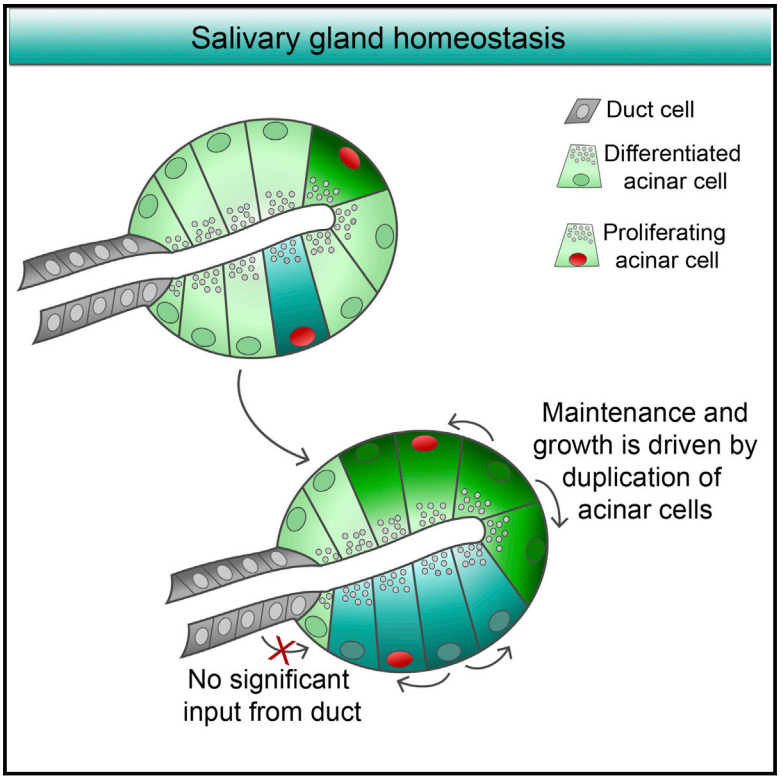
Short Article

Developmental Cell

Salivary Gland Homeostasis Is Maintained through Acinar Cell Self-Duplication

Graphical Abstract



Highlights

- Main mechanism of salivary gland maintenance is self-duplication of acinar cells
- Contribution of stem cells to acinar cell maintenance in salivary glands is minimal
- Acinar cells surviving injury are involved in regeneration of salivary glands

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In Brief

The work of Aure et al. supports a model for salivary gland homeostasis based predominantly on self-duplication of secretory cells, rather than on differentiation of stem cells. The proliferative capacity of differentiated secretory cells may prove critical in the implementation of cell-based strategies to restore damaged salivary glands after radiation.

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Short Article

Salivary Gland Homeostasis Is Maintained through Acinar Cell Self-Duplication

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SUMMARY

Current dogma suggests that salivary gland homeostasis is stem cell dependent. However, the extent of stem cell contribution to salivary gland maintenance has not been determined. We investigated acinar cell replacement during homeostasis, growth, and regeneration, using an inducible CreER^{T2} expressed under the control of the *Mist1* gene locus. Genetic labeling, followed by a chase period, showed that acinar cell replacement is not driven by the differentiation of unlabeled stem cells. Analysis using *R26$^{Brainbow2.1}$* reporter revealed continued proliferation and clonal expansion of terminally differentiated acinar cells in all major salivary glands. Induced injury also demonstrated the regenerative potential of pre-labeled acinar cells. Our results support a revised model for salivary gland homeostasis based predominantly on self-duplication of acinar cells, rather than on differentiation of stem cells. The proliferative capacity of differentiated acinar cells may prove critical in the implementation of cell-based strategies to restore the salivary glands.

INTRODUCTION

Rapid advances in tissue bioengineering and stem cell biology have opened the way for regenerative approaches to restore the function of damaged tissues. One such approach is cell-based, in which the delivery of cells to a damaged organ may lead to functional regeneration. However, the identity of tissue-specific regenerative cells is not always known. Tissue turnover in many adult organs depends on the contribution and differentiation of stem cells, but it is also well established that some tissues are maintained independently of stem cells (Dor et al., 2004; Sangiorgi and Capecchi, 2009; Teta et al., 2007; Yanger et al., 2014).

Severe loss of salivary gland function is a frequent outcome of radiation therapy used to treat head and neck cancers. Decreased saliva production leads to dry mouth, a chronic condition associated with oral infections, increased dental caries, and other often debilitating consequences. Saliva is collectively produced by three major pairs of glands, the parotid (PG), submandibular (SMG), and sublingual (SLG), which although composed of different cell types, share the common make-up of secretory acinar cells linked by a ductal tree. The major cause of dry mouth is a dramatic loss of the secretory acinar cells (Hall, 2000; Vissink et al., 2010), which is irreversible and for which no cure currently exists.

