Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Monell Chemical Senses Center

2. **Reporting Period (start and end date of grant award period):** 01/01/12-12/31/2012

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):**Gary K. Beauchamp, PhD

4. **Grant Contact Person's Telephone Number:** 267-519-4700

5. **Grant SAP Number:** 4100057669

6. **Project Number and Title of Research Project:** 1 **-** Characterization of the Lgr5-expressing Adult Taste Stem Cells.

7. **Start and End Date of Research Project:** 01/01/12-12/31/2012

8. **Name of Principal Investigator for the Research Project:** Peihua Jiang, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $110,000

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Jiang	Assistant Member	10%	$8,740.92
Li	Postdoctoral Fellow	50%	$21,710.00
Redding	Research Specialist	16.6%	$8,868.77

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
None		

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No____X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No_____X_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert “not funded” in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
None	☐NIH ☐ Other federal (specify:_____ _____) ☐ Nonfederal source (specify: _____)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes_____X____ No_____

If yes, please describe your plans:

We plan to submit two R21 grants to NIH to further characterize the Lgr5-expressing adult taste stem cells this year.

12. Future of Research Project. What are the future plans for this research project?

We are continuing this line of research in the lab which is partly funded by the startup funds from Monell to Dr. Jiang. If we are successful in obtaining more funding, we hope to develop a program on adult taste stem cells.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes_____ No____X_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male				
Female				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				
Unknown				
Total				

	Undergraduate	Masters	Pre-doc	Post-doc
White				
Black				
Asian				
Other				
Unknown				
Total				

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No____X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes_____X____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

This project led to a major breakthrough in the taste stem cell field, and our findings were reported in Stem Cells journal this February. This project also allowed us to recruit a new postdoctoral fellow, Dr. Wenwen Ren, and she will continue to work on the Lgr5-expressing taste stem cells.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No____X_____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes_____ No____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Project Goals and Specific Aims

The overall goal of this project was to characterize the Lgr5-expressing cells in taste tissue in posterior tongue of mice to test the hypothesis that Lgr5 marks adult taste stem cells. We also examined if Lgr5 homologue- Lgr6 also marks adult taste stem cells.

In this project, we had three specific aims. First, we set out to determine if Lgr5, Lgr4 and Lgr6 and their recently identified ligands (R-Spondins) are expressed in or in the immediate vicinity of taste cells. We used immunostaining, in situ hybridization and genetic reporters in mouse models to characterize Lgr5+ (positive) cells in circumvallate and foliate papillae, as well as assessed if Lgr4, Lgr6 and R-Spondins are expressed in taste cells or surrounding cells. Using double immunostaining with BrdU and Ki67, we set out to determine if these cells are capable of cycling.

The second aim set out to determine if Lgr5+ cells in the posterior portion of the tongue give rise to taste cells. The Lgr5-GFP-IRES-creERT2 mouse line was crossed with Rosa26-lacZ mice to perform lineage tracing. This technique was used to demonstrate that Lgr5+ cells give rise to all types of gut cells, therefore establishing that Lgr5+ cells are intestinal stem cells. Our tracing experiments suggested that Lgr5+ in circumvallate tissue gave rise to taste cells after 1 month. We completed tracing experiments by obtaining data from different time points after induction by tamoxifen, including 1 day, 2 days, 1 week, 2 weeks, 1 and 2 months.

The third aim set out to determine which types of taste cells are progeny of Lg5+ stem cells. We used the mice from the tracing studies (1 or 2 months after tamoxifen induction) to determine the identity of LacZ positive cells in taste buds. We performed double-immunostaining using antibodies against beta-galactosidase (LacZ) and taste cell markers (e.g., trpm5 for type II cells, SNAP25 for type III cells, and NTPDase2 for type I cells) to determine if Lgr5+ cells give rise to all types of taste cells, indicative of true “stemness” of Lgr5+ cells.

Experimental Design/Overview

Reverse transcription PCR

cDNA from circumvallate (CV) papilla, the surrounding non-taste (NT) epithelial tissue devoid of taste cells, and intestinal tissue (duodenum) was made using Clontech smart cDNA technology. Lgr5 primers for PCR are 5’ gtccacatgctcctgtcctt 3’ (forward) and 5’ agactctccagggtggcagt 3’ (reverse). The amplified fragment (983 bp) was confirmed by

DNA sequencing. *GAPDH* was used as a control reference gene for the amount and quality of cDNA in reverse-transcription PCR (RT-PCR).

Lineage Tracing

Transgenic mice were obtained from Jackson Labs. *Lgr5-EGFP-IRES-creERT2* mice were crossed with *Rosa26-LacZ* or *Rosa26-tdTomato* mice to generate *Lgr5*$^{+/-}$, *Rosa26-LacZ*$^{+/-}$ and Lgr5$^{+/-}$, Rosa26-tdTomato$^{+/-}$ progeny. Genotyping used primer sets recommended by Jackson Labs. The expression of GFP further confirmed the genotype. Lineage-tracing experiments used 6- to 16-week-old male mice. For five injections at 1-day intervals (5 days of injections), we followed a procedure described previously (Okubo et al., 2009), with a slight modification of a daily intraperitoneal injection of 0.22 mg/g tamoxifen (Sigma, T5648) which was dissolved in sunflower seed oil (Sigma, S5007). For a single tamoxifen injection (1-day injection), 0.22 mg/g tamoxifen was injected intraperitoneally. At the end of 1-day or 5-day tamoxifen induction, mice were sacrificed after 1 day, 2 weeks, 1 month, 2 months, and 6 months. *Lgr5*$^{+/-}$, *Rosa26-LacZ*$^{+/-}$ mice were given either 1-day or 5-days of tamoxifen injections, whereas all Lgr5$^{+/-}$, Rosa26-tdTomato$^{+/-}$ mice were given just one tamoxifen injection (Table. S1). All experiments were performed under National Institutes of Health guidelines for the care and use of animals in research and approved by the Institutional Animal Care and Use Committee of the Monell Chemical Senses Center.

β-Galactosidase staining

Immediately after removal, the tongues and duodena from the tamoxifen-induced mice were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h. After extensive washes with PBS, tissues were immersed in 30% sucrose overnight and embedded in OCT. Sections 10 µm thick were stained in X-gal solution (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl, 0.02% NP-40, 0.1% cholate, 2 mg/ml X-gal) overnight at room temperature and counterstained with nuclear fast red.

