sox11 Ybx1, Etv4,* and *Hmga2*) progressively decreased in expression during development (Figures 6D, S5A, and S5B).

Our earlier analysis showed two distinct proacinar populations defined by *Smgc* and *Bpifa2* expression. Interestingly, similar TFs were decreased in expression at P1 for both populations including *Sfpq, Hnrnpk, Cebpb,* and *Ybx1* (Figure S5B). In contrast *Etv1* and *Zbtb20* were enriched in P1 *Bpifa2+* proacinar cells, whereas *Atf3, Arid5b,* and *Hopx* were enriched in *Smgc+* proacinar cells (Figure S5B). *Smgc+* cells from P30 continued to show enriched expression of *Junb, Cebpd, Fos, Atf3,* and *Egr1* postnatally, but the acinar-correlated gene *Spdef* was decreased (Figure 6E). Because serous acinar cells were not identified at P30, we could not determine TFs potentially involved in this transition from P1 SMG.

The postnatal maturation of seromucous acinar cells is not well understood, and TI analysis did not predict a direct lineage relationship with proacinar cells. Thus, it is unclear which of the two populations of proacinar cells at P1 (*Smgc+* and *Bpifa2+*), if either, give rise to seromucous acinar cells. To predict a potential lineage relationship, we compared the transcriptional profile of proacinar cells and seromucous acinar cells
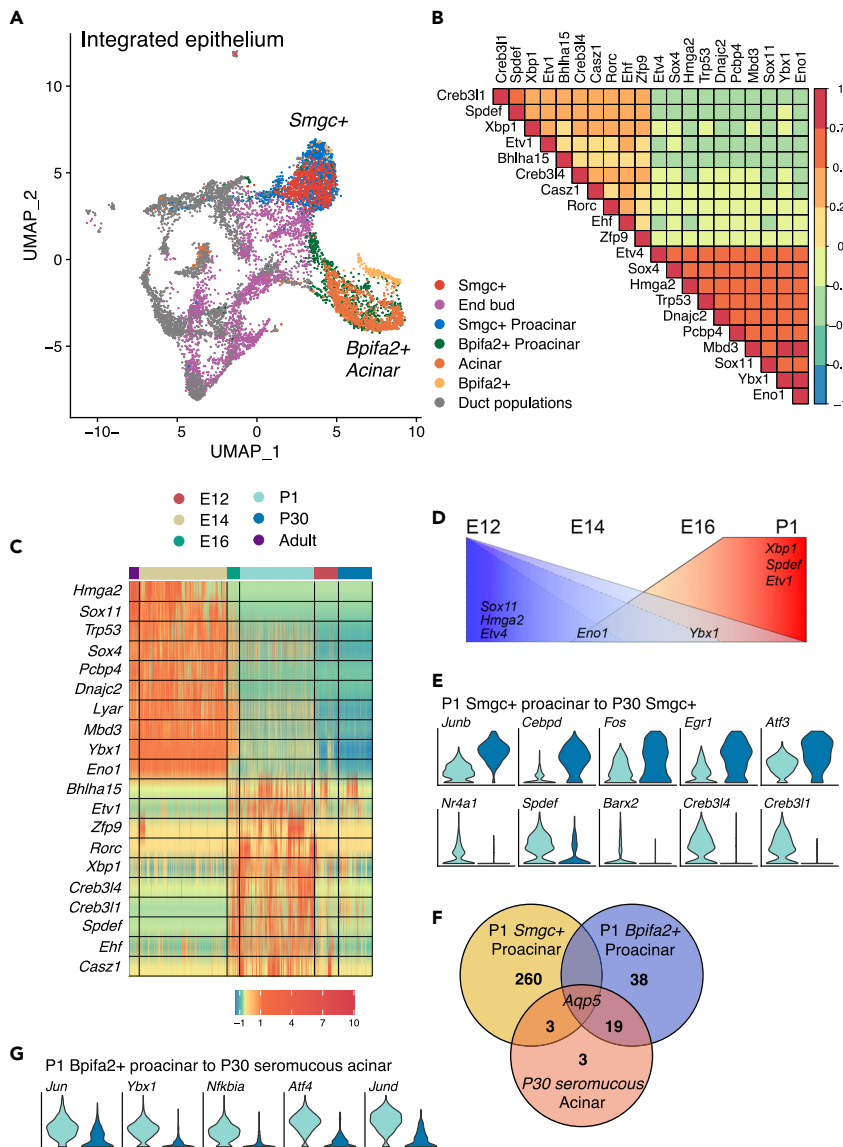
Figure 6. Transcriptional Regulators of Acinar Differentiation

(A) UMAP of integrated SMG epithelium highlighting end bud, proacinar, and acinar populations colored by cell type.

(B) Analysis of TFs correlated with *Bhlha15*. Color scale represents correlation scores (p < 0.05).

(C) Heatmap showing scaled expression of *Bhlha15*-correlated genes in end bud, proacinar, and acinar clusters. The colored bars represent developmental stages.

(D) Schematic summarizing gene expression changes of selected genes throughout development. Blue area shows genes that decrease in expression, whereas red indicates genes that increase during acinar differentiation.

(E) Violin plots of top differentially expressed TFs between P1 *Smgc+* proacinar and P30 *Smgc+* cells. Color scale consistent with Figure 6C.

(F) Venn diagram of the comparison between defining genes for P1 proacinar populations and seromucous acinar cells at P30.

(G) Violin plots of top differentially expressed TFs between P1 *Bpifa2+* proacinar and P30 seromucous acinar cells. Color scale consistent with Figure 6C.

to identify transcriptional similarities. Only 4 of the 267 defining genes for *Smgc+* proacinar cells were also enriched in seromucous acinar cells at P30, whereas 50% of the defining genes for *Bpifa2+* proacinar cells were enriched in seromucous cells (Figure 6F). Accordingly, 20 of the 26 seromucous acinar-defining genes were enriched in *Bpifa2+* proacinar cells suggesting that *Bpifa2+* proacinar cells are more closely related to

mature seromucous acinar cells in mouse SMG. Differential expression analysis between *Bpifa2+* proacinar and seromucous acinar cells showed reduced expression of the TFs *Jun, Ybx1, Nfkbia, Atf4,* and *Jund* in mature acinar cells (Figure 6G).

Taken together with our previous analyses, these data suggest that *Bpifa2+* and *Smgc+* proacinar cells may differentiate from a common progenitor around E16 but subsequently undergo distinct developmental programs: one that shares the transcriptional signature of mature acinar cells (*Bpifa2+*) and one that undergoes further differentiation into a distinct phenotype (*Smgc+*). Future lineage tracing experiments are required to confirm these predictions.

Putative TFs Involved in Duct-Acinar Differentiation Include *Atf3* and *Jun*

Krt5+ cells have also been reported to generate acinar cells upon severe injury (Weng, et al., 2018), and lineage tracing studies have shown that *Krt5+* BD cells act as progenitors for GCTs and SD cells (Aure, et al., 2019). Little is understood about the factors driving BD cell differentiation and maturation, as well as those involved in their plasticity to regenerate acinar cells. Thus, using a similar strategy to our previous analysis of the acinar lineage, we aimed to identify TF potentially involved in development of duct populations (Figure 7A).

Early development of *Krt19+* ducts involved increased expression of *Cebpd, Klf6, and Tfcp2l1* at both E14 and E16 (Figure 7B). Interestingly, decreased expression of *Ybx1, Hmga2, Sox11, and Eno1* also occurred, similar to our observations of end bud development (Figure S5A). Analysis of BD populations across developmental stages showed that 70% of BD-defining genes at embryonic stages (156 of 225) are maintained postnatally but the number of defining genes increases 5-fold to 1,135 genes in postnatal BD cells (Figure 7C), suggesting further specialization. The top TFs involved in BD development are shown in Figure 7D. To explore putative transitions from BD to GCT, SD, and acinar cells in adult SMG, we performed differential expression analysis between these populations (Figure 7E). *Bola3* and *Id2* were enriched in SD compared with BD cells, and *Carhsp1, Mafb, Bola3, Eno1,* and *Phb* were enriched in GCT compared with BD. Last, *Bhlha15, Etv1,* and *Creb3l1* were the only enriched TFs in acinar cells compared with BD populations, suggesting that they are potentially involved in the plasticity of *Krt5+* basal cells to generate acinar cells. The genes *Junb, Nfkbia,* and *Klf6* were enriched in BD compared with GCT, SD, and acinar cells, suggesting that these genes may be important for the BD phenotype. Other TFs enriched in BD cells included *Atf3* and *Fos*.

Many of the TFs involved in duct development were present in the developmental transitions of the acinar lineage (Figure 5), including *Ybx1, Eno1, Atf3,* and *Jun*. Notably, *Ybx1* and *Eno1* were decreased during early differentiation of both duct and acinar lineages and may therefore represent factors involved in global epithelial differentiation not exclusive to a specific lineage. In contrast, *Atf3* and *Jun* were decreased in the transition from either proacinar cells at P1 or BD cells to acinar cells. In pancreas, *Atf3* activation is associated with loss of acinar cell phenotype (Fazio, et al., 2017). Thus, these results warrant further investigation to determine whether downregulation of *Atf3* and *Jun* is required for acinar specification and maturation. The complete list of DEGs for each transition is provided in Data S4.

Subpopulations of ID in Mouse SMG Are Defined by Expression of *Gstt1* and *Gfra3*

Smgc, a splice variant of *Muc19,* has been described as an embryonic mucin in mucous proacinar cells (Das, et al., 2009). It is hormonally regulated and declines during postnatal development persisting only in female SMG (Kusakabe, et al., 2016). Our analyses consistently showed that *Smgc* delineated a discrete population in the P30 and adult glands, separate from acinar cells (Figure 3B). Accordingly, we found Smgc localized to IDs adjacent to *Mist1+Aqp5+* acinar cells in P30 and adults but did not express Mist1, unlike *Smgc+* cells at P1 (Figure 8A). Surprisingly, although Smgc+ protein was not detected in male SMG by immunostaining, our P30 scRNAseq, which contained male and female glands showed a cluster of Smgc+ cells in the male SMG (Figure S6A).