A potential long-term treatment for dry mouth would be replacement of the acinar cells. The ground-breaking demonstration that transplantation of gland germ cells derived from embryonic tissue can produce functional salivary glands showed that regeneration can be accomplished in the adult animal (Ogawa et al., 2013). The challenge is to identify an adult source of cells with similar regenerative properties for use in cell-based therapy. A logical starting point is to investigate the mechanism of salivary gland maintenance. The current view is that stem cells residing in the ducts replace both duct and secretory acinar cells in the adult glands (Pringle et al., 2013). However, to date there has been no definitive lineage analysis demonstrating stem cell contribution to maintenance of the glands. Using a genetic pulse-chase experiment, in combination with single cell clonal analysis, we have investigated acinar cell replacement in adult murine salivary glands.

RESULTS

Acinar Cells Are Maintained by Self-Duplication of Pre-existing Acinar Cells

The PG is comprised predominantly of serous secretory cells, organized in acini, which produce a watery secretion. The SMG contains seromucous cells, which produce a viscous secretion of mucin glycoproteins, whereas the SLG is composed predominantly of mucous cells, each acinus capped by serous cells. The transcription factor *Mist1* (Bhlha15), a marker for salivary gland acinar cells, is expressed from the onset of cell differentiation and persists throughout adulthood (Lemercier et al., 1997; Pin et al., 2000; Yoshida et al., 2001). Immunohistochemistry (IHC) showed Mist1 exclusively localized to acinar cells, with no evidence of expression in duct cells (Yoshida et al., 2001) (Figure 1A). This was confirmed by co-localization of Mist1 with the acinar cell markers, aquaporin 5 (Aqp5) (Figure S1A), and $Na^+/K^+/2Cl^-$ co-transporter (Nkcc1) in all three major salivary glands (Figure S1B).

Acinar cell-specificity of Mist1 expression was reiterated in the *Mist1^{CreERT2}* mouse strain, which carries a tamoxifen-inducible Cre recombinase (CreERT2), inserted at the *Mist1* locus

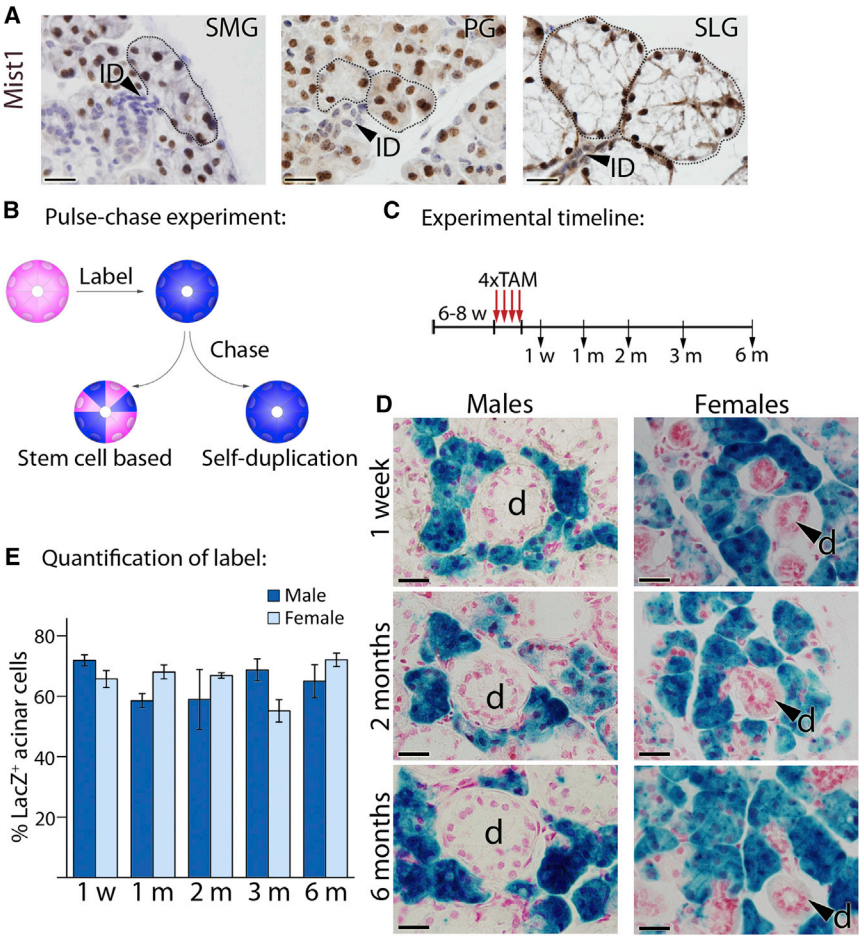

Figure 1. Acinar Cells Are Maintained by Self-Duplication of Pre-existing Acinar Cells

(A) IHC with antibody to the nuclear transcription factor, Mist1, stains acinar cells (brown) in the major salivary glands. Intercalated duct (ID); acinus is outlined.

(B) Schematic diagram depicts potential outcomes of pulse-chase experiment. See text for details.

