Hyperacetylation of Microtubules in Mesenchymal Cells Increases Cytokeratin 14-Positive Epithelial Progenitors in Developing Salivary Glands

Journal of Dental Research
2016, Vol. 95(13) 1518–1527
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DOI: 10.1177/0022034516662450
jdr.sagepub.com

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Abstract

Cells engage in bidirectional communication with their surroundings. This reciprocal dialogue between cells and their cellular microenvironments often governs the maintenance and differentiation of stem/progenitor cells. Here, the authors present evidence that in developing salivary gland explants, a single posttranslational change in microtubules in mesenchymal cells alters the mesenchymal microenvironment and promotes the maintenance and differentiation of a subset of epithelial progenitor cells that impairs branching morphogenesis. Specifically, the authors report that hyperacetylation of microtubules in mesenchymal cells increased cytokeratin 14-positive (K14+) progenitors and their differentiated progeny, myoepithelial cells, in epithelial basal and suprabasal layers in the distal endbud region of developing salivary glands. Mechanistically, this process engages the transforming growth factor β 1 protein and Notch signaling pathways. This report establishes that a simple posttranslational change in the cytoskeletal system of mesenchyme dictates the maintenance and differentiation of adjacent epithelial progenitor cells to alter branching morphogenesis of the epithelium.

Keywords: salivary physiology, morphogenesis, cell differentiation, cell signaling, epithelial-mesenchymal interaction, extracellular matrix (ECM)

Introduction

The communication between cells and other cells, and of cells with their surroundings, is essential for successful embryonic development in many multicellular organisms (Nelson and Bissell 2006; Xu et al. 2009). This bidirectional dialogue between cells and their cellular microenvironments, which is often referred to as reciprocity, can also be observed in developing branching organs, such as salivary glands, lungs, and kidneys (Watt and Hogan 2000; Morrisey and Hogan 2010; Patel and Hoffman 2014). Not surprisingly, miscommunication or dysregulation of reciprocity can lead to pathogenesis, including defects in embryogenesis and growth, as well as development of cancer (Nelson and Bissell 2006; Pickup et al. 2014). Thus, understanding the nature of such reciprocity will be a key step in deciphering not only these biological phenomena but also for stimulating regeneration of damaged and/or diseased organs.

One of the most powerful model systems to study the effects of the cellular microenvironment is organ culture. Such *ex vivo* cultures retain almost all biological architecture and *in vivo* functions for several days in organ culture, including the maintenance and differentiation of their progenitor cells within native cellular microenvironments. Developing salivary glands provide a classical model organ culture system that possesses the required cell and matrix constituents for studying the role of cellular microenvironment in the maintenance and differentiation of epithelial progenitor cells (Lombaert et al. 2011; Knox et al. 2012; Patel and Hoffman 2014). Several epithelial

progenitor cell populations have been identified in developing salivary glands that are uniquely influenced and regulated by their own immediate microenvironment. For example, basal epithelial cytokeratin 5-positive (K5+) epithelial progenitors require a functioning parasympathetic ganglion to maintain their population and then to differentiate further into ductal cytokeratin 19-positive (K19+) epithelial cells (Knox et al. 2010, 2013). In contrast, KIT-positive (KIT+) cells coexpressing cytokeratin 14 (K14+), which are distal endbud epithelial

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A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

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progenitors, require inputs from the surrounding mesenchyme to maintain and expand their population (Lombaert et al. 2013).

Cytoskeletal proteins are crucial for the survival and differentiation of cells, yet how these essential proteins influence and regulate the maintenance, expansion, or differentiation of epithelial progenitor cell populations is not clearly understood. This is particularly true for posttranslationally modified molecules, such as acetylated microtubules. Although numerous *in vitro* studies have demonstrated potential effects of microtubule acetylation on secretion, cell migration, cancer metastasis and prognosis, and neurological disorders, there is little *in vivo* evidence for its role in embryonic development (Hammond et al. 2008; Tanabe and Takei 2009; Janke and Bulinski 2011; Boggs et al. 2015; Song and Brady 2015; Joo and Yamada 2016). While studying the crosstalk between the actin cytoskeleton and microtubule network, we found that increasing microtubule acetylation in salivary glands increased fibronectin (FN) and $\alpha 5\beta 1$ integrin (Joo and Yamada 2014). We now find that hyperacetylation of microtubules modulates transforming growth factor $\beta 1$ (TGF $\beta 1$) protein level and Notch signaling in the mesenchyme, which govern the maintenance of K14⁺ epithelial progenitors and their differentiation into myoepithelial cells in developing salivary glands.

