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# Concise Review: A Critical Evaluation of Criteria Used to Define Salivary Gland Stem Cells

**Pei-Lun Weng**[a,b], **Marit H. Aure**[c], **Catherine E. Ovitt**[d]

[a]Department of Dermatology, Yale University, New Haven, Connecticut, USA;

[b]Department of Pathology, Yale University, New Haven, Connecticut, USA;

[c]Matrix and Morphology Section, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland, USA;

[d]Center for Oral Biology, Department of Biomedical Genetics, University of Rochester School of Medicine and Dentistry, Rochester, New York, USA

## Abstract

In the effort to develop cell-based therapies to treat salivary gland dysfunction, many different populations of cells in the adult salivary glands have been proposed as stem cells. These cell populations vary, depending on the assay used, and are often nonoverlapping, leading to the conclusion that salivary glands harbor multiple stem cells. The goal of this review is to critically appraise the assays and properties used to identify stem cells in the adult salivary gland, and to consider the caveats of each. Re-evaluation of the defining criteria may help to reconcile the many potential stem cell populations described in the salivary gland, in order to increase comparability between studies and build consensus in the field.

## Graphical Abstract

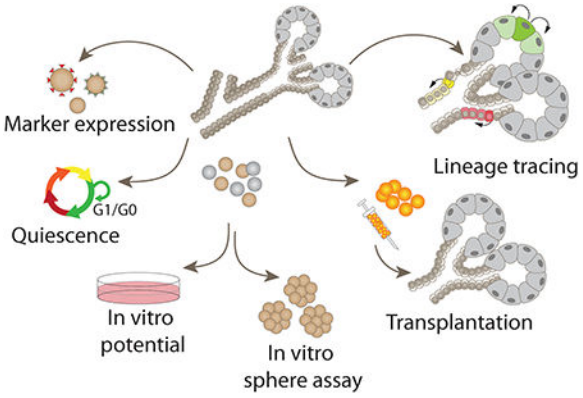
Correspondence: Catherine E. Ovitt, Ph.D., Center for Oral Biology, Department of Biomedical Genetics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, USA. Telephone: 585-275-2920; catherine_ovitt@urmc.rochester.edu.

Author Contributions

P.-L.W.: conception and design, manuscript writing, final approval of manuscript; M.H.A.: conception and design, manuscript writing, final approval of manuscript; C.E.O.: conception and design, financial support, manuscript writing, final approval of manuscript

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.




**Keywords**

Stem cell; Salivary gland; Progenitor cell; Lineage-restricted; Acinar

## Introduction

Salivary gland dysfunction, as a result of radiation therapy for head and neck cancer, or of disease, such as Sjögren's Syndrome, is a permanent and debilitating condition. Regenerative approaches are focused on cell-based strategies, which require identification of cells with the potential to replace the salivary gland duct and secretory acinar cell types. Salivary gland maintenance and regeneration has been widely held to depend on adult stem cells [1]. Many studies have reported the identification of often nonoverlapping, potential stem cell populations in mouse, rat, and human salivary glands [2]. To reconcile the various reports, it is often concluded that the salivary glands harbor multiple stem cell populations [1, 2].

No clear consensus exists on what criteria should be applied for the identification of putative salivary gland stem cells. Those used have included expression of stem cell-associated markers, ability to proliferate or differentiate in vitro, ability to form spheres, rescue of salivary function following transplantation into irradiated glands, and in vivo lineage tracing (Fig. 1). Although several of these features are consistent with the definition of a stem cell, singly each of these assays has caveats and are open to alternative interpretations. We propose that the number of potential stem cell populations identified in the salivary glands may reflect the uneven application of criteria used to define a stem cell. The purpose of this review is to critically evaluate the properties and assays on which salivary gland stem cell identification has been based, with the goal of reconciling the various reports and building a consensus in the field.

## Defining and Distinguishing Stem and Progenitor Cells

Classically, there are two key properties that define a stem cell: (a) the unlimited ability to self-renew, and (b) the ability to differentiate into more than one mature cell type [3]. To date, adult stem cells that meet these criteria have been found in only a few tissues [4, 5], such as the intestine and hematopoietic system [6, 7]. It is now recognized that adult stem

cells from different tissues do not share identical properties [8]. For example, quiescence is a defining characteristic of hematopoietic, satellite muscle, and neural stem cells [8], while hair follicle and intestinal stem cells undergo rapid and continuous proliferation [9]. This variability in stem cell characteristics has made it difficult to establish rigorous criteria for defining adult stem cells.