(C) Experimental timeline.

(D) Male and female SMGs stained for LacZ and counterstained with nuclear fast red at 1 week, and 2 and 6 months following tamoxifen induction.

(E) Quantification of LacZ$^+$ acinar cells per total number of acinar cells counted in glands of males and females isolated at 1 week (w), and 1, 2, 3, and 6 months (m) after tamoxifen administration. Error bars represent ± SEM.

See also Table S1 and Figure S1. Scale bars represent 20 μm.

(Shi et al., 2009). When crossed with the *Rosa26*^{LacZ} (*R26*^{LacZ}) reporter strain, β-galactosidase (LacZ) expression is induced exclusively in Mist1-expressing cells by Cre-mediated removal of a transcriptional stop sequence (Figure S1C). One week after tamoxifen treatment, LacZ was detected specifically in acinar cells of all major salivary glands (Figures S1D–S1F). Untreated *Mist1*^{CreERT2};*R26*^{LacZ} mice showed no LacZ activation (Figure S1G). The *Mist1*^{CreERT2};*R26*^{LacZ} model is therefore cell-type specific, inducible, and heritable.

To determine the extent of acinar cell replacement by stem cells in the adult SMG, we adapted a pulse-chase experiment (Dor et al., 2004) based on genetic labeling of the differentiated acinar cells (see Figure 1B). Following a period in which cell turnover occurs, there are two possible outcomes: (1) a decrease of label over time is expected if replacement of acinar cells originated from unlabeled stem cells, or (2) the percentage of labeled cells will remain constant if labeled acinar cells are undergoing self-duplication. Due to the sexual dimorphism of rodent salivary glands, including differences between male and female SMG in the rates and mechanisms of cell maintenance (Denny and Denny, 1999; Denny et al., 1993), both sexes were included in the analysis.

Mist1^{CreERT2};*R26*^{LacZ} mice were given tamoxifen for 4 consecutive days (Figure 1C). Starting at 1 week following the last tamoxifen treatment and at indicated time points thereafter, SMG were harvested and sections stained for LacZ. In glands from both sexes, acinar cells were labeled at the 1-week point, but duct cells were not (Figure 1D). This pattern was consistent at all time points examined. The percentage of LacZ-positive cells, which included acinar cells and their descendants, was calculated from the number of labeled cells per total number of acinar cells on individual sections (five sections per animal). At 1 week, 72% ± 1.8% acinar cells were labeled in males, and 66% ± 2.8% in females (Figure 1E). At 6 months, the values were 65% ± 5.4% in males, and 72% ± 2.2% in females. Quantification at 1, 2, and 3 months was consistently similar in glands of both sexes, and there was no significant change in the percentage of labeled cells over time (Table S1). Estimates of acinar cell turnover range from 50 to 125 days in salivary glands (Vissink et al., 2010; Zajicek et al., 1985) and acinar cell replacement is expected to occur within the 6-month chase period. Thus, our results indicate that the majority of newly formed acinar cells do not arise from unlabeled stem cells.

Acinar Cells Proliferate and Divide in Adult Salivary Glands

Although acinar cells are frequently described as post-mitotic, early labeling studies consistently reported proliferation in the acinar compartment (Denny and Denny, 1999; Denny et al., 1993; Klein, 1982; Redman and Sreebny, 1970). To confirm proliferation in differentiated acinar cells, we labeled cells with the thymidine analog EdU. Male and female mice (6–8 weeks) were injected with EdU, followed by a 2 hr chase. Sections were probed with antibodies to EdU and Nkcc1. In SMG, 0.9% ± 0.3 and 1.4% ± 0.6% of acinar cells were EdU-positive in males and females, respectively (Figure S2A). In the PG, 1.0% ± 0.2% and 0.6% ± 0.1% cells were positive for both markers in males and females, respectively (Figure S2B). In the SLG, 0.3% ± 0.02% and 0.6% ± 0.08% of both serous and