Results

Hyperacetylation of Microtubules Increases K14⁺ Epithelial Progenitors and Their Differentiated Myoepithelial Progeny

Previously, we established that substantial levels of acetylated microtubules are present in the peripheral zone of epithelial bud cells and that elevated levels of acetylated microtubules increase FN and $\alpha 5\beta 1$ integrins at the junction between epithelium and mesenchyme in developing salivary glands (Joo and Yamada 2014). Since it is well documented that maintenance and differentiation of progenitor cells are greatly influenced by their surroundings (Watt and Hogan 2000; Fuchs et al. 2004), and because these increases in FN and $\alpha 5\beta 1$ integrins would potentially alter the microenvironment of salivary gland progenitor cells, we tested whether salivary gland epithelial progenitor cells are altered by their new microenvironment induced by microtubule acetylation. As a baseline analysis, we visualized several known progenitor cells in the epithelium of developing mouse salivary glands using their known markers and found that overexpression of an acetyl-mimetic mutant of tubulin (HyperAcMT, K40Q) increased K14⁺ progenitors in distal endbuds without affecting either K5⁺ progenitors or their K19⁺ related progeny (Fig. 1A, B). To quantify these changes in progenitor (K5⁺, K14⁺, K5+K14⁺) and differentiated populations (K5+K19⁺, K14+K19⁺, K5+K14+K19⁺, K5–K14–K19– or Neg) in developing salivary glands expressing different levels of acetylated microtubules, we counted the numbers of cytokeratin-positive cells by fluorescence-activated cell sorting (FACS) analysis as described in Lombaert et al. (2013). As shown in Appendix Figure 1A, increased expression

of acetylated microtubules significantly increased the number of K14⁺ progenitor cells. Furthermore, this overexpression of acetylated microtubules in salivary glands also increased the expression of K14 messenger RNA (mRNA) as indicated by quantitative polymerase chain reaction (qPCR) analysis (Appendix Fig. 1B, C). Interestingly, the mRNA level of K5 was also increased (Appendix Fig. 1B), yet no significant changes were observed in its protein level, as indicated by the immunostaining of K5 and its quantification (Fig. 1A, B), or in the number of K5⁺ cells, as indicated by FACS analysis (Appendix Fig. 1A), suggesting that K5⁺ progenitors were not affected significantly by the changes in microtubule acetylation. Moreover, there was no change in the other endbud progenitors in glands with hyperacetylated microtubules, as indicated by KIT and transcription factor SOX10 (Lombaert et al. 2013) immunostaining (Appendix Fig. 1D–F), or in the level of cytokeratin 7 mRNA, a marker for terminally differentiated progeny of K5⁺ and K19⁺ ductal cells. Together, these results indicate that an increase in microtubule acetylation promotes the expansion of K14⁺ progenitors in developing salivary glands.

We next evaluated whether the increase in K14⁺ progenitors in salivary glands resulted in any increase in downstream differentiated cells, such as myoepithelial and acinar cells (Knox et al. 2010, 2013; Lombaert et al. 2013). As shown in Figure 1C and D, salivary glands that overexpressed acetylated microtubules showed increased K14⁺ progenitors and their progeny cells destined to become myoepithelial cells according to alpha smooth muscle myosin (α SMA) staining, especially in the endbuds. Interestingly, there were no significant changes in levels of AQP5⁺ acinar cells (Appendix Fig. 1E). Thus, these results indicate that increased microtubule acetylation alters the microenvironment of the epithelium and increases a subpopulation of progenitor cells that lead to differentiated myoepithelial cells.