It is critical to recognize the difference between stem cells and progenitor cells, which although frequently mentioned interchangeably, are not equivalent and exhibit distinct properties [10]. Stem cells can replicate indefinitely and produce both undifferentiated and differentiated progeny. Progenitor cells undergo only a finite number of cell divisions, do not selfrenew, and are often limited in the number of cell types they can generate [11]. This difference is difficult to experimentally distinguish, but critical to recognize. Long-term self-renewal and multipotent differentiation capacity are functional properties that require rigorous analysis of the cells within their native tissue niche. Because it is difficult to identify stem cells meeting these criteria in vivo, the trend has been toward loosening the criteria to those that describe progenitor cells. However, the removal of stem cells from their niche for in vitro analysis can result in alteration of cell properties [10], leading to observations, which may not reflect in vivo behavior. Ultimately, in vitro evidence of stem cell properties must be corroborated in vivo to unambiguously identify a stem cell. The ability to contribute to salivary gland repair may not require a bona fide stem cell, but the fundamental differences between stem and progenitor cells could be important when using cells that should ideally last the lifespan of the patient. In theory, and very likely in practice, stem and progenitor cells will not be equal in their long-term capacity to repair damaged or diseased tissue. Thus, a common consensus on the criteria used to define stem cells is needed.

## Stem Cell Marker Expression

The identification of stem cells in the salivary gland has often been based on the expression of specific markers associated with stem cells in other organs [2]. c-KIT (CD117) and stem cell antigen 1 (SCA-1), surface markers of hematopoietic stem cells [12], are both expressed by subpopulations of cells in the salivary glands [13]. Cells expressing these markers were observed to increase in number after duct ligation injury, supporting the idea that they are stem cells involved in gland repair [13]. Keratin 5 (K5) and Keratin 14 (K14) are cytoskeletal proteins expressed in basal epithelial cells of many adult tissues, which in trachea and olfactory epithelium act as stem cells [14, 15]. In the developing salivary glands, K5 and K14 positive cells are embryonic progenitors of acinar and duct cells [16, 17], leading to the proposal that they act as stem cells in adult glands [16, 17]. The leucinerich repeat containing G-protein coupled receptor 5 (LGR5), is an established marker of adult stem cells in numerous tissues including the intestine [6] and hair follicles [18]. LGR5-expressing cells in human parotid and submandibular glands have been proposed to be stem cells [19].

Although there is some overlap, the salivary gland cell populations expressing general stem cell markers such as c-KIT, K5, or LGR5 are diverse. Notably, all these proposed stem cells are located in the salivary gland ducts. The assumption that salivary gland stem cells are

localized in the ducts originated with early thymidine labeling studies and was based primarily on anatomical proximity to labeled acinar cells [20, 21]. The expression of stem cell markers such as SCA-1 by duct cells supported this hypothesis [1]. However, identification of a stem cell based on gene expression has several caveats. No universal stem cell marker has been identified [5, 22], and expression of general stem cell markers is not strictly limited to stem cells. For example, LGR5, well-recognized as a stem cell marker, is also expressed in the olfactory bulb in a large subset of postmitotic neurons [23]. In support of these arguments, recent assays have shown that duct cells expressing c-KIT or K14 do not function as multipotent stem cells in adult salivary glands [24, 25].

The discovery of cells in adult salivary glands that express mesenchymal stem cell (MSC) surface antigens, including CD44, CD49f (integrin), CD90, and CD105, prompted suggestions that they are stem cells [19, 26–28]. These cells can differentiate into chondrocytes, osteoblasts, and adipocytes in vitro and have been analyzed for their ability to contribute to salivary gland acinar and duct cell lineages [19, 26, 27]. Transplantation of the MSC-like cells partially rescues radiation-induced salivary gland dysfunction [19, 27], but in vivo lineage tracing to acinar and duct cells remains to be explored.