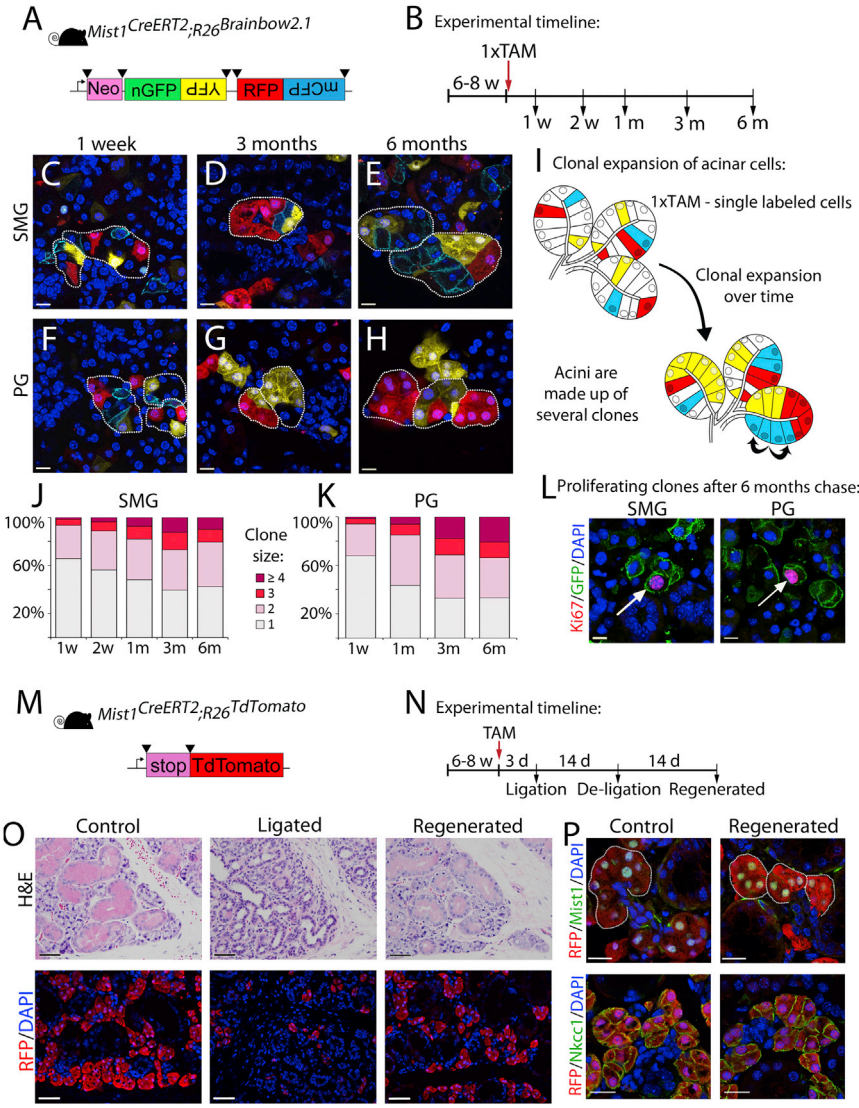

Figure 2. Clonal Analysis and Regeneration of Acinar Cells in the SMG and PG

(A) *Mist1Cre*^{ERT2} was crossed with the *R26*$^{Brainbow2.1}$ reporter strain (schematic). Black triangles, LoxP sites.

(B) Experimental timeline.

(C) Labeled cells in the SMG at 1 week following tamoxifen treatment.

(D and E) Clonal expansion of labeled cells in the SMG at 3 and 6 months after tamoxifen treatment.

(F) Single labeled cells in the PG 1 week following tamoxifen treatment.

(G and H) Clonal expansion of labeled cells in the PG at 3 and 6 months.

(I) Model of acinar cell proliferation and clonal expansion in adult SMG and PG.

(J) Quantification of clone sizes over time in adult SMG shows decrease of single cells and increase in clones of 3 cells or more (see Table S2).

(K) Quantification of clone sizes over time in adult PG (see Table S2).

(L) Proliferation in labeled clones in adult female SMG and PG 6 months after tamoxifen induction detected by Ki67 (red) and GFP antibody (green) (arrows). Scale bars represent 10 μm. See also Figures S2 and S3; Movies S1A and S1B).

(M) *Mist1Cre*^{ERT2} was crossed with the *R26*^{TdTomato} reporter strain (schematic). Black triangles, LoxP sites.

(N) Experimental timeline.

(O) Top: H&E staining of sections from control SMG (left) and ligated SMG (middle) after 14 days ligation; and of ligated SMG (right) after 14 days of regeneration. Bottom: RFP antibody staining (red) of control SMG (left) and ligated SMG (middle) after 14 days ligation; and ligated SMG (right) after 14 days of regeneration. Scale bars represent 50 μm.

(P) Co-localization of RFP (red) and Mist1 (green) or Nkcc1 (green) in acinar cells of control and ligated SMG after 14 days regeneration. Scale bars represent 20 μm.

mucous Nkcc1-positive acinar cells were EdU-positive in males and females, respectively (Figure S2C). Labeling with Nkcc1 and Ki67, a marker of actively dividing cells, (Scholzen and Gerdes, 2000), revealed a slightly higher proliferation rate. This is consistent with Ki67 protein expression throughout the active cell cycle, whereas EdU is only incorporated during the S phase (Kee et al., 2002). Quantification of the total number of Nkcc1/Ki67-positive acinar cells indicated that 2.6% ± 0.8% and 3.8% ± 1.6% in the SMG, 3.5% ± 0.8% and 2.4% ± 0.4% in the PG, and 0.6% ± 0.09 and 0.9% ± 0.2% in the SLG were in active phases of the cell cycle in male and female glands, respectively (Figures S2D–S2F). These results confirm the active proliferation of differentiated acinar cells in the adult salivary glands.