Immunohistochemistry

For co-imaging taste cell markers and β-galactosidase or intrinsic tdTomato fluorescence, we used mice that were sacrificed 1-2 months after tamoxifen induction. Tissues were processed and sectioned as above. To detect β-galactosidase, we used a goat anti-β-galactosidase antibody (Biotrend; 1:500) and anti-goat secondary antibody Alexa-Fluo 555, and then treated tissues with the following specific primary antibodies against taste cell markers and secondary antibodies: type I taste cells, rabbit anti-NTPDase2 (nucleoside triphosphate diphosphohydrolase-2; Centre de Recherche du CHUL; 1:500); type II taste cells, guinea pig anti-Trpm5 (transient receptor potential cation channel subfamily M member 5; gift from Dr. Emily Liman; 1:500); and type III taste cells, anti-5-HT (serotonin; Immunostar; 1:100). For serotonin detection, mice were injected with 5-HTP (Sigma) 1 h before being sacrificed to allow detection of 5-HT in type III cells. Species-specific Alexa-Fluo 647-conjugated secondary antibodies were used to visualize specific taste cell markers. For co-labeling of Lgr5-GFP with proliferation biomarkers, a rabbit anti-Ki67 antibody (Novus; 1:100) or a mouse monoclonal antibody against K14 (Developmental Studies Hybridoma Bank; 1:100) was used with paraffin-embedded tissues after antigen retrieval. A chicken anti-GFP antibody (Abcam; 1:500) was used to detect

Lgr5-GFP. A goat polyclonal antibody against KCNQ1 (Santa Cruz; 1:500) was used to visualize taste bud cells. Appropriate secondary antibodies were applied accordingly. Secondary antibodies alone were used as negative controls. To visualize the nuclei, 4',6-diamidino-2-phenylindole (DAPI)-containing mounting medium (Vector Laboratories) was used to cover the tissue sections. All images were acquired by either a Nikon Eclipse E800 microscope or Leica Sp2 confocal microscope. Confocal images were compressed z-stacks, and single optical sections gave rise to the same results as the z-stacks.

Results

Aim 1 Results:

Lgr5 is selectively expressed in taste tissue of the posterior tongue

We used reverse-transcription PCR to determine if mRNA of the intestinal stem cell marker Lgr5 is also expressed in adult taste tissue. cDNA was prepared from circumvallate (CV) papilla tissue as well as "non-taste" (NT) lingual epithelium devoid of taste cells. Using exon-spanning primers, an *Lgr5* transcript was amplified from cDNA from CV papilla and intestinal tissue, but not from NT cDNA (Fig. 1A), indicating that Lgr5 is selectively expressed in taste tissue but not in the surrounding epithelium.

To determine which types of tongue epithelial cells express Lgr5, we used heterozygous mice with one wild-type *Lgr5* allele and one allele in which *GFP* (green fluorescent protein) has been inserted into the *Lgr5* gene (a knock-out/knock-in strain). Thus, GFP serves as a surrogate marker for Lgr5 expression. The fidelity of the Lgr5-GFP knock-in reporter has been validated in multiple tissues. Strong GFP signals were detected in cells in the bottom of the trench area below the CV papilla and adjacent to the opening of the ducts of Von Ebner's glands (Fig. 1B,C), as well as below the foliate papillae and adjacent to the opening of ducts there. Less intense GFP signals were detected at the base of taste buds of the CV (Fig. 1B,C) and foliate papilla immediately below and surrounding the mature taste bud cells marked by expression of the voltage-gated potassium channel KCNQ1 or Trpm5. No GFP signal was detected in other portions of Von Ebner's glands or in tongue epithelium devoid of taste tissue. Furthermore, no GFP signal was detected in the fungiform papillae or soft palate, therefore, we focused on analysis of the Lgr5 expressing cells in posterior tongue.

Next, we determined if Lgr5-GFP-positive cells (referred to hereafter as "Lgr5$^+$") are capable of proliferating in taste tissue like Lgr5$^+$ cells in the small and large intestine. Using Ki67 as a cell proliferation marker to identify all actively dividing cells, we observed that some green Lgr5$^+$ cells at the trench below the CV papilla and at the base of CV taste buds also displayed red Ki67 immunoreactivity (yellow-orange cells in Fig. 1D). However, many of the Lgr5$^+$ cells in the trench area were Ki67 negative (green cells in Fig. 1D,E), indicating that most of these Lgr5$^+$ cells are not actively dividing. The non-uniform Ki67 expression in these Lgr5$^+$ cells suggests that there are at least two pools of Lgr5$^+$ cells in taste tissue: Ki67-negative quiescent and Ki67-positive actively cycling cells.

K14 is expressed in basal epidermal cells in taste tissue as well as the surrounding epithelium in the tongue, and K14$^+$ cells have been shown to give rise to the taste bud cells and surrounding keratinocytes in gustatory tissues. If Lgr5 marks adult taste stem or

progenitor cells, we would expect Lgr5$^+$ cells to constitute a subset of K14$^+$ epithelial cells. Double immunostaining demonstrated that almost all Lgr5$^+$ cells at the base of the CV papilla also expressed K14 (Fig. 1F, G), and very few Lgr5$^+$ cells appeared to have weak or no K14 immunoreactivity. Interestingly, expression of K14 was stronger in the cells in the trench below the CV papilla than in the basal regions of the taste buds at the base of the CV papilla (Fig. 1F, G), similar to the pattern observed with Lgr5-GFP (Fig. 1B).

Using *Lgr6-EGFP-IRES-CreERT2* knockout/knockin mice, we were able to detect GFP signals in cells immediately surrounding taste buds in fungiform papillae in anterior tongue, which indicates that Lgr6 is expressed in cells surrounding taste buds as GFP is a surrogate marker for Lgr6 (Fig. 2).

We haven't finished the experiments to determine the expression pattern of Lgr4 and R-Spondins as expected because our progress in characterizing the Lgr5- and Lgr6-expressing cells required more effort than we originally anticipated.

