

SARS-CoV-2 Receptor ACE2 Is Enriched in a Subpopulation of Mouse Tongue Epithelial Cells in Nongustatory Papillae but Not in Taste Buds or Embryonic Oral Epithelium

Zhonghou Wang, Jingqi Zhou, Brett Marshall, Romdhane Rekaya, Kaixiong Ye, and Hong-Xiang Liu*

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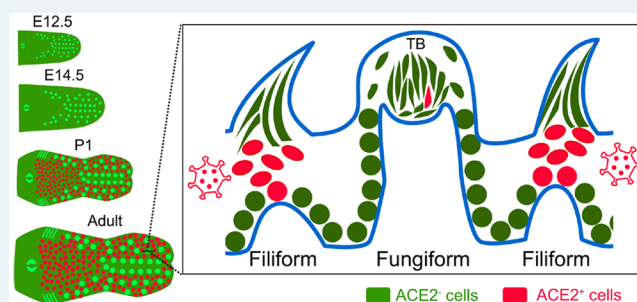
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ABSTRACT: As a result of the COVID-19 pandemic, evidence revealed that SARS-CoV-2 infection caused taste loss at a rate higher than that of influenza. ACE2, the entry receptor of SARS-CoV-2, has been identified in the oral epithelium; however, it is unclear at what developmental stage ACE2 expression emerges and whether ACE2 is expressed in taste buds. To identify the specific developmental stage, we analyzed RNA-Seq data from embryonic and newborn mouse oral tissue. We found that robust ACE2 expression was observed in the newborn oral epithelium. In contrast, only extremely low levels, if any, of ACE2 transcripts in the embryonic stage oral tissue were found (E12.5 and E14.5).

Analyses of three public scRNA-seq data sets of adult mouse tongue epithelial cells showed that receptors for various viruses were enriched in distinct clusters of tongue epithelial cells. ACE2 was enriched in a subpopulation of epithelial cells in the basal region of nongustatory filiform papillae but not in the taste papillae or taste buds. Expression of ACE2 was detected in a small proportion of type III taste cells. Our results indicate that when applied across species, nongustatory papilla epithelial cells are the prime targets for SARS-CoV-2 infection in the tongue; thus, taste loss in COVID-19 patients is likely not caused by a direct infection of SARS-CoV-2 to taste bud cells. Additionally, fetuses at different stages of development may have distinct susceptibility to SARS-CoV-2 infection.

KEYWORDS: COVID-19, SARS-CoV-2, ACE2, taste bud, taste loss, tongue epithelium



Taste buds are the sensory organs for taste that transduce gustatory stimuli into neural signals conveyed to the central nervous system. In mammals, taste buds are located in the oral cavity, primarily in the lingual taste papillae and the soft palate. Mammalian taste bud cells are post-mitotic and have a short life span (on average 10–12 days in mice).^{1–5} Thus, it is essential that progenitor cells in the surrounding tissue compartments continuously differentiate into the specific types of taste cells (types I, II, and III) for taste bud homeostasis and intact taste sensation.

Deficiencies of taste buds and/or their progenitors cause taste dysfunctions. These deficiencies may be caused by genetic factors, injuries, medical treatments (e.g., radiotherapy and chemotherapy), and illness, including infections in the oral cavity (see review by Feng et al.).⁶ Upper respiratory and oral cavity viral infections are among the most common causes of taste dysfunction and are frequently associated with taste loss.^{7–11} For example, hypogeusia and dysgeusia are often a complaint in patients following an influenza-like illness.¹¹

Evidence collected during the COVID-19 pandemic revealed that taste and smell loss occurs at a higher rate in patients with COVID-19 symptoms compared to those with influenza-like symptoms.^{12–17} Angiotensin-converting enzyme 2 (ACE2), the receptor that mediates the entry of SARS-CoV-