Proliferating Acinar Cells Undergo Clonal Expansion

To directly demonstrate the proliferative activity of individual acinar cells, we used the *Rosa26*$^{Brainbow2.1}$ (*R26*$^{Brainbow2.1}$) reporter strain, which carries four reporter genes in head to tail arrangement, flanked by loxP sites (Figure 2A). When crossed with *Mist1*^{CreERT2}, Cre activation will mediate a recombination event that stochastically juxtaposes one of the four reporter genes directly downstream of the *R26* promoter. As a result, individual acinar cells will randomly express one of the four fluorescent reporter proteins. This recombination event is heritable and allows parallel observation of multiple clones.

Adult female mice (6–8 weeks old) were given a single dose of tamoxifen to restrict labeling to individual cells. Glands were analyzed at indicated time points (Figure 2B). After 1 week, labeled single cells or doublets were randomly scattered in the SMG and PG (Figures 2C, 2F, S3A, and S3B). Clones were defined as clusters of cells of the same color in direct contact with each other within a single acinus. After 3 and 6 months chase, multicellular clones were found in the SMG and PG (Figures 2D, 2E, 2G, 2H, S3C, and S3D). Many acini were completely composed of labeled cells, demonstrating turnover of unlabeled cells and clonal expansion. In both the SMG (Figures 2D and 2E; Movie S1A) and PG (Figures 2G and 2H;

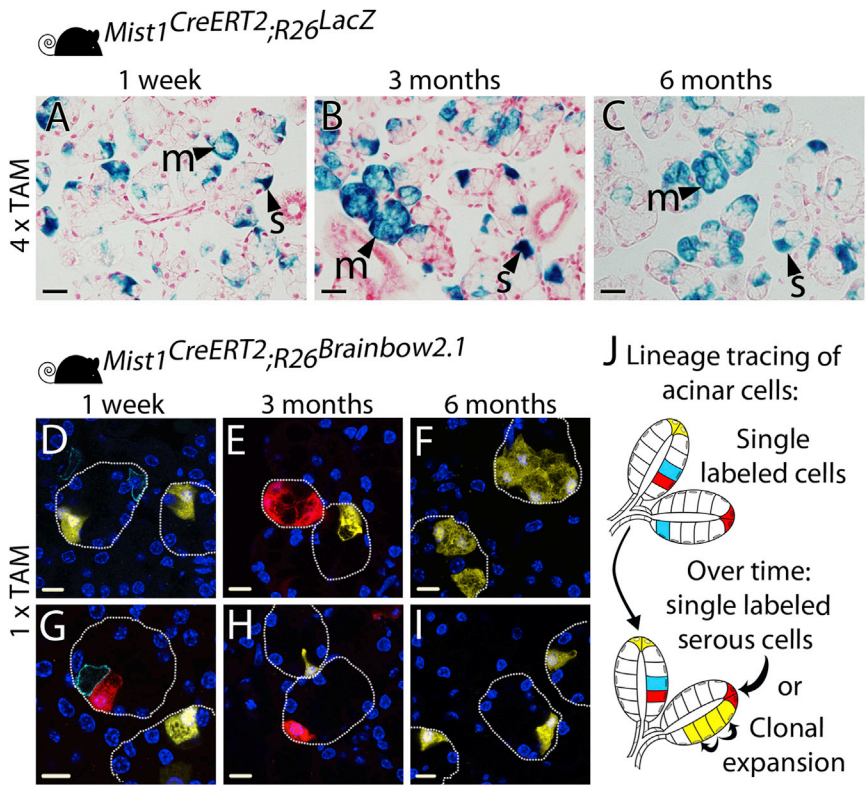

Figure 3. Clonal Expansion of Mist1-Expressing Cells in the SLG

(A) *Mist1*^{CreERT2};*R26*^{LacZ} mice were given tamoxifen for 4 consecutive days. LacZ expression labels serous (s) and mucous (m) acinar cells at 1 week after tamoxifen induction.

(B and C) At 3 (B) and 6 months (C), LacZ positive cells were present as single cells or larger clusters.

(D and G) Single labeled cells in SLG of *Mist1Cre*^{ERT2};*R26*$^{Brainbow2.1}$ mice at 1 week after a single tamoxifen treatment.

(E and F) After 3 and 6 months chase, some acinar cells in the adult SLG were present in monoclonal clusters (see Movie S2A).

(H and I) At 3 and 6 months chase, most labeled cells in the SLG are single cells (see Movie S2B).

(J) Model of acinar cell proliferation and clonal expansion in adult SLG.

(A–C) Scale bar represents 20 μm. (D–I) Scale bar represents 10 μm.

Movie S1B), labeled acini frequently included more than one clone, and clones varied in size. These observations indicate that replication is not dependent on a single cell progenitor and the expansion of each clone proceeds independently. Importantly, the extent of each clone appears to be restricted by the acinus boundary (see Figure 2I).

