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Salivary Glands: Stem Cells, Self-duplication, or Both?

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Abstract

Understanding the intrinsic potential for renewal and regeneration within a tissue is critical for the rational design of reparative strategies. Maintenance of the salivary glands is widely thought to depend on the differentiation of stem cells. However, there is also new evidence that homeostasis of the salivary glands, like that of the liver and pancreas, relies on self-renewal of differentiated cells rather than a stem cell pool. Here, we review the evidence for both modes of turnover and consider the implications for the process of regeneration. We propose that the view of salivary glands as postmitotic and dependent on stem cells for renewal be revised to reflect the proliferative activity of acinar cells and their role in salivary gland homeostasis.

Keywords: thymidine labeling, tissue homeostasis, stem/progenitor cells, acinar cells, proliferation, regeneration

Introduction

Salivary glands are perhaps most appreciated by those who suffer the loss of their secretory function. Normally, the 3 major and numerous minor salivary glands work to produce continuous saliva secretion essential for the biological and functional health of the oral mucosa (reviewed in Grundmann et al. 2009). However, in patients treated for head and neck cancer or those developing Sjögren's syndrome, decreased saliva secretion results in a permanent condition known as xerostomia, which is associated with numerous adverse consequences (Saleh et al. 2015). Although the causes leading to xerostomia are diverse, the decrease in saliva production is due to a dramatic loss of functional secretory acinar cells (reviewed in Grundmann et al. 2009).

Management of xerostomia represents a demanding and unresolved clinical challenge. The design of restorative therapies requires knowledge of how the salivary glands normally replace aging cells or repair damaged tissue. The prevailing view is that new secretory acinar and duct cells are generated from resident adult stem cells (reviewed in Pringle et al. 2013). However, the relative contributions of stem cells to salivary gland cell turnover have been unclear. In a recent study, we found that maintenance of acinar cells is derived through self-duplication, with little evidence of stem cell contribution (Aure et al. 2015). This result, although surprising, has been observed in studies that highlighted proliferation as a factor in salivary gland maintenance (Dardick and Burford-Mason 1993; Redman 1995). Although there is significant evidence for stem cells, maintenance of the salivary glands appears to resemble that of the liver and the pancreas, which rely on self-renewal of differentiated cells rather than a stem cell pool. The goal of this review is to reconsider the current view of salivary glands as postmitotic and dependent on stem cells for renewal. We examine the evidence for salivary gland stem cells and the origin of the stem cell–based model, as well as the evidence for simple duplication of differentiated cells in light of the possibility that maintenance and regeneration may depend on stem cells, self-duplication, or both.

The Proliferating Salivary Gland

The assumption that salivary glands rely on stem cells to contribute to tissue turnover has prevailed for years, but this view was not always dominant. The salivary glands are primarily composed of secretory acinar cells, which produce the saliva that drains first into the smallest intercalated ducts (IDs) and is then conducted through larger connecting ducts to the oral cavity. Developmental studies on salivary glands in the mouse and rat routinely showed that all parenchymal cell types have the ability to undergo cell division (Fig. 1; Redman and Sreebny 1970, 1971; Chang 1974; Srinivasan and Chang 1979). In developing rat parotid and submandibular glands (SMGs), mitotic activity of the secretory acinar cells was widely observed (Redman and Sreebny 1970; Cutler and Chaudhry 1974). Redman and Sreebny (1970) specifically noted that acinar cells in adult rat parotid glands contained mitotic figures even after differentiation, as determined by the presence of secretory vesicles. Proliferation of acinar cells in adult salivary glands has subsequently been documented in rat (Redman and Sreebny 1970; Schwartz-Arad et al. 1988; Redman 1995), mouse (Denny et al.