Aim 2 results:

Lineage tracing of Lgr5$^+$ cells in CV papilla

By definition, adult stem cells give rise to multiple types of terminally differentiated cells. We used an established lineage-tracing protocol to identify terminally differentiated taste cells derived from Lgr5$^+$ cells. *Lgr5-EGFP-IRES-creERT2* mice were crossed with *Rosa26-LacZ* mice to generate *Lgr5*$^{+/-}$, *Rosa26-LacZ*$^{+/-}$ mice in which tamoxifen-induced Cre generates β-galactosidase activity to mark cells from the Lgr5$^+$ lineage. We examined β-galactosidase$^+$ cells at different time points after tamoxifen induction. Two injection regimens (5 days of injections vs. a single 1-day injection) were used for lineage tracing. Because of the expectation of a low stochastic probability of inducing Cre in cells in taste tissue by tamoxifen, as well as the previous results of Okubo et al. (2009) with *K14-creERT* tracing studies, we first injected tamoxifen five times during a span of 5 days. Mice were sacrificed at different time points to determine if β-galactosidase activity was present only in those Lgr5$^+$ cells strongly expressing GFP in the trench area and/or weakly expressing GFP at the base of CV papilla taste buds. If β-galactosidase is detected in intragemmal taste bud cells that do not express Lgr5, then the results provide strong evidence that Lgr5$^+$ cells in CV tissue can act as stem cells to give rise to other types of cells.

One day after the 5-day tamoxifen induction, β-galactosidase$^+$ cells were readily detected in the CV papilla at the bottom of the trench area and in basal layers and the pore region of taste buds (Fig. 3A,B). Importantly, at this time point we rarely found β-galactosidase$^+$ intragemmal cells (i.e., cells within taste buds; Fig. 3A, B). However, 2 weeks after the five-injection regimen, more β-galactosidase$^+$ cells were found within and surrounding the taste buds, including intragemmal cells with typical taste cell morphology, as well as perigemmal cells (Fig. 3C, D). Thus, Lgr5$^+$ cells can give rise to multiple cell types both within the taste buds and in the immediately surrounding epithelium. One month after tamoxifen induction, we detected even more β-galactosidase$^+$ intragemmal cells within taste buds (Fig. 3E, F). Importantly, cells at the bottom of the trench area and at the base of the taste buds remained β-galactosidase$^+$, indicating that the Lgr5$^+$ cells here are capable of self-renewing. β-Galactosidase$^+$ taste bud cells could be detected even 6 months after the

5-day tamoxifen induction (Fig. 4). In contrast, we never detected any β-galactosidase$^+$ cells within the surrounding NT epithelium or in glandular tissue, indicating that the Lgr5$^+$ cells generate only taste cells and perigemmal cells in taste tissue. No β-galactosidase$^+$ staining was detected in fungiform papillae taste tissue. Duodena were used as positive controls for lineage tracing of Lgr5$^+$ intestinal stem cells (data not shown). As a negative control, no β-galactosidase$^+$ activity was found in littermates with an *Lgr5*$^+/^+$, *Rosa26-LacZ*$^{+/-}$ genotype.

We also performed a single tamoxifen injection and then examined β-galactosidase expression 1 day later. As expected, the induction efficiency from a single tamoxifen injection was low; however, we could detect a few β-galactosidase$^+$ cells predominantly in the bottom of the trench area and adjacent to the associated duct (Fig. 5), presumably due to the higher expression level of Lgr5 in the bottom of the trench area. Examination of β-galactosidase$^+$ cells from single injections at 2 weeks, 1 month, and 2 months gave patterns of staining similar to those observed with the 5-day injection regimen, albeit with fewer total cells labeled in the CV papilla (Fig. 5) or in the foliate papilla (Fig. 6). Altogether, these data indicate that the Lgr5$^+$ cells give rise to intragemmal and perigemmal cells as well as cells in the pore region.

After a long lineage tracing period some cells in the trench area below the CV papilla of *Lgr5*$^{+/-}$, *Rosa26-LacZ*$^{+/-}$ mice are β-galactosidase$^+$ (Fig. 3E & 4). However, due to the architecture of the trench area and the associated duct, we cannot reliably determine if these β-galactosidase$^+$ cells are also Lgr5-EGFP$^+$. To address this question, *Lgr5-EGFP-IRES-creERT2* mice were crossed with *Rosa26-tdTomato* mice to generate *Lgr5*$^{+/-}$, *Rosa26-tdTomato*$^{+/-}$ mice in which tamoxifen-induced Cre generates expression of tdTomato fluorescence protein (red) to mark cells from the Lgr5$^+$ lineage, allowing us to directly visualize (a) Lgr5$^+$ singly positive cells (EGFP green but not induced for tdTomato), (b) Lgr5$^+$/tdTomato$^+$ doubly positive cells (green and red in respective channels and yellow or orange in merged image) and (c) tdTomato$^+$ singly positive cells (red, representing progeny of the Lgr5$^+$ lineage that have been induced to express CRE and tdTomato but no longer express Lgr5$^+$). Our lineage tracing data (1 injection) using *Lgr5*$^{+/-}$, *Rosa26- tdTomato*$^{+/-}$ mice gave similar results to our LacZ tracing data, albeit with much higher induction efficiency and improved resolution. One day or two days after a single tamoxifen injection, we detected induced tdTomato red fluorescent cells among both the strong Lgr5$^+$ cells at the bottom of the trench area and the weak Lgr5$^+$ cells in the base of taste buds, indicating that both subpopulations of Lgr5$^+$ cells can be induced by tamoxifen (Fig. 7A-F). At this short time after induction we rarely detected cells showing tdTomato red fluorescence without also displaying EGFP green fluorescence. One week (Fig. 7G-I) after a single tamoxifen induction, a few intragemmal taste cells showed intrinsic tdTomato red fluorescence. Two weeks (Fig. 7J-L) and one month (Fig. 7M-O) after tamoxifen induction (1 injection), many intragemmal taste cells showed intrinsic tdTomato red fluorescence of varying intensity, but never with EGFP green fluorescence, indicating that these cells are progeny of Lgr5$^+$ cells. Interestingly, many Lgr5$^+$ cells were also tdTomato$^+$ even long after the induction, indicating that they may be capable of self-renewal. At the bottom of the trench area and in the immediately adjacent ducts we observed a few cells 2 weeks or 1 month after induction that were Tdtomato$^+$ but Lgr5$^-$ (Fig. 7J-O), suggesting that these cells may be progeny of the neighboring strong Lgr5$^+$ cells at the bottom of the

trench area. No green or red fluorescence was found in littermates with an *Lgr5*$^{+}/^{+}$, *Rosa26-tdTomato*$^{+/-}$ genotype 1 month after a single tamoxifen injection (Fig. 7P-R).