Clone sizes of approximately one cell thickness from random areas within the *Mist1*^{CreERT2};*R26*$^{Brainbow2.1}$ glands were analyzed using confocal imaging to quantify proliferation and expansion. At 1 week after tamoxifen induction, most labeled cells were singles or doublets in the SMG (93%) and PG (94%) (Figures 2J and 2K). The proportion of single cells was decreased after 2 weeks and 1 month, and after 3 months, was 40% and 33% of all labeled cells counted in the SMG and PG, respectively (Figures 2J and 2K). Some loss of single labeled cells may be due to cell turnover. However, there was a concomitant increase in the proportion of clones composed of three cells or more (27% in the SMG, and 33% in the PG). These results clearly demonstrate that differentiated acinar cells in both the SMG and PG continue to proliferate and clonally expand.

We noted that the size distribution of labeled clones did not change between the 3 and 6 months chase periods (Figures 2J and 2K). Continued proliferative activity in labeled cells was however confirmed by co-localizing Ki67 with the GFP reporter (Figure 2L). We suggest that the limitation of clonal expansion by acinar boundaries eventually leads to a steady state between cell turnover and clonal expansion within each acinus. Once the acinar limit is reached, there is no more increase in the expansion of individual clones.

To test if acinar cell replacement occurs under regenerative conditions, we performed ductal ligation, an induced injury that results in loss of acinar cells, leaving the ducts intact (Tamarin, 1971). These experiments were done using *R26*^{tdTomato} reporter mice to maximize acinar cell labeling efficiency (Figure 2M). Mice were tamoxifen treated and after 3 days, duct ligation was performed on the left SMG, with the contralateral gland serving as control (n = 4). The ligation was removed after 2 weeks, and regeneration proceeded for 14 days (Figure 2N). Histological staining shows loss of the acinar cells after 2 weeks of ligation in comparison to control, and recovery of acinar cells in the regenerated gland (Figure 2O). Notably, after 2 weeks of duct ligation, some labeled acinar cells still survive. After 14 days of regeneration, an increase in labeled cells suggests that they have resumed proliferation and expanded. The labeled cells in the regenerated gland co-localize with antibodies to Mist1 and Nkcc1, indicating that they are acinar cells (Figure 2P). We conclude that duplication of surviving acinar cells contributes to salivary gland regeneration.

Acinar Clonal Expansion in Sublingual Gland

In the SLG, serous and mucous acinar cells are labeled in *Mist1*^{CreERT2};*R26*^{LacZ} mice at 1 week after tamoxifen induction (Figure 3A). In contrast to the SMG and PG, most labeled cells in the SLG remained single or doublets after 3 and 6 months chase (Figures 3B and 3C), although completely labeled acini were also observed. To investigate whether acinar cells clonally expand in the SLG, limited labeling was induced with tamoxifen in the *Mist1*^{CreERT2};*R26*$^{Brainbow2.1}$ mice at 1 week (Figures 3D and 3G). After 3 and 6 months chase, monoclonal clusters of labeled mucous acinar cells were observed (Figures 3E and 3F; Movie S2A), confirming that SLG cells do proliferate and expand in a clonal manner. In contrast, the majority of labeled serous cells remained single even after 6 months chase (Figures 3H–3J; Movie S2B).

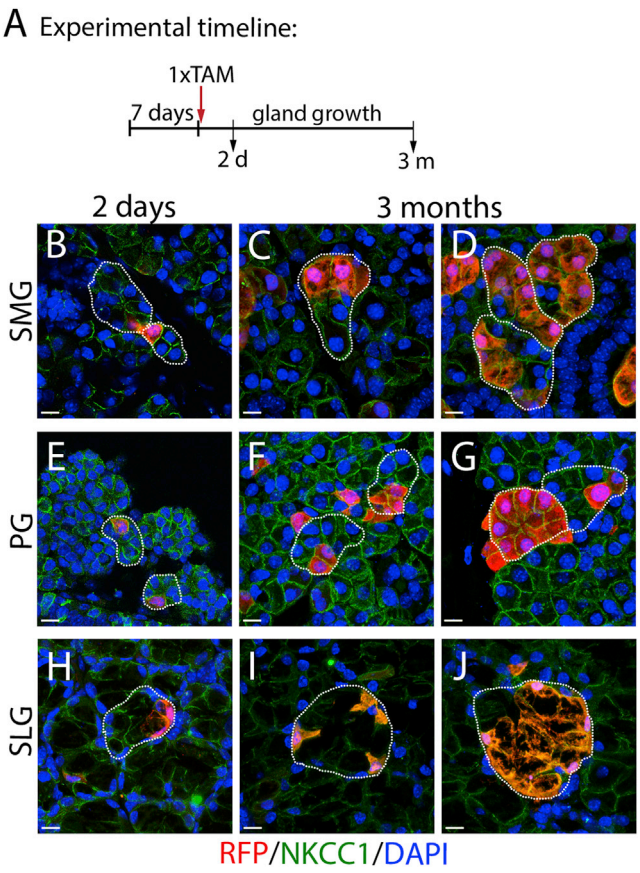

Figure 4. Self-Duplication of Acinar Cells Drives Postnatal Salivary Gland Expansion

(A) Experimental timeline.

