Supplementary Information

ROCK Inhibitor Increases Proacinar Cells in Adult Salivary Gland Organoids

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2. Materials and Methods

2.1. Mouse Strains

CD-1 (Charles River) or C57BL/6 (JAX #000664), adult (8-12 weeks old) or timed-pregnant female mice were obtained from Charles River Laboratories, and are used as indicated in the figure legend. *Mist1*^{CreERT2} mice were gifted to us by Dr. Catherine Ovitt from University of Rochester with permission from Dr. Stephen Konieczny, Purdue University. *Mist1*^{CreERT2}mice were bred to the ROSA26^{TdTomato} reporter strain (JAX #007909), and genotyped by PCR to detect Cre and TdTomato, as previously described (Le and Saur, 2000; Inoue et al, 2010; Fujiwara et al, 2016). Mice were induced with tamoxifen (Sigma cat# T5648) (1 mg/ml) injected intraperitoneal (IP) with one 5mg/kg of body weight dose delivered every other day for three days, and tissue harvested one week from the first induction. 5 mg of tamoxifen was dissolved in 450 µL of corn oil and 50 µL of 100% v/v ethanol (10 mg/ml stock) to make the 1 mg/ml stock solution. To confirm success of tamoxifen injection, RFP fluorescence of live SMG tissue was examined with an inverted fluorescent microscope (EVOS, Invitrogen). All CD-1 or C57BL/6 mice were aged 7-11 weeks prior to harvest. *Mist1*^{CreERT2}; ROSA26^{TdTomato} mice were aged 18 months. SMG from female mice were primarily used but males were used as indicated in specific experiments. All animals were maintained and handled using protocols approved by the University at Albany Institutional Animal Care and Use Committee (IACUC).

2.2. Salivary Gland Removal and Primary Epithelial Cell Cluster Isolation

E16 mouse SMGs were extracted by removing a mandible slice from the head with a scalpel and removing the glands from the slice with sterile forceps under a dissecting microscope. E16 salivary glands were processed as previously described (Hosseini et al, 2018 and 2019). For adult glands, cells were similarly digested as with embryonic tissue but with the addition of 30 µg/mL DNAse I for 10 minutes. (Stem Cell Technologies #07900). Time zero (T0)

gland preparations were prepared as follows. After digestion, 10^5 cells were mixed in a 1:1 mixture of cells in Media to Matrigel®, placed on a Nuclepore filter in a 10 µL volume, and incubated for 30 minutes at 37C. T0 were immediately fixed and prepped for ICC as described below. For organoid culture, epithelium went through an adhesion depletion stage in a 35 mm dish for two hours to reduce contaminating stromal-derived cells, as previously described (Hosseni et al, 2018, 2019).

2.3. Preparation of enriched primary E16 SMG mesenchyme for organoid assays

Primary mesenchyme cells were isolated as previously described (Hosseini et al, 2018, 2019). Briefly, E16 SMG were treated with enzymes to release epithelial clusters and subjected to gravity sedimentation. Cells in the supernatant were filtered through a 70 µM filter (Falcon 352350) followed by a 40 µM filter (Fisherbrand 22363547). To deplete $EpCAM^+$ epithelial cells from enriched mesenchymal cell preparations for use in organoid experiments, EpCAM microbeads (130-105-958, Miltenyi Biotech) were incubated with the cells and collected with a MACS sorting column, as previously described in Kwon et al, 2017 and Hosseini, et al, 2019.

2.4. Culture Media

Simple serum-containing medium was prepared using Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (ThermoFisher 11039047) supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin (Pen-Strep, ThermoFisher #15140163), and 10% v/v Fetal Bovine Serum (FBS), certified, heat-inactivated (ThermoFisher 10082-147). “Salisphere” (Sali) medium was prepared using DMEM/F12 together with the following components: 100 ng/mL Fetal Growth Factor 2 (FGF2 Peprotech #450-33), 100 ng/mL Epidermal Growth factor (EGF, PeproTech, #AF100-15), 10 µg/mL Insulin (Sigma #I882), 1.25 µg/mL Hydrocortisone (Sigma #H0135), 1% v/v N2 Serum Supplement (ThermoFisher #17502048), L-Glutamine (ThermoFisher #25030081), and Pen-Strep (Life Technologies #15140122) as in Lombaert et al, 2008. Y27632 was obtained from Sigma- Aldrich (CAS #146986-50-7) dissolved in

DMEM:F12 media and stored as frozen single-use aliquots. Y27632 was added to the culture media for a final concentration of 10 µM.