Aim 3 results:

Lgr5^{+} cells give rise to multiple types of mature taste bud cells

Our data indicate that Lgr5^{+} cells are the adult stem or progenitor cell source for at least some mature taste cells. A key question is whether the Lgr5^{+} cells are multipotent stem cells in the parental lineages of all types of mature taste bud cells or unipotent or oligopotent progenitor cells that give rise to one or only a few taste bud lineages. Thus, we determined whether the Lgr5^{+} cells generated tdTomato^{+} or β-galactosidase^{+} taste cells that also express specific markers of all three taste cell subtypes. Type I taste cells are supporting cells that can be marked with NTPDase2 (nucleoside triphosphate diphosphohydrolase-2). Type II taste receptor cells respond to sweet, bitter, and umami tastants and express Trpm5. Type III taste receptor cells respond to sour and salty stimuli and can be marked by their expression of serotonin (5-HT). At 1 month after tamoxifen induction, we simultaneously visualized intrinsic tdTomato fluorescence and immunostained with individual taste-type-specific antibodies. From this set of experiments, we observed type I (NTPDase2^{+}), type II (Trpm5^{+}), and type III (serotonin^{+}) taste cells that were also tdTomato^{+} (Fig. 4). Comparable results were obtained using *Lgr5*$^{+/-}$, *Rosa26-LacZ*$^{+/-}$ mice at 1-2 months after tamoxifen induction (Fig. 8). Mature taste cells were generated even 6 months after the 5-day tamoxifen induction (Fig. 9). As noted above Lgr5^{+} cells give rise also to perigemmal cells, but no cells within the surrounding NT epithelium or in glandular tissue. These results are consistent with Lgr5 marking adult taste stem cells and less consistent with marking more limited progenitor cells.

Conclusion

In summary, we have shown that Lgr5 is strongly expressed in cells at the bottom of trench areas at the base of circumvallate and foliate papillae, and weakly expressed in the basal area of taste buds. Our lineage tracing data demonstrate that Lgr5-expressing cells can give rise to taste cells and perigemmal cells. Using subtype-specific taste markers, we have shown that the progeny of Lgr5-expressing cells include all three major subtypes of mature taste cells. All together, our results indicate that Lgr5 marks adult taste stems and/or progenitor cells in the posterior tongue. To our knowledge this is the first identification of the location and nature of adult taste stem cells.

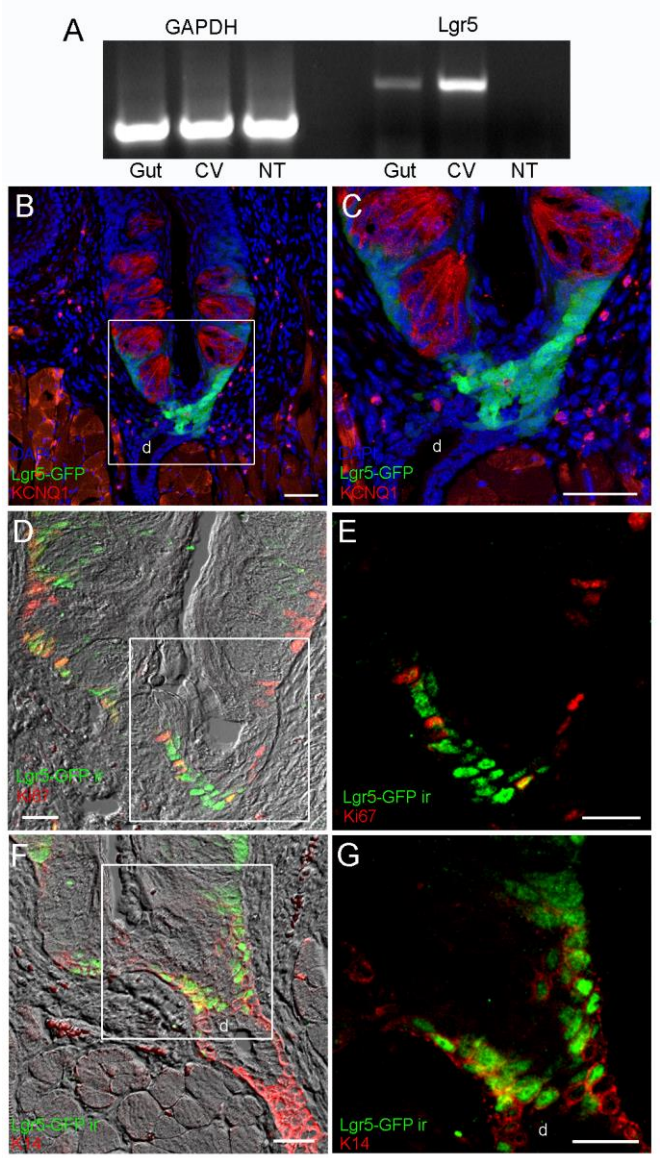

Fig. 1. Lgr5 is expressed in circumvallate papilla
A) RT-PCR demonstrates *Lgr5* expression in gut (positive control) and circumvallate (CV) papilla taste tissue but not in the surrounding non-taste (NT) epithelium. The *GAPDH* RT-PCR controls confirm equivalent template amounts from gut, CV, and NT cDNA. B-G) Confocal images of Lgr5$^+$ cells in CV papilla. B and C) The *Lgr5-GFP* transgene was detected by intrinsic fluorescence (green). The strongest GFP signal is at the bottom of the trench area below the CV papilla and adjacent to the opening of the ducts of Von Ebner's glands. A weaker GFP signal is found in cells at the base of the taste buds immediately below and between the mature taste cells (red), which were immunodetected with a KCNQ1 antibody. D and E) Dual immunostaining shows that only some Lgr5$^+$ cells (green, anti-GFP antibody) at the bottom of the CV trench area are also immunoreactive for proliferation marker Ki67 (red, anti-Ki67). F and G) Dual immunostaining shows that all Lgr5$^+$ cells (green, anti-GFP) at the bottom of the CV trench area are also immunoreactive for K14 (red, anti-K14). All images are compressed confocal z-stacks (~5µm thickness). d, duct of Von Ebner's gland. Scale bars: B=80 µm, C=10 µm, and D-G=20µm.