2 into cells,^{18–22} is expressed in the oral mucosa and highly enriched in the tongue epithelial cells in humans, thus providing evidence for the infectious susceptibility of the oral cavity.^{23,24} More recent data showed that the ACE2-expressing (ACE2⁺) cells in the tongue of humans are a subpopulation of keratinocytes.²³ In mice, ACE2 is detected in both gustatory and nongustatory tongue epithelium.²⁵ Quantitative data from cellular analyses are currently unavailable. In spite of the valuable knowledge gained recently about ACE2 and COVID-19 infections, several relevant questions remain unanswered: (1) Where are ACE2⁺ cells localized in the tongue epithelium? (2) Is ACE2 expression more enriched in taste bud cells such that viral infection directly causes taste bud cell death and subsequently taste loss? (3) How does ACE2 expression in oral epithelium change during different stages of embryonic development?

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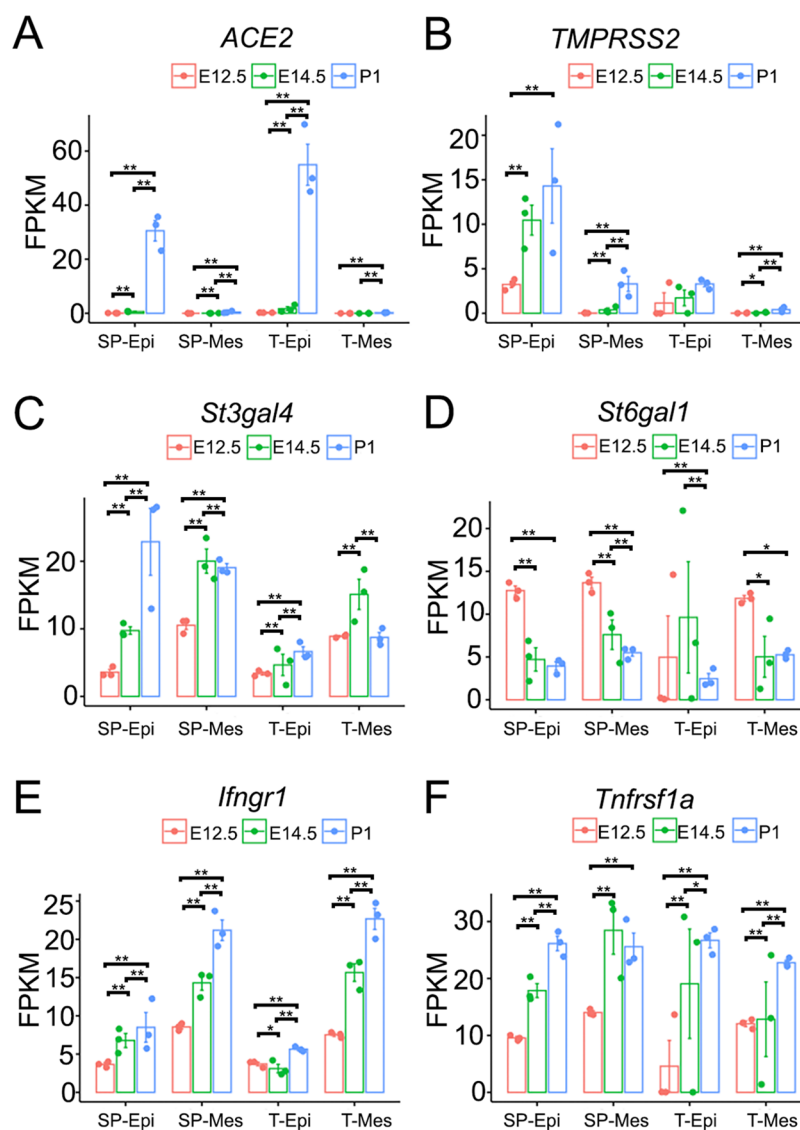


Figure 1. Histograms of fragments per kilobase million reads (FPKM) values (mean \pm SE, $n = 3$) to illustrate the expression of genes related to SARS-CoV-2 (A and B), influenza (C and D), and inflammation (E and F). The epithelium (Epi) and mesenchyme (Mes) were separately collected from E12.5, E14.5, and P1 mouse tongue (T) and soft palate (SP), the two main regions that host taste buds. Dots in each histogram represent the individual data points of the biological replicates. Statistical analysis of differential expression in the same tissue compartment across stages was performed based on read counts using DESeq2. *, $P < 0.05$; **, $P < 0.01$ (adjusted P value).