(B, E, and H) At 9 days old, single labeled acinar cells were found in all major salivary glands.

(C, F, and I) Single labeled cells or small clones in the salivary glands after 3 months chase.

(D, G, and J) Larger clones in all glands were also found after 3 months chase. Images show anti-RFP (red) and Nkcc1 (green) double antibody staining. Acini are indicated by outline. Scale bars represent 10 μm.

A population of Sox2-positive cells has been identified in the SLG, which are thought to represent an adult stem cell (Arnold et al., 2011). We investigated whether the single labeled cells are related to the Sox2-positive precursor cells present in the SLG. IHC revealed that many, but not all, of the single labeled cells express Sox2 (Figure S4). Thus, there is a population of long-lived single labeled cells that is not correlated with Sox2 expression. Taken together, these results indicate that acinar cells in the SLG self-duplicate and clonally expand, but they also reveal a population of single serous cells, which appear to be very long-lived.

Self-Duplication of Acinar Cells Occurs throughout Postnatal Development

Salivary gland development is initiated at embryonic day 11.5 and continues postnatally (Tucker, 2007). The early postnatal period involves extensive growth and differentiation of the glands (Das et al., 2009; Jayasinghe et al., 1990; Ribeiro et al., 2006). Embryonic organogenesis is driven by progenitor cells (Knox et al., 2010; Lombaert et al., 2013), which may also contribute to postnatal development. However, [^{3}H]thymidine labeling studies in the rat have attributed postnatal gland expansion to the proliferation of the acinar cells themselves (Taga and Sesso, 2001). We therefore investigated whether the self-duplication of acinar cells contributes to the postnatal development phase.

Mist1^{CreERT2};*R26*$^{Brainbow2.1}$ mice were treated with a single dose of tamoxifen at postnatal day 7 and analyzed after 2 days or 3 months (Figure 4A). Sections were stained using antibodies to the RFP reporter and Nkcc1. As expected, single- or double-labeled cells were found in all three glands at 2 days following treatment (Figures 4B, 4E, and 4H). After 3 months, the labeled clones had increased in cell number confirming that self-duplication of acinar cells occurs during postnatal gland growth. As seen in the adult glands, both single cells and small clones were labeled (Figures 4C and 4F). In the SMG and PG, most labeled cells were part of larger clones (Figures 4D and 4G). In contrast, after 3 months chase, most labeled cells in the SLG remained single, with the morphology of serous cells (Figure 4I). This pattern, also observed in the adult SLG, reinforces our conclusion that a subpopulation of SLG serous acinar cells is very long-lived. Occasional, larger clones detected in the SLG (Figure 4J) indicated that the acini were generated from a single precursor. Thus, we conclude that self-replication of acinar cells is the central means of expansion and maintenance in the postnatal salivary gland.

DISCUSSION

We report that the primary mechanism of adult salivary gland maintenance is duplication of differentiated acinar cells. In contrast to the widely held view that acinar cells are replaced through stem cell differentiation (Pringle et al., 2013), our data show that the contribution from stem cells is minimal. We demonstrate that acinar cell proliferation accounts for postnatal growth and expansion of the salivary gland, as well as for maintenance and regeneration of the adult organ. Thus, the salivary glands, like the liver and pancreas, are tissues in which growth and maintenance proceed without direct support of a stem cell pool (Dor et al., 2004; Teta et al., 2007; Yanger et al., 2014).

The prevailing idea that nascent acinar cells arise from stem cells residing in the intercalated ducts is based on varied interpretations of [^{3}H]thymidine labeling experiments (Denny et al., 1999; Man et al., 2001; Schwartz-Arad et al., 1988; Zajicek et al., 1985). Although numerous reports demonstrated active proliferation in acinar cells of the postnatal and adult glands (Dardick and Burford-Mason, 1993; Denny et al., 1993; Man et al., 2001; Taga and Sesso, 2001), experimental limitations and the inability to determine lineage relationships resulted in conclusions that fit a stem cell model. Using genetic labeling to directly trace cell lineages allows us to definitively follow individual labeled cells, and clearly links the proliferation of differentiated acinar cells to the formation of expanded clones.

Clonal analysis in all three glands shows active acinar cell proliferation, and labeling with Ki67 confirms that the differentiated acinar cells continue to be mitotically active. After 3 months, most acini consist of a mix of labeled and non-labeled cells. The

absence of large, dominant clones derived from a single precursor argues against a small population of highly replicative cells, and suggests that all acinar cells are equally able to proliferate. Notably, the expansion of clones is limited to daughter cells within the same acinus, and does not extend beyond acini boundaries, resulting in a steady state of labeling between 3 and 6 months. We suggest that acini act as independent units with regard to cell turnover.

