Biology Open

Supplementary Material

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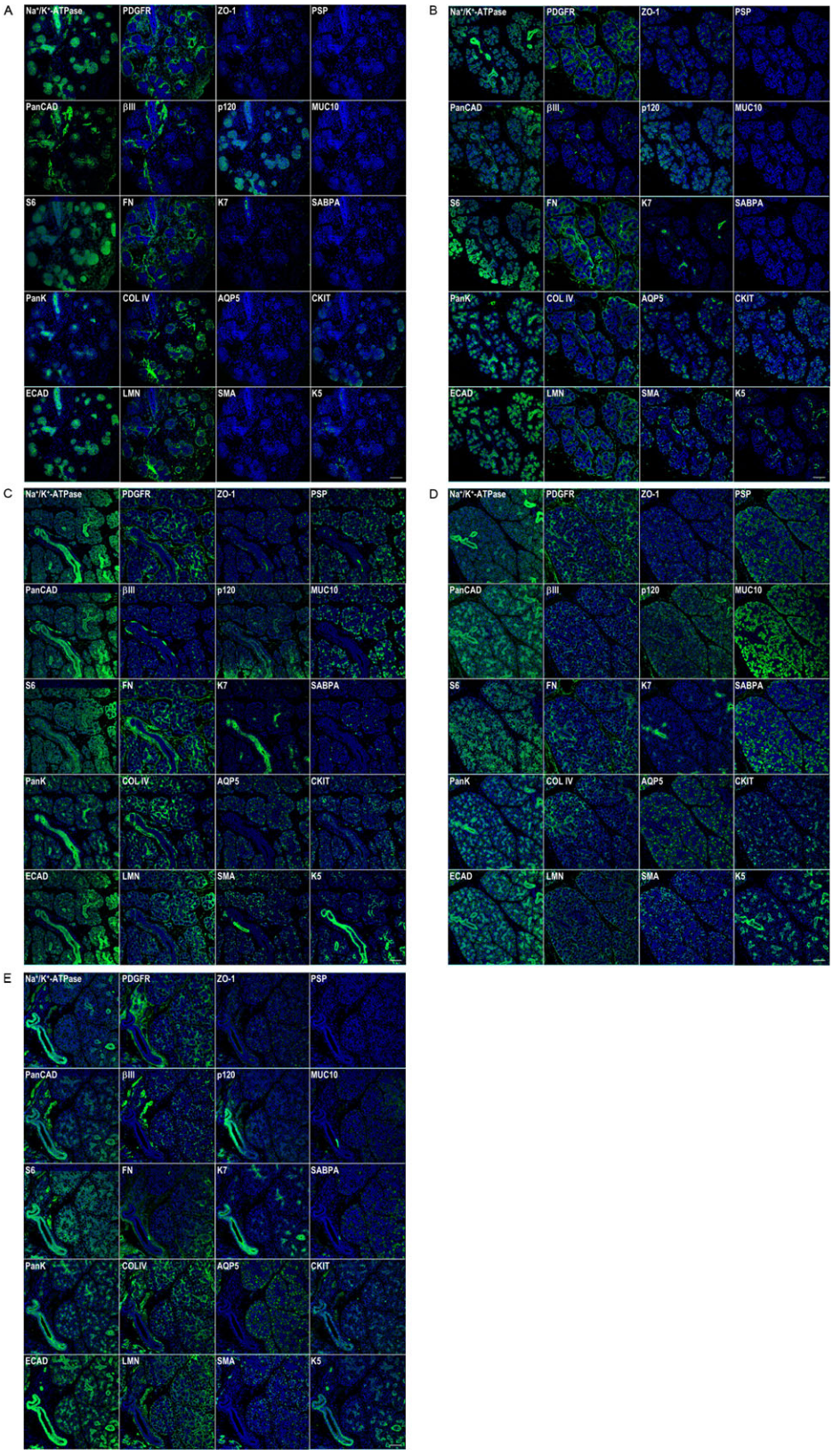


Fig. S1. Single channel images of all 20 markers at all developmental stages are shown as overlays with DAPI. Embryonic stages E14 (**A**), E16 (**B**), E18 (**C**) and postnatal stages P5 (**D**) and P20 (**E**).