Sexual dimorphism in mouse SMG by P25 is well documented but little is known about transcriptional differences at single cell level (Mukaibo, et al., 2019; Gresik, 1994). Thus, we performed differential expression analysis between male and female cell populations using our P30 dataset. The complete list of identified sexually dimorphic genes is provided in Data S5. Surprisingly, GCTs, which hold the most distinct morphologic difference, did not show a significant number of DEGs with the exception of *Smgc*, which was detected in female GCT cells (Figures S6B and S6C). On the other hand, the *Smgc+* population was the
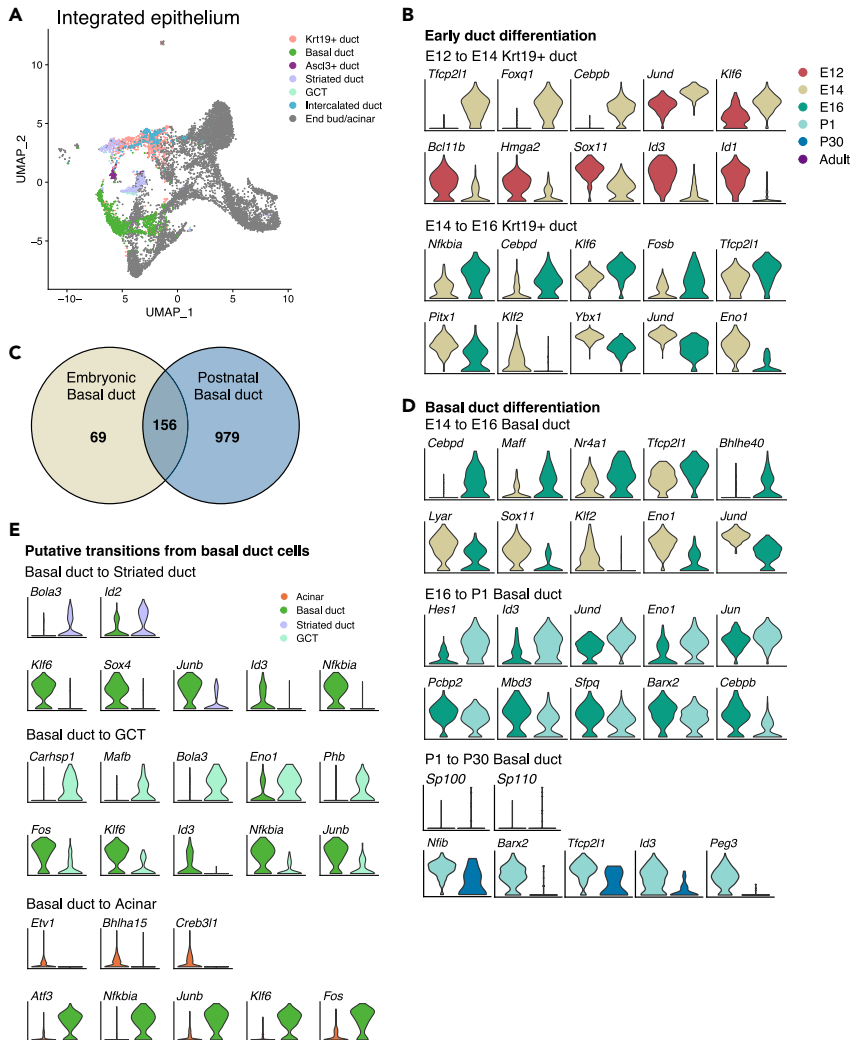
Figure 7. Transcriptional Regulators of Duct Differentiation
(A) UMAP of integrated SMG epithelium highlighting duct populations colored by cell type.
(B and D) Differential expression analysis between pairs of clusters from contiguous developmental stages as indicated by the color scheme and cell type labels. Top differentially expressed TFs (p < 0.05) are shown. TFs are sorted by fold change.
(C) Venn diagram of the comparison between defining genes for basal duct in embryonic versus postnatal stages.
(E) Differential expression analysis from BD to SD, GCTs, and acinar cells. Top differentially expressed TFs (p < 0.05) are shown.

most sexually dimorphic at a transcriptional level (Figure S6C). To date, *Smgc* is the only known marker for this population. Therefore, to better characterize these sexually dimorphic ID cells, we evaluated DEGs identified in our analysis. *Dcdc2a* was enriched in female *Smgc+* ID, whereas *Serpinb11* was enriched in males, and *Gstt1* was a common gene in both sexes (Figure S6D). Sex-dependent expression of these genes was detected by PCR between P20 and P30 and became more pronounced over time (Figure 8B). *Cdkn1c* was enriched in females in our scRNAseq data, but the gene was also detected in males by qPCR (Figures 8B and S6D). *Gstt1* protein was also detected in IDs of both male and female glands (Figure 8C). We propose that *Gstt1* is a defining gene for this ID population in both males and females.

The ID segment has long been proposed to contain a reservoir of progenitors with regenerative potential and several reports have identified *Kit* as a marker for an ID subpopulation (Kwak, et al., 2016; Katsumata, et al., 2009; Man, et al., 2001). Indeed, we identified a specific cluster of *Kit+* cells in the P30 and adult SMG. The *Kit+* ID population was defined by expression of *Gfra3*, *Nkd2*, and *Esp18* (Figures 8D–8F). These markers were not sexually dimorphic, and Gfra3 protein was detected within a subset of ID cells adjacent
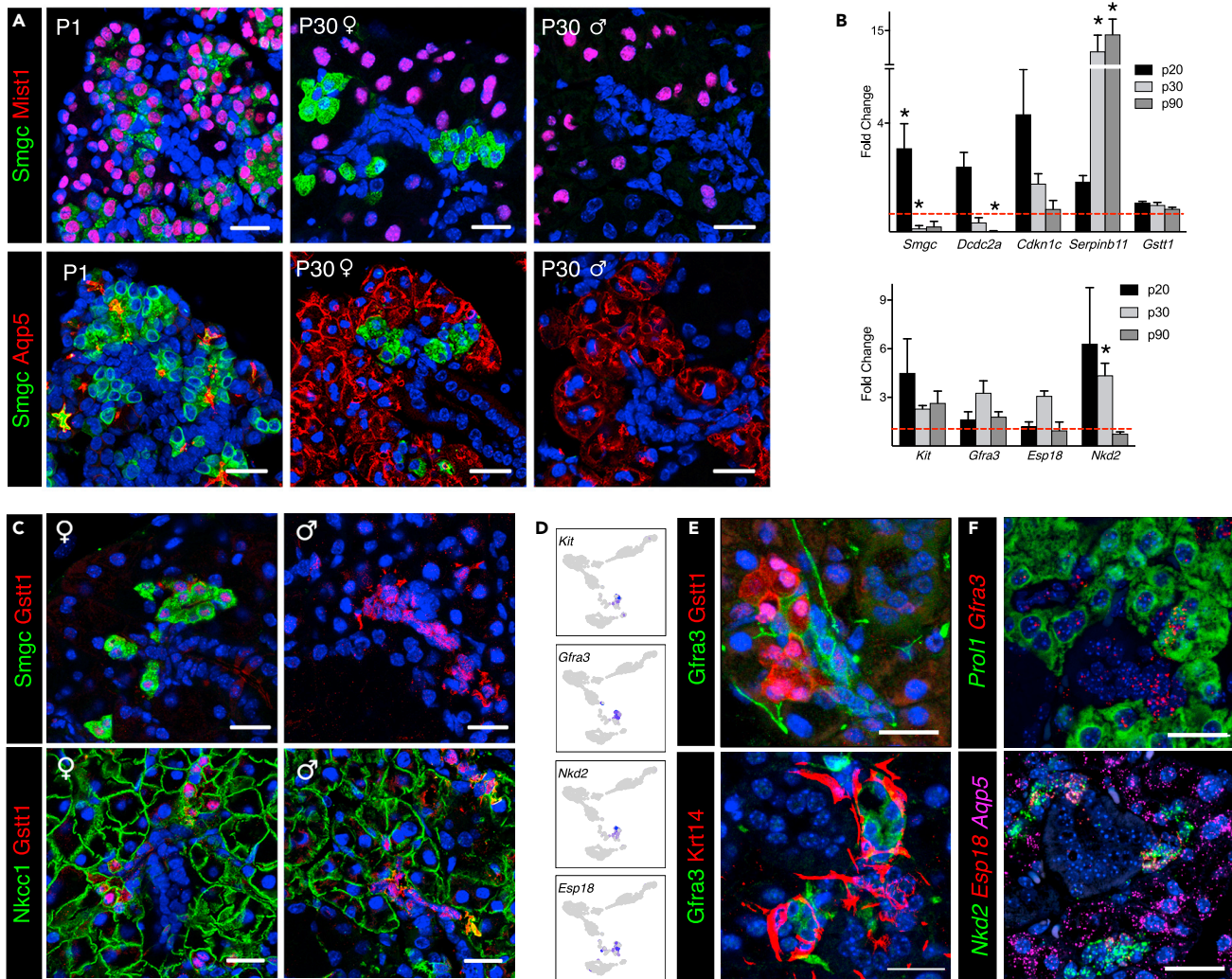
Figure 8. Subpopulations of SMG ID Are Defined by Expression of *Smgc*, *Gstt1*, and *Gfra3*

(A) Immunofluorescence staining showing localization of Smgc (green) in P1 and P30 SMG. Proacinar and acinar cells are labeled with Mist1 and Aqp5 (red).

(B) qPCR for genes enriched in *Smgc+* (top panel) and *Kit+* ID cells (bottom panel). Data are normalized to *Rsp29* and age-matched female SMG (dotted line). Data are represented as mean ± SEM, and asterisks show statistical significance (p < 0.05) compared with age-matched female controls (n = 3; two-tailed t test).

(C) Immunofluorescence staining showing expression of Gstt1 (red) in Smgc+ cells (green, top panel) and Nkcc1+ cells (green, lower panel) in P30 SMG from male and female mice.

(D) UMAPs showing selected DEGs in *Kit+* ID cells in P30 glands.

(E) Immunofluorescence shows distinct ID populations with no overlap between Gfra3, Gstt1, and Krt14 protein.