Overexpression of acetylated microtubules was previously shown to increase FN and $\alpha 5\beta 1$ integrins (Joo and Yamada 2014), while the present results show that it unexpectedly increases K14⁺ progenitors and differentiated myoepithelial cells. Consequently, we tested whether experimentally increasing FN and/or $\alpha 5\beta 1$ integrin activity could phenocopy the salivary gland alterations resulting from elevated acetylated microtubules. As shown in Figure 2A, addition of exogenous FN or manganese cation to hyperactivate $\alpha 5\beta 1$ integrins (Gailit and Ruoslahti 1988) increased branching, as predicted by published studies (Sakai et al. 2003). However, the coaddition of FN with manganese significantly decreased branching (Fig. 2A, right column), thus phenocopying the gross morphological changes observed when microtubule acetylation was induced. We immunostained for the different cytokeratins in glands treated with FN and/or manganese and found that only when glands were incubated with both exogenous FN and manganese were there increases in K14⁺ progenitors in the endbuds (Fig. 2B, C). There also appeared to be a small increase in myoepithelial cells in the glands with experimentally elevated FN and manganese cation but not in KIT⁺, SOX10⁺, or AQP5⁺ cells in

Figure 1. Hyperacetylation of microtubules in salivary gland increases K14+ progenitor cells. Representative confocal images of untreated salivary glands (Control–no virus) or glands expressing green fluorescent protein (GFP) (Control–GFP) or GFP-tagged wild-type tubulin (GFP–WT–MT), acetyl-null tubulin K40A (GFP–HypoAcMT–K40A), or acetyl-mimetic tubulin K40Q (GFP–HyperAcMT–K40Q) as indicated and **(A)** immunostained for cytokeratin 14 (K14), cytokeratin 5 (K5), or cytokeratin 19 (K19) or **(C)** immunostained for cytokeratin 14 (K14), alpha smooth muscle myosin (α SMA), or E-cadherin (Ecad). Arrowheads point to basal and suprabasal layers of the epithelium in distal endbuds. Significantly increased K14+ and/or α SMA+ immunostaining of cells in the distal endbuds is observed in HyperAcMT (K40Q) expressing glands. Fluorescence intensity of the cytokeratins (K5, K14, or K19, **B**) or α SMA (**D**) in the basal and suprabasal layers of the epithelium as outlined by the dotted white lines in panels A or C, respectively, was quantified and expressed as relative to the GFP control ($N > 3$ experiments, $n > 12$ glands). Data are mean $\pm$ SEM. * $P < 0.05$ by statistical analysis using 2-way analysis of variance followed by Tukey posttest. Scale bars, 200 μ m (A) and 50 μ m (C).

Figure 2. Exogenous addition of soluble fibronectin and manganese cation decreases branching morphogenesis and increases K14+ progenitor cells. **(A)** Representative brightfield images of salivary gland explants incubated with 4 mg/mL soluble fibronectin and/or 1 nM manganese cation. **(B)** Confocal images of salivary gland explants incubated with 4 mg/mL soluble fibronectin and/or 1 nM manganese cation for 48 h and then immunostained for cytokeratin 14, 5, or 19. A significant increase of K14+ immunostaining of cells in the distal endbuds is observed in glands treated with both fibronectin and manganese cation compared with the control or fibronectin-treated glands. **(C)** Quantification of fluorescence intensity of K14 in the basal and suprabasal layers of the epithelial area outlined by the dotted white line in panel B relative to the green fluorescent protein control ($N > 3$, $n > 10$). Data are mean $\pm$ SEM. $*P < 0.05$ using 2-way analysis of variance followed by Tukey posttest. Scale bars, 200 μ m.

the epithelium (Appendix Fig. 2A–C). This is consistent with the interpretation that our original observation of microtubule acetylation-induced increases in FN and $\alpha 5\beta 1$ integrin is, in part, a mechanism contributing to the elevated K14+ endbud progenitors and decreased branching morphogenesis.

