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Salivary gland cell aggregates are derived from self-organization of acinar lineage cells

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Abstract

Objective: The objective of this study was to characterize the mechanism by which salivary gland cells (SGC) aggregate *in vitro*.

Design: Timelapse microscopy was utilized to analyze the process of salivary gland aggregate formation using both primary murine and human salivary gland cells. The role of cell density, proliferation, extracellular calcium, and secretory acinar cells in aggregate formation was investigated. Finally, the ability of cells isolated from irradiated glands to form aggregates was also evaluated.

Results: Salivary gland cell self-organization rather than proliferation was the predominant mechanism of aggregate formation in both primary mouse and human salivary gland cultures (SGC). Aggregation was found to require extracellular calcium while acinar lineage cells account for ~80% of the total aggregate cell population. Finally, aggregation was not impaired by irradiation.

Conclusions: The data reveal that aggregation occurs as a result of heterogeneous salivary gland cell self-organization rather than from stem cell proliferation and differentiation, contradicting previous dogma. These results suggest a re-evaluation of aggregate formation as a criterion defining salivary gland stem cells.

Keywords

aggregates; salivary gland; radiation damage; acinar cells

Introduction

To systematically investigate salivary gland biology and pathophysiology in a controlled manner, a culture platform is critical (Gvazava, Vasil'ev, Balan, & Terskikh, 2011; Maimets et al., 2016; Pringle, Nanduri, Marianne, Ronald, & Coppes, 2011; Redman, Ball, Mezey, & Key, 2009; Rugel-Stahl, Elliott, & Ovitt, 2012; Tran et al., 2005). Salivary gland culture is also fundamental to expand cells for tissue engineering strategies to treat gland dysfunction (Feng, van der Zwaag, Stokman, van Os, & Coppes, 2009; Lombaert, Brunsting, Wierenga, Kampinga, et al., 2008). The three major salivary glands, parotid, sublingual, and submandibular (SMG), include multiple cell types, but are predominantly composed of acinar and duct cells. Acinar cells produce the fluid and protein content of saliva while ductal cells modify the ionic composition of saliva and form a network of sequentially organized intercalated and striated ducts, which converge into the major excretory duct and drain to the oral cavity (Nauntofte, 1992). Conventional cell isolation and culture methods disrupt normal tissue function, homeostasis, and epithelial polarity, resulting in cell dysfunction and loss of the secretory phenotype (Szlavik et al., 2008). As with other tissues, monolayer culture methods and cell lines exist for the salivary glands, and have been used with varying degrees of success. However, cell lines have significant limitations, including poor recapitulation of native gland phenotype and function (Capes-Davis et al., 2010; Lin et al., 2018; Liu, Liao, & Ambudkar, 2001; Nelson, Manzella, & Baker, 2013).

Following the discovery that single stem cells form spheres or aggregates through proliferation and differentiation, "sphere" cultures have been widely used for culture of intestine, liver, mammary gland, and neural tissue, and have also been adapted for salivary glands (Cao et al., 2011; Feng et al., 2009; Ishiguro et al., 2017; Lathia, Mack, Mulkearns-Hubert, Valentim, & Rich, 2015; Lombaert, Brunsting, Wierenga, Faber, et al., 2008; Maimets, Bron, de Haan, van Os, & Coppes, 2015; Manuel Iglesias et al., 2013; Min, Lee, Bak, & Kim, 2015; Nanduri et al., 2011; Reynolds & Weiss, 1992; Shubin, Felong, Graunke, Ovitt, & Benoit, 2015; Smart et al., 2013). Using rigorous techniques, sphere culture enables isolation and expansion of clonogenic stem/progenitor cell populations with growth only through proliferation (Chen et al., 2012; Reynolds & Weiss, 1992). The expansion of a single salivary gland cell following Wnt stimulation has been shown to give rise to multicellular spheres or 'salispheres' (Maimets et al., 2016). However, the term 'salisphere' has been applied more liberally to all cell aggregates that form within 48 hours following culture of dissociated gland cells (Lombaert, Brunsting, Wierenga, Faber, et al., 2008; Maimets et al., 2016; Pringle, Van Os, & Coppes, 2013; Shubin et al., 2015; Shubin et al., 2017). In fact, the use of sphere assays as an indicator of stem cells in neuron cultures has

been challenged, with timelapse data providing direct demonstration that self-organization contributes to the increase in size of cultured neural spheres (Mori et al., 2006; Singec et al., 2006).