## Quiescent or Proliferative Stem Cells

Due to uncertainty over their identity, the search for salivary gland stem cells has focused on both quiescent and rapidly dividing cell populations. Label-retaining assays are based on the idea that quiescent stem cells are slowly cycling and retain a DNA label over time in pulse-chase experiments, whereas continued division of nonquiescent cells will eventually dilute the label. Label retaining cells (LRCs) identified in the salivary glands have been suggested to be quiescent stem cells [29–31]. The advantage of label retaining assays is the unbiased approach to stem cell identification, independent of protein markers. A caveat is that the DNA label will be retained by cells undergoing terminal differentiation, as well as by potential quiescent stem cells [32, 33]. Consistent with this, LRCs are found in all parenchymal compartments of the salivary glands [29–31, 34], and can colocalize with markers of differentiated acinar or duct cells [30, 31, 34, 35]. Due to the low rate of cell turnover in the adult salivary gland, cells labeled during an earlier proliferative phase will be retained for long periods. Another limitation of stem cell identification based on label-retaining assays is that the outcome varies with the labeling strategy (age of animal at labeling) and the experimental design (length of chase time). Labeling done during embryonic development identified LRCs after long-term chase that had low proliferative potential in vitro and did not actively proliferate following injury [34]. In contrast, labeling postnatally followed by a shorter chase identified LRCs that showed self-renewal capacity in vitro [31] and in vivo proliferation in response to injury [30, 35]. Labeling at embryonic or postnatal stages likely marked different populations of dividing cells. For unambiguous classification of the LRCs as stem cells, characterization of their in vivo lineage potential needs to be conducted.

In contrast to LRCs, many studies have searched for rapidly dividing stem cells in the salivary gland. Several populations of potential stem cells have been designated based on in vitro proliferation of dissociated primary cells [19, 26–28, 36, 37]. These cells are a

heterogeneous population that often expresses c-KIT, K5, or K14, suggesting a ductal cell origin. The combination of in vitro proliferation and expression of these general stem cell markers is taken as proof that they are stem cells. However, in vitro proliferation is not a characteristic unique to stem cells [10]. Many differentiated cell types, including primary salivary gland cells, continue to proliferate in vitro for several cell divisions [38]. Long-term proliferative potential should be confirmed in vivo within the native niche environment of the potential stem cell.

## Assessing In Vitro Potential

Several potential salivary gland stem cells have been identified based on the in vitro potential to generate acinar, duct, and myoepithelial cell types [19, 36, 39–43]. A major caveat of using in vitro assays to determine stem cell potential is the removal of a cell from its native location [44]. Most cells are capable of changing their phenotypic properties in response to the surrounding microenvironment [5], and particularly under stress, may exhibit the plasticity to transition to intermediate, dedifferentiated, or alternate cell types [45]. For example, the stem cell marker LGR5 is not expressed in the adult pancreas, but is induced when pancreatic duct cells are cultured in vitro [46]. Thus, stem cell-like characteristics may be an artifact of in vitro culture [5]. Studies in which the stem cell properties appear only after several passages in culture [47] are particularly suspect, as more time in culture introduces the likelihood of alterations in cellular properties. The use of in vitro assays to define stem cell potential should involve rigorous demonstration that cell differentiation is accompanied by a decrease in stem cell marker expression, and increased expression of differentiation markers. Differentiated cells generated in vitro should not express markers of more than one cell type, and should not continue to express stem cell markers.

Some potential stem cell populations isolated from salivary glands have the capacity to differentiate into chondrogenic, osteogenic, and adipogenic cell types, a characteristic of MSCs [19, 26, 27]. Culture of these cells in Matrigel yields branched and aggregated structures resembling native salivary gland acini and ducts [28, 48]. Similar structures have been generated by mouse and human salivary gland cells cultured in Matrigel [36, 40]. However, it is necessary to determine if cells in these structures express acinar and duct cell-specific markers.

## Sphere Assays to Determine Self-Renewal

The ability to form spheres in vitro has been used to evaluate stem cell self-renewal and multipotency [49]. The use of this assay assumes that each sphere originates from a single cell, and that only self-renewing stem cells can form spheres [49]. In vitro sphere formation by salivary gland cells is well established [37, 40–43, 50, 51]. However, in many cases, sphere assays have been performed using the heterogeneous mixture of cells obtained after dissociation of an entire gland [36, 37, 42, 43, 50, 51]. Video analysis of dissociated salivary gland cells cultured under nonadherent conditions showed that the cells rapidly aggregated within 24–48 hours [52]. The cell aggregates formed included differentiated cells, and did not originate from single stem cells. To insure that spheres are clonal and derived from a single initiating cell, it is necessary to exclude the possibility of random cellular aggregation

[40, 41, 49]. This has been done using flow cytometry followed by plating single cells in Matrigel [40, 41, 50]. Even so, in one study, four isolated and distinct populations of mouse salivary gland cells each formed spheres [50], suggesting that sphere formation may be a general property of many cell types in vitro.