Hyperacetylation of Microtubules in the Mesenchymal Niche Alters Epithelial K14+ Progenitors in the Endbuds

Our experimental approach to this point involved dissection and treatment of intact salivary glands. Whole dissected developing salivary glands are known to contain multiple tissues, including mesenchyme, epithelium, parasympathetic ganglion (PSG), and blood vessels, requiring more in-depth analysis to identify which tissue(s) is responsible for the decreased epithelial branching. A definitive method to decipher the source is to mechanically separate mesenchyme, epithelium, and PSG tissues and then to reconstitute them one at a time. Briefly, dissected developing salivary glands were infected with lentivirus to exogenously express different tubulin acetylation mutants, followed by separation of the mesenchyme, epithelium, and PSG, and then reconstitution of each with uninfected tissues from untreated salivary glands (Appendix Fig. 3A). As shown in Figure 3A, B and Appendix Figure 3B, only when infected mesenchyme was reconstituted with other uninfected salivary gland tissues was it possible to observe the same morphological changes as in the whole gland; that is, decreased epithelial branching morphogenesis was induced only by lentiviral infection that produced hyperacetylation of microtubules in the mesenchyme. Furthermore, there appeared to be an increase in K14+ progenitors in the endbuds of the reconstituted glands containing mesenchyme expressing hyperacetylated tubulin compared with the glands containing mesenchyme expressing hypoacetylated tubulin (Fig. 3C D). Further support for these findings is the fact that FN and $\alpha 5\beta 1$ integrin staining were mostly found in the mesenchyme (Joo and Yamada 2014).

TGF β 1 Protein Levels and Notch Signaling Are Increased, and Inhibition of Both Signals Rescues Hyperacetylation-Disrupted Salivary Glands

We searched for a mechanism that might explain how an increase in FN and $\alpha 5\beta 1$ integrin resulting from hyperacetylation of microtubules in the mesenchyme could reduce branching morphogenesis in the epithelium, a spatially separated tissue. We speculated that the increases in FN and $\alpha 5\beta 1$ integrins might provide a platform for mesenchyme-derived growth factors to concentrate and activate their downstream signaling pathways (Hynes 2009). We reasoned that the factors or related cell signaling components involved in the morphogenic changes resulting from increased microtubule acetylation should be present during the developmental stages studied (i.e., E13–15). Based on the publicly available salivary gland gene expression atlas (<http://sgmap.nidcr.nih.gov/sgmap/sgexp.html>), we screened all 17 of these candidate factors. Only

Tgf β 1 and *Hey2* mRNA were altered in glands infected with the acetyl-mimetic mutant of tubulin K40Q (HyperAcMT; Fig. 4A, Appendix Fig. 4A). This increased expression of *Tgf β 1* and *Hey2* genes by qPCR was confirmed by immunostaining (Fig. 4B, C; Appendix Fig. 4B, C). Interestingly, changes in microtubule acetylation did not significantly alter total cell proliferation as indicated by Ki67 immunostaining (Fig. 4C).

These results suggested that TGF β 1 protein levels and Notch signaling were upregulated and in part responsible for the decrease in salivary gland morphogenesis. To test this interpretation, we hypothesized that inhibiting TGF β and Notch signaling pathways in glands overexpressing acetylated microtubules should rescue the observed decrease in branching morphogenesis if both TGF β and Notch pathways were involved. Our results confirmed this predicted rescue: when glands overexpressing acetylated microtubules were treated with reagents to inhibit TGF β and Notch signaling pathways (Fig. 4D, E), branching morphogenesis was rescued. Coinhibition of TGF β and Notch signals also reverted the levels of K14 at the endbuds in glands overexpressing hyperacetylated versus hypoacetylated microtubules (Appendix Fig. 4D, E). Moreover, TGF β 1 protein levels were increased only in glands treated with excess amounts of FN and manganese (Appendix Fig. 2D). Last, a recent publication reports that increased FN can induce Notch signaling via integrins (Wang and Astrof 2016). Together, these findings support a model in which hyperacetylation of microtubules in the mesenchyme of salivary glands increases FN and $\alpha 5\beta 1$ integrins, which in turn increases TGF β and Notch signaling, which together decrease epithelial branching morphogenesis. Taken together, these data imply that a simple alteration in postpolymerized microtubules in the mesenchyme of salivary glands influences the behavior of epithelial progenitors.

Discussion

Our surprising findings establish that altered microtubule acetylation in the mesenchyme induces the expansion and differentiation of K14+ progenitors in the endbuds. Another unexpected finding was that the total numbers of K5+ and KIT+ progenitors were not significantly altered by the altered microtubule acetylation, suggesting that microtubule acetylation specifically affects a subpopulation of K14+ endbud progenitors.