Although mouse *ACE2* is not susceptible to SARS-CoV-2,²⁶ its expression may still provide insights into the development of pathological changes in humans given that mice and humans share similar gene expression patterns. In this study, analyses of available RNA-Seq data from our lab and recent publications^{27,28} indicate that *ACE2* expression was detected in the oral epithelium at birth and not in embryos at E12.5 and E14.5. In adult mice, *ACE2* is enriched in a subpopulation of epithelial cells in the nongustatory filiform papillae but not in taste papillae or taste buds. Our mouse data, together with other reports, if true in humans, suggest that (1) taste loss in COVID-19 patients is likely not directly caused by the transfection of SARS-CoV-2 to taste bud cells, (2) embryos at different stages of development have distinct susceptibility to the disease, and (3) the risk of a maternal–fetal transmission exists given the expression of *ACE2* in the organs of late embryos and newborns, placenta, and the female reproductive system.

RESULTS

Expression Dynamics of SARS-CoV-2, Influenza Virus, and Inflammation-Associated Genes in Mouse Oral Cavity at Early Stages (E12.5, E14.5, and P1). To predict how early oral tissues can be susceptible to SARS-CoV-2 and influenza virus, we retrieved our bulk RNA-Seq data regarding the expression of genes associated with SARS-CoV-2 (*ACE2*, *TMPRSS2*),²⁹ influenza virus (*St3gal4* and *St6gal1*),³⁰ and inflammation (*Ifngr1* and *Tnfrsf1a*). Four tissue compartments (epithelium and underlying mesenchyme in the tongue and soft palate) were collected at three developmental stages (E12.5, E14.5, and P1). The selected genes showed distinct expression patterns across the four tissue compartments and during the different stages of development. In general, the expression level increased for all genes during tissue development except for *St6gal1*, which was decreased at later development stages. Among the examined genes, *ACE2* was