In theory, if derived from a stem cell, salivary spheres should include cells expressing acinar, duct, and myoepithelial markers, as well as stem cell markers. However, expression of the differentiated cell markers should be mutually exclusive, such that single cells do not express markers of more than one cell type. Continued expression of a general stem cell marker by all cells in a sphere raises doubts about the nature of the spheres, and the conclusion that they represent stem cells.

Secondary sphere formation is also used as proof of stem cells, but has similar caveats. Dissociation of a primary sphere will generate a heterogeneous population of cells that includes differentiated cell types, which can continue in vitro proliferation for several generations [38]. As with primary spheres, it is therefore necessary to establish clonality and rule out cell aggregation. In addition, once generated, secondary and tertiary spheres formed from potential stem cells should be reanalyzed for evidence of differentiation to acinar, duct, and myoepithelial cells.

## In Vivo Transplantation

The ability of a single cell to repopulate a tissue provides definitive proof of self-renewal and multilineage potential. Evidence that a single cell could permanently repopulate the entire hematopoietic system established the identity of the HSCs [53]. Similarly, transplantation of a single basal mammary stem cell led to generation of a fully functional mammary gland [54]. Interestingly, additional studies have uncovered more than one cell population with this multipotency [55].

Transplantation has frequently been used as an assay for defining salivary gland stem cells by testing whether saliva secretion can be restored following cell injection into irradiated glands [27, 40, 41, 43, 48, 50, 56–61]. However, in most studies, cell populations rather than single cells were injected and trans-plantation protocols vary widely. Potential stem cells have been transplanted as early as one day [27], or as much as 90 days after irradiation [43], and in one study, cells were transplanted multiple times [60]. Such variability in the experimental time-lines compounds the difficulty of comparing studies. Additional discrepancies include the radiation dose used for the transplantation recipients, which ranges from 2 Gy to 18 Gy [27, 60], resulting in widely different degrees of salivary gland damage [62]. To demonstrate that potential stem cells can rescue irradiated salivary glands, it should first be established that the radiation dose used has caused measurable damage to the gland, including a sustained decrease in saliva secretion.

A frequent expectation in these studies is that the transplanted cells survive, engraft in the target tissue, and differentiate into acinar and duct cells. Although most studies show improved saliva secretion and reduced acinar cell loss, there is limited evidence for cell engraftment or for significant contribution of the transplanted cells to restore salivary gland

tissue. An alternative possibility is that the transplanted cells promote the survival or regeneration of irradiated endogenous cells through paracrine signaling. In support of this, a recent study demonstrated that MSCs, immobilized through encapsulation in hydrogel and transplanted into irradiated glands, could improve functional saliva secretion and restore acinar cell mass [63]. Not surprisingly, one potential stem cell population identified in the salivary glands expresses elevated levels of at least seven growth factors, including glial cell line-derived neurotrophic factor (GDNF) [41]. Direct injection of these cells into irradiated salivary glands promoted saliva secretion and preserved acinar cells, as did injection of the GDNF factor alone [41]. Other studies have reported that injection of keratinocyte growth factor (KGF) or insulin-like growth factor 1 (IGF1) can rescue salivary glands from radiation damage [64–67]. These results suggest that irradiated glands can respond to paracrine signals and that endogenous cells may be induced to repair and restore gland function. Given these findings, stem cells identified through rescue of salivary gland function after transplantation should be re-evaluated to explore potential paracrine activity.

## In Vivo Lineage Tracing

Heritable genetic labeling reveals the in vivo relationship between precursor and progeny, and is considered the gold standard for establishing stem cell potential [5]. Typically, lineage tracing experiments rely on Cre recombinase activity to drive heritable expression of a reporter in progeny cells. Although this powerful tool has offered a better understanding of in vivo line-age relationships, lineage tracing hinges on the specific Cre used. Caution must therefore be used when analyzing the outcome of such experiments, as Cre drivers may have dynamic expression patterns or label heterogenous cell populations.