Despite widespread reports using salivary gland cell aggregates, the mechanism by which they form is poorly characterized. A greater understanding of this process is necessary for maximizing the utility of aggregates, which has been demonstrated through restoration of secretory function in radiation-damaged salivary glands following transplantation (Lombaert, Brunsting, Wierenga, Faber, et al., 2008; Nanduri et al., 2013). The *ex vivo* expansion of donor cells within aggregates could be applied to autologous glandular tissue, which would be subsequently transplanted back into patients, avoiding complications related to rejection.

In this study, timelapse microscopy of dissociated salivary gland cells was utilized to investigate the process of aggregate formation with respect to cell density and self-organization. Furthermore, the role of proliferation in aggregate formation was directly evaluated. Heterogeneous populations of primary cells from murine or human glands were also used to determine the requirements for aggregate formation under normal conditions and, for mouse tissue, after *in vivo* irradiation. The data provide direct evidence that self-organization drives aggregate formation rather than proliferation, and that aggregates are composed of a significant proportion of acinar lineage cells. These findings challenge the paradigm that salivary gland cell aggregates are derived from stem cells.

Materials and Methods

Salivary gland cell culture and aggregation:

All animal experiments were approved by the University Committee on Animal Resources at the University of Rochester, and complied with the National Institutes of Health guide for the care and use of Laboratory animals. To generate salivary gland cell aggregates, submandibular glands (SMGs) were aseptically isolated from 6–12 week old C57/Bl6 female mice. Due to known sexual dimorphism between male and female mice in salivary gland composition, only female mice were used for this study, as they more closely model human SMGs (Pinkstaff, 1998). After fine mincing with a razor blade for 10 minutes, tissue was incubated with 1 mg/mL collagenase II and 5 µg/mL hyaluronidase in Hank's Balanced Salt Solution (HBSS) on a shaker plate at 37 °C for 1 hr. Following one wash with PBS, tissue was incubated for 5 minutes with 0.05% w/v Trypsin-EDTA and strained through a 40 µm filter. Serum-free cell culture media was made as described previously (Shubin et al., 2015; Shubin et al., 2017). Briefly, basal media consisted of 1:1 Dulbecco's Modified Eagle Medium (DMEM) and F12 (GIBCO), supplemented with Glutamax (1X ; GIBCO), antibiotic solution (100 IU/mL penicillin, 100mg/mL streptomycin, 0.25 mg/mL amphotericin B; GIBCO), N2 supplement (1X Invitrogen), 10 mg/mL insulin (Life Technologies), 1 mM dexamethasone (Sigma), 20 ng/mL epidermal growth factor (EGF; Life Technologies), and 20 ng/mL basic fibroblast growth factor (bFGF; Life Technologies). After counting using a hemocytometer with Trypan Blue exclusion, cells were seeded first at a range of cell densities from 5×10^1 to 5×10^6 cells/mL for data shown in Figure 1 then at

5×10^5 cells/mL thereafter in 24-well suspension culture plates for aggregate formation over 48–72 hours.

Imaging and image analysis:

A Nikon T1 epifluorescence microscope was used for phase contrast and fluorescent time-lapse imaging. After allowing cells to equilibrate for 4 hours, a tabletop incubation chamber (LiveCell, Pathology Devices) was used to maintain 37 °C, > 75% humidity, and 5% CO₂. Images were collected at 4x magnification, every 20 minutes, for up to 72 hours using NIS-Elements Viewer (Nikon) software. All timelapse data was processed and quantified using ImageJ. Up to 20 timelapses of at least 72 hours duration were processed for each experimental group. To identify and enumerate cells/aggregates, thresholding and segmentation were performed using automatic threshold (Huang method) and watershed functions (Wang et al., 2010). Particle analysis was then used to detect and quantify aggregates and the maximum size of aggregates per field of view (FOV, which is 3.8 mm² for image analyses herein) with a lower threshold of 150 μm² to avoid quantification of debris.

