Journal of Dental Research
DS1–DS3

DOI: 10.1177/0022034514559251
jdr.sagepub.com

Establishment of Functional Acinar-like Cultures from Human Salivary Glands

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Appendix

Materials and Methods

Human Salivary Gland Tissue and Cell Cultures

Biopsy of minor salivary gland tissue was obtained from the consenting healthy volunteers and Sjögren syndrome patients (pSS) from the Clinical Center of National Institute of Dental and Craniofacial Research (NIDCR) at the National Institutes of Health (NIH), through NIH institutional review board–approved protocols. The primary human minor salivary gland epithelial cells (phmSG) were obtained using the explant outgrowth technique. Unless indicated, phmSG cells from healthy controls were used in this study. The tissue was rinsed with cold phosphate-buffered saline (PBS) containing 1× antibiotic-antimycotic solution (penicillin, 100 IU/mL; streptomycin, 100 µg/mL; and fungizone, 2.5 µg/mL; Invitrogen, Carlsbad, CA, USA), minced, and transferred into a culture flask and incubated with supplemented basal epithelial medium (s-BEM; Dulbecco's modified Eagle's medium [DMEM]/Ham's F12: 1/3 mixture; Invitrogen), supplemented with glutamine (1×; Invitrogen), antibiotic-antimycotic solution, epidermal growth factor (EGF; 10 ng/mL), hydrocortisone (0.4 µg/mL), insulin (0.5 µg/mL), and 2.5% heat-inactivated fetal bovine serum (FBS; Invitrogen; Dimitriou et al. 2002). Cells were detached by Trypsin/EDTA (0.05%, Invitrogen), plated on collagen-coated dishes, and maintained in keratinocyte growth medium (KGM; Lonza, Basel, Switzerland) supplemented with bovine pituitary extracts (BPEs), recombinant human epidermal growth factor (hEGF), insulin (INS), hydrocortisone (HC), gentamicin, epinephrine, and transferrin (referred to as "KGM" in the article). The calcium concentration in KGM was adjusted to either 0.05 mM (KGM-L) or 0.8 mM (KGM-H). All the experiments were typically conducted using phmSG cells between passages 3 and 5. In the initial study, the mammalian epithelial basal medium (MEBM; Lonza) was included for optimization of cell growth conditions. The immortalized human salivary gland (HSG) cell lines were gifted from Bruce Baum's laboratory at the NIDCR/NIH and maintained in DMEM/F12 (Invitrogen) supplemented with 5% of heat-inactivated FBS and 1× antibiotic-antimycotic solution (Invitrogen).

Western Blot and Quantitative Real-Time RT-PCR

Total cellular extracts were isolated and Western blot was performed as previously described (Jang et al. 2007). Briefly, equal amounts of cellular lysates (50–80 µg) were mixed with sample buffer (1×; Invitrogen), resolved in NuPAGE 4% to 12% polyacrylamide gels (Invitrogen), and transferred onto PVDF membranes. After incubation with blocking buffer containing 5% nonfat dry milk in Tris-buffered saline with 0.1% of Tween-20 solution (TBS-T) for 30 min at room temperature, the membranes were incubated with indicated primary antibodies in blocking buffer overnight at 4 °C. After washing, the membranes were blotted with secondary horseradish peroxidase (HRP) antibody in blocking buffer for 1 h at room temperature. Protein bands were detected using the SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA).

To monitor the gene expression, total RNAs were obtained using miRCURY RNA isolation kit (Exiqon, Vedbaek, Denmark) and used for complementary DNA (cDNA) synthesis with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time polymerase chain reaction (qPCR) was performed using indicated Taqman probes with StepOnePlus (Applied Biosystems). In 1 study, a mix sample was made for PCR reaction by combining an equal amount of cDNA from each sample and served as a control for each transcript of relative comparison (Figure 1). The housekeeping gene *GAPDH* was used as an internal control for normalization of input cDNA, and the difference of the cycle threshold (Ct) of the gene of interest was calculated with the ΔCt method ($\Delta Ct = (Ct_{sample} - Ct_{GAPDH})$) and used