(F) *In situ* hybridization showing expression and localization of *Gfra3*, *Prol1*, *Nkd2*, *Aqp5*, and *Esp18* mRNA in P30 SMG. Scale bars, 20 μm.

to and distinct from Gstt1+ IDs and in adult glands, consistent with scRNAseq data (Figure 8E). Coexpression and localization with *Nkd2* and *Esp18* mRNA was confirmed by *in situ* hybridization (Figure 8F). *Gfra3+* ID cells did not overlap with *Krt14+* cells (Figure 8E), indicating that they are also a distinct population. Interestingly, *Kit*, *Nkd2*, and *Esp18* are coexpressed in the same clusters at P1, but *Gfra3* is not expressed in the epithelium (Figure S6E). qPCR analysis of intact SMGs shows that expression of *Gfra3* and *Nkd2* is markedly increased at P8 (Figure S6F), suggesting that differentiation of these cells occurs around this stage. Gfra3/Ret signaling occurs in nerves following binding of the neurotrophic factor artemin (Baloh, et al., 1998). As expected, *in situ* hybridization showed that *Gfra3* was co expressed with its co-receptor *Ret* in the parasympathetic ganglia of P1 mice (Figure S7). However, in the adult gland where Gfra3 is expressed in the ID epithelium, we found that *Ret* was broadly expressed in acinar cells, but not in the ID (Figure S7), suggesting a different signaling mechanism in epithelial cells that remains to be determined.
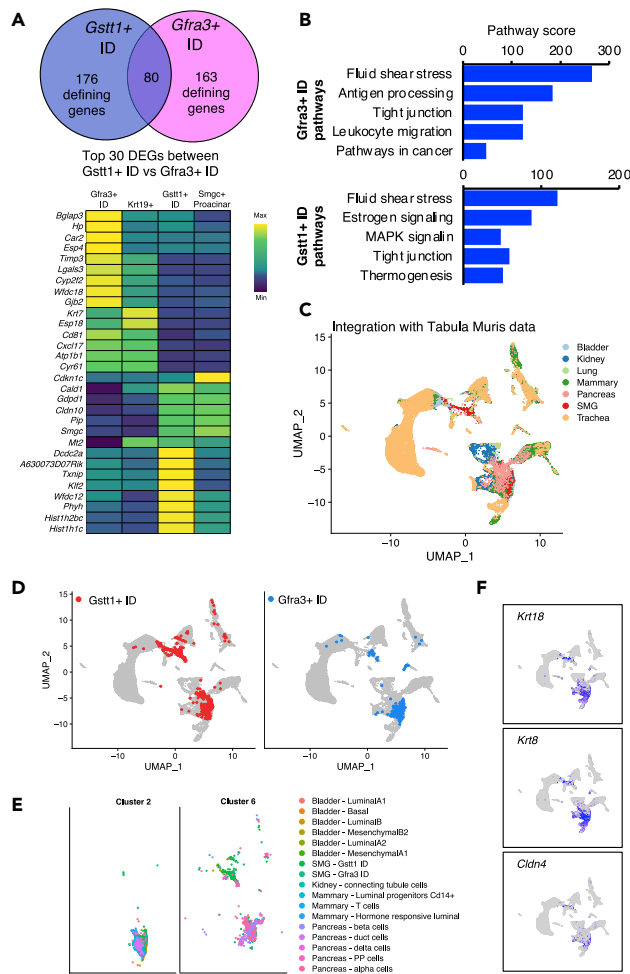
Figure 9. Subpopulations of SMG ID Are Defined by Expression of *Smgc, Gstt1,* and *Gfra3*

(A) Venn diagram of the comparison between defining genes for *Gstt1+* and *Gfra3+* ID cells. Top 15 defining genes for each population are shown in the heatmap.

(B) Pathway analysis of defining genes from ID populations.

(C–E) UMAP of SMG ID cells integrated with selected populations from the Tabula Muris colored by tissue of origin and cell type as indicated in the legend.

(F) UMAP showing expression of *Krt18, Krt8,* and *Cldn4.*

Our observations indicate that *Gstt1+* and *Gfra3+* cells define two ID subpopulations with potentially distinct functions. We compared the defining genes for both populations, and found that they share a third of their transcriptional profile (Figure 9A). Interestingly, the top defining genes for *Gfra3+* ID are also expressed by *Krt19+* ducts from P1, whereas the top defining genes for *Gstt1+* ID cells are shared by Smgc+ proacinar cells (Figure 9A, heatmap). KEGG pathway analysis showed that both populations have functions related to fluid sheer stress and tight junctions, which may be related to their duct phenotype (Figure 9B). *Gstt1+* cells were also enriched for genes associated with estrogen and MAPK signaling, whereas the top pathways in *Gfra3+* cells were antigen processing, leukocyte migration, and cancer pathways.

To gain further insight into the potential biological functions of these cell types, we combined *Gstt1+* and *Gfra3+* ID cells with the Tabula Muris scRNAseq database, which contains cells from multiple non-salivary tissues including mammary gland, lung, trachea, pancreas, bladder, and kidney (Tabula Muris, et al., 2018). We integrated and re-clustered cells with SEURAT to identify transcriptional similarities between salivary gland ID and other cell types. Salivary ID cells were in two of the resulting 22 unsupervised clusters, namely, clusters 2 and 6 (Figures S8A and S8B). Cluster 2 also contained luminal cells from

bladder and mammary gland, connecting tubule cells from the kidney, and duct pancreatic cells, according to the original annotations from the Tabula Muris (Figures 9C–9E, S8C, and S8D), whereas cluster 6 contained alpha, beta, delta, and polypeptide pancreatic (PP) cells; mammary T cells; and mesenchymal bladder cells. Evaluation of the defining genes for clusters 2 and 6 revealed variation in gene expression in subsets of cells within these clusters (Figure S8E), likely reflecting tissue-specific differences. Nonetheless, we identified a small group of genes that were conserved across most cells from clusters 2 and 6, including *Krt8, Krt18, Epcam*, and *Cldn4* (Figures 9F and S8E). In addition, *Wfdc2, Krt7, Foxq1, Sfn, Krt19*, and a riken gene (1600029D21Rik) were widely expressed across cells in cluster 2 (Figure S8E). Given that most cell types in cluster 2 are luminal or ductal in nature, it is possible that these markers are involved in duct or luminal specification across tissues. Future investigation using genetic approaches will be needed to determine whether these markers are involved in specification of *Gstt1+* and *Gfra3+* ID cells in salivary glands and to determine whether ID cells share functional similarities with luminal and duct cells in other tissues.

Cell type annotations in the P30 and adult SMG were updated based on our findings and are provided along with their defining genes as supplementary material (Data S6). Ready-to-use SEURAT files are available from https://figshare.com/s/01778d0ed37fabf61b8e (embryonic SMG integrated) and https://figshare.com/s/134e9898ef8a20ff5c68 (postnatal SMG integrated).

DISCUSSION

A Resource for Development and Salivary Gland Research

Although previous scRNAseq studies in salivary glands have started to uncover heterogeneity at defined timepoints (Sekiguchi, et al., 2020; Oyelakin, et al., 2019; Song, et al., 2018), a major advance of our scRNA-seq resource is the inclusion of discrete developmental landmarks that will inform future studies on the mechanisms that regulate the development of branching organs, particularly salivary glands. These data add to our previous microarray analysis of SMG development and are available in the Salivary Gland Molecular Anatomy Project (SGMAP, https://sgmap.nidcr.nih.gov). The SGMAP includes microarray and bulk RNAseq of salivary gland from mice and humans. Incorporation of this resource and other available scRNA-seq datasets from parotid gland and salivary tumors (Praktiknjo, et al., 2020) to the SGMAP will allow for more comprehensive analyses available to the scientific community.

One caveat of our atlas is that stromal and neuronal cell populations were not well-represented in the adult gland, despite attempts to isolate these cells using multiple dissociation techniques and digestion enzymes. This limitation has been reported in other scRNAseq studies and is potentially due to the morphological complexity and abundance of extracellular matrix surrounding these cell types. One way to circumvent this issue is through single nuclei isolation to recover populations hard to dissociate (Nguyen, et al., 2019), which may provide a complementary technique for future studies seeking to build upon the SGMAP. Nonetheless, the strengths of this resource are highlighted through the identification of cell-defining genes and heterogeneity in the SMG epithelium throughout development, and we further demonstrate its potential applications by identifying TFs (*Ybx1, Eno1, Sox11 and Atf4*) and cell populations potentially involved in acinar cell differentiation. We predict a lineage relationship between *Bpifa2+* proacinar cells and seromucous acinar cells, and we characterized two distinct subpopulations of ID cells: a *Kit+* population defined by *Gfra3* and a proacinar-derived subset characterized by *Gstt1* with sexually dimorphic *Smgc* co-expression in females and high *Serpinb11* co-expression in males.

SMG scRNAseq Atlas Will Inform Regenerative Approaches

Irreversible loss of acinar cells occurs during treatment of head and neck cancer with radiation and as a consequence of autoimmune diseases, such as Sjögren syndrome (Jensen, et al., 2019). This leads to hyposalivation, dry mouth, and decreased quality of life with only palliative therapies available (Chibly, et al., 2014). As a result, a major goal in the field is to identify cell populations and cell-fate-determinant factors that drive specification and development of acinar cells to design effective regenerative therapies.

Mist1 is a well-known marker of acinar differentiation necessary and sufficient to induce and maintain secretory cell architecture of the stomach, pancreas, and salivary glands (Lo, et al., 2017). Consistent with previous reports, our dataset revealed the presence of a seromucous population of acinar cells with high

expression of the Mist1 gene *Bhlha15,* and other secretory markers including *Car6, Lpo,* and *Prol1.* This population was transcriptionally similar to *Bpifa2+* proacinar cells that appeared at E16, suggesting a potential lineage relationship. *Bpifa2+* proacinar cells persisted at P1, and they have also been identified at P5 (Nelson, et al., 2013). Bpifa2 was absent at P30, similar to a report showing lack of Bpifa2 staining in P20 SMG (Nelson, et al., 2013). These finding may suggest that seromucous differentiation from *Bpifa2+* proacinar cells occurs between P5 and P20. Interestingly, a serous-like population characterized by expression of *Bpifa2* and *Dcpp* genes reappeared in adult SMG. A similar cluster was also described in a single-cell RNA study of salivary gland cancer (Praktiknjo, et al., 2020).

Lineage tracing of *Bhlha15* (Mist1) demonstrated the ability of salivary acinar cells to undergo self-renewal with little or no input from progenitors (Aure, et al., 2015); however, *Bhlha15* KO mice are still able to produce acinar cells, indicating that additional factors are required for acinar specification (Lo, et al., 2017; Huh, et al., 2010; Pin, et al., 2001). Our analysis revealed TFs that become upregulated at the onset of acinar differentiation and correlate with *Bhlha15* expression, including *Spdef, Xbp1,* and *Etv1.* Interestingly, *Spdef* is required for terminal differentiation of mucous secreting cells in lungs and the intestinal tract (Horst, et al., 2010; Chen, et al., 2009), and *Xbp1* is upstream of Mist1 and is associated with the unfolded protein response, which is prominent in secretory cells (Hess et al., 2016). In addition to these genes, we observed that acinar differentiation was characterized by a gradual downregulation of numerous TFs, including *Ybx1, Eno1, Atf3, Jun,* and *Atf4.* This is consistent with a study showing that *Atf3* inhibits acinar specification (Fazio, et al., 2017).