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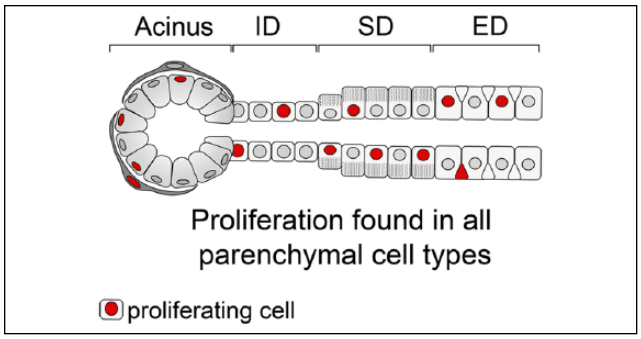

Figure 1. Schematic overview of salivary gland parenchymal cells. The secretory units, acini, are composed of the secretory acinar cells and myoepithelial cells. The ducts are generally classified, in ascending order of size, as intercalated duct (ID), striated duct (SD), and excretory duct (ED). Multiple studies have found active proliferation in all parenchymal cell types.

1993), and human (Dardick et al. 1990; Dardick and Burford-Mason 1993; Ihrler et al. 2002), leading to the suggestion that the acinar compartment need not be dependent on a source of duct stem cells (Redman 1995; Ihrler et al. 2004). In fact, analysis in the mouse SMG yielded the conclusion that "most, if not all, proliferative activity leading to acinar cell population maintenance occurs by self-proliferation" (Denny et al. 1993). Despite this convincing body of evidence, as stem cells moved to central stage, the idea that salivary glands are maintained by stem cells became accepted (Pringle et al. 2013). It is thus important to clarify the role of both stem cells and differentiated cells in maintaining the salivary glands.

The Streaming Salivary Gland

The origin of the stem cell hypothesis can be traced to studies that investigated cell proliferation and differentiation in rat and mouse salivary glands using ^{3}H-thymidine labeling (Chang 1974; Zajicek et al. 1985; Schwartz-Arad et al. 1988; Denny et al. 1993; Denny and Denny 1999; Man et al. 2001; Taga and Sesso 2001). Since ^{3}H-thymidine is incorporated only into dividing cells, all labeled cells should be proliferating. Consistent with the results mentioned above, cells of all types are labeled after short pulse times, although at different frequencies (Chang 1974; Zajicek et al. 1985; Schwartz-Arad et al. 1988; Denny et al. 1993; Denny and Denny 1999; Man et al. 2001; Taga and Sesso 2001). The labeling index of each salivary gland cell type was calculated from the number of labeled nuclei per total nuclei counted. In most cases, the gland compartment with the highest labeling index was the ID (Denny and Denny 1999; Man et al. 2001; Zajicek et al. 1985). With increasing chase time, the number of labeled cells in the ID decreased, and more labeled cells were detected in the wider granulated ducts (Zajicek et al. 1985; Denny et al. 1993). The observation that mitotically active ID cells were located directly adjacent to the striated ducts fueled speculation that they were precursor cells (Chang 1974). A concurrent increase in the number of labeled acinar cells was likewise interpreted to suggest that labeled ID cells differentiate into acinar cells (Zajicek et al. 1985; Denny and Denny 1999; Man et al. 2001).