Isolation and aggregation of human salivary gland cells:

The University of Rochester Institutional Review Board approved all tissue acquisition from patients and experiments were carried out in accordance with The Code of Ethics of the World Medical Association. Following informed, signed patient consent, freshly excised parotid or submandibular gland tissue was received from the University of Rochester Department of Otolaryngology. Initial processing involved careful debridement to separate adipose and electrocautery debris from salivary gland tissue (Chan, Huang, Young, & Lou, 2011). Subsequent cell dissociation and seeding at 5×10^5 cells/mL was performed as described for murine SMG tissue, except that cells were incubated in 2 mg/mL collagenase II and 10 μg/mL hyaluronidase for primary dissociation.

Analyzing salivary gland cell proliferation during aggregation:

SMG cells from mice were dissociated and cultured as previously described with media supplemented with 10 μM EdU (5-ethynyl-2'-deoxyuridine, Thermofisher). After 48 hours, aggregates were collected in Eppendorf tubes, centrifuged, and fixed with 4% paraformaldehyde for 20 minutes. The cell suspensions were centrifuged and washed with PBS before gently resuspending in 50 μl optimal cutting temperature solution (OCT, Tissue-Tek) pigmented with red dye (Rit Dye Fuchsia, Phoenix Brands) to aid in locating aggregates during cryosectioning. All centrifugation steps were done at 400 g for 3 minutes. The OCT/cell suspension was placed in a plastic cryomold, frozen at –20 °C, and embedded further with unpigmented OCT. The blocks were frozen and kept at –20 °C prior to cryosectioning. Sections (10 μm) were cut using a CM 1850 UV cryostat (Leica) and mounted on Superfrost slides (Thermofisher) and dried overnight at 37 °C prior to staining. Sections were then permeabilized with 0.5% Triton X-100 in PBS for 30 minutes, washed twice with PBS, and labeled with AlexaFluor® 488 using Click-iT® Plus EdU AlexaFluor® 488 imaging kit with DAPI as a nuclear counterstain. Images were taken using an Olympus DX41 microscope and DSL camera, with DSL software at 10x magnification. To represent the frequency of aggregates with EdU positive nuclei, 30 images containing one to three

aggregates from three separate slides were compiled using Adobe Photoshop. Confocal images of aggregates were taken with an FV1000 laser scanning confocal microscope (Olympus) at 40x magnification with 4x zoom.

Investigating the effect of extracellular calcium on aggregate formation:

Concentrated (1.25 M) stock solutions of Ca^{2+} and ethylenediaminetetraacetic acid (EDTA), a potent calcium chelator commonly used for cellular dissociation (Tokiwa, Hoshika, Shiraishi, & Sato, 1979), were prepared in Ca^{2+} and Mg^{2+} free 1x HBSS followed by sterile filtration. Immediately following isolation, dissociated SMG cells were cultured for 72 hrs in basal sphere media supplemented with 1.25 mM Ca^{2+} or 1.25 mM EDTA, as diluted from the stocks, in 24-well suspension culture plates.

Evaluating the role of acinar lineage cells in aggregate formation:

To enable tracing of acinar lineage cells, an inducible acinar cell-specific reporter mouse was utilized. Briefly, the $\text{R26}^{\text{tdTomato}}$ (Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J) reporter strain (Jackson Laboratory) was crossed to $\text{Mist1}^{\text{CreERT2}}$ on a C57/BL6 background, as described previously (Aure, Konieczny, & Ovitt, 2015), to generate the $\text{Mist1}^{\text{CreERT2}};\text{R26}^{\text{tdTomato}}$ strain for acinar lineage tracing. Tamoxifen was dissolved in 10% ethanol and 90% corn oil at 60 mg/mL. Oral gavage with tamoxifen was performed for two consecutive days on 6–12 week old female mice, each at a dose of 0.25 mg/g, to induce Cre activation. SMG were harvested for culture one week following tamoxifen administration. Cell isolation and seeding were performed as previously described.