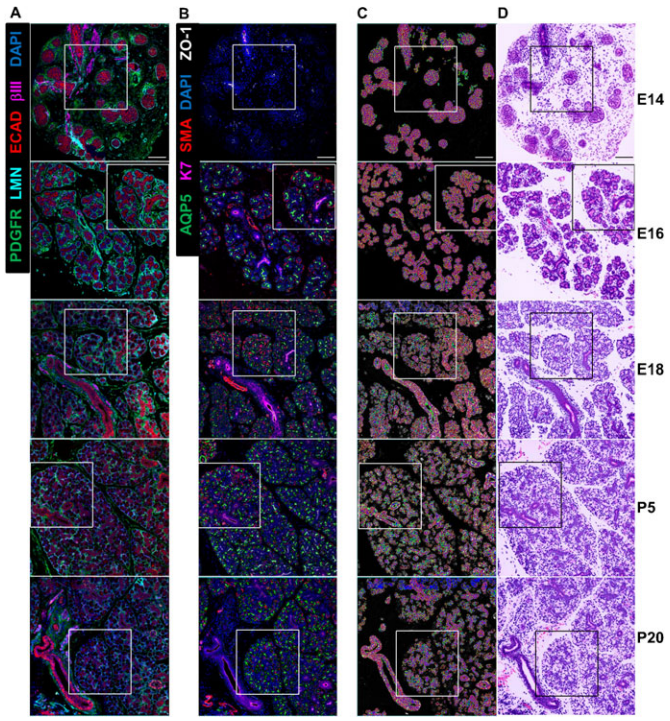
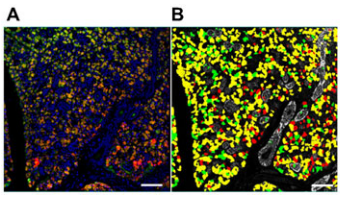


Fig. S2. MxIF analysis of mouse submandibular salivary gland morphogenesis. MxIF of a developmental TMA including embryonic stages (E14, E16, E18) and postnatal stages (P5 and P20) was performed using sequential application of directly conjugated antibodies to detect multiple markers of tissue structures and cell types on the same tissue sections. (**A**) Tissue compartments. The epithelium, mesenchyme, neurons, and basement membranes was detected using antibodies directed towards E-cadherin (ECAD, red), platelet-derived growth factor (PDGFR, green), βIII tubulin (bIII, magenta), and laminin (LMN, cyan), respectively. (**B**) Epithelial differentiation. Maturation of the proacinar, ductal, and myoepithelial cell types was detected using antibodies directed towards aquaporin 5 (AQP5, green), cytokeratin 7 (K7, magenta), and smooth muscle α-actin (SMA, red); maturation of the cell–cell adhesions was monitored using an antibody to zonula occludens-1 (ZO-1, white). DAPI was used to stain the nuclei in both A and B. (**C**,**D**) Single cell segmentation of the epithelium and comparison with simulated hematoxylin and eosin (H&E) stained images. (C) Using a developmental TMA, MxIF was used to identify markers of cell type and cell subcompartments: epithelium (E-cadherin and pan-keratins), plasma membrane (Na^+/K^+-ATPase and pan-cadherin), cytoplasm (S6), and nuclei (DAPI) (supplementary material Fig. S1). An epithelial mask was computed using the E-cadherin and pan-cytokeratin stains and used to identify cells with the algorithm that uses the cell membrane, cytoplasm, and nuclei stains to identify individual cells. Computationally segmented epithelial cell membranes are displayed in (red), cytoplasm (green), and nuclei (blue), and are displayed on top of an image of E-cadherin to identify the epithelial cells. Each cell was assigned a unique identifier (data not shown) for quantification. (D) The cell segmentation in C was used to computationally generate corresponding simulated histological H&E images, which are displayed for comparison to illustrate the tissue morphology. Scale bars: 100 µm zoom level one and 50 µm zoom level two.