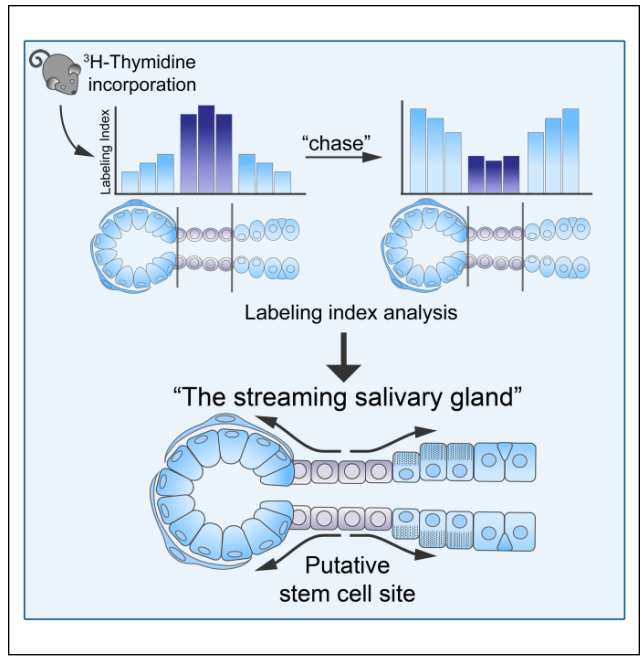

Figure 2. The streaming salivary gland. A series of reports used ^{3}H-thymidine incorporation to investigate proliferation and differentiation in rat and mouse salivary glands. Proliferating cells were labeled with ^{3}H-thymidine, and the number of labeled cells per total cells was used to calculate labeling indices for each cell type (acinar, intercalated duct [ID], and duct). After a chase period, the labeling index in ID was lower, while that in acinar and duct cells was increased. These changes were interpreted as due to the movement and differentiation of stem cells from the ID to acinar or duct cells. Collectively, these reports led to the view that the ID is the site of salivary gland stem cells.

It was also noted that ID cells retain morphology and molecular expression patterns resembling undifferentiated stem cells (Chang 1974; Man et al. 1995; Redman 1995). Based on what was known of stem cells in the intestine and skin, a model was developed, which postulated that a population of undifferentiated stem cells located in the ID is the source of newly differentiated acinar and duct cells. It posited the movement or lineal "streaming" of stem cells from the ID to replenish differentiated cells in the ducts as well as the acinar compartment (Fig. 2; Chang 1974; Zajicek et al. 1985; Schwartz-Arad et al. 1988; Denny and Denny 1999; Man et al. 2001). This concept was adopted by subsequent research groups, and it framed the prevailing view of salivary gland homeostasis and regeneration.

Evidence for Salivary Gland Stem and Progenitor Cells

In line with the idea that renewal and regeneration depend on stem cells, substantial evidence for the presence of stem or progenitor cell types in the salivary glands has been accrued (reviewed in Pringle et al. 2013). Cell populations identified by the expression of markers often associated with stem cells—including cKit, Sca1 (Hisatomi et al. 2004), integrin α6β1/CD49f (Matsumoto et al. 2007; Sato et al. 2007; David et al. 2008), and CD24^{hi}/CD29^{hi} (Nanduri et al. 2014)—have all been implicated as salivary gland stem or progenitor cells.

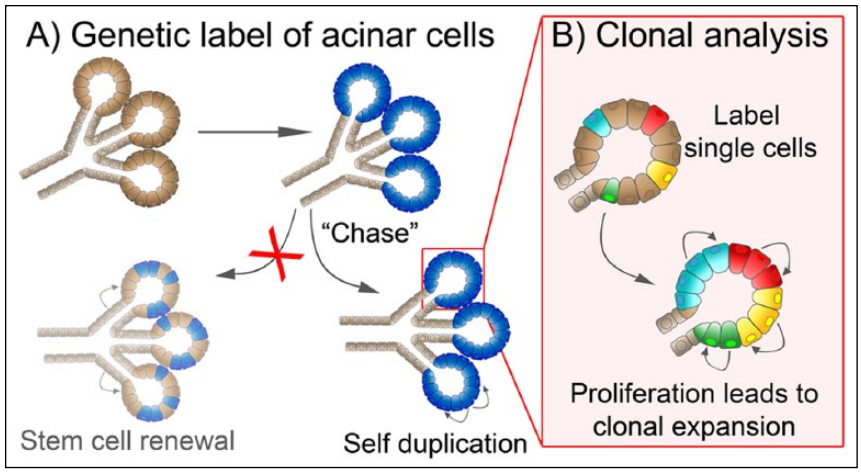

Figure 3. Salivary gland acinar cells are self-duplicating. (**A**) Genetic labeling of differentiated acinar cells followed by a chase period was used to detect the contribution of unlabeled stem cells to acinar cell replacement. Putative stem cells in the intercalated duct should replace labeled acinar cells with unlabeled as the glands age. No decrease in the number of labeled cells was observed, even after 6 mo, indicating self-duplication as the main source of newly formed acinar cells. (**B**) Expression of fluorescent reporter proteins was randomly activated in single differentiated acinar cells. After a chase period, the individual acinar cells had proliferated and expanded into clones, directly demonstrating that new acinar cells are derived from self-duplication.