After severe damage, *Krt5+*-expressing cells have shown limited ability to repopulate acinar cells (Weng, et al., 2018), but the regulatory mechanisms remain unknown. Our SMG atlas allows us to speculate about the potential factors involved in acinar cell generation from *Krt5+* basal cells, which included *Bhlha15, Etv1, Atf3,* and *Jun.* Further research into the contribution of these TFs to acinar development and regeneration post-injury is warranted.

Characterization of Salivary ID Subpopulations

It has been widely speculated that ID cells may have varying degrees of plasticity to give rise to acinar cells following injury due to their proliferative capacity, label-retaining properties, and *Kit* expression (Kwak, et al., 2016; Katsumata, et al., 2009; Man, et al., 2001). *Kit* lineage tracing failed to demonstrate contribution to acinar cells during homeostasis (Kwak, et al., 2018), whereas recent studies suggest plasticity of multiple populations after severe injury (Ninche, et al., 2020). In this regard, it is interesting that *Kit+* ID cells are defined by expression of *Gfra3,* a neurotrophic factor receptor that could potentially modulate their function upon binding to its cognate ligand artemin, which is expressed in the neurons within the gland. A precedent for the potential therapeutic effects of neurotrophic factors in injured salivary glands has already been demonstrated *in vivo* with administration of GDNF or Neurturin, which bind Gfra1 and Gfra2 receptors and improve saliva secretion post-irradiation (Ferreira, et al., 2018; Xiao, et al., 2014; Knox, et al., 2013). This is also reminiscent of acinar regeneration of SMGs *in vivo* after experimental duct ligation, which requires input from the nerves (Carpenter, et al., 2009). Whether innervation of ID plays a role in regeneration through Gfra3 remains to be explored.

Our data also allowed us to characterize an ID population defined by *Gstt1* with sexually dimorphic expression of *Smgc* in females and *Seprinb11* in males indicating that these cells have sex-dependent functions. This is supported by previous studies that report a pheromone-like function of Smgc when secreted into saliva of female mice (Isogai, et al., 2018). Whether *Gstt1+* or *Gfra3+* ID cells have the ability to repopulate acinar cells in the adult gland remains to be determined. Our atlas provides with cell-specific markers in these populations that will inform future studies.

In summary, this transcriptional atlas will benefit future studies to understand the mechanisms of SMG development, functions of specific cell populations, and lineage relationships and will enable comparisons with other tissues to identify evolutionarily conserved or unique cell types and functions. A combination of scRNAseq with lineage tracing models informed by the results in this study will be essential to answer questions about lineage and regeneration (Wagner and Klein, 2020). This atlas can be built upon by the addition of scRNAseq analysis of salivary gland disease and damage models, such as duct ligation, radiation, and partial gland extirpation, which will allow comparison of the cells that regenerate the gland after damage in each of these models.

Limitations of the Study

Due to the dissociation technique, few mesenchymal cells are represented in P30 and adult SMG, and neuronal cells were not recovered from all postnatal samples including P1, P30, and adult SMG. In addition, current methods for trajectory analysis like the one used in this study, are not yet fully optimized for complex datasets where divergent developmental processes are expected (i.e., multiple lineages) across multiple time points. Thus, results from trajectory analysis should be carefully interpreted and further validated.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Alejandro Chibly (chiblyaa@nih.gov).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The accession number for the single-cell RNAseq libraries reported in this paper is GEO: GSE150327. The code used for analysis is provided in Data S7 and also available through github: https://github.com/chiblyaa/Salivary-Gland-Development. Ready-to-use SEURAT objects are also available via figshare:

Embryonic SMG integrated dataset: https://doi.org/10.6084/m9.figshare.13157687.

Postnatal SMG integrated dataset: https://doi.org/10.6084/m9.figshare.13157726.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101838.

AUTHOR CONTRIBUTIONS

Conceptualization, methodology, writing and editing, B.R.H., M.H.A., M.P.H., and A.M.C.; Software, A.M.C., M.C.K., and GCBC; Resources, M.P.H. and A.M.C.; Visualization, B.R.H., M.H.A., and A.M.C.; Data Curation, Project Administration, and Supervision, A.M.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Generation of a Single-Cell RNAseq