Evaluating aggregate formation following gland irradiation:

Following tamoxifen administration to label all acinar lineage cells in 6–12 week old $\text{Mist1}^{\text{CreERT2}};\text{R26}^{\text{tdTomato}}$ female mice at 6–12 weeks of age, a 1440 projection whole body computed tomography (CT) scan was performed to identify the SMGs. The Small Animal Radiation Research Platform (SARRP) (xstrahl, Suwanee, GA) was used to irradiate (15 Gy of X-ray) both SMG using separate 5 mm x 5 mm windows (Supplemental Figure 1). Mice were anesthetized with continuous isoflurane during imaging and irradiation. Cell isolation and seeding were performed as previously described at 2 weeks and 1 month following irradiation. After 48 hours of culture during which timelapse microscopy was performed, aggregates were fixed in 4% PFA for 20 minutes prior to centrifugation at 400 g for 3 minutes, and resuspension in PBS containing DAPI (1:1000) for 5 minutes. Aggregates were resuspended in Immumount, and applied to a microscope slide with coverslip prior to imaging.

Statistical analysis:

All statistical tests were performed using GraphPad Prism 6.0 software. T-test, one-way ANOVA, or two-way ANOVA was used with appropriate post-hoc testing to correct for multiple comparisons as indicated within figure legends to assess significant differences between means with $\alpha = 0.05$. For all plots, the mean is represented with standard deviation shown as error bars.

Results

Murine salivary gland cell aggregates form through self-organization

To assess the dependency of aggregate formation on seeding density, initial SMG cell seeding concentration was varied from 5×10^1 cells/ml to 5×10^6 cells/ml and aggregate formation was observed at 48 h. As shown in Figure 1, minimal sphere formation was observed at densities below 5×10^4 , suggesting that a critical cell seeding density is required for aggregate formation. To temporally observe aggregate formation, phase contrast timelapse microscopy was used to track cells following tissue dissociation and seeding at a constant seeding density of 5×10^5 cells/ml at which aggregates reproducibly formed by 48 h. Imaging revealed the formation of aggregates by 48 hours after murine SMG cell isolation (Figure 2A-D). Following thresholding and segmentation to identify and demarcate salisphere/cell boundaries (Figure 2E-H), ImageJ was used to count cell aggregates. The number of aggregates significantly decreased from 2500 to 1500 per field of view (FOV) over the timelapse from 4 to 72 hours, and the area of the maximum aggregates significantly increased over 2-fold from $3600 \mu\text{m}^2$ at 4 hours to $7600 \mu\text{m}^2$ by 24 hours then stabilized through 48 hours and further increased at 72 hours to $11000 \mu\text{m}^2$. Full length phase contrast timelapse is provided in Supplemental Video 1. The combined cell density effects, increase in sphere size, and decrease in overall number of spheres are consistent with aggregate formation through self-organization.

Human parotid and submandibular gland cells form aggregates *in vitro*

To investigate whether human salivary gland cells demonstrate aggregation behavior, phase contrast timelapse microscopy was performed using freshly isolated human parotid (Figure 2A-D) or SMG tissues (Figure 2E-H) immediately following dissociation and seeding at 5×10^5 cells/mL. Timelapses, as shown in Supplemental Videos 2 and 3, showed that human parotid and SMG cells self-organized to form aggregates over 72 hours, which is consistent with data from murine SMG.

Proliferation is not a dominant factor in salivary gland cell aggregation

To directly evaluate the contribution of proliferation to increases in aggregate size, EdU was added to the media during aggregate formation over 48 hours. Aggregates were subsequently fixed and counterstained with DAPI. As shown in Figure 4, imaging revealed that only a small number of cells within aggregates are EdU positive and not all aggregates contain EdU positive cells. This result suggests that proliferation does not significantly contribute to aggregate formation from salivary gland cells.

Calcium is critical for aggregate formation

To test whether sphere formation is affected by calcium, dissociated murine SMG cells were cultured in media with EDTA, a powerful Ca^{2+} chelator, or in media supplemented with Ca^{2+} . Compared to untreated controls (Figure 5A-D, M), EDTA prevented aggregation as highlighted by no changes over 72 h in cell area per field of view (Figure 5E-H, M). In contrast, calcium supplementation did not have a significant effect on aggregate formation (Figure 5I-L, M) compared to untreated controls (Figure 5A-D, M), as each demonstrated a

decrease in cellular area of approximately 25% by 72 hours, suggesting that cells were aggregating. Notably, cell viability, as measured by DNA content, was not affected by either treatment (Supplemental Figure 2). Taken together, these results suggest that aggregate formation is dependent on calcium.