2.5. Salisphere Culture

Epithelial clusters derived from two adult glands were prepared as described in 2.2 and seeded in 2 mL of media in a Falcon® 6 Well Clear Flat Bottom TC-Treated Multiwell Cell Culture Plate, with Lid,

(Corning, #353046) at $2X10^6$ cells per well (approximately 0.5 glands/well). Adult epithelium was cultured for three days to generate salispheres. At 72 hours, the salispheres were sedimented by gravity in an Axygen MaxyClear Standard Microcentrifuge Tube, 1.7mL, (Axygen, #MCT-175-C) similarly described in previous protocols (Hosseni et al, 2018, 2019) 2-3 times and subjected to adhesion depletion to remove stromal-derived cells. Roughly 1000 salispheres were seeded on top of 0.1 µm pore porous polycarbonate filters (Nuclepore, Whatman #0930051) and floated on top of 200 µL media (vehicle control or media with 100ng/ml FGF2) in 50 mm glass-bottom dishes (MatTek, #P50G-1.5-14F) for 7 days.

2.6. Organoid Assay

Salispheres were collected by media removal, and 10 µl of cold of 1:1 media: Matrigel® added to the clusters and gently pipetted. Where indicated, $2*10^4$ primary mesenchyme cells derived from E16 SMGs were mixed with 1000 salispheres or to 1.25 glands worth of E16 epithelium ($2.75*10^5$ cells) in either control media or 100 ng/mL FGF2. Salispheres were cultured for 7 days unless otherwise specified and media was changed every 72 hours to form organoids.

2.7. ICC and Confocal Imaging

Salispheres were either fixed in 4% v/v Paraformaldehyde (PFA) (Electron Microscopy Sciences) at 4 degrees overnight or 100% v/v Methanol at -20 degrees for 18 minutes. PFA-

fixed cells for ICC were performed as described previously as in Hosseni et al, 2018, and Hosseni et al, 2019, secondary antibody incubations were performed at room temperature for 2 hours. Samples were then blocked in a mix of 3% v/v BSA in PBS with 5% v/v donkey serum containing Mouse on Mouse (M.O.M) blocking agent (Vector Laboratories MKB-2213), for 2 hours. The primary antibodies were utilized as follows: FITC-EpCAM (1:200, eBiosciences #11-5791-82), Vimentin (1:1000, Sigma #V2258), Cytokeratin 5 (K5) (1:200 BioLegend # 905501), Cytokeratin 14 (K14) (1:400 BioLegend # 906001), AQP5 (1:200. Alomone #AQP-005), c-Kit (1:200 R&D Systems #AF1356), Ki67 (1:200 AbCAM #AB15580), CC3 (1:200, #Cell Signaling 9661), RFP (1:200, Abcam #ab62341), Mist1 (1:100, Invitrogen #MA1-517) with DAPI (Life technologies # D1306 1:1000) to label nuclei. Secondary antibodies including Cyanine and Alexa dye-conjugated AffiniPure $F(ab')_2$ fragments were all purchased from Jackson ImmunoResearch Laboratories and used at a dilution of 1:200. Nuclepore filters were mounted on glass slides using Fluorogel with a Tris buffer-based mounting media (Electron Microscopy Sciences #17985-11). Images for quantification were acquired using the Zeiss Cell Observer inverted fluorescent microscope. Confocal microscopy was performed using a Zeiss LSM710 laser scanning confocal microscope at 20X-40X magnification using matched acquisition parameters within an experiment for each channel from which maximum projection images were generated.

2.8. Image Analysis and Statistical Analysis

ImageJ was used to generate maximum intensity projections of confocal images. Image J was used to perform quantification from widefield fluorescent images, as previously reported (Gervais et al, 2015; Hosseni et al, 2018). The percent of cells positive for a specific marker was determined by counting the # of cells positive for the marker relative to number of cells expressing DAPI. To quantify the intensity of a marker expressed by cells, pixel intensity of the marker being tested was normalized to pixel levels of EpCAM. Area of a specific marker was

quantified from thresholded images of that marker are normalized to the thresholded $DAPI^+$ area of the sample. For statistical analysis one-way ANOVA was carried out for multiple comparisons using Vassar-Stats with Tukey post-hoc test with $p^* < 0.05$ considered to be statistically significant for comparisons within the ANOVA. Student's two-tailed T tests were carried out for 2-way comparisons. At least three experiments were quantified to generate each graph with triplicate samples. Graphs of quantified data were made using Excel. Error bars are standard error of the mean (SEM).