Atlas of Murine Salivary Gland Development

Belinda R. Hauser, Marit H. Aure, Michael C. Kelly, Genomics and Computational Biology Core, Matthew P. Hoffman, and Alejandro M. Chibly
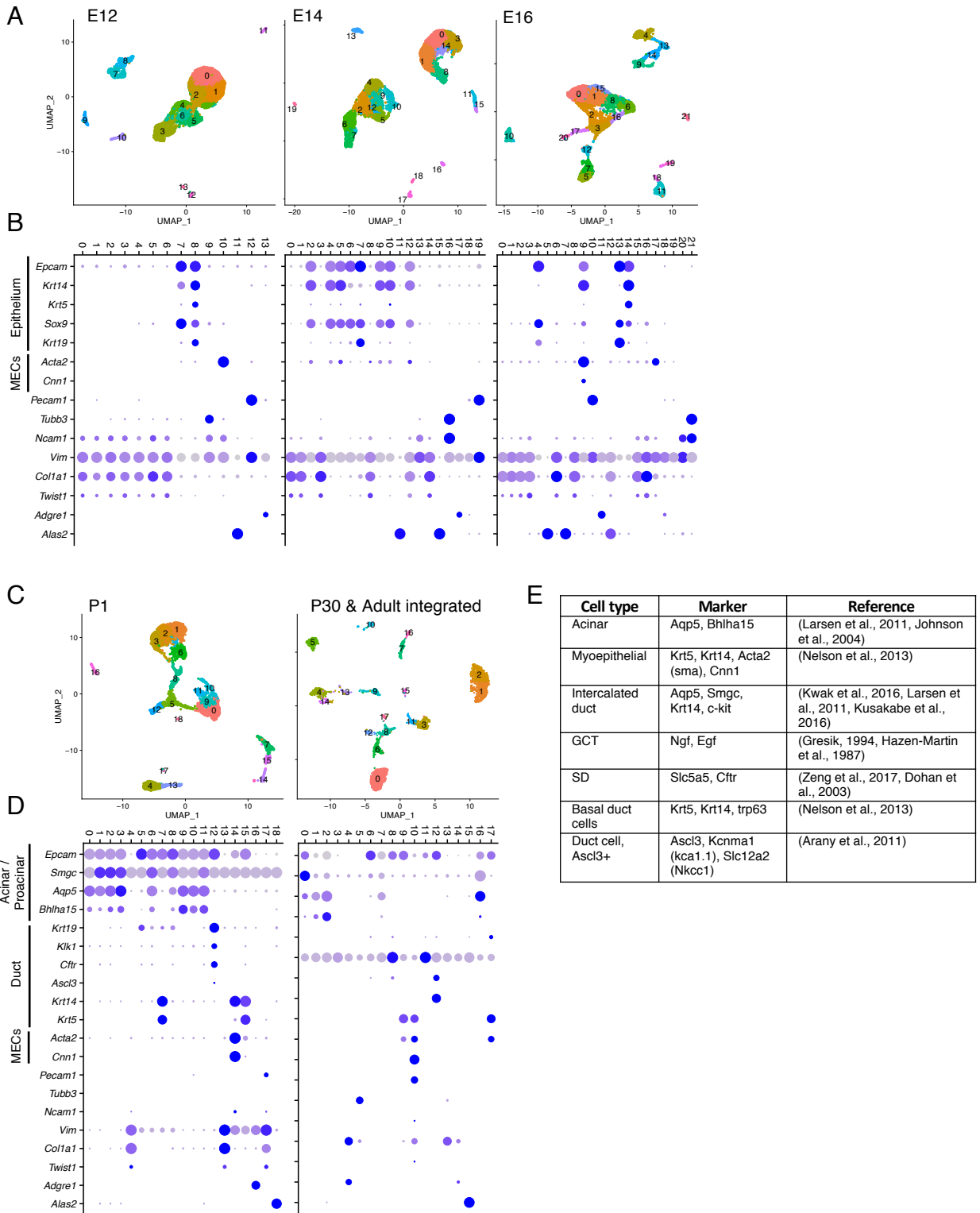
Supplementary Figure 2. Annotation strategy.
Cell clustering was performed for individual developmental stages to annotate cell populations based on known cell markers prior to data integration.
A) UMAPs showing unsupervised clustering of scRNAseq from embryonic SMG.
B) Dot plot showing expression of markers used for annotation of embryonic SMG (MECs = myoepithelial cells).
C) UMAPs showing unsupervised clustering of scRNAseq from postnatal SMG.
D) Dot plot showing expression of markers used for annotation of postnatal SMG
E) Table with references to known epithelial markers for specific cell types in adult salivary glands.
Non-epithelial markers: *Pecam1*: Endothelial; *Tubb3*: Nerves; *Ncam1*: Nerves and Glial cells; *Vim, Col1a1 & Twist1*: Mesenchymal and stromal cells;
Adgre1: Macrophages; *Alas2*:Erythroid; *Acta2*: Myoepithelial and smooth muscle cells.
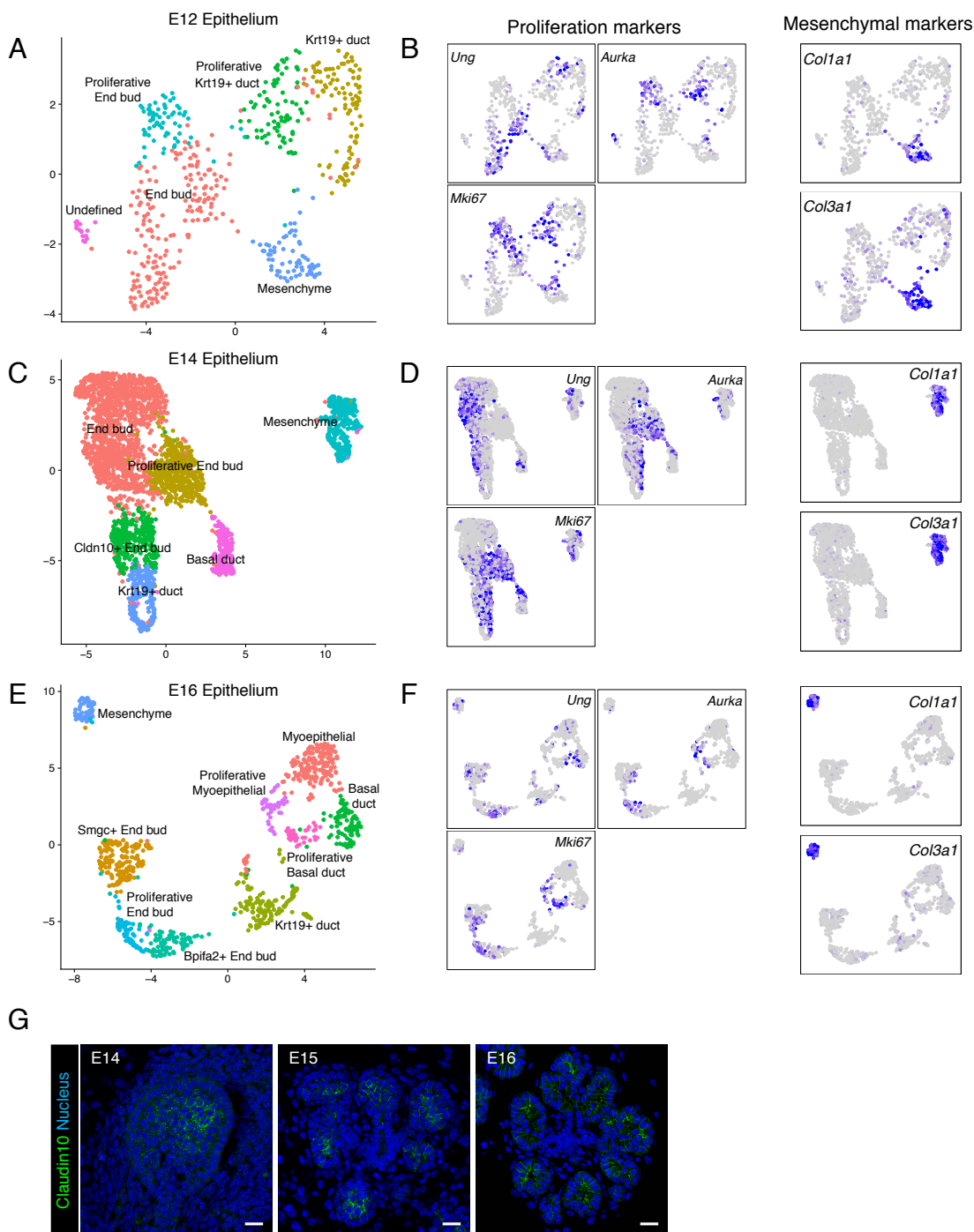
Supplementary Figure 3: Proliferative epithelial subpopulations in embryonic SMG
A) Annotated subpopulations in E12 SMG epithelium.
B) UMAP showing subpopulations of proliferative and mesenchymal markers in E12 SMG epithelial cells.
C) Annotated subpopulations in E14 SMG epithelium.
D) UMAP showing subpopulations of proliferative and mesenchymal markers in E14 SMG epithelial cells.
E) Annotated subpopulations in E16 SMG epithelium.
F) UMAP showing subpopulations of proliferative and mesenchymal markers in E16 SMG epithelial cells.
G) Immunostaining for claudin 10 (green) in embryonic SMG
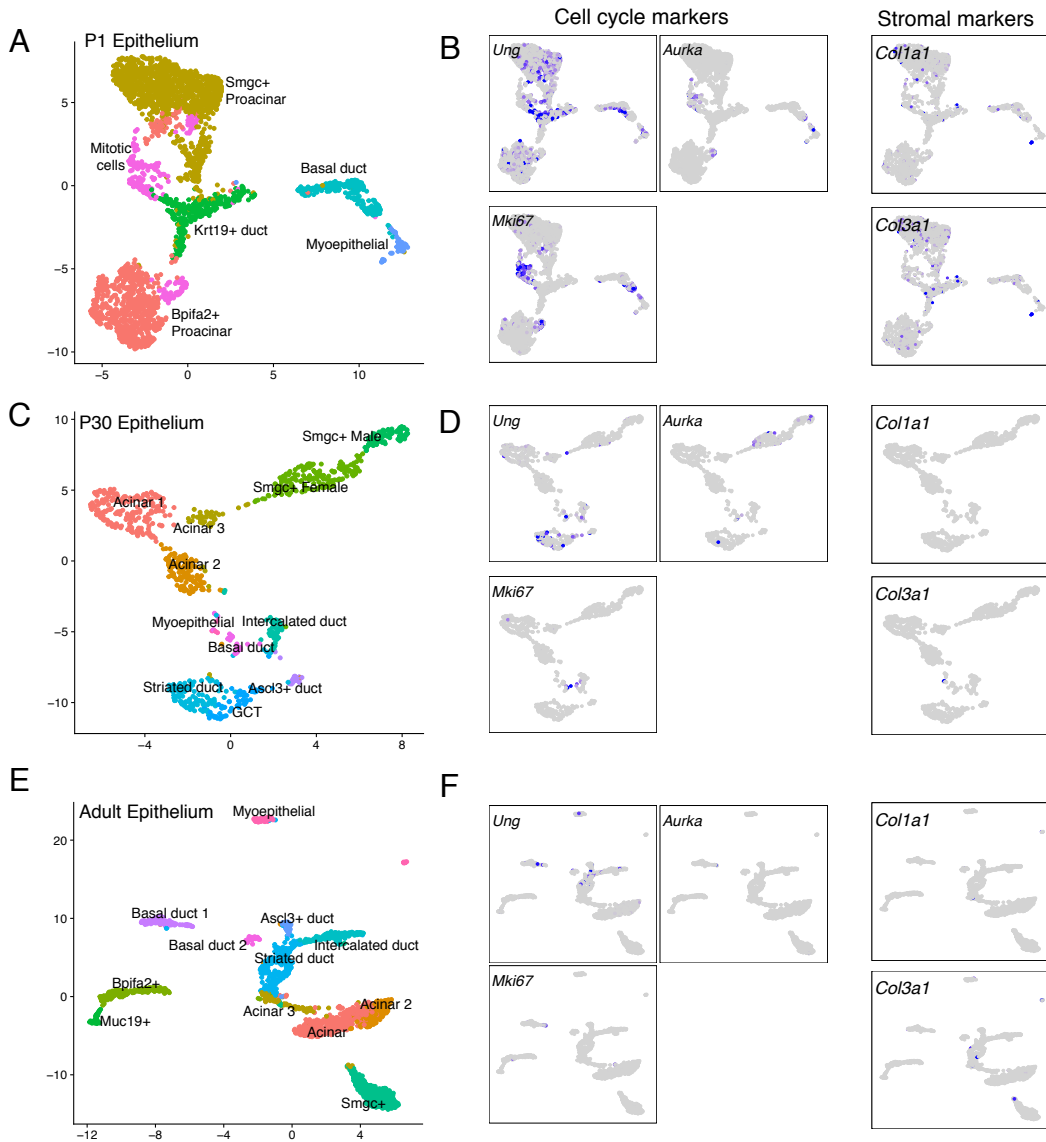
Supplementary Figure 3: Proliferative epithelial subpopulations in postnatal SMG
A)   Annotated subpopulations in P1 SMG epithelium.
B)   UMAP showing subpopulations of proliferative and stromal markers in P1SMG epithelial cells.
C)   Annotated subpopulations in P30 SMG epithelium.
D)   UMAP showing subpopulations of proliferative and stromal markers in 30 SMG epithelial cells.
E)   Annotated subpopulations in Adult SMG epithelium.
F)   UMAP showing subpopulations of proliferative and stromal markers in Adult SMG epithelial cells.

Krt14

Kit
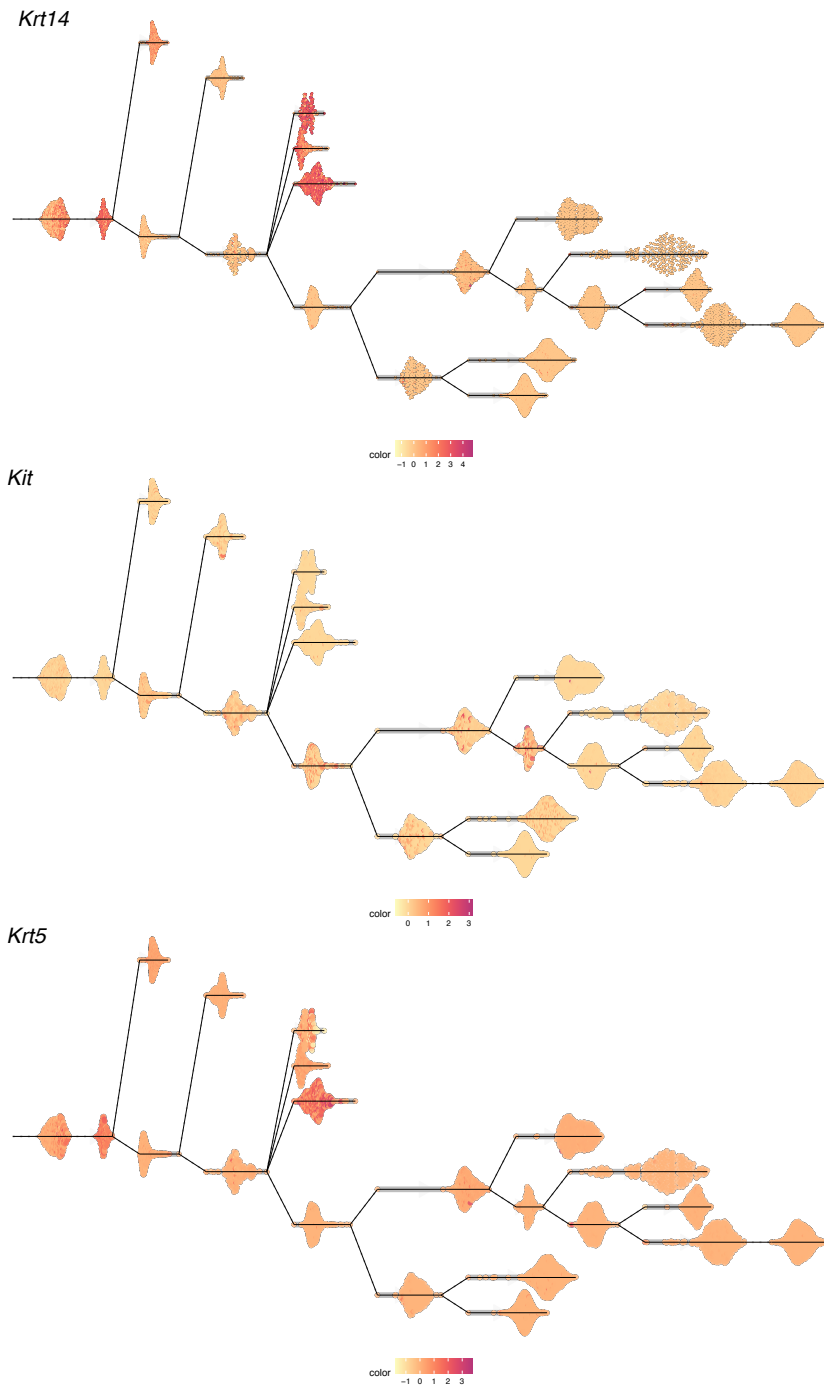
Krt5

Supplementary Figure S4. Expression of Krt14, Kit, and Krt5 along pseudotime trajectory. Intensity of color is relative to the scaled gene expression across all cells in the integrated dataset