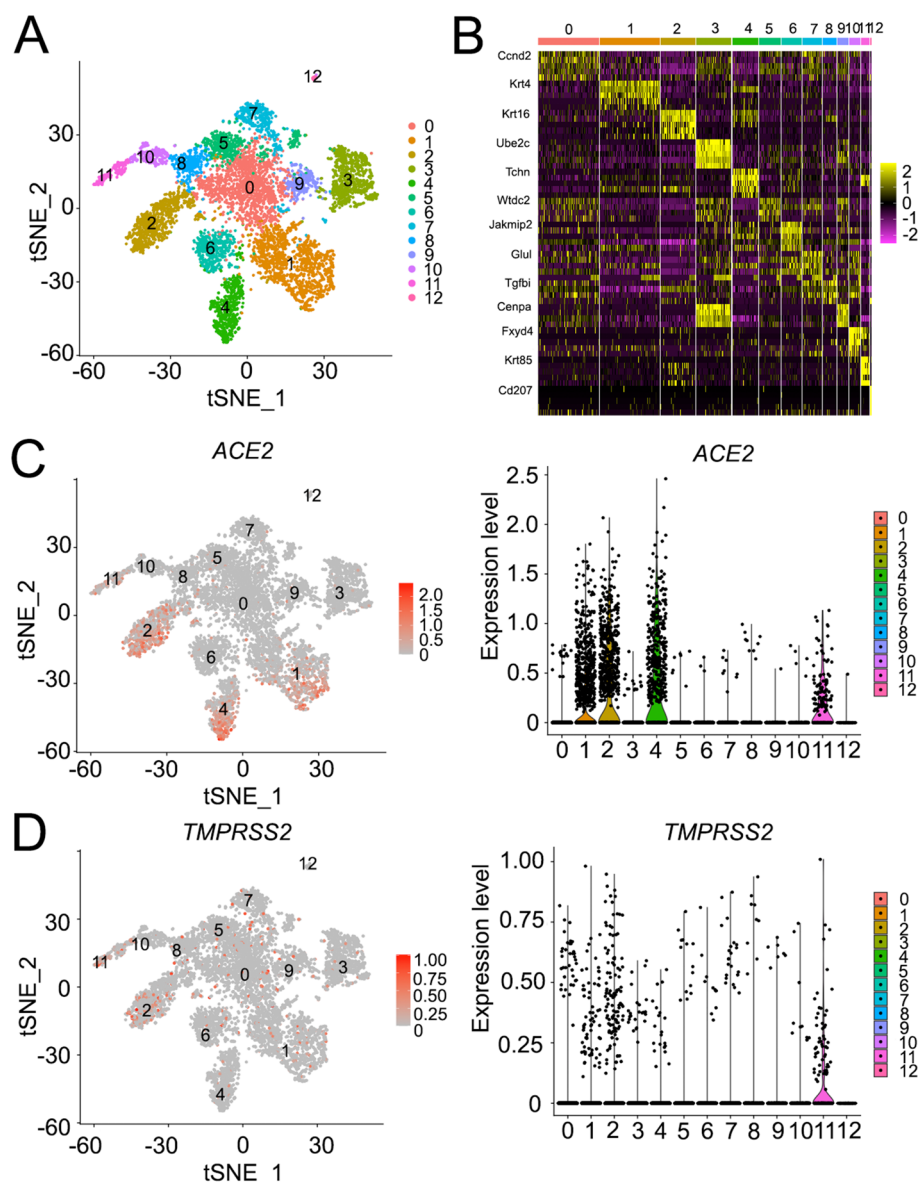


Figure 2. Distribution of SARS-CoV-2-associated genes, *ACE2* and *TMPRSS2*, in the cell clusters of anterior tongue epithelium. (A) t-SNE map to illustrate the 13 cell clusters that were identified in a scRNA-seq data set (7538 anterior tongue epithelial cells in total) from Schaum et al.²⁷ Each dot represents a single cell in this data set. (B) Heatmap to show the 13 cell clusters identified with the most significantly different gene from other clusters. The colors represent expression ($\log(\text{UMIs count}/10\,000 + 1)$) of marker genes as the legend indicates. (C, D) t-SNE maps and violin plots to illustrate the expression ($\log(\text{UMIs count}/10\,000 + 1)$) of *ACE2* (C) and *TMPRSS2* (D). In the t-SNE maps, each dot represents an individual cell in the data set, and the color gradients of dots represent the expression levels of *ACE2* (C) or *TMPRSS2* (D) in cells. In the violin plots, each dot represents an individual cell that expressed *ACE2* (C) or *TMPRSS2* (D).

exclusively found in the epithelium, while all other genes were detected in both the epithelium and the mesenchyme.

The expression of *ACE2* was rarely detected in any of the four tissue compartments in embryos (E12.5 and E14.5); however, robust expression of *ACE2* transcripts was found in the epithelium at P1 but not the mesenchyme of the soft palate and tongue (Figure 1A). Expression of the protease *TMPRSS2*, which mediates the initial viral entry of SARS-CoV-2, showed a progressive increase over developmental stages in both the epithelium and mesenchyme of the soft palate and in tongue epithelium. Furthermore, the transcripts of *TMPRSS2* were the most abundant in the soft palate epithelium (Figure 1B). *St3gal4* and *St6gal1*, which are essential for synthesis of sialic acid recognized by influenza virus,³⁰ were detected across all tissue compartments at all three developmental stages. The

pattern of expression of these two genes varied across the different developmental stages characterized by a general increased expression for *St3gal4* and decreased abundance of *St6gal1* transcripts (Figure 1C,D). *Ifngr1* and *Tnfrsf1a*, genes that encode two essential receptors for inflammatory cytokines, were detected in all examined tissue compartments as early as E12.5 and their expression increased during development (Figure 1E,F).