Growth factors such as fibroblast growth factor (FGF) and stem cell factor (SCF, also called KIT ligand, KitL) are necessary for the expansion of the KIT+ epithelial progenitors in developing salivary glands (Lombaert et al. 2013). Furthermore, other growth factors such as members of the TGF β , Notch, and Wnt superfamilies are known to be important for the maintenance or differentiation of stem/progenitor cells (Klüppel and Wrana 2005; Dziedzic et al. 2014; Lien and Fuchs 2014). Thus, it was puzzling to find that both TGF β 1 protein level and Notch pathways are upregulated in salivary glands overexpressing acetylated microtubules, because TGF β is known to facilitate maintenance of progenitor cells by promoting inhibition of their differentiation (Hardman et al. 1994), while the Notch signaling pathway is critical for promoting differentiation of progenitor cells (Klüppel and Wrana 2005; Garcia-Gallastegui

Figure 3. Hyperacetylation of microtubules in the mesenchyme increases K14+ progenitor cells. Examples of recombined developing salivary glands with infected mesenchyme expressing the indicated proteins with uninfected normal epithelium and parasympathetic ganglion. Images were acquired by **(A)** brightfield microscopy or **(C)** confocal microscopy after immunostaining for cytokeratin 5, 14, or 9. **(B)** Quantification of numbers of buds at 49 h as shown in panel A relative to the green fluorescent protein (GFP) control ($N > 3$, $n > 3$). **(D)** Quantification of the fluorescence intensity of K14 immunostaining in the basal and suprabasal layers of the epithelium relative to the GFP control ($N > 3$, $n > 3$). Slightly stronger K14 staining is observed in glands recombined with mesenchyme expressing HyperAcMT (K40Q).

et al. 2014). However, we observed that not only did the numbers of K14+ progenitors increase, but also the numbers of the differentiated form of these cells, myoepithelial cells, increased—suggesting that these seemingly opposing processes can proceed in parallel. Importantly, most of the α SMA+ cells were positive for K14, but not all K14+ cells were

Figure 4. Hyperacetylation of microtubules increases transforming growth factor β 1 (TGF β 1) protein level and Notch signaling. **(A)** Quantitative polymerase chain reaction (qPCR) analysis of developing salivary glands expressing green fluorescent protein (GFP) (Control-GFP) or GFP-tagged wild-type tubulin (GFP-WT-MT), acetyl-null tubulin K40A (GFP-HypoAcMT-K40A), or acetyl-mimetic tubulin K40Q (GFP-HyperAcMT-K40Q) as indicated. A trend of increased *Tgfβ1* and *Hey2* messenger RNA (mRNA) is observed in glands expressing HyperAcMT (K40Q; $N > 3$). **(B)** Confocal images of salivary gland explants expressing GFP or different tubulins as indicated and immunostained for Ki67, TGF β 1, or E-cadherin. **(C)** Quantification of fluorescence intensity of TGF β 1 and Ki67 in the basal and suprabasal layers of the epithelium in panel B relative to the GFP control ($N > 3$, $n > 11$). A significant increase in TGF β 1 immunostaining intensity in the distal endbuds is observed in the HyperAcMT (K40Q) expressing glands. **(D)** Representative examples of brightfield images of salivary glands expressing GFP or different tubulins, then treated with TGF β inhibitor (SB431542) and/or Notch inhibitor (DAPT). Only concurrent treatment by both inhibitors could restore branching morphogenesis in glands expressing HyperAcMT (K40Q). **(E)** Area of epithelium in panel D expressed relative to the GFP control. Note that the significant inhibition of branching due to HyperAcMT was abolished by treatment with both inhibitors. Data are mean $\pm$ SEM. * $P < 0.05$ using 2-way analysis of variance followed by Tukey posttest. Scale bars, 200 μ m.

positive for α SMA⁺, further indicating that K14⁺ progenitors had increased. The factors inducing these very selective growth factor changes, altered progenitors, and suppression of morphogenesis may be complex, with roles for the previously described increased levels of FN and integrin activation and/or decreased tissue contractility.