A

E12 to E14 end bud

Cebpb *Klf6* *Fos* *Klf2* *Jund*

Id2 *Sox11* *Hmga2* *Barx2* *Id3*

E14 to E16 end bud

Creb3l1 *Ehf* *Spdef* *Nr4a1* *Xbp1*

Meis2 *Hmga2* *Sox4* *Sox11* *Eno1*

B

E16 End bud to Bpifa2+ Proacinar

Hes1 *Jund* *Etv1* *Zbtb20* *Rfxap*

Pcbp2 *Sfpq* *Hnrnpk* *Cebpb* *Ybx1*

- E12
- E14
- E16
- P1
- P30
- Adult

E16 End bud to Smgc+ Proacinar

Jund *Atf3* *Jun* *Arid5b* *Hopx*

Barx2 *Sfpq* *Hnrnpk* *Cebpb* *Ybx1*

C

Smgc+ Proacinar
Bpifa2+ Proacinar
Acinar

Min *Max*

Percent
- 25
- 50
- 75
- 100

2310057J18Rik / Car6 / Ssgb2b26 / Muc1 / Ssgb1b27 / Ssgb2b27 / Prol1 / Muc2 / Wfdc12 / Gm5886 / Lpo / Kcnn4 / Agt / Pip / Bglap3 / Clrt1 / Muc5 / Agp5 / Grn4439 / Smox4 / Etv1 / Tcn2 / Gjb1 / Dleft3 / P4hb / Esp8

Figure S6: Differential expression analysis of end bud populations during early embryonic development

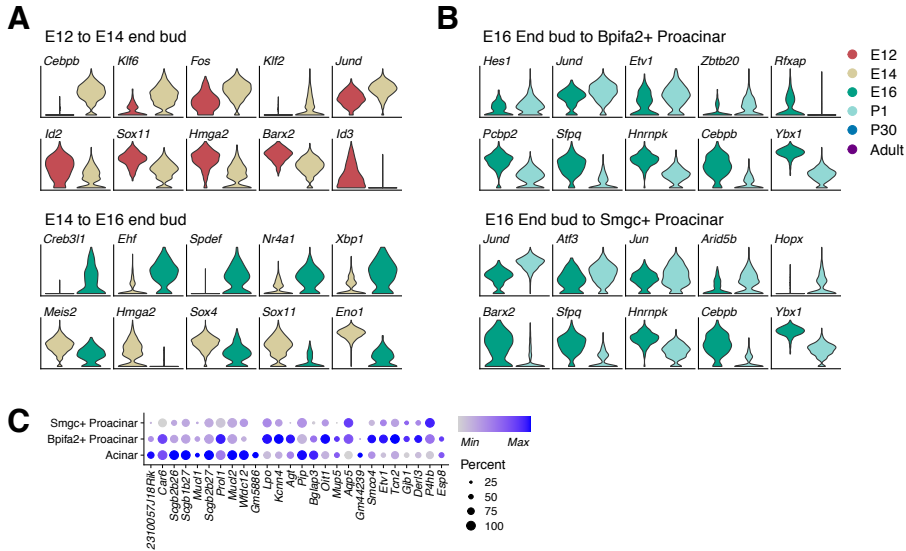A)   Top transcription factors (TFs) differentially expressed in end buds from E12 to E14 (top panel), and E14 to E16 (bottom panel).

B)   Top TFs differentially expressed in the transition from end buds at E16 to proacinar cells at P1.

C)   Expression levels of the 26 defining genes for seromucous acinar cells from P30 SMG across acinar and proacinar cells. Gene list is associated with the Venn Diagram in Figure 6F. The size of the dot is relative to the percentage of cells expressing a gene, and the color scale is relative to the level of expression.
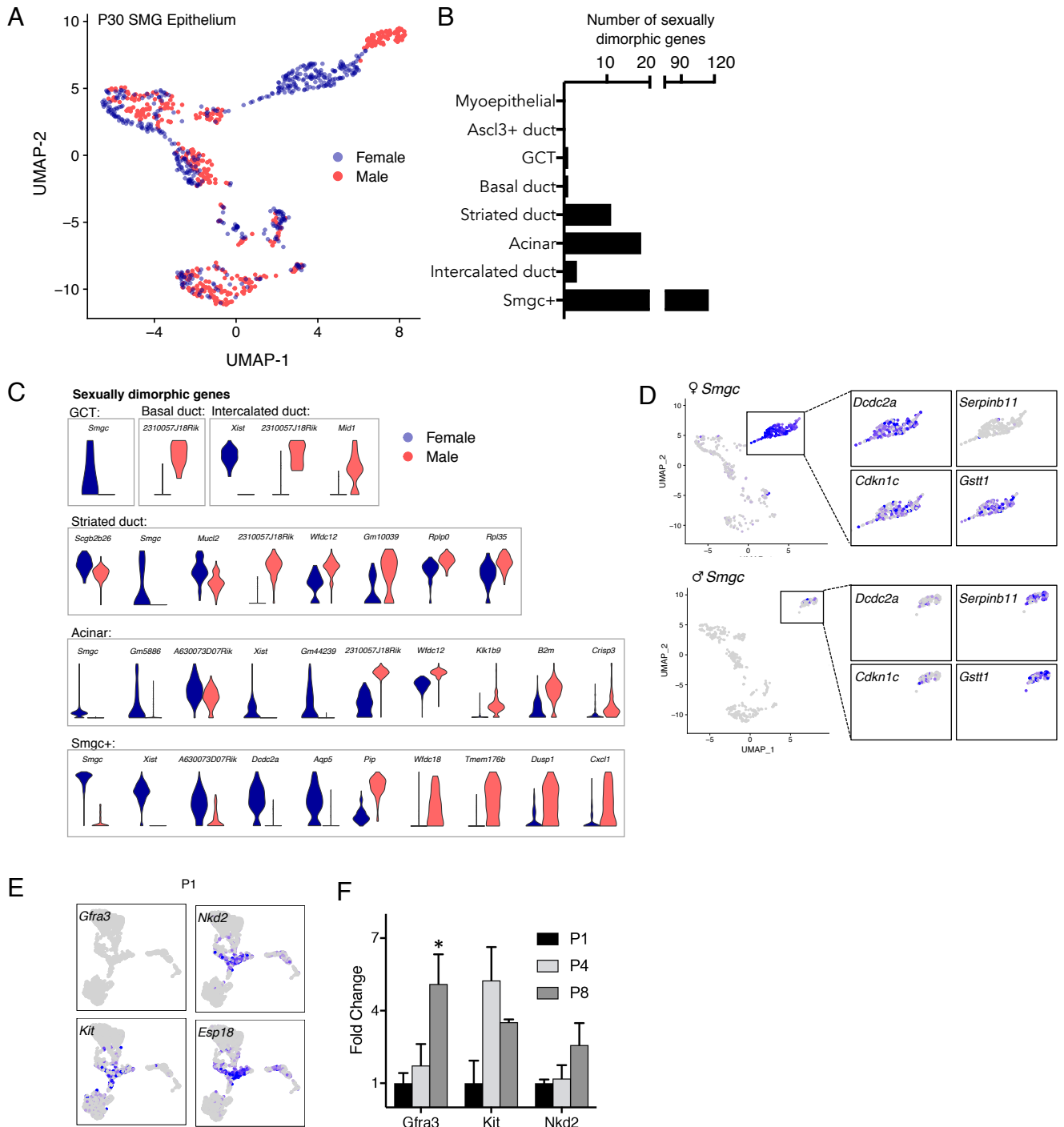
Figure S4: Sexually dimorphic genes in P30 SMG

A) UMAP of P30 SMG separated by sex.

B) Graph showing number of differentially expressed genes (DEGs) identified per cell type in female vs males.

C) Violin plots showing top 10 sexually dimorphic genes identified per cell type. All genes are shown for groups with less than 10 DEGs.

D) Representative female and male-specific genes in Smgc+ cells, as well as *Gstt1* which is common for both.

E) UMAP of expression of Gfra3+ID defining genes in P1 SMG

F) PCR results of *Gfra3, Kit,* and *Nkd2* expression in postnatal SMG normalized to P1 glands. Data are shown as the mean +/- SEM and asterisks represent statistical significance (p<0.05) compared to P1 SMG (n=3, two tailed t-test).
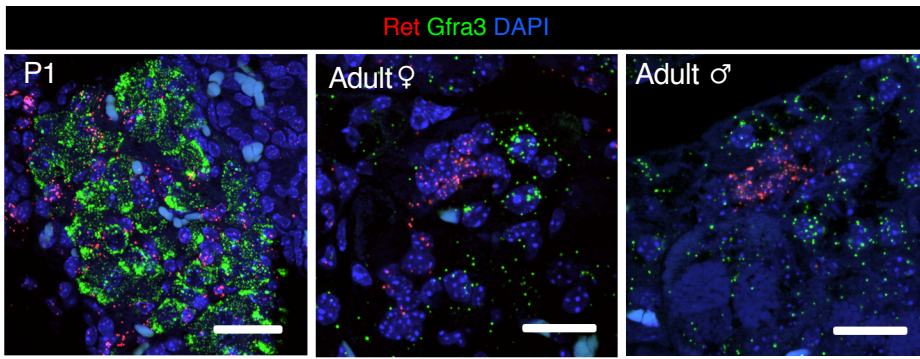
Figure S7
In situ hybridization of *Ret* (red) and *Gfra3* (green). At P1, these genes are co-expressed in ganglia. In the adult gland both male and females express *Gfra3* in ID, while *Ret* is found in acinar cells. All scale bars = 20μm.