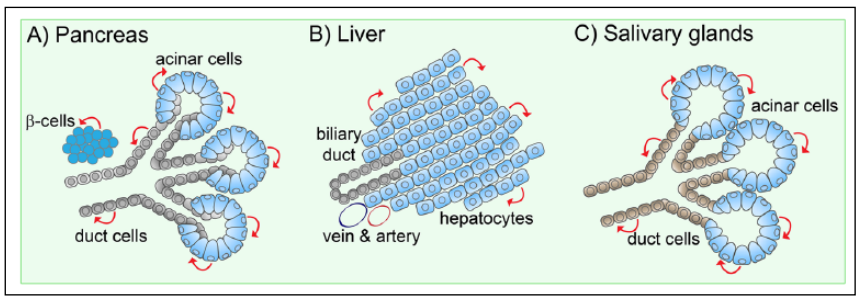

Figure 4. Self-duplication as a common strategy for tissue maintenance. Extensive investigation has established that the secretory exocrine cells of the pancreas and liver as well as endocrine β cells are maintained through self-renewal rather than by differentiation of a stem cell pool (**A** and **B**; reviewed in Tetteh et al. 2015). Tissue maintenance in salivary glands is analogous to pancreas and liver, as self-duplication of differentiated cells is evident (**C**).

Label retaining cells, thought to be slow-cycling stem cells, have been detected in all parenchymal compartments (Kim et al. 2008; Kimoto et al. 2008; Chibly et al. 2014) and shown to colocalize with some stem cell markers (Chibly et al. 2014). Recent data based on a histone H2BGFP model characterized mitotically active cells in the ID and excretory duct (Kwak and Ghazizadeh 2014), sites of cKit-expressing cell populations thought to represent stem cells (Pringle et al. 2013). Although many of these cell populations display stem or progenitor cell potential in vitro (Hisatomi et al. 2004; Matsumoto et al. 2007; Sato et al. 2007; David et al. 2008; Lombaert et al. 2008) and some demonstrate regenerative properties when transplanted into irradiated glands (Lombaert et al. 2008; Nanduri et al. 2014), their in vivo differentiation potential must still be established.

Lineage-based analysis has demonstrated that progenitor cell populations present in the embryonic gland give rise to multiple cell types (Bullard et al. 2008; Knox et al. 2010; Arnold et al. 2011; Lombaert et al. 2013). In postnatal glands, however, direct evidence for stem cell activity through lineage tracing is not yet available for most of the candidate cell populations, and their role in salivary gland maintenance remains unclear. To date, the only lineage tracing in adult tissue showed that Sox2-expressing cells generated both acinar and duct cells in sublingual glands, suggesting that they are multipotent adult stem or progenitor cells (Arnold et al. 2011). Previous work in our laboratory showed that the transcription factor Ascl3 marks progenitors that generate acinar and duct cells (Bullard et al. 2008). However, these data were based on a constitutively active Cre allele, which most likely labels acinar and duct cells due to prenatal expression of Ascl3. Although we have demonstrated that Ascl3-expressing cells isolated from adult glands have the ability to generate both cell types in vitro (Rugel-Stahl et al. 2012), their in vivo potential in adult glands has not yet been confirmed. In summary, several stem and progenitor cell populations have been identified in the salivary glands, but what remains to be clarified is which of the candidate populations may contribute to gland upkeep and under what conditions.

Determining the Stem Cell Contribution to Acinar Cell Homeostasis

To determine the contribution of duct stem cells to acinar cell replacement in adult salivary glands, our laboratory recently performed fate-mapping experiments (Aure et al. 2015). In contrast to the previously described isotope-labeling experiments, our studies relied on genetic methods of lineage tracing, allowing us to directly follow descendants of the labeled cells. According to the stem cell model, genetic labeling of differentiated acinar cells, followed by a chase period, should lead to a decrease in labeled cells, due to input from the unlabeled stem cell source (Fig. 3A). However, over a period of 6 mo, we observed no decrease in the number of labeled acinar cells, suggesting that there is little contribution from stem cells to replacement. Based on the heritable nature of the lineage label, these data implied that acinar cells are self-duplicating.