Supplementary References

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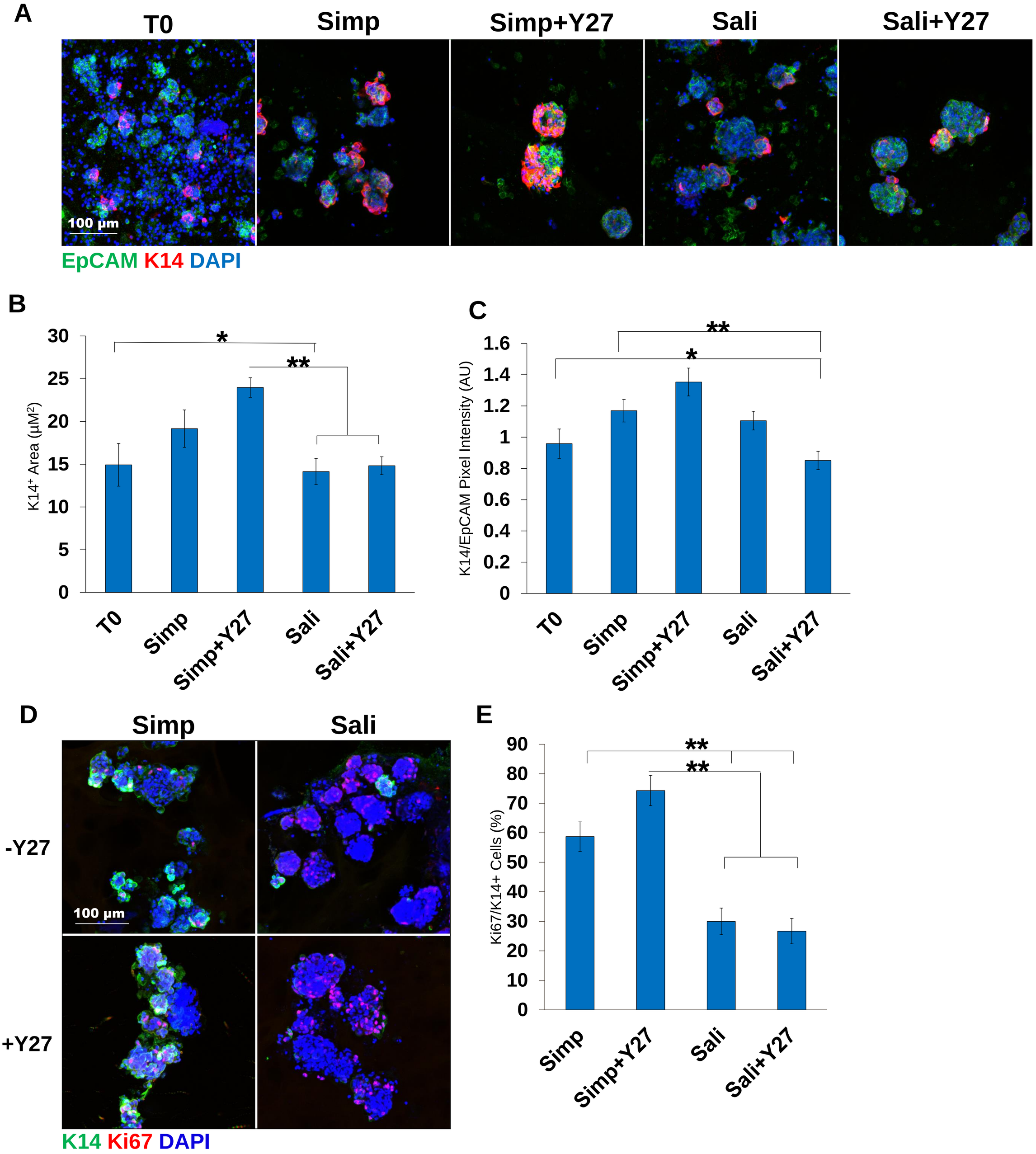
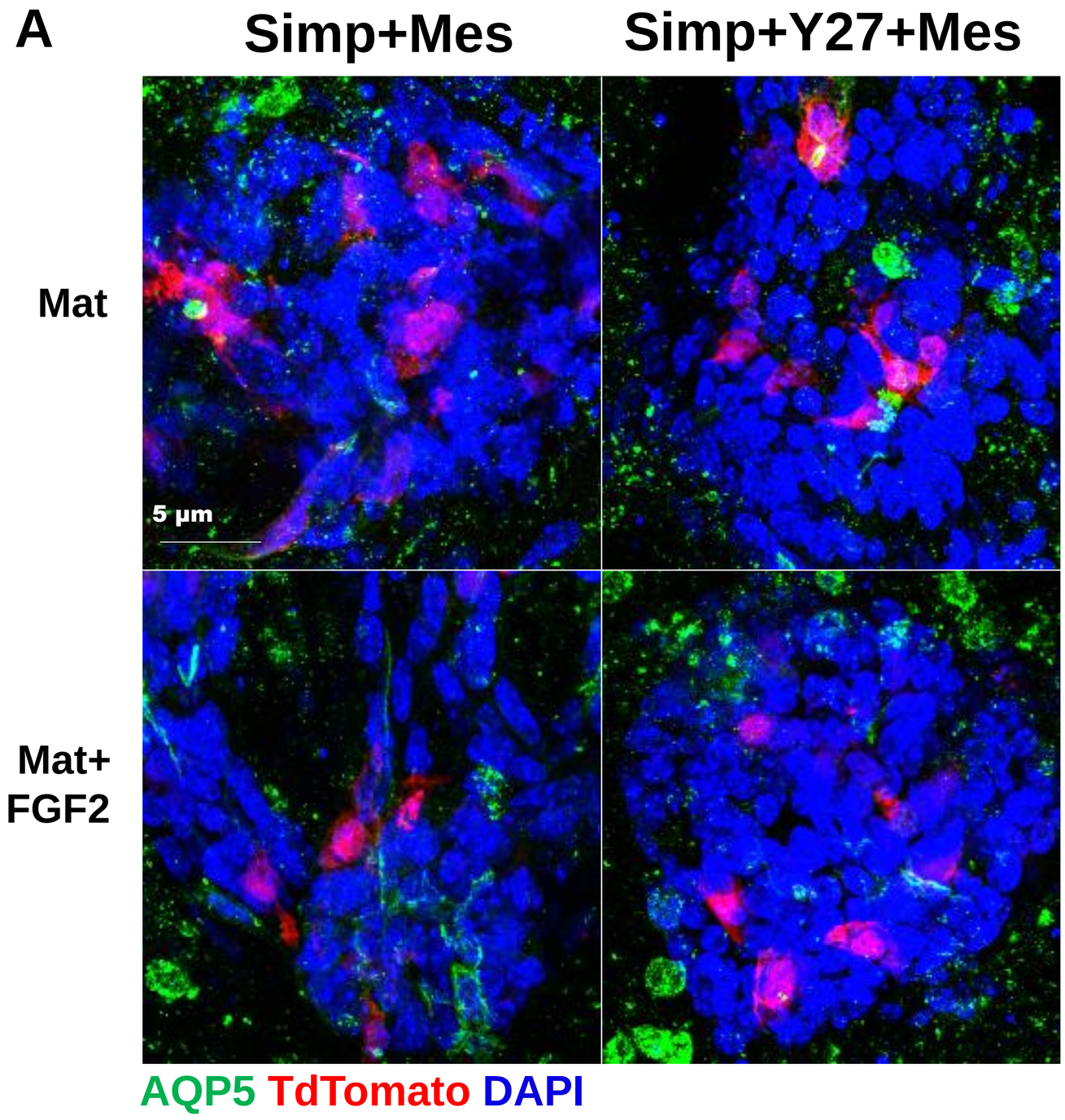