A

B

Cluster #	Gfra3 ID cells	Gstt1 ID cells
0	3	23
2	137	184
3	0	3
5	0	2
6	42	391
7	2	1
8	1	2
9	0	2
10	2	0
16	8	0
17	0	1

C

Subset clusters 2 & 6

- Cluster 2
- Cluster 6

D

Original annotation from Tabula Muris	Tissue	Cluster 2 (number of cells)
LuminalB	Bladder	372
LuminalA1	Bladder	262
Basal	Bladder	256
Luminal epithelial progenitors Cd14+	Mammary	214
LuminalA2	Bladder	210
Gstt1 ID	SMG	184
Hormone responsive luminal epithelial cells	Mammary	175
connecting tubule cells	Kidney	169
Gfra3 ID	SMG	137
duct cells	Pancreas	112

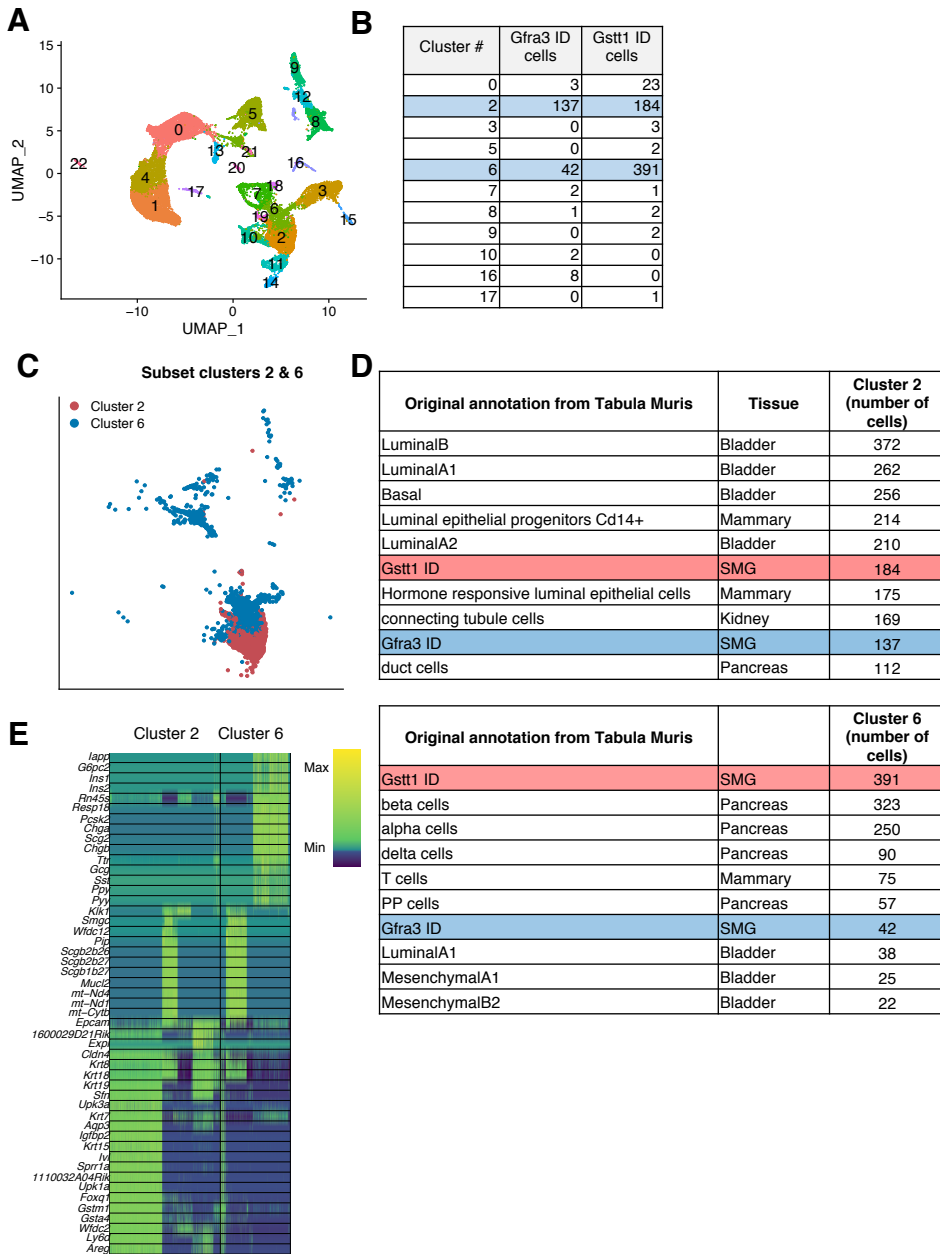| Original annotation from Tabula Muris		Cluster 6 (number of cells)
Gstt1 ID	SMG	391
beta cells	Pancreas	323
alpha cells	Pancreas	250
delta cells	Pancreas	90
T cells	Mammary	75
PP cells	Pancreas	57
Gfra3 ID	SMG	42
LuminalA1	Bladder	38
MesenchymalA1	Bladder	25
MesenchymalB2	Bladder	22

E

Figure S8. Integration of ID cells with Tabula Muris data.
A)   UMAP of unsupervised clustering after integration of ID cells with selected datasets from the Tabula Muris
B)   Table showing cluster localization of Gstt1+ and Gfra3+ ID cells. Clusters 2 and 6 contain most ID cells.
C)   UMAP of clusters 2 and 6
D)   Table showing the 10 most abundant cell types in clusters 2 and 6 based on original annotations from Tabula Muris
E)   Heatmap showing expression of the top 25 genes expressed by clusters 2 and 6 compared to all other clusters

TRANSPARENT METHODS

ICR mice

Timed-pregnant ICR females were purchased from Envigo at gestational day E9. From this point onwards mice were cared for and maintained at the NIDCR Veterinary Resource Core in accordance with institutional and IACUC guidelines. All mice were fed ad libitum and kept under 12-hour light/12-hour dark cycle. Pregnant females used for collection of mouse embryos were housed in pairs while those required for collection of P1 pups were individually housed to prevent overcrowding. Mouse embryos were collected from ICR pregnant females at embryonic days E12, E14, and E16, and newborn pups were collected at P1. Additional 30-day old (P30) male and female ICR mice were purchased from Envigo for experimentation.

C3H mice

Adult (10-month old) C3H female mice were obtained from Jay Chiorini's laboratory at NIDCR. Prior to experimentation, these mice were also housed and kept at the NIDCR Veterinary Resource Core in accordance with IACUC guidelines. These mice were used for experimentation upon receipt and were not maintained by our laboratory.

Single-cell Dissociation. SMGs from seven E12 and E14 littermate embryos were separated, isolated and incubated at 37°C for 30 min with 500ul of papain (20U/ml in 1mM L-cysteine with 0.5mM EDTA (Worthington biochemical, New Jersey, USA) in 200ul Eppendorf tubes. Glands were dissociated with a pipette in 5-minute intervals. After 30 min, Papain was inactivated with (1:1) albumin-inhibitor solution (Worthington biochemical, New Jersey, USA) and the cell suspension was collected by centrifugation at 400g at 4°C for 10 min, washed twice and resuspended in Hanks balanced salt solution (HBSS 1x, Gibco, USA) with 1% Bovine serum albumin solution (BSA, Sigma-Aldrich).

SMGs from 2-4 E16 embryos and P1 mice were isolated and enzymatically dissociated in 5ml of digestion cocktail containing Collagenase II (100mg/ml), Hyaluronidase (50mg/ml), deoxyribonuclease (40mg/ml), and neutral protease (50mg/ml) in HBSS-BSA. Dissociation was performed in a 15ml gentleMACS C tube using a miltenyi gentleMACS Octo Dissociator following their preset dissociation program A (24 seconds of clockwise-counterclockwise spin cycles) followed by 30 min incubation at 37°C. Dissociated cells were centrifuged (400xg) for 10 min and resuspended in HBSSA-BSA. Then, cells were passed through 100 μm, 40 μm, and 20 μm filters centrifuging (400xg) for 5 min and resuspending in HBSS-BSA after each filtration step. The final cell suspension was centrifuged (400xg) 5 min and treated with Papain as described above.

For P30 ICR and adult C3H mice, glands were dissociated in a 15ml gentleMACS C tube with 5ml of digestion cocktail prepared with the human tumor dissociation kit (#130-095-929, Miltenyi Biotech, Auburn CA) in RPMI 1640 w/L-Glutamine (Cell applications, Inc, USA). 2 SMGs from male and female ICR mice, and 2 SMG from adult female C3H mice were processed individually. Similar to previous dissociations, digestion was performed in a Miltenyi gentleMACS Octo Dissociator but following the preset 37C_h_TDK_2 program. We added 5ml of RPMI media to the dissociated cells and centrifuged (400xg) for 10 min. Cells were resuspended in RPMI 1640 w/L-Glutamine with 5% PenStrep (Gibco, USA) and washed twice with RPMI. Next, cells were centrifuged (400g) for 5 min and washed with RPMI medium before each filtration step through 70 μm and 40 μm filters. Apart from the enzymatic digestion, all

steps were performed on ice. Single-cell dissociation was confirmed by microscopic examination and cell concentration determined with a Cellometer (Nexcelom Biosciences). Cell concentration was adjusted to $5 \times 10^5 - 1 \times 10^6$ cells/ml prior to analysis with a 10X genomics Next GEM Chromium controller.

Library prep and sequencing: Single-cell RNA-seq library preparation was performed at the NIDCR Genomics and Computational Biology Core using a Chromium Single Cell v3 method (10X Genomics) following the manufacturer's protocol. Pooled single-cell RNA-seq libraries were sequenced on a NextSeq500 sequencer (Illumina). Cell Ranger Single-Cell Software Suite (10X Genomics) was used for demultiplexing, barcode assignment, and unique molecular identifier (UMI) quantification using the mm10 reference genome (Genome Reference Consortium Mouse Build 38) for read alignment.

Computational analysis: Cell Ranger files were imported to SEURAT v3 using R & R Studio software and processed for clustering following their default pipeline. As a quality control measure, cells with fewer than 200 genes were not included in subsequent analyses, and those with >5% of UMIs mapping to mitochondrial genes were defined as non-viable or apoptotic and were also excluded. A threshold of >10% was used for P30 and adult glands because inherently higher percentage of mitochondrial genes was detected in specific duct populations, and the 5% threshold would therefore filter out these specific cells disproportionately. A similar approach has been recommended for human tissues because a threshold of 5% fails to accurately discriminate between low-quality and healthy cells in ~30% of the evaluated tissues (Osorio and Cai, 2020). Normalization and scaling were performed following SEURAT's default pipeline. All developmental stages were processed individually except for P30 and adult glands, which were integrated prior to assigning cell annotations. The optimal number of PCs for clustering was determined for each individual file using the 'ElbowPlot' function. Cell type labels were assigned based on the expression of known cell type markers summarized in supplementary figure S1. After cell type annotations, data integration with SEURAT was performed for embryonic stages and postnatal stages separately to generate the UMAPs in Figure 1 for visualization only. Data integration of all datasets was used for trajectory inference analysis (Figure 5) with Dynverse (Saelens et al., 2019) and PAGA (Wolf et al., 2019) packages, and for differential expression across developmental stages with SEURAT (Figures 6-7).