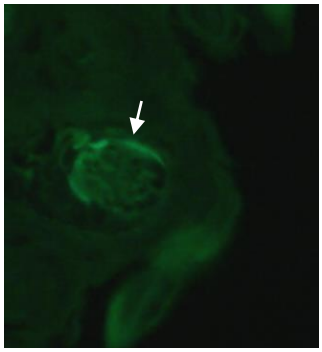

Fig. 2. Lgr6-EGFP signals are detected in cells surrounding taste bud cells in a fungiform papilla in anterior tongue. Representative images from a single bud from a Lgr6-EGFP-IRES-CreERT2. Magnificence 20x

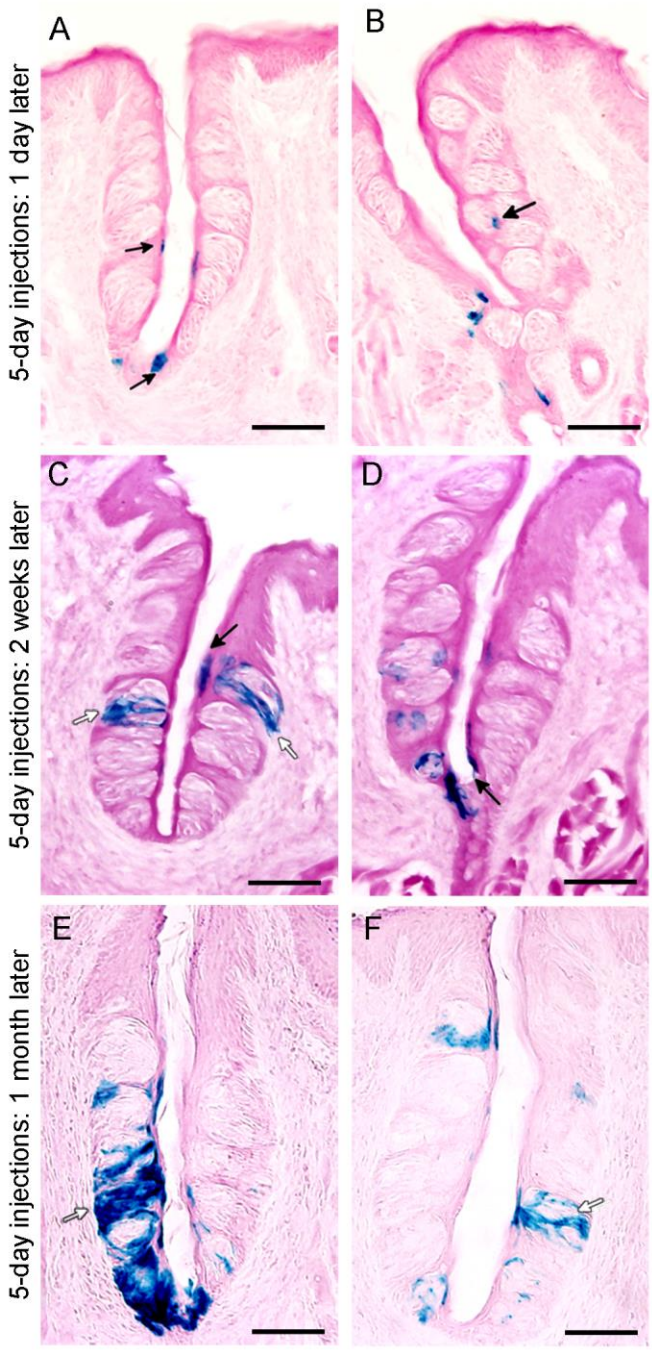

Fig. 3. Lgr5-expressing cells give rise to mature taste cells
Tamoxifen-induced Lgr5-Cre generates β-galactosidase activity to mark cells from the Lgr5$^+$ lineage. A and B) Representative images of β-galactosidase-stained taste cells (blue) of the Lgr5$^+$ lineage present in the trench at the base of the circumvallate papilla, at the taste pore, and at the basal layer of the taste buds (arrows), one day after 5-days of tamoxifen induction of Lgr5-Cre. C and D) β-Galactosidase-stained taste cells (blue) of the Lgr5$^+$ lineage mark both intragemmal (within the taste bud; white arrows) and perigemmal (surrounding the taste bud; black arrows) cells, two weeks after 5-days of tamoxifen induction of Lgr5-Cre. E and F) β-Galactosidase-stained taste cells (blue) of the Lgr5$^+$ lineage mark many intragemmal taste cells (white arrows), one month after 5-days of tamoxifen induction of Lgr5-Cre. Scale bars: A-F=40 µm.

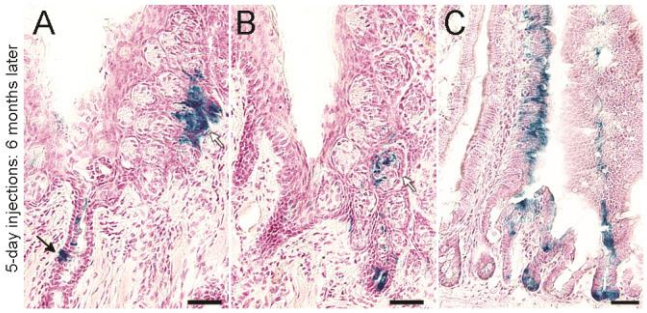

Fig. 4. Lgr5-expressing cells continue to generate mature taste bud cells 6 months after induction.
(A and B) β-Galactosidase$^+$ cells from the Lgr5$^+$ lineage are found in circumvallate papilla taste bud cells as well as in perigemmal cells (white arrows) 6 months after 5-days of tamoxifen induction of Lgr5-Cre. In addition, β-galactosidase$^+$ cells are found in the trench area at the opening of the Von Ebner's gland (black arrow). (C) 6 months after 5 days of tamoxifen induction many β-galactosidase-stained mucosal cells of the Lgr5$^+$ lineage are present in crypts and villi of the small intestine. d, duct of Von Ebner's gland. Scale bars: 40 um.