Enrichment of SARS-CoV-2 Receptor Gene *ACE2* Occurs in a Subpopulation of Tongue Epithelial Cells of Nongustatory Papillae but Not in Taste Papillae or Buds. Recent studies indicated that *ACE2* is enriched in the tongue epithelial cells in humans.²³ To understand which subpopulation(s) of tongue epithelial cells are the likely target of SARS-CoV-2, we scoured the data in the recently published

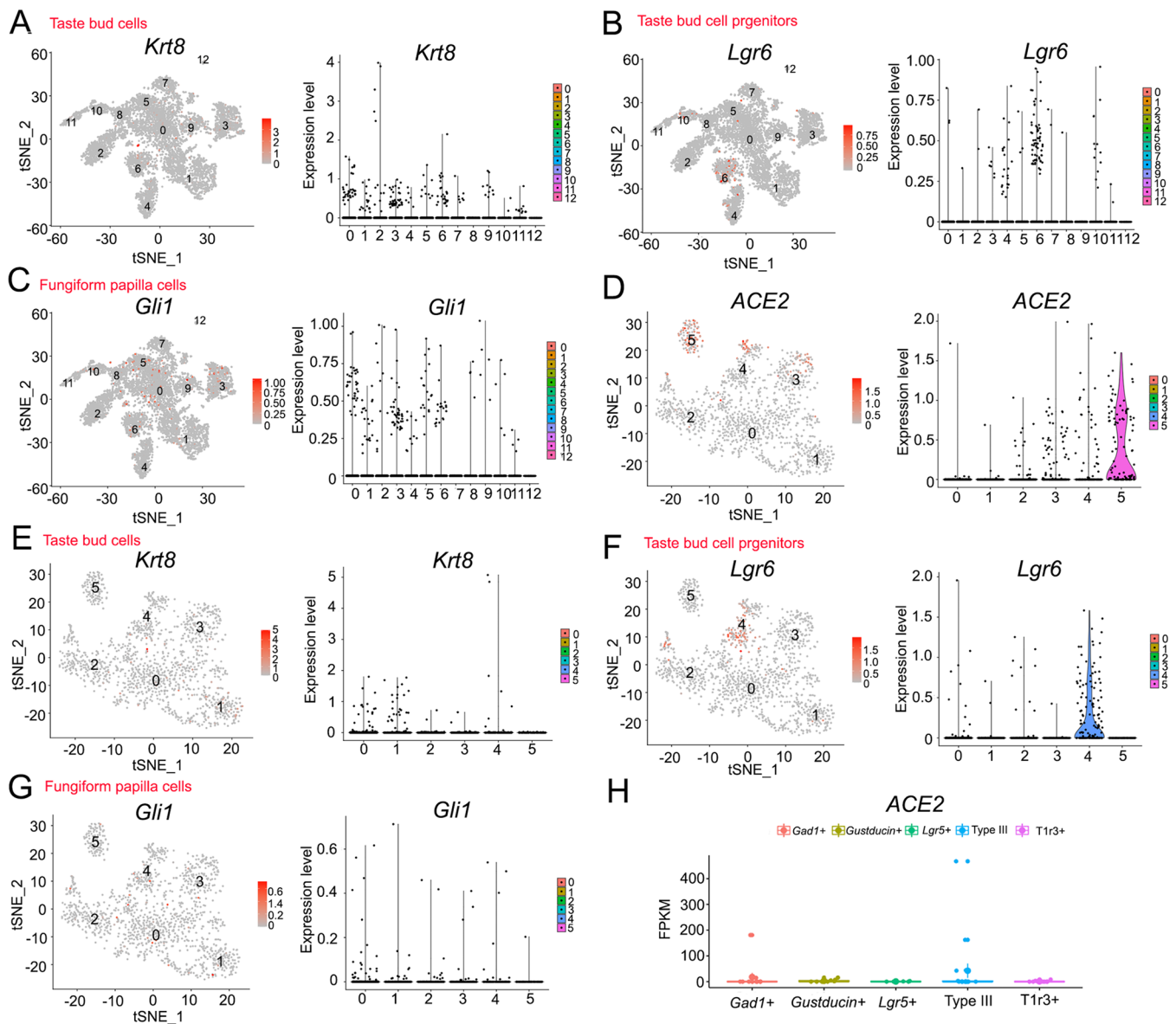


Figure 3. *ACE2* is not enriched in taste papilla epithelium and taste buds. (A–C) t-SNE maps and violin plots to illustrate expression (log(UMIs count/10 000 + 1)) of taste bud cell marker *Krt8* (A), taste bud cell progenitor marker *Lgr6* (B), and fungiform papilla cell marker *Gli1* (C) in distinct cell clusters in data set from Schaum et al. (7538 cells in total).²⁷ (D–G) t-SNE maps and violin plots to illustrate expression (log(read counts/10 000 + 1)) of *ACE2* (D), taste bud cell marker *Krt8* (E), taste bud cell progenitor marker *Lgr6* (F), and fungiform papilla cell marker *Gli1* (G) in Schaum et al. data set (1432 cells in total). In the t-SNE maps, each dot represents an individual cell in the data set and the color gradients of dots represent the expression levels of the gene indicated in the title. In the violin plots, each dot represents an individual cell that expressed the gene indicated in the title. (H) Boxplot to illustrate expression (FPKM) of *ACE2* of 47 taste bud cells and 5 taste bud progenitors in the data set from Sukumara et al.²⁸ Almost all the lines standing for first quartile, median, and third quartile of boxplots stack at zero.