Murine aggregates are composed predominantly of acinar cells

To determine the role of acinar cells in aggregate formation, fluorescence timelapse microscopy was performed using genetically labelled acinar cells isolated from *Mist1^{CreERT2} R26^{tdTomato}* mice (Figure 6A-B). Fluorescence timelapse images excluded non-labelled cells and debris, evaluating only Mist1 (tdTomato)-positive cellular aggregates (Figure 6C-F). Using thresholding and segmentation, as in phase contrast (Figure 6G-J) experiments, the number of aggregates significantly decreased from 1100 to 300 per field of view (FOV) by 24 hours (Figure 6K), and the largest detectable aggregates significantly increased in area from 3100 μm^2 to 6000 μm^2 , a 2-fold change, by 24 hours, which stays constant until 72 hours (Figure 6L). Full length fluorescence timelapses with phase overlay are provided in Supplementary Video 4. The abundance of genetically labeled cells in the aggregates demonstrates that acinar lineage cells contribute to the majority of aggregates.

Aggregate formation and acinar cell contribution is not affected by gland irradiation

To determine whether radiation affects the formation and composition of salivary gland cell aggregates, the SMG of *Mist1^{CreERT2} R26^{tdTomato}* mice were irradiated with a single dose of 15 Gy one week following tamoxifen gavage. SMGs were removed at 2 weeks or at 1 month post-irradiation. Dissociated SMG cells were cultured and plated to allow aggregate formation for 48 hours. Aggregates were subsequently fixed as whole mounts for tdTomato quantification with DAPI nuclear staining (Figure 7A-C). The number of aggregates formed from irradiated SMG cells at 2 or 4 weeks following irradiation was not significantly lower than that from non-irradiated controls (Figure 7D, Supplemental Videos 5 and 6). After 48 hours, maximum aggregate area increased significantly from 4000 μm^2 for aggregates derived from control unirradiated glands to nearly identical 16,000 μm^2 for aggregates derived from cells isolated either 2 weeks or 1 month post-irradiation (Figure 7E). There were no significant differences in the percent of tdTomato labelled cells to non-labeled cells in aggregates formed from non-irradiated controls and irradiated mice (Figure 7F). Therefore, radiation treatment does not interfere with the ability of acinar cells to form aggregates.

Discussion

The goal of this work was to investigate the mechanism of salivary gland cell aggregation, as well as to examine the contribution of acinar lineage cells to aggregates. A large body of literature suggests that aggregates are generated by proliferation of ductal stem or progenitor cells. In this study, timelapse microscopy followed by characterization of proliferative cells within aggregates clearly demonstrate that cell self-organization rather than proliferation is the primary mechanism by which aggregate formation occurs in mouse and human salivary gland cell cultures, as depicted in the model shown in Figure 8. Genetic labeling of acinar cells *in vivo*, prior to aggregation supports this assertion, demonstrating that the majority of

cells in newly formed aggregates are of acinar cell lineage. Aggregates can form without the isolation of a distinct stem cell population, and the majority of the resulting aggregate composition is non-stem cell derived. This indicates that, like embryonic salivary gland cells (Wei, Larsen, Hoffman, & Yamada, 2007), dissociated cells from adult salivary glands have the intrinsic capacity to self-assemble.

Aggregate formation is also observed with both freshly isolated human parotid and SMG cells. The aggregation of both human parotid and SMG cells continues in culture for up to 6 days (Supplementary Videos 2, 3). Acinar cell proliferation has been demonstrated both *in vivo* and *in vitro* but in very few cells and at very low rates (Aure et al., 2015; Shubin et al., 2015). This is similar to our findings where the percentage of proliferative cells in aggregates is low and not all aggregates are positive for the proliferative marker, EdU (Figure 4). Together, these results clearly show that cell self-organization plays a major role in aggregation in both murine and human salivary gland cell cultures.