To directly examine whether acinar cells are maintained by self-duplication, we analyzed acinar cell proliferation through multiplex lineage tracing using the *Rosa26*$^{Brainbow2.1}$ reporter mouse strain (Aure et al. 2015). The Brainbow system enables stochastic expression of multiple fluorescent proteins from a single transgene (Livet et al. 2007). By crossing the *Rosa26*$^{Brainbow2.1}$ reporter strain with an acinar-specific, inducible

Cre^{ERT2} mouse strain (Shi et al. 2009), individual acinar cells are labeled in a mosaic pattern. Over time, clusters of unicolored cells were detected, directly demonstrating the proliferative activity and clonal expansion of mature differentiated acinar cells in the adult salivary gland (Fig. 3B). Furthermore, analysis after ductal ligation showed that duplication of surviving acinar cells also contributes to salivary gland regeneration (Aure et al. 2015). Together, these results demonstrate that differentiated acinar cells are self-duplicating and are maintained without a significant input from stem/progenitor cells.

Revised Model of Salivary Gland Homeostasis

In support of the streaming salivary gland model (see Fig. 2), recent evidence indicated that a population of stem/progenitor cells, marked by expression of keratin 14, can differentiate into ductal cell types (Kwak and Ghazizadeh 2014). However, differentiation of ID cells to acini has not yet been clearly demonstrated. Our conclusion that acinar cells are maintained by self-renewal contradicts the stem cell model but is consistent with a large body of evidence documenting acinar cell proliferation in adult glands (Redman and Sreebny 1970; Dardick et al. 1990; Dardick and Burford-Mason 1993; Denny et al. 1993; Redman 1995; Ihrler et al. 2002; Ihrler et al. 2004). With longer chase times, the number of labeled acinar cells increased in the thymidine-labeling studies (Zajicek et al. 1985; Schwartz-Arad et al. 1988; Denny et al. 1993; Denny and Denny 1999; Man et al. 2001) and was interpreted as movement of cells from one compartment to another based on the stem cell model. However, in light of recent direct evidence, the increase of labeled acinar cells is also consistent with the conclusion that they are proliferating, leading to the generation of twice as many labeled cells. We therefore propose that the prevailing view of salivary glands as postmitotic and dependent on stem cells for renewal be revised to reflect the proliferative activity of acinar cells and their role in tissue turnover. This view does not rule out a role for stem cells but requires a modification of the classical salivary gland stem cell model.

Mechanism of Salivary Gland Regeneration

Although radiation damage is generally not reversed, regeneration does occur in the salivary glands after some injuries. Ligation of the main excretory duct, the injury model most commonly used, results in acinar cell loss, while leaving ducts relatively intact (Takahashi et al. 2004). Removal of the ligation is followed by regeneration and replacement of acinar cells, which is thought to occur through the activation of stem cells present in the surviving ducts (Cotroneo et al. 2008; Cotroneo et al. 2010).

Consistent with earlier reports (Cotroneo et al. 2010), we observed that acinar cells can survive the ligation injury and subsequently proliferate and expand during regeneration (Aure et al. 2015). It has also been reported that regeneration after partial excision of the rat SMG is characterized by a very high rate of acinar cell mitosis (Boshell and Pennington 1980). Proliferation of mature acinar cells in human salivary glands is also significantly increased in response to the injury of chronic sialadenitis (Ihrler et al. 2004). Thus, regeneration of the salivary glands in rodents and humans involves self-renewal of acinar cells. However, it has not yet been established whether acinar cells serve as the source of all regenerating cells or if reserve stem cells are activated during salivary gland regeneration. Although a recent study has ruled out the presence of quiescent stem cells in the ducts of mouse SMG (Kwak and Ghazizadeh 2014), further investigation is required to delineate the respective roles of stem and differentiated cells in gland regeneration.