Computational separation and analysis of epithelial clusters: Epithelial populations were computationally separated from the previously annotated datasets containing cell type labels using SEURAT's subset function. Epithelial subsets from each stage were re-normalized and scaled to generate new SEURAT objects. Cluster-defining genes were determined using the 'FindAllMarkers' function for unsupervised epithelial clusters.

Trajectory inference analysis: Data integration was performed for all annotated epithelial subsets with SEURAT. This function automatically determined the 2000 most variable genes used as anchors for the analysis. The resulting gene expression matrix and the genes used for integration were then used as input for trajectory analysis with Dynverse. Based on Dynverse guidelines, trajectory inference analysis was performed with the Partition-based graph abstraction (PAGA) algorithm, which determines a pseudotime score and trajectory topology starting from a selected cell population. Because E12 salivary progenitors give rise to the entire salivary epithelium in the adult, E12 epithelial cells were manually selected as the 'root' of the inferred trajectory.

Bhlha15-correlation analysis: End bud, proacinar, and acinar populations were separated from the integrated SEURAT file to extract a gene expression matrix for these populations. Correlation to Bhlha15 gene expression was performed using the 'cor.test' function in R. The resulting correlation scores and p-values are provided in Supplementary File 3 and the top 10 positive and negative correlations were plotted using the 'corrplot' package.

Analysis of developmental transitions: Differential expression between specific populations from the integrated file containing epithelial cells from all stages was performed using the SEURAT 'FindMarkers' function. In order to identify transcription factors, we cross-referenced the resuling differentially expressed genes agains a database of mouse transcription factors (Schmeier *et al*, 2017).Only the top 5 transcription factors (if present) are shown in violin plots using an adjusted p value <0.05 as a measure of significance. The complete list of identified markers is provided in Supplementary File 4.

Immunohistochemistry: Isolated glands were fixed in 4% paraformaldehyde overnight at 4°C. The tissue was dehydrated with gradient ethanol (2 hours for each dehydration step) and placed in 70% Ethanol until the day of standard paraffin embedding. 5μm sections were deparaffinized with xylene substitute for 10 minutes and rehydrated with reverse ethanol gradient for 5 minutes each. Tissue sections were washed twice in each eathanol concentration (90, 70, and 50%) and then placed in distilled water for 10 min. Following rehydration, heat induced antigen retrieval was done in a pressure cooker for 20 min using a pH 9.0 Tris-EDTA homemade prepared with Tris Base (1.21g), ETDA (0.37g) in 100mL of distilled waster. Sections were washed for 5 minutes with homemade 0.1% PBS-Tween (PBST) using 1XPBS (Quality Biologiocal, Gaithersburg, MD) and Tween20 (Quality Biological, Inc). Non-specific binding sites were blocked using the M.O.M.® (Mouse on Mouse) Immunodetection Kit (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature followed by overnight incubation at 4°C with primary antibodies. Tissue sections were washed 3 times for 5 minutes each with PBST and inclubated in secondary antibodies at room temperature for 45 min. Nuclear staining was done with Hoechst (Thermo Fisher Scientific, Marietta, OH) and coverslips were mounted with Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA). A complete list of antibodies used and their respective dilutions is shown in the Key Resource Table associated with this manuscript.

Wholemount staining: SMGs from E12, E14, and E16 mouse embryos were dissected and fixed with 1:1 Acetone-Methanol at -20°C for 10 minutes. After fixation, glands were washed with 1XPBS and blocked with M.O.M kit for an hour at room temperature. Incubation in primary antibody was performed overnight at 4°C followed by a 1-hour incubation in secondary antibody at room temperature. Glands were mounted on standard microscopy glass slides with Fluoro Gel mounting media. A complete list of antibodies used and their respective dilutions is shown in the Key Resource Table associated with this manuscript.

qRT-PCR: Gene expression of selected markers was measured by qRT-PCR. DNase-free RNA was isolated from whole gland SMG lysates from P20, P30, and P90 male and female ICR mice using the RNAqueous-4PCR kit and DNase removal reagent (Ambion, Inc. Austin, TX). cDNA (20ng) was generated and analyzed by qPCR using SuperScript™ III First-Strand Synthesis System (ThermoFisher Scientific). Melt curve analysis was used to verify the generation of a single amplicon. Expression levels were normalized by the delta-delta Ct method to the housekeeping gene *Rsp29* and aged-matched female glands. Four biological replicates for each group were processed in duplicates except when otherwise specified. Primers were designed using Beacon Designer software and sequences are available in Supplementary File 8.

RNAprobes: Adult p30 and p1, freshly dissected tissue and collected in 200ul eppedorf tubewas and then washed in 1x PBS RNAse free solution. All tools were cleaned with 70% ethanol and wiped before use. All tissue must be preserved in RNAse free solution. Freshly prepared tissue was placed in 4% PFA for no longer 36hrs and sent to ACD with for RNA in situ hybridization with respective probes. Sample were also accompanied by dehydration pockets to remove mosture from prepared slides and slide were immediately placed in 4°C prior to imaging. Specific probe sequences are proprietary and generated with RNAscope® technology by Advanced Cell Diagnostics.

Quantification and statistical analysis

For computational analyses with SEURAT, all statistics were performed using their pipeline's default statistical test, which is based on non-parameteric Wilcoxon rank sum test. Adjusted p-values of <0.05 were chosen as a measure of significance. Correlation analysis was performed using the 'cor.test' function, which uses Pearson's method for significance. For statistical analysis of qRT-PCR data, log-transformed fold changes were compared between male glands and age-mathched female samples using a two-tailed unpaired t-test for each gene individualy.

KEY RESOURCES TABLE

REAGENT or RESOURCE		SOURCE	IDENTIFIER
Antibodies			
AQP5	1:200	Alomone labs	AQP-005
Cytokeratin-5	1:200	Covance	PRB-160P
Cytokeratin-14	1:200	Covance	PRB-155P
GFR alpha-3/GDNF R alpha-3	1:80	R&D systems	AF2645
GSTT1	1:200	Lifespan Bioscience	LS-B10781
Epcam	1:200	Abcam	Ab71916
NKCC1	1:200	Santa Cruz Biotechnology	sc-21547
Vimetin	1:200	Abcam	Ab8978
Smgc	1:100	Lifespan Bioscience	LS-C154825
Mist1	1:200	Cell signaling	14896
Mist1	1:100	Santa Cruz Biotechnology	sc-80984
PSP/Bpifa2	1:100	Lifespan Bioscience	LS-B9833
Lpo	1:100	Thermo Fischer Scientific	PA1-46353
Claudin10	1:200	Thermo Fischer Scientific	38-8400
Alexa Fluor® 488 AffiniPure F(ab')$_2$ Fragment Donkey Anti-Goat IgG (H+L)	1:250	Jackson Immunoresearch Laboratories	705-546-147
Alexa Fluor® 647 AffiniPure F(ab')$_2$ Fragment Donkey Anti-Goat IgG (H+L)	1:250	Jackson Immunoresearch Laboratories	705-606-147
Alexa Fluor® 488 AffiniPure F(ab')$_2$ Fragment Donkey Anti-Rabbit IgG (H+L)	1:250	Jackson Immunoresearch Laboratories	711-546-152
Cy™3 AffiniPure F(ab')$_2$ Fragment Donkey Anti-Rabbit IgG (H+L)	1:250	Jackson Immunoresearch Laboratories	711-166-152
Cy™3 AffiniPure F(ab')$_2$ Fragment Donkey Anti-Mouse IgG (H+L)	1:250	Jackson Immunoresearch Laboratories	715-165-150
Hoechst 33342	1:1000	Thermo Fisher Scientific	H3570
Oligonucleotides – Sequences provided in Supplementary File 8			
Smgc		Life Technologies	N/A
Dcdc2a		Life Technologies	N/A
Serpinb11		Life Technologies	N/A
Gstt1		Life Technologies	N/A
Kit		Life Technologies	N/A
Gfra3		Life Technologies	N/A
Esp18		Life Technologies	N/A
Nkd2		Life Technologies	N/A
Rs29		Life Technologies	N/A
RNA probe for Aqp5		ACDBio	N/A
RNA probe for Esp18		ACDBio	N/A
RNA probe for Gfra3		ACDBio	N/A
RNA probe for Nkd2		ACDBio	N/A
RNA probe for Prol1		ACDBio	N/A
RNA probe for Ret		ACDBio	N/A

Chemicals, Peptides, and Recombinant Proteins		
Hanks balanced salt solution (HBSS) no calcium, no magnesium, no phenol red	Gibco/ Life technologies	14175-103
PBS 1X	Quality Biological, Inc	114-058-101
Tween 20	Quality Biological, Inc	A611-M147-13
Fluoro-Gel with TRIS mounting media	Electron Microscopy Sciences	1798510
Bovine serum albumin solution (BSA)	Sigma Aldrich	A9205
16% Paraformaldehyde (formaldehyde) Aqueous solution	Electron Microscopy Sciences	15700
Xylene substitute	Sigma Aldrich	A5597-1GAL
Ethanol 100%	Sigma Aldrich	E7023
Ethanol 95%	Sigma Aldrich	493538
Acetone	Sigma Aldrich	179973
Methanol	ThermoFisher	A412-4
Tris Base	Millipore Sigma	648310-M
EDTA	Millipore Sigma	E1161
RPMI 1640	Cell application	185-500
Neutral Protease	Worthington Biochemical Corp	LS02109
Deoxyribonuclease I	Worthington Biochemical Corp	LS002145
Hyaluronidase	Worthington Biochemical Corp	LS002594
Collagenase, Type 2	Worthington Biochemical Corp	LS00476
Critical Commercial Assays		
Human tumor dissociation kit	Miltenyi Biotech	130-095-929
M.O.M (Mouse on Mouse) Immunodetection Kit	Vector Laboratories	BMK-2202,
Superscript III First-Strand Synthesis System	Life Technologies	18080051
Papain Dissociation system	Worthington Biochemical	LK003150
Deposited Data		
scRNAseq of murine SMG at multiple developmental stages	This paper	GSE15032
Experimental Models: Organisms/Strains		
Timed-pregnant ICR Female Mice	Envigo	ICR (CD-1®)
ICR postnatal mice	Envigo	ICR (CD-1®)
C3H adult female mice	From Jay Chiorini's lab	N/A
Software and Algorithms		
R & R studio	https://rstudio.com/	N/A
Cell Ranger	10X Genomics	N/A
SEURAT	Stuart, Butler *et al* (2019)	N/A
Dynverse	Saelens *et al* (2019)	N/A
PAGA	Wolf A *et al* (2019)	N/A
Other		
MACS SMART Strainers 70*µ*m	Miltenyi Biotech	130-110-916

Supplementary references

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