C

Percent Positive	
SABPA:	38.4
MUC10:	35.9
BOTH:	29.4

Fig. S3. Mixed seromucous phenotype of mouse submandibular secretory acinar cells. (**A**) Immunostaining for SABPA (green) and MUC 10 (red) in P20 glands depicting co-expression of the serous and mucinous secretory proteins, respectively. (**B**) Statistical outputs from A were overlaid on E-cadherin-stained images (white) to produce overlays of the computationally identified SABPA- (green) and MUC 10 (red)-expressing epithelial progenitor cell populations. Cells that segregate as expressing both as SABPA and MUC 10 are shown in yellow. Note the largely overlapping expression in both A and B. (**C**) Quantification shown in B.

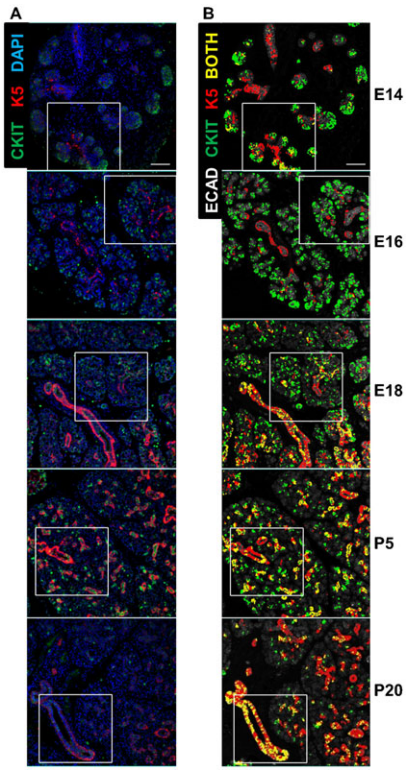


Fig. S4. Dynamic expression of epithelial progenitor cell markers during submandibular gland development. (**A**) The spatio-temporal distribution of the functional epithelial progenitor markers c-kit (CKIT, green) and keratin 5 (K5, red) are shown throughout SMG salivary gland development, with total nuclei stained with DAPI (blue) in a low and high magnification view. Inset areas are shown with white lines. (**B**) Statistical outputs from A were overlaid on E-cadherin-stained images (white) to produce overlays of the computationally identified CKIT-(green) and K5-(red) expressing epithelial progenitor cell populations. Cells that segregate as expressing both as c-kit and K5 are shown in yellow. Note the early expression of c-kit primarily in the end buds followed by progressive partitioning to the ducts, whereas the K5 expressing cells are largely ductal throughout development. Developmental stages are as indicated. Numerical data for the quantitative cell analysis is shown in Table 4. Scale bars: 100 μm.

Table S1. Order of application of directly conjugated antibodies. The imaging round is indicated by number with the protein target of the Cy3- and/or Cy5-directly conjugated antibody used in each round indicated, as applicable. No antibodies were applied in round 1. In round 2, c-kit was applied by indirect immunocytochemistry. In round 16, an Alexa^{647}-labeled antibody was generated by zenon labeling. Bleaching was performed after every step (not shown).

Imaging order	Cy3 channel	Cy5 channel
1	Background	Background
2		c-kit (indirect)
3		S6
4	E-cadherin	Na^+/K^+-ATPase
5	Keratin 5	Keratin-pan
6		Cadherin-pan
7	Keratin 7	Aquaporin 5
8	SABPA	Fibronectin
9	PSP	
10		βIII-tubulin
11	SMA	Collagen IV
12		Laminin
13		p120 catenin
14	Mucin 10	
15		ZO-1
16		PDGFR (zenon)