Simple Duplication Is a Common Strategy for Tissue Maintenance

The maintenance of salivary gland acinar cells is analogous to that in the pancreas and the liver, where differentiated cells are maintained by self-renewal (Fig. 4; reviewed in Tetteh et al. 2015). In a hallmark study (Dor et al. 2004), genetic labeling was used to prove that endocrine β cells arise from preexisting β cells and that replication of β cells is the primary mechanism of postnatal growth and maintenance (Georgia and Bhushan 2004) without the requirement for specialized progenitors (Brennand et al. 2007; Teta et al. 2007). It has subsequently been established that homeostasis of pancreatic acinar and duct cells, as well as α and β cells under normal conditions, is maintained through self-duplication without drawing on a stem cell pool (Desai et al. 2007; Strobel et al. 2007; Solar et al. 2009; Kopinke and Murtaugh 2010). Although clearly involved in embryonic development of the pancreas, the presence of stem cells in the adult organ has not been definitively established (Jiang and Morahan 2014). As a result of this uncertainty, the regenerative capacity of the pancreas has attracted much interest, and studies have revealed that differentiated cell types can function as reserve cells to repair tissue damage (Puri et al. 2015). In fact, it has been demonstrated that after injury, pancreatic acinar cells can transdifferentiate into insulin-secreting β cells (Pan et al. 2013), suggesting a high degree of cellular plasticity.

Controversy still exists over the presence of stem cells in the liver (reviewed in Tetteh et al. 2015). Studies have demonstrated that mature hepatocytes self-renew under normal conditions, as well as regenerate or even transdifferentiate following injury (Yanger et al. 2013; Schaub et al. 2014; Tanimizu et al. 2014; Tarlow et al. 2014; Yanger et al. 2014). In fact, all mature and differentiated liver cell types participate in liver regeneration after injury without contribution from stem cells (Tarlow et al. 2014; Yanger et al. 2014). What remains unclear is whether facultative progenitor cells exist in the adult liver.

The plasticity exhibited by differentiated cells in these and other organs challenges classically defined stem cell hierarchies. Rather than a 1-way path from a stem/progenitor cell to a functionally mature differentiated cell, it is becoming clear

that cell fate is much more flexible (reviewed in Puri et al. 2015; Tetteh et al. 2015). Although it is not known whether salivary gland cells also possess this plasticity, it is intriguing to note that Sca-1/cKit-positive progenitor cells isolated from adult mouse salivary glands after injury were found to differentiate into pancreatic and hepatic cell types (Hisatomi et al. 2004). In addition, the ablation of Ascl3-positive cells, which give rise to both acinar and duct cell types, yielded no discernable phenotype (Arany et al. 2011), implying that other cells in the salivary gland have the potential to compensate. It will be interesting to explore whether fully differentiated salivary gland acinar cells have transdifferentiation potential that could be useful in a therapeutic setting.

Concluding Remarks

Active proliferation of differentiated acinar cells in the salivary glands has been widely observed, and acinar cell self-duplication has now been directly visualized (Aure et al. 2015). This suggests that the model for salivary gland homeostasis be modified to include acinar cell self-duplication as a means for replacement of aging or injured cells. However, the evidence for stem cells in the salivary gland is also convincing. Although we found little stem cell contribution to normal homeostasis (Aure et al. 2015), it is not yet known whether stem cells are required for regeneration of the salivary glands. Experiments to determine the source of regenerated cells after salivary gland injury could answer this question and have become a high priority.

In the meantime, recent advances in our knowledge of potential stem cell populations, as well as the intrinsic proliferative capacity of differentiated acinar cells, offer exciting options for strategies to treat salivary gland dysfunction and xerostomia. In vitro demonstrations of stem cell potential should be followed by further investigations into their therapeutic ability. At the same time, recognition of the proliferative capacity of differentiated acinar cells could shift emphasis away from the requirement for stem cells in cell-based approaches. In addition, investigation into the potential plasticity of salivary gland cells might reveal exciting and previously unrecognized abilities. Such studies could have a profound effect on potential repair strategies.

Author Contributions

M.H. Aure, contributed to conception, design, and data interpretation, drafted and critically revised the manuscript; S. Arany, contributed to conception, drafted and critically revised the manuscript; C.E. Ovitt, contributed to conception and data interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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