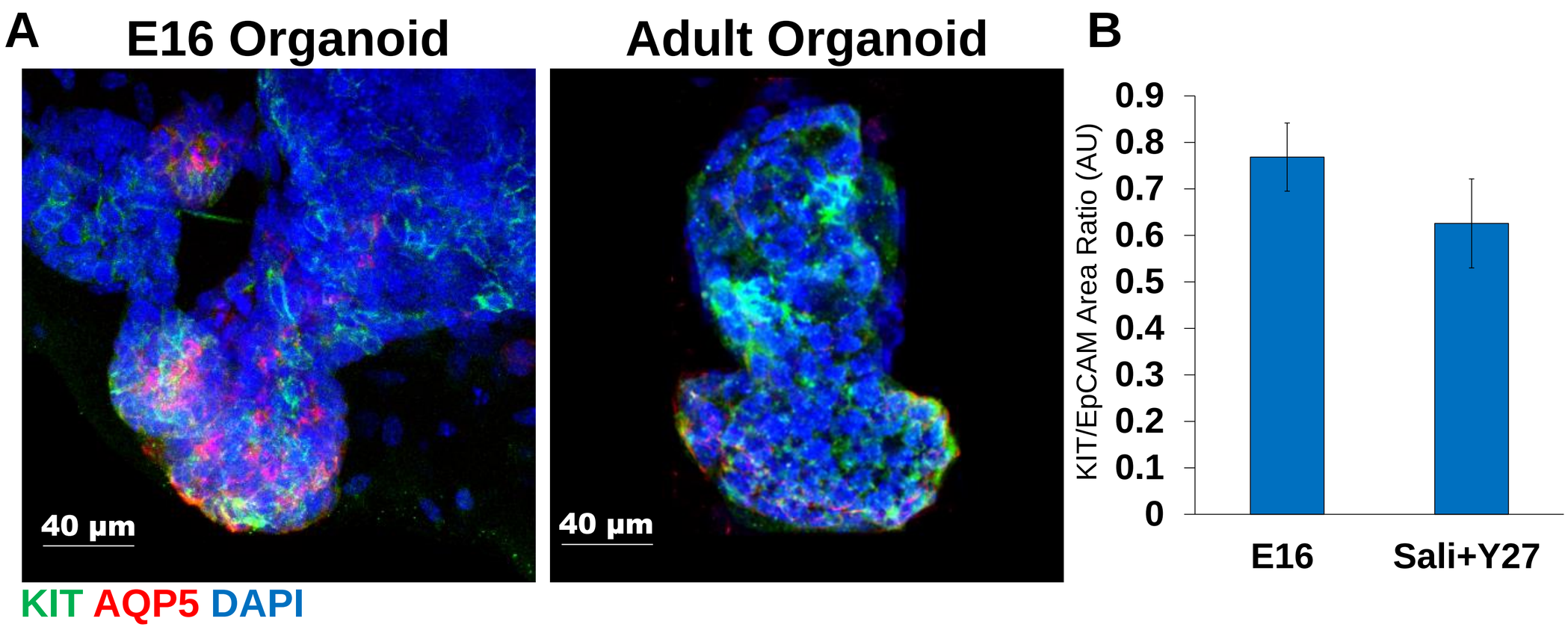
Supplemental Figure 1: K14 is increased in Salispheres Grown in Simple Medium