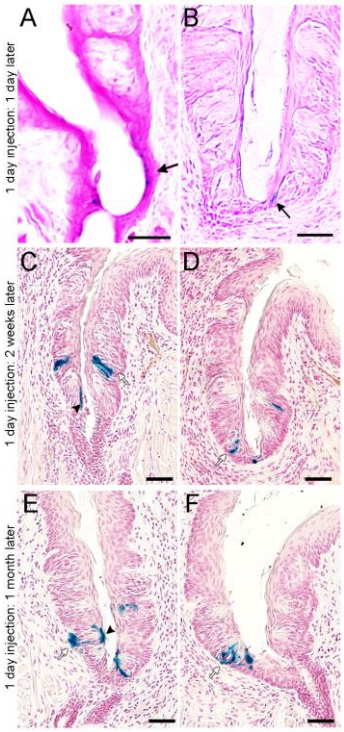

Fig. 5. Lgr5-expressing cells generate mature taste cells after a single tamoxifen injection.
(A, B) One day after 1-day single tamoxifen injection, a small number of β-galactosidase-stained taste cells (blue) of the Lgr5$^+$ lineage are present only at the bottom of the trench area (black arrows). β-Galactosidase$^+$ cells from the Lgr5$^+$ lineage are found in circumvallate papilla taste bud cells (white arrows) as well as in perigemmal cells (arrowheads) 2 weeks (C, D) and 1 month (E, F) after a single tamoxifen injection. Scale bars: 20 µm.

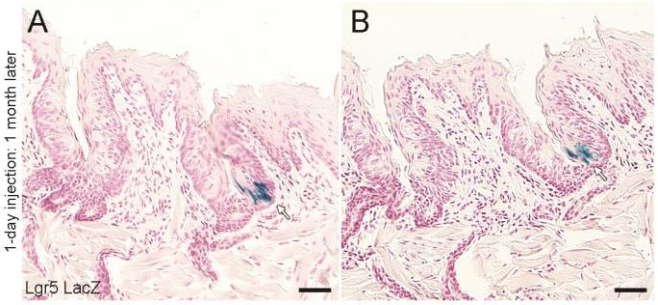

Fig. 6. Lgr5-expressing cells generate mature taste cells after a single tamoxifen injection.
(A, B) One day after 1-day single tamoxifen injection, a small number of β-galactosidase-stained taste cells (blue) of the $Lgr5^+$ lineage are present only at the bottom of the trench area (black arrows). β-$Galactosidase^+$ cells from the $Lgr5^+$ lineage are found in circumvallate papilla taste bud cells (white arrows) as well as in perigemmal cells (arrowheads) 2 weeks (C, D) and 1 month (E, F) after a single tamoxifen injection. Scale bars: 20 μm.

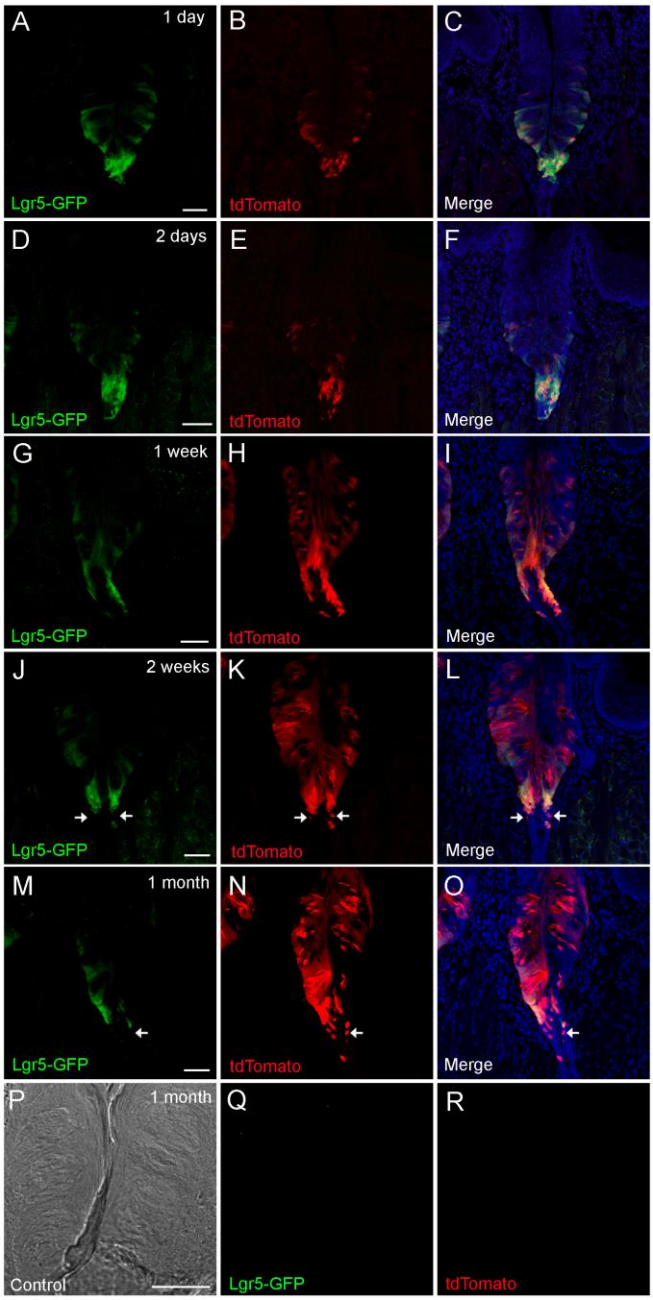

Fig. 7. Lgr5-expressing cells give rise to mature taste cells and cells in the associated ducts