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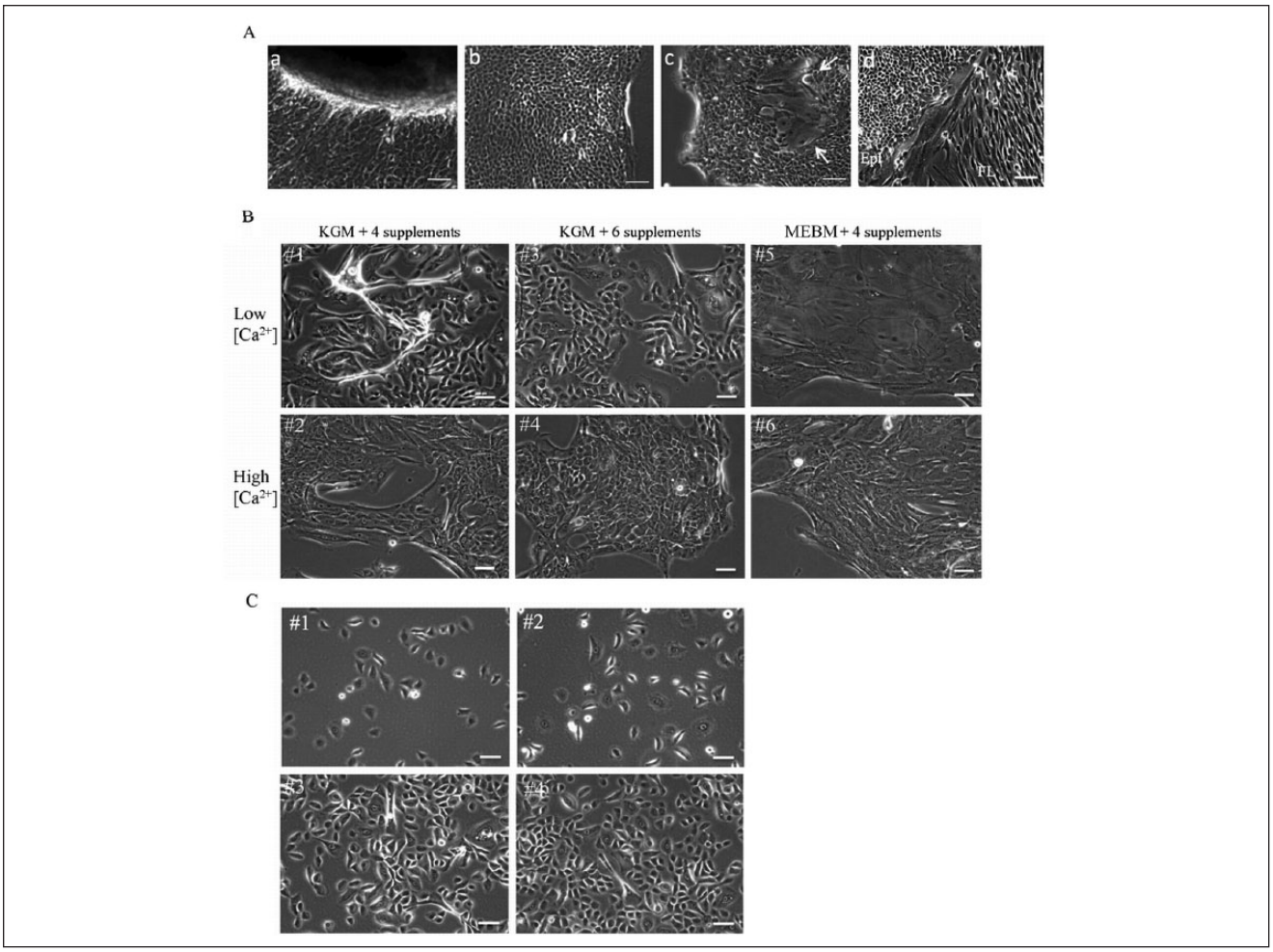
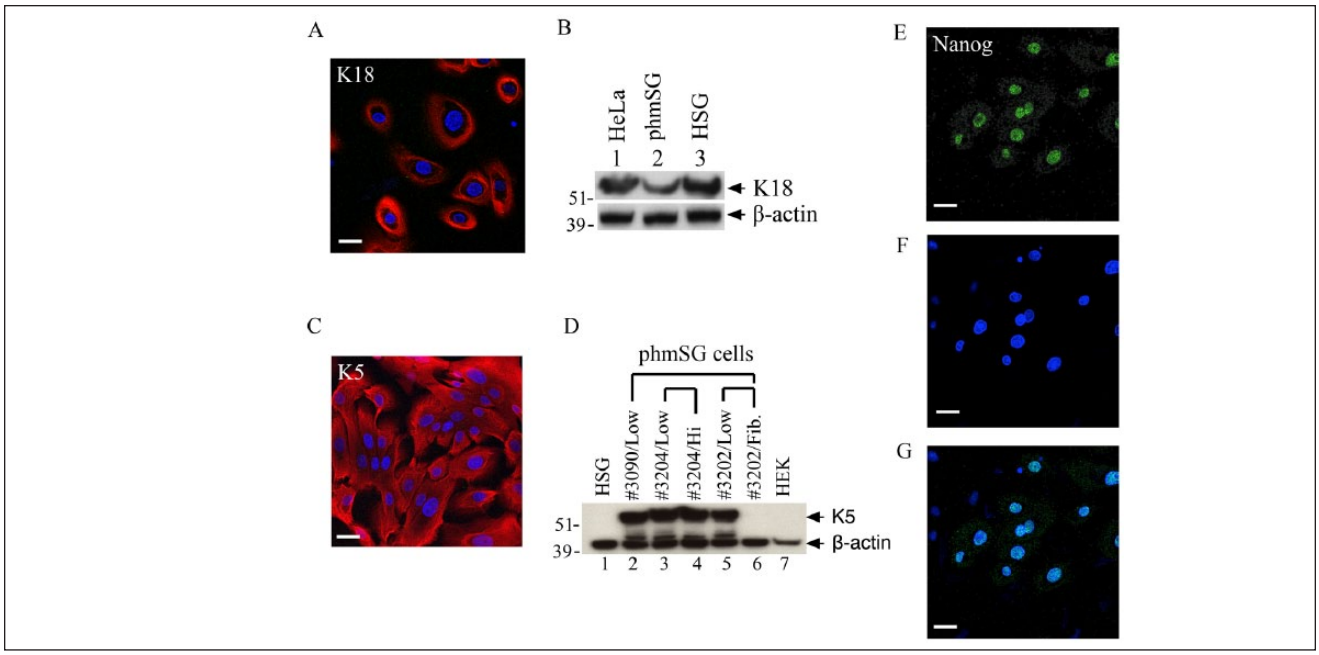