A) ICC of K14 in Salispheres. B) K14 pixel area relative to DAPI. C) K5 pixel area relative to EpCAM. D) ICC of salispheres with K14 and Ki67. E) K14/Ki67 proliferation. (%) A-C: N=6 experiments (2 experiments with male BL6 salivary glands). D-E N=3 experiments (Bl6 mice). *p < 0.05, ** p < 0.01, One-Way ANOVA



Supplemental Figure 2: In organoids derived from salispheres expanded in simple medium, Mist1-derived, TdTomato$^+$ cells show minimal AQP5 expression.

A) ICC of salispheres grown +/-Y27632 in Simp medium in the salisphere phase and in the presence or absence of primary E16 salivary mesenchyme cells (Mes) in Matrigel +/- FGF2 in the organoid phase. Images are zoomed in Max projections at 40X. AQP5 is Green, TdTomato is Red, and DAPI is blue. Scale Bar is 5 uM. B) % of TdTomato positive cells that are AQP5$^+$ and AQP5$^-$. N=3 experiments



Supplemental Figure 3: KIT Levels are similar in embryonic organoids and adult organoids derived from salispheres grown in Sali medium + Y27632.

A) ICC of E16 derived organoids vs Adult Salisphere derived organoids grown in optimal conditions. KIT is Green, AQP5 is Red, and DAPI is blue. Images are Max projections at 40X taken from confocal images B) Kit pixel intensity relative to EpCAM in Organoids. N=3 experiments. One Way ANOVA