Tamoxifen-induced Lgr5-Cre generates tdTomato fluorescent protein (red) to mark cells from the Lgr5^{+} lineage. Representative confocal images of the circumvallate papilla, one day (A-C), two days (D-F), one week (G-I), two weeks (J-L) and one month (M-O) after a single tamoxifen induction of Lgr5-Cre. Images in the left column (A, D, G, J, M) show Lgr5-GFP intrinsic fluorescence (green) at the indicated times. Images in the middle column (B, E, H, K, N) show tdTomato^{+} (red) cells. The right column (C, F, I, L, O) shows the merged images. Note that increasing numbers of tdTomato^{+} intragemmal taste cells and perigemmal cells are generated over time after a single tamoxifen induction. At all dates examined after a single tamoxifen induction many Lgr5-GFP^{+} cells are also tdTomato^{+} in both the trench area and at the base of the taste buds. A few cells (white arrows) that are tdTomato^{+} but not Lgr5-GFP^{+} are present in the ductal area under the trench two weeks or 1 month after a single tamoxifen induction. P-R) A transmitted light image of a circumvallate section (P) of an *Lgr5*$^{+}$/$^{+}$, *Rosa26-tdTomato*$^{+/-}$ mouse (negative control littermate 1 month after a single tamoxifen induction) shows no EGFP (Q) or tdTomato (R) fluorescence when scanned at the same confocal settings. All images are compressed confocal z-stacks (~8µm thickness). Scale bar: 20 µm.

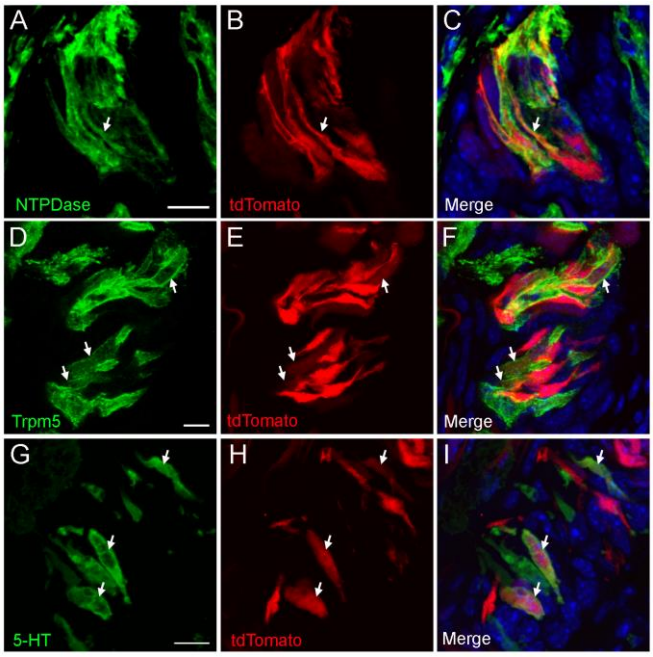

Fig. 8. Lgr5^{+} stem/progenitor cells generate all three types of taste bud cells.
Mature taste bud cells marked by Lgr5-Cre-generated tdTomato 1 month after tamoxifen induction were stained with markers for each of the three types of taste cells. A-C) tdTomato (red) is co-expressed in type I taste cells with NTPDase2 (pseudo-colored green). D-F) Lgr5-Cre-generated tdTomato (red) is co-expressed in type II taste receptor cells with Trpm5 (pseudo-colored green). G-I) Lgr5-Cre-generated tdTomato (red) is co-expressed in type III taste receptor cells with serotonin (pseudo-colored green). Arrows show colocalization of tdTomato and taste specific markers. All images are compressed confocal z-stacks (~3 µm thickness). Scale bars: 10 µm.

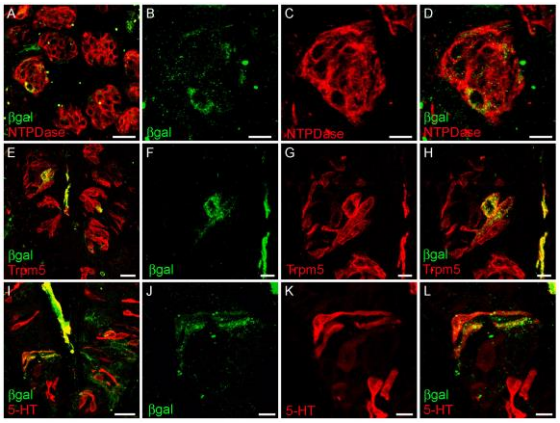

Fig. 9. Lgr5^{+} stem cells generate all three types of taste cells.
Mature taste bud cells marked by Lgr5-Cre-generated β-galactosidase 1-2 months after tamoxifen induction were double stained with anti-β-galactosidase and markers for each of the three types of taste cells. A-D) β-Galactosidase immunoreactivity (green) is co-expressed in type I taste cells with NTPDase2 (red), two months after a single tamoxifen induction. E-H) Lgr5-Cre-generated β-galactosidase immunoreactivity (green) is co-expressed in type II taste receptor cells with Trpm5 (red), 2 months after 5-days of tamoxifen injections. I-L) Lgr5-Cre-generated β-galactosidase immunoreactivity (green) is co-expressed in type III taste receptor cells with serotonin (red), six weeks after a single tamoxifen injection. All sections are compressed Z-stacks (4-10 µm thickness). Scale bars: A, E, and I=20 µm; B-D, F-H, and J-L=8 µm.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
__X___No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics

_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and

an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1.Lgr5-EGFP Marks Taste Bud Stem/Progenitor cells in Posterior Tongue	Karen Y. Yee Yan Li Kevin M. Redding Ken Iwatsuki Robert F. Margolskee Peihua Jiang	Stem Cells	08/12	☐Submitted ☐Accepted ■Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes____X_____ No_____

If yes, please describe your plans:

We are finishing up our work on the Lgr6-expressing taste stem/progenitor cells in anterior tongue. We expect to submit a manuscript in the coming months to report our findings. The PA health department will be acknowledged for partial support of the work.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be

single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

The major discovery of the project was the identification of a marker for adult taste stem cells in posterior tongue. Until this work, taste stem cells were elusive. This work demonstrated that the Lgr5-expressing cells are adult taste stem/progenitor cells in posterior tongue and these cells can give rise to all subtypes of taste bud cells.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:

Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No____X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

NAME	POSITION TITLE
Peihua Jiang	Assistant Member
eRA COMMONS USER NAME (credential, e.g., agency login)	
JIANGPH	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	MM/YY	FIELD OF STUDY
Nankai University, Tianjin, China	B.S.	07/92	Biology
Chinese Academy of Sciences, Beijing, China	M.S.	07/95	Biophysics
University of Pittsburgh, Pittsburgh, PA	Ph.D.	08/01	Neurobiology
University of Pittsburgh, Pittsburgh, PA	Postdoctoral	07/02	Neurology
Mount Sinai School of Medicine, New York, NY	Postdoctoral	07/05	Neuroscience

A. Personal Statement

Until recently, it was thought that all mammals can detect the five basic tastes that humans can. Our work and others have showed that there are many exceptions to this general belief. Many mammalian species show specific taste loss due to the pseudogenization of taste receptor genes and loss of taste receptor function appears directly related to a change in diet. Understanding the precise relationship among taste receptor structure, dietary choice and the associated metabolic pathways constitutes one of my two main research interests.