Since both the maintenance and differentiation of K14⁺ progenitors were promoted in salivary glands overexpressing acetylated microtubules, the effects on branching morphogenesis could theoretically have been either positive or negative. We actually observed smaller and less-branched salivary glands when acetylated microtubules were overexpressed. Interestingly, a recent publication showed that ablation of DLK1 from mouse salivary glands reduced salivary gland size while increasing the presence of epithelial progenitor cells, specifically K14⁺ progenitor cells (Garcia-Gallastegui et al. 2015). Thus, this DLK1-deficient mouse model supports the notion that it is possible to have more progenitor cells even in smaller salivary glands. Interestingly, DLK is a member of the Notch superfamily (Garcia-Gallastegui et al. 2014, 2015). Our findings also suggest that there is no simple relationship between the number of progenitors or differentiated derivatives and the extent of branching, because regulators such as the balance of growth factors can play critical roles in governing overall morphogenesis. Alternatively, the observed inhibition of epithelial branching in glands overexpressing acetylated microtubules could have resulted from delayed epithelial growth. However, we did not see notable changes in cell proliferation rate or cell death as indicated by nuclear fragmentation (data not shown) or cell shape.

Microtubules segregate chromosomes during mitosis, and many pharmacological reagents that disrupt microtubules halt cell division. Indeed, increased levels of an isoform of tubulin can disrupt proliferation (Bhattacharya and Cabral 2004). However, none of our lentivirus constructs designed to express exogenous wild-type, acetyl-mimetic, or acetyl-null forms of tubulins altered observed rates of proliferation, suggesting that the tubulin expression levels were low; however, they were sufficient for the selective growth factor, progenitor, and morphogenic effects. Because lentiviral infection can itself sometimes affect sensitive developmental systems such as embryonic salivary glands, we emphasize the importance of directly comparing the effects of hyperacetylation with hypoacetylation or with the effects of inhibitors within each particular experiment.

In summary, we discovered unexpectedly that 1) numbers of progenitor cells, particularly K14⁺ progenitors, can be regulated by microtubule acetylation, and 2) growth factors and/or signaling known to be regulators of branching morphogenesis are altered and can be shown to be causal and biologically significant for morphogenesis. Interesting future studies could involve further analysis of the roles of this subpopulation of K14⁺ progenitors in salivary gland development and whether a similar subpopulation of K14⁺ progenitors is present in other branching organs.

Materials and Methods

Organ Culture

All animal maintenance and procedures were in accordance with guidelines of the Intramural Program of the National Institutes of Health under protocol number 14–745 of the National Institute of Dental and Craniofacial Research Animal Care and Use Committee. Submandibular salivary glands (SMGs) were dissected, grown, treated, or infected with viruses exactly as previously described (Joo and Yamada 2014). Briefly, dissected SMGs were grown on either 0.1- or 0.2- μ m polycarbonate membrane filters (Whatman) and were treated with purified human plasma FN (Miekkka et al. 1982) and/or 1 nM MnCl₂ for 48 h, treated with SB 431542 and/or DAPT (R&D Systems), or infected with lentiviruses encoding wild-type or mutant tubulins using a virus titer of 2×10^8 for each set of glands. Images were acquired using an inverted brightfield microscope.

Immunofluorescence Studies

Cultured and treated SMGs were either 1) fixed with 4% paraformaldehyde for 15 min and then permeabilized with 0.5% Triton X-100/100 mM glycine at room temperature for 3 to 10 min or 2) fixed with a methanol/acetone (1:1) mixture at -20°C for 15 min with no further permeabilization. Glands were then incubated with blocking buffer (0.05% Tween-20, 5% donkey serum, 1% bovine serum albumin [BSA], and M.O.M. blocking reagent; Vector Laboratories) in phosphate-buffered saline (PBS) before incubating with either primary or secondary antibodies in PBS supplemented with 0.05% Tween-20, 5% donkey serum, and 8% M.O.M. at 4°C to 23°C for 2 to 18 h. The primary antibodies used for immunostaining were anti-cytokeratin 14 (1:300; Covance PRB-155P-100), anti-cytokeratin 5 (1:2,000; Covance PRB-160P-100), anti-cytokeratin 19 (1:300; DSHB Troma-III-c), anti-Sox 10 (1:200; Santa Cruz D-20sc-17343), anti-KIT (1:200; mSCF MAB1356), anti-AQP 5 (1:400; Alomone AQP-005), anti-smooth muscle actin (1:200; Sigma A5228), anti-Ki67 (1:300; BD Bioscience 550609), anti-TGF β (1:200; Abcam ab66043), anti-Hey2 (1:100; Sigma HPA030205), anti-collagen IV (1:400; Millipore AB769), anti-E-cadherin (1:100; Invitrogen 131900), anti-E-cadherin (1:200; BD Biosciences 610182), and anti-E-cadherin (1:100; Cell Signaling 3195S). Confocal microscopy images were obtained using an LSM 710 confocal microscope (Carl Zeiss) as described (Joo and Yamada 2014) or an A1R confocal microscope (Nikon) with similar settings. To capture the middle cross section of the epithelium of each gland, Z-stacks that spanned the entire epithelium were acquired, and the middle 3 to 10 μ m of each epithelium was maximally projected for visualization and analysis. These processed middle cross sections were prepared for quantifying immunostaining by thresholding the image for the epithelium and using the Euclidian distance option of Metamorph (Molecular Devices) to outline the basal and suprabasal layers of the distal endbuds of the epithelium.