Calcium is important for salivary gland cellular signaling and cell-cell interactions (Ambudkar, 2016) including, as shown here, salivary gland cell aggregation. While media supplementation with calcium had no effect, calcium chelation with EDTA completely abrogated aggregation. Salivary gland acinar cells have both calcium dependent (via E-cadherin) and calcium independent (via epithelial cell adhesion molecule (EpCAM)) mechanisms for intercellular adhesion (Davis & Reynolds, 2006; Phattarataratip, Masorn, Jarupoonphol, Supatthanayut, & Saeoweiang, 2016). EDTA-mediated inhibition of aggregate formation may occur directly by blocking calcium dependent contacts between cells, similar to earlier studies showing that antibody inhibition of E-cadherin alters the organization of embryonic salivary gland tissues (Wei et al., 2007). EDTA may also disrupt calcium signaling required for aggregation. Interestingly, however, calcium chelation with EDTA does not impede juxtaposition of cells. This effect is shown in Figure 5, as cell density of EDTA-treated cultures is approximately 55% of the field of view at 4 hours, whereas controls and calcium treated cultures show an initial cell density of approximately 38%. Unlike controls and calcium treated cultures, EDTA treatment does not decrease cell area over time, indicating inhibition of cell aggregation.

Salivary gland cell aggregates are often assumed to be generated from ductal stem/progenitor cells, the loss of which are said to contribute to gland dysfunction following irradiation (Nanduri et al., 2011; van Luijk et al., 2015). Together with the assumption that aggregates are enriched in, and dependent upon, ductal stem cells, this suggests that aggregate formation will be impaired following irradiation (Nanduri et al., 2013; Nanduri et al., 2011; Pringle et al., 2011; Pringle et al., 2013; van Luijk et al., 2015). However, aggregate formation proceeds normally (Supplemental Videos 5, 6), and in fact, yields larger aggregates compared to non-irradiated control cells (Figure 7). This may be a result of increased expression of specific tight junction complexes due to salivary gland radiation injury (Yokoyama et al., 2017), which results in larger initial cell clusters after isolation, culminating in larger aggregates after timelapse. Moreover, it is clear that, even following 15 Gy irradiation, aggregates predominantly consist of acinar lineage cells. These findings underscore the importance of rigorous characterization of cell aggregate formation. Similar to recent findings in aggregate/sphere cultures from other tissues (Pastrana, Silva-Vargas, &

Doetsch, 2011; Singec et al., 2006), our data demonstrate clear cellular heterogeneity rather than (ductal) stem cell clonality (Pastrana et al., 2011) and suggest that aggregate formation should not be used as an indicator of stem cell activity.

Conclusions

Salivary gland cell culture presents challenges for fundamental biology studies and expansion of cells for tissue regeneration approaches. Much work has focused on the use of salivary gland cell aggregates purportedly derived from stem or progenitor cells through proliferation. Here, we provide direct evidence that cellular self-organization rather than proliferation drives salivary gland cell aggregation. Furthermore, our data show a significant acinar cell contribution to newly formed aggregates. These findings challenge the paradigm that salispheres are derived from stem cells and represent heterogenous cells generated by differentiation. The significant percentage of acinar cells within the spheres is relevant for future tissue regeneration approaches, which seek to restore gland secretory function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Salivary gland cell aggregates form via self-organization.
- Primary aggregates are predominantly composed of acinar lineage cells.
- Extracellular calcium is necessary for the formation of aggregates.
- Salivary gland irradiation does not prevent aggregate formation.

Figure 1. Phase contrast images taken at 48 h show seeding density directly contributes to aggregate formation. Scale bars represent 200 μm .

Figure 2.

Phase contrast timelapse microscopy shows aggregate formation over 72 hours. (A-D) Phase contrast images at 4, 24, 48, and 72 hours. (E-H) Thresholded images at 4, 24, 48, and 72 hours. (I) Density of aggregates (aggregates/field of view (FOV); FOV=3.8 mm²) detected at 4, 24, 48, and 72 hours as measured using ImageJ processing. (J) Maximum aggregate area at 4, 24, 28, and 72 hours (Mean $\pm$ SD, n > 10, *, +, #, ! p < 0.05 versus 4 hrs, 24 hrs, 48 hrs, and 72 hrs, respectively, by one-way ANOVA with Tukey's post hoc test). Scale bars represent 200 μm .