The other line of my research aims to study adult taste stem cells. Taste cells regenerate constantly during an animal's life, yet the identity of adult taste stem cells for replenishing taste epithelium remains elusive. I am interested in identifying reliable markers for adult taste stem cells and characterizing such cells subsequently. We utilize a broad range of approaches in these studies, including molecular, genetic, cellular, computational and imaging techniques.

B. Positions and Honors

Positions and Employment

1996-2001	Research Assistant, University of Pittsburgh, PA
1997-2000	Teaching Assistant, University of Pittsburgh, PA
2001-2002	Postdoctoral Fellow, University of Pittsburgh, PA
2002-2005	Postdoctoral Fellow, Mount Sinai School of Medicine, NY
2005-2007	Instructor, Mount Sinai School of Medicine, NY
2007-2009	Senior Scientist, Redpoint Bio Corporation, NJ
2009-2012	Research Associate, Monell Chemical Senses Center, PA
2012-present	Assistant Member, Monell Chemical Senses Center, PA

Honors

1991	Honored Student, Nankai University
1999	CNUP Travel Award, University of Pittsburgh
2002-2004	NIDA Training in Pharmacology of Drugs of Abuse
2004-2005	National Research Service Award (NRSA)
2005-2007	Revson Senior Fellow in Biomedical Research

C. Selected Peer-reviewed Publications

1. Damak S, Rong M, Yasumatsu K, Kokrashvili Z, Varadarajan V, Zou S, Jiang P, Ninomiya Y, Margolskee RF. (2003) Detection of sweet and umami taste in the absence of taste receptor T1R3. *Science* 301, 850-3.
2. Jiang P, Ji Q, Liu Z, Snyder LA, Benard LM, Margolskee RF, Max M. (2004) The cysteine-rich region of T1R3 determines responses to intensely sweet proteins. *Journal of Biological Chemistry*. 279(43):45068-75. Epub Aug 06, 2004.
3. Jiang P, Cui M, Zhao B, Liu Z, Snyder LA, Benard LM, Osman R, Margolskee RF, Max M. (2005) Lactisole interacts with the transmembrane domains of human t1r3 to inhibit sweet taste. *Journal of Biological Chemistry.* 280(15):15238-46. Epub Jan 24, 2005.
4. Jiang P, Cui M, Ji Q, Snyder LA, Liu Z, Benard LM, Margolskee RF, Osman R, Max M. (2005) Molecular mechanisms of sweet receptor function. *Chemical Senses* 30 Suppl 1:i17-i18.
5. Jiang P, Cui M, Zhao B, Snyder LA, Benard LM, Osman R, Max M, Margolskee RF. (2005) Identification of the cyclamate interaction site within the transmembrane domain of the human sweet taste receptor subunit t1r3. *Journal of Biological Chemistry.* 280(40):34296-305. Epub 2005 Aug 2.
6. Cui M, Jiang P, Maillet E, Max M, Margolskee RF, Osman R. (2006) The heterodimeric sweet taste receptor has multiple potential ligand binding sites. *Current Pharmaceutical Design*. 12(35):4591-4600.
7. Romanove RA, Rogachevskaja OA, Bystrova MF, Jiang P, Margolskee RF, Kolesnikov SS. (2007) Afferent neurotransmission mediated by hemichannels in mammalian taste cells. *EMBO J*. 26:647-656.
8. Lin DF, Jiang P, Hu Y, Max M, Wang DC. (2008) Crystal structure of mabinlin II: a novel structural type of sweet proteins and the main structural basis for its sweetness. *J Struct Biol.* 162(1):50-62. Epub 2008 Jan 11.
9. Liu B, Ha M, Meng XY, Kaur T, Khaleduzzaman M, Zhang Z, Jiang P, Li X, Cui M. (2011) Molecular mechanism of species-dependent sweet taste toward artificial sweeteners. J. Neurosci. 31(30):11070-6.
10. Jiang P*, Josue J, Li X, Glaser D, Li W, Brand JG, Margolskee RF, Reed DR, Beauchamp GK*. (2012) Major taste loss in carnivorous mammals. *PNAS*. 109 (13): 4956-61. Epub 2012 Mar 12 (* co-corresponding authors)
11. Jiang P*, Josue J, Li X, Glaser D, Li W, Brand JG, Margolskee RF, Reed DR, Beauchamp GK*. (2012) Reply to Zhao and Zhang: Loss of taste receptor function in mammals is directly related to feeding specializations. *PNAS*. 109(23) E1465 (*co-corresponding authors)
12. Tordoff MG, Alarcon LK, Valmeki S, Jiang P. (2012) T1R3: A human calcium taste receptor. *Scientific Reports*. 2:496.
13. Lee RJ, Xiong G, Kofonow JM, Chen B, Lysenko A, Jiang P, Abraham V, Doghramji L, Adappa ND, Palmer JN, Kennedy DW, Beauchamp GK, Doulias P, Ischiropoulos H, Kreindler JL, Reed DR, Cohen N. (2012) Genetics of the bitter taste receptor T2R38, underlie susceptibility to upper respiratory infection. *The Journal of Clinical Investigation.* 122 (11): 4145-59.

14. Karen YY, Li Y, Redding KM, Iwatsuki K, Margolskee RF, Jiang P* (2013). Lgr5-EGFP marks taste bud stem/progenitor cells in posterior tongue. *Stem Cells*, in press. (*corresponding author)