Appendix Figure 1. Explant culture of human submandibular gland primary epithelial cells. (**A**) Phase contrast images of outgrowth epithelial cells with cobblestone morphology (a and b). Some large sizes of cells were found in outgrowth culture (c, arrows). Outgrowth culture contains both epithelial (d, left, Epi) and fibroblast-like (d, right, FL) cells. Bar = 100 µm. (**B**) Morphology of first passage of phmSG cells maintained in either KGM (#1, #2, #3, #4) or MEBM (#5, #6) containing 4 supplements of BPE, hEGF, insulin, and hydrocortisone (#1, #2, #5, #6) or 6 supplements (above 4 supplements plus transferrin and epinephrine) (#3, #4). The $[Ca^{2+}]$ in medium was either 0.05 mM (#1, #3, #5) or 0.80 mM (#2, #4, #6). (**C**) Time course growth of phmSG cultures. Frozen phmSG cells were thawed and maintained in a collagen-coated dish with KGM-L. Contrast images were taken after seeding for 2 h (#1), 12 h (#2), 48 h (#3), and 56 h (#4). Bar = 50 µm.

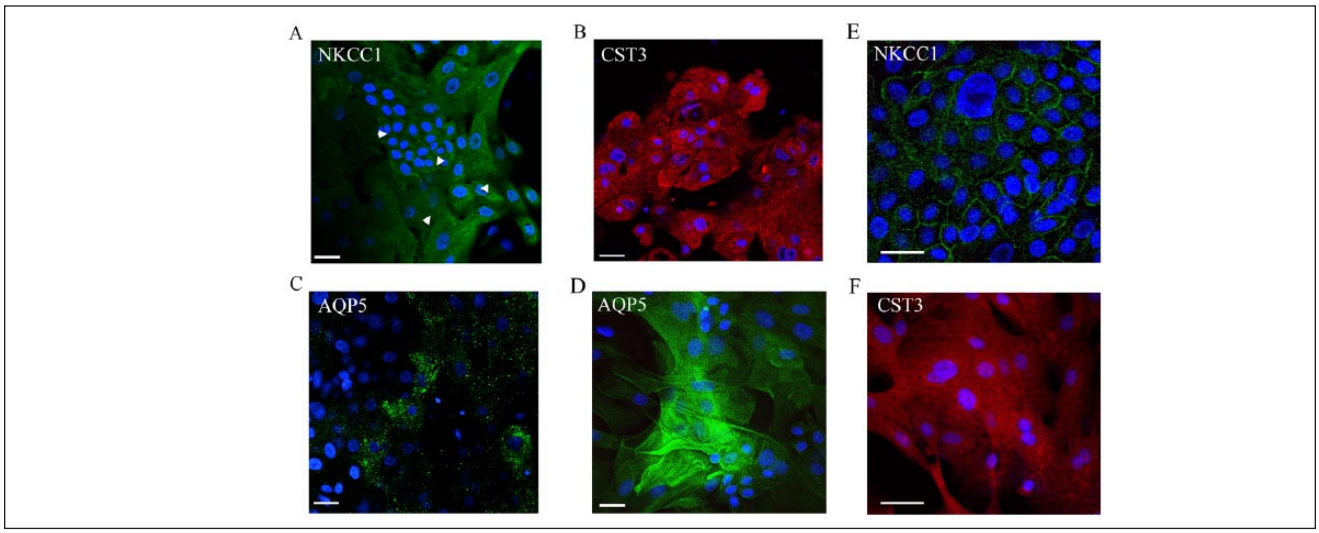
to determine the relative quantitation (RQ) values ($2^{-\Delta\Delta Ct}$), which represent the relative level of fold change over control.

Immunofluorescence and Confocal Microscopy Imaging

The phmSG culture was maintained in either KGM-L or KGM-H for 3 d and fixed with cold methanol for 10 min at –20 °C. Immunocytostaining was conducted as reported previously (Jang et al. 2007). The primary antibodies included aquaporin 5 (AQP5), keratin 5, keratin 8, cystatin C (CST3), and nanog (all from Abcam, Cambridge, MA, USA), as well as α-amylase (Cell Signaling, Danvers, MA, USA) and keratin 18 (Sigma, St. Louis, MO, USA). Secondary antibodies were goat antibody conjugated with either fluorescein isothiocyanate (FITC) or rhodamine (Molecular Probes, Invitrogen). The nucleus was stained with 4′,6-diamidino-2-phenyindole dihydrochloride (DAPI; Vector Laboratories, Burlingame, CA, USA). The fluorescence images were captured using laser scanning confocal microscope (IX81; Olympus, Tokyo, Japan) coupled with the Fluoview (FV1000) software.



Appendix Figure 2. Expression of epithelial markers in phmSG cells. Immunofluorescence staining of keratin 18 (**A;** K18), keratin 5 (**C;** K5), and nanog (E-G) in phmSG cells maintained in KGM-L (6 supplements, 0.05 mM [Ca^{2+}]). Western blotting of keratin 18 (**B**) and keratin 5 (**D**) in phmSG cells (#3090, #3202, #3204), primary fibroblasts from human minor salivary gland (#3202/Fib), HeLa, HSG, and HEK cultures. Low: KGM-L; Hi: KGM-H. HeLa cells were used for a positive control of keratin 18 expression, and β-actin was used as a loading control. (**E–G**) The phmSG cells were stained with anti-nanog antibody (E). DAPI was for nuclei staining (F). (G) Merged image of both E and F. Bar = 50 μm.



Appendix Figure 3. Detection of sodium/potassium/chloride ion channel (SLC12A2/NKCC1), cystatin C (CST3), and aquaporin 5 (AQP5) in phmSG cells. The phmSG culture (passage 3: **A–D;** passage 7: **E–F**) was grown in KGM-L (C, E) or KGM-H (A, B, D, F) for 3 d before fixation. The anti-SLC12A2 (NKCC1) (A, E, green), anti-CST3 (B, F, red), and anti-AQP5 (C, D, green) antibodies were used for immunofluorescence staining. DAPI was for nuclei staining (blue). Bar = 50 μm.

Appendix Reference

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