Although we find that acinar cells show regenerative potential even after injury, our data cannot rule out some contribution from adult stem cells in the salivary glands. The pulse chase experiment is dependent on the efficiency of the acinar cell-specific *Mist1*^{CreERT2}, and a small contribution of stem cells to the unlabeled pool may go undetected in this model. Several studies have identified potential stem/progenitor cells in the salivary gland based on in vitro differentiation, self-renewal, or transplantation (summarized in Lombaert and Hoffman, 2010). Lineage-based analysis has identified progenitor cells for all cell types in the embryonic gland (Arnold et al., 2011; Bullard et al., 2008; Knox et al., 2010; Lombaert et al., 2013). However, in the adult, only Sox2-positive cells in the SLG are known to contribute to both acinar and ductal cell types (Arnold et al., 2011). *Mist1*^{CreERT2} labels Sox-2 positive acinar cells in the SLG, which remain single after 6 months chase. Thus, this population of Sox2-positive cells does not make a marked contribution to the steady-state maintenance of the gland. We conclude that although stem cells may play a role, acinar duplication is the predominant mechanism of salivary gland homeostasis.

Radiation induces DNA damage, which impairs or prevents cell division and can result in the death of cells attempting mitosis. The sensitivity of salivary glands to radiation has been considered an anomaly, because acinar cells are thought to be post-mitotic (Vissink et al., 2010). We suggest that the basis of salivary gland radiosensitivity is acinar cell duplication required for cell renewal and regeneration. We speculate that a compromised ability to undergo mitosis will impair gland maintenance and result in overall loss of secretory acinar cells from irradiated glands. Knowing that acinar cells are the primary targets of radiation-induced DNA damage will be crucial for formulating radioprotective strategies.

An important implication of these data is that therapies to treat radiation-induced dry mouth in head and neck cancer patients may not require the identification of a stem cell. We speculate that a better understanding of acinar cell replication could lead to successful in vitro expansion, and use for in vivo repair. In combination with the ability of dissociated salivary gland cells to self-organize (Wei et al., 2007), the proliferative capacity of differentiated acinar cells may enable design of a viable cell-based strategy to repair dysfunctional salivary glands.

EXPERIMENTAL PROCEDURES

Animals and Genotyping

All mouse strains were maintained on a C57/Bl6 background. *Mist1*^{CreERT2} mice were produced as described (Shi et al., 2009) and crossed with the reporter strains *R26*^{LacZ} (*Gt(ROSA)26Sor*^{tm1Sor}), *R26*$^{Brainbow2.1}$ (*Gt(ROSA)26Sor*$^{tm1(CAG-Brainbow2.1)Cle}$/J), or *R26*^{TdTomato} (*Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)Hze}$/J) (all from Jackson Labs). Standard genotyping was performed. All procedures and protocols were approved by the University Committee on Animal Resources at the University of Rochester.

Immunohistochemistry

For single labeling with Mist1 antibody, salivary gland slides were stained following standard protocol for the peroxidase Rabbit IgG Vectastain ABC kit (Vector Labs). For double labeling, fluorescent secondary antibodies were used. An extra blocking step using unconjugated donkey anti-rabbit Fab fragment (Jackson Labs) was added for double staining with same-species primary antibodies. For quantification of double-labeled cells, five random areas (430 × 330 μm) were chosen within each gland, from three biological replicates. See the Supplemental Experimental Procedures for details and antibodies.

β-Galactosidase Staining

Salivary gland slides were stained for β-galactosidase following standard protocol. Five pictures from random areas (20× objective) within each gland were taken from at least three biological replicates. See the Supplemental Experimental Procedures for details.

Clonal Analysis

Labeled cells were identified by co-localization of DAPI staining and endogenous fluorescence from the *R26*$^{Brainbow2.1}$ reporter genes. Five random areas (40× objective) within each gland were chosen for confocal imaging (stacks of ~10 μm thickness), from at least three biological replicates. From merged stacks, clone sizes of RFP-, YFP-, and CFP-labeled cells were analyzed using ImageJ (NIH). An average of 400 and 500 clones per gland at each time point were counted in the SMG and PG, respectively. See the Supplemental Experimental Procedures for details.

Data Analysis

Adobe Illustrator CS6 and Photoshop CS5 (Adobe Systems) were used to make illustrations and perform image adjustments. Changes in contrast and brightness were applied to entire image. All graphs are presented as mean ± SE. At least three biological replicates were included in each group. Statistical significance between two groups was determined using Student's t test ($p < 0.05$).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, two tables, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.02.013.

AUTHOR CONTRIBUTIONS

M.H.A. performed experiments and data analysis and contributed to writing the paper. S.F.K. provided the *Mist1*^{CreERT2} mice and contributed to writing the paper. C.E.O. designed the study and contributed to data interpretation and writing the paper.

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