Lineage tracing has been used to determine the in vivo potential of several proposed stem cell populations in the adult salivary glands [24, 25, 68–74]. Under normal physiological conditions, the proposed K5-expressing and K14-expressing stem cell populations labeled only duct cells [16, 17, 25, 71, 73]. However, K14 cells undergo continuous cycling in vivo, contribute to differentiated cells in the granular ducts, and retain label after 28 weeks, indicating that they are a lineage-restricted unipotent stem cell population [25]. Lineage tracing also revealed that potential stem cells expressing c-KIT [24, 70, 73] and Wntresponsive Axin2 [71] are restricted to generating duct cells in adult. In contrast to these results, lineage tracing of p63-positive cells, which contribute to all salivary gland cell lineages during embryonic development, labeled a small number of acinar cells, in addition to duct and myoepithelial cells [72]. Multiple signaling pathways are known to control p63 expression [75] and dynamic extracellular signals may influence lineage-tracing outcomes. The role of p63-expressing cells in salivary gland cell maintenance and injury repair therefore requires further investigation.

In an alternative approach, lineage tracing was used to determine how much potential stem cells in the ducts contribute to acinar cell replacement [76]. Labeling and long-term chase of all mature acinar cells showed no evidence that new cells are generated from potential duct stem cells under normal homeostasis. Furthermore, use of the versatile $R26^{Brainbow2.1}$ reporter, to label single acinar cells in distinct colors, demonstrated that individual acinar cells generate clones, indicating that the acinar cell lineage is maintained by acinar cell self-

duplication [76]. Consistent with this finding, lineage tracing showed that SOX2-expressing acinar cells in murine sublingual glands generate more acinar cells [70]. In combination with the lineage tracing results of proposed duct stem cells, current evidence suggests that in adult glands, acinar and duct cell line-ages are maintained separately and not through a multipotent stem cell.

## Conclusion

The number of proposed stem cell populations in the adult salivary glands suggests a broad interpretation of the criteria used to define a stem cell. The purpose of this review is to highlight the caveats associated with assays used to define stem cells (Table 1). Proposed stem cells, originally identified through marker gene expression, in vitro proliferation, or sphere formation, have more recently been shown through lineage tracing to be lineage-restricted, demonstrating that they do not meet the basic stem cell criteria. Thus, the outcomes vary depending on the assays used and suggest caution when comparing published results. To date, using the assays described, no single cell meeting the stringent definition of a stem cell has been unambigu-ously identified in adult salivary glands.

Collectively, the evidence indicates that there are several cell populations in adult glands with varying degrees of potential, to proliferate, to differentiate, or to stimulate in a paracrine fashion. Results of lineage tracing show that acinar and duct cell lin-eages are maintained separately in the adult glands, but under conditions of severe cell loss, both cell lineages can contribute to acinar cell regeneration [71]. Evidence that differentiated cells can display cellular plasticity, particularly under stressful conditions, may help in reconciling the various stem cell populations proposed in the salivary glands. Perhaps, therapeutic approaches should be less focused on the identity of a specific stem cell and more on a cellular state that may be manipulated. Understanding cell–cell interactions that lead to plasticity, and whether this is a process that can be applied to salivary gland regeneration are critical areas for investigation.

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## Significance Statement

A number of diverse and nonoverlapping cell populations have been designated as adult stem cells in the salivary glands. The present study is focused on the criteria used to define these cell populations and highlights the limitations associated with each. A critical re-evaluation of how the various cell populations were characterized may serve to clarify which cells may be useful for regenerative therapy.