Intensities were quantified using identical instrument settings for all experimental conditions. For quantification of K19, however, the intensity of K19 immunostaining in the entire epithelium was measured after determining the middle cross sections and maximal intensity projections.

qPCR

RNA from SMGs was isolated using RNeasy-Micro kits (Ambion) and treated with DNase according to the manufacturer's instructions. Using iScript cDNA synthesis kits (Bio-Rad), complementary cDNA (cDNA) was synthesized from the RNA. Subsequently, cDNA was amplified using real-time PCR for 40 cycles of 95 °C (10 s) and 62 °C (30 s). The generation of single amplicon products for each primer set was confirmed by melt curve analysis. Fold changes in gene expression were then determined by normalizing to the housekeeping gene *Rps29*. Data from a minimum of 3 biological samples were plotted as mean $\pm$ SEM. The list of primer sets used for qPCR is provided in the supplementary information.

Fluorescence-Activated Cell Sorting Analysis

Cultured SMGs were dissociated with 0.1% trypsin-EDTA for 15 min to obtain single cell suspensions. Cells were centrifuged, washed in 1% BSA-Hank's balanced salt solution (HBSS), and fixed in cold acetone-methanol (1:1) at -20 °C for 10 min. After 3 washes with HBSS, cells were incubated for 20 min at 4 °C with fluorescently labeled rabbit anti-cytokeratin 14 (Covance, RBP-155P-100), anti-cytokeratin 5 (Covance, RBP-160P-100), rat anti-cytokeratin 19 (DHSB, Troma-III-c), and anti-E-cadherin (BD Bioscience, 610182). Fluorescent labeling was performed with Lightning-link conjugation kits (Innova Biosciences), according to the manufacturer's protocol. After labeling, the cells were washed and resuspended in 1% BSA-HBSS buffer. Subsequently, cells were analyzed on a LSRII Flow Cytometer (BD Biosciences), acquiring at least 20,000 events for each measurement. The data were analyzed by FlowJo software (TreeStar), and gates were set according to appropriate controls (Knox et al. 2013; Lombaert et al. 2013).

Statistics

Sample sizes for each statistical analysis were based on current literature standards and are listed for each analysis. Multiple-group comparisons were first analyzed by 2-way analysis of variance (ANOVA) followed by the Tukey posttest. For qPCR analyses, data were log-transformed and 1-way ANOVA with Dunnett's post hoc test was used to compare more than 2 experimental groups. Differences were considered significant at $P < 0.05$ with N indicating the number of independent experiments and n indicating the number of glands.

Author Contributions

E.E. Joo, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; I.M.A. Lombaert, contributed to design, data acquisition, analysis, and interpretation, critically revised the manuscript;

K.M. Yamada, contributed to conception, design, and data interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

This work was supported by the Intramural Research Program of the National Institute of Dental and Craniofacial Research, National Institutes of Health. EEJ and KMY designed studies and EEJ performed experiments. IMAL designed and performed qPCR, recombination, and fluorescence activated cell sorting assays. EEJ, IMAL, and KMY wrote the manuscript. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article. IMAL is currently affiliated with the Biologic and Material Sciences, School of Dentistry and Biointerfaces Institute, University of Michigan, Ann Arbor, MI, USA.

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