Figure 3. Human salivary gland cells form aggregates via self-organization *in vitro*. (A-D) Phase contrast images of human parotid gland cells at 4, 24, 48, and 72 hours. (E-H) Phase contrast images of human SMG cells at 4, 24, 48, and 72 hours. Scale bars represent 200 μ m.

Figure 4. Proliferation within aggregates is modest and does not significantly contribute to aggregate formation. (A) Aggregates incubated with EdU (green) were fixed, sectioned, co-stained with DAPI, and imaged after 48 hours. (B) Thresholded images of aggregates. Arrows indicate aggregates that contain cells positively stained for EdU. Scale bars represent 100 μm .

Figure 5.

Calcium is necessary for salivary gland cell aggregation. (A-D) Phase contrast images of mouse aggregate controls at 4, 24, 48, and 72 hours. (E-H) Phase contrast images of mouse aggregate cultures in the presence of the calcium chelator, EDTA, at 4, 24, 48, and 72 hours. (I-L) Phase contrast images of aggregate cultures in the presence of Ca^{2+} at 4, 24, 48, and 72 hours. (M) Temporal percentage of area occupied by aggregates over the timelapses. The EDTA condition is significantly different from untreated and calcium treatment conditions while no difference is detected between untreated and calcium treated cells. (Mean $\pm$ SD,

which is represented by color-matched dotted lines, $n = 15$, **** $p < 0.0001$ by two way ANOVA with Tukey's post hoc testing). Scale bars represent 200 μm .

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Figure 6.

Acinar cells contribute to the formation and composition of aggregates over 72 hours. (A) *Mist1Cre^{ERT2}* was crossed with the *R26^{TdTomato}* reporter strain (schematic) to enable tracing of isolated acinar cells. Black triangles, LoxP sites. (B) Following tamoxifen administration by oral gavage, reporter mice permanently and heritably express tdTomato in acinar cells. (C-F) TdTomato signal in sphere cultures at 4, 24, 48, and 72 hours. (G-J) Thresholded and segmented TdTomato images at 4, 24, 48, and 72 hours. (K) Density of aggregates (aggregates/field of view (FOV); FOV=3.8 mm²) detected at 4, 24, 48, and 72 hours as measured using ImageJ processing. (L) Maximum aggregate area at 4, 24, 28, and 72 hours

(Mean $\pm$ SD, $n > 10$, *, +, #, ! $p < 0.05$ versus 4 hrs, 24 hrs, 48 hrs, and 72 hrs, respectively, by one-way ANOVA with Tukey's post hoc test). Scale bars represent 200 μm .

Figure 7.

Representative aggregates containing acinar lineage cells marked by TdTomato fluorescence and labeled with DAPI (nuclei). Aggregates form from cells isolated from non-irradiated, control glands (A), glands 2 weeks after irradiation, (C) and glands 1 month after irradiation. (D) Density of aggregates (salispheres/field of view (FOV); FOV=3.8 mm²) detected at 48 hours after isolation. (E) Maximum aggregate area 48 hours after isolation (data represented as mean $\pm$ SD; n > 100, * indicates statistical significance at an alpha = 0.05 by one-way ANOVA and Tukey's post hoc test). (F) Percentage of aggregate cells exhibiting co-localization with TdTomato. (Scale bar = 50 μm) (Mean $\pm$ SD, n > 20, comparisons are not statistically significant at an alpha of 0.05 by one-way ANOVA and Tukey's post hoc test).

Figure 8.

Working model for formation of salivary gland cell aggregates. Aggregate formation from heterogeneous cells isolated mechanically and enzymatically from salivary glands was examined using timelapse microscopy. The data showed that self-organization predominates over proliferation to generate aggregates. Furthermore, use of genetically-labeled acinar lineage cells showed that acinar lineage cells contribute to the majority of cells within mature aggregates and that this process is not inhibited by radiation damage prior to gland isolation.