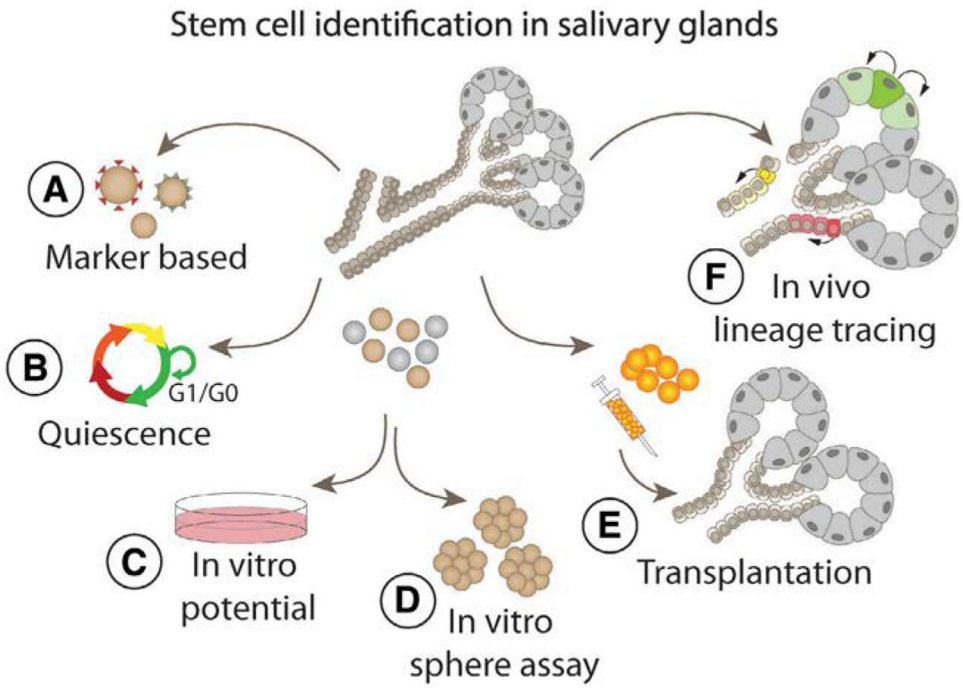


**Figure 1.**

Assays used for the identification of potential stem cells in adult salivary glands have included (A) expression of stem cell markers, (B) proliferation or quiescence, (C) in vitro differentiation, (D) sphere formation, (E) rescue of salivary gland function following transplantation, and (F) in vivo lineage tracing.

**Table 1.**

Assays used for the identification of potential salivary gland stem cells are listed with expected outcomes, the caveats associated with each assay, and possible alternative explanations

| Stem cell assay | Expected outcome | Caveat | Alternative outcomes |
|---|---|---|---|
| Expression of stem cell markers | Cells expressing stem cell markers found in other tissues are considered stem cells | No universal marker for stem cells is known | Stem cell marker expression is not always limited to stem cells |
| Morphology and localization | Proliferating intercalated duct cells located in appropriate proximity to acini are designated as stem cells | Static analysis of cell proximity does not demonstrate a lineage relationship | Rapid division of duct cells generates only duct cells |
| Label-retaining assay | LRCs are slow cycling stem cells | Label can also be retained by postmitotic cells | LRCs can be long-lived terminally differentiated cells |
| In vitro proliferation | In vitro proliferation for more than one passage is an exclusive capability of stem cells | **a.** Removal of cells from in vivo niche can change properties<br>**b.** Many differentiated cell types can continue to proliferate in vitro | Rapidly dividing and expanding cells may be an in vitro artifact |
| In vitro differentiation potential | Evidence of acinar, ductal, and myoepithelial markers or morphology indicates differentiation of a stem cell | **a.** Most assays are performed on dissociated cells of whole gland which includes differentiated cell types<br>**b.** Differentiation into adipocyte, chondrocyte, and osteogenic cells is characteristic for mesenchymal stem cells, but does not demonstrate differentiation into acinar, duct, or myoepithelial cells of salivary gland | **a.** Presence of differentiated cells in starting culture confounds analysis<br>**b.** Stress may cause cultured cells to transition to intermediate, dedifferentiated, or alternate cell types |
| Sphere formation | The formation of spheres in culture is evidence of stem cell activity | **a.** If not plated singly, cells can aggregate, a property of many cell types<br>**b.** Use of dissociated glands for sphere formation includes large numbers of differentiated cells | Cells aggregated into spheres can include differentiated cells from original dissociation, and dividing cells that may not be stem cells |
| Transplantation | Rescue of salivary gland function by transplanted cells is due to regeneration by stem cells | **a.** Little evidence for cell engraftment or contribution to repair<br>**b.** Injected cells may stimulate repair through paracrine signaling | Rescue of salivary gland function may be due to paracrine activity of injected cells |
| In vivo lineage tracing | Tracing a multipotent stem cell lineage should produce acinar, duct, and even myoepithelial cells | Cell labeling is based on the promoter chosen to drive Cre, which may be dynamically regulated or expressed in multiple cell types | Lineage tracing shows that acinar and duct cells are lineage restricted, except under conditions of extreme injury |

Abbreviation: LRCs, label retaining cells.