mouse scRNA-seq atlas.²⁷ Using the best high-throughput data set (7538 cells in total), we identified 13 anterior tongue epithelial cell clusters (Figure 2A) and their associated signature genes (Figure 2B, Table S1) using a machine learning approach. Consistent with human data,²³ SARS-CoV-2 entry receptor coding gene, *ACE2*, is detected in mouse tongue epithelium and *ACE2*⁺ cells are specifically enriched in 4 of the 13 clusters (Nos. 1, 2, 4, and 11) of anterior tongue epithelial cells (Figure 2C). In contrast, the expression of *TMPRSS2* is broadly scattered at a low frequency across all cell clusters (Figure 2D).

To identify the type(s) of *ACE2*⁺ cells and answer the question of whether taste bud cells express *ACE2*, we empirically analyzed the *ACE2*⁺ cell-enriched clusters using

markers for taste bud cells (*Krt8*⁺), taste bud cell progenitors (*Lgr6*⁺), and basal epithelial cells in fungiform papillae (*Gli1*⁺) in three independent scRNA-seq data sets. In the first data set (7538 cells in total from the anterior tongue epithelium), *Krt8*⁺, *Lgr6*⁺, and *Gli1*⁺ cells were not enriched in *ACE2*⁺ cell-enriched clusters (Nos. 1, 2, 4, and 11) (Figure 3A–C). To confirm the previous results, a second data set (1432 anterior tongue epithelial cells) was analyzed. Consistent with the results shown above (Figure 2C,D), *ACE2*⁺ cells were especially enriched in one cell cluster (No. 5), while some positive cells were found in other clusters (Figure 3D). Again, *ACE2*⁺ cell-enriched clusters had few or no cells expressing *Krt8*, *Lgr6*, or *Gli1* (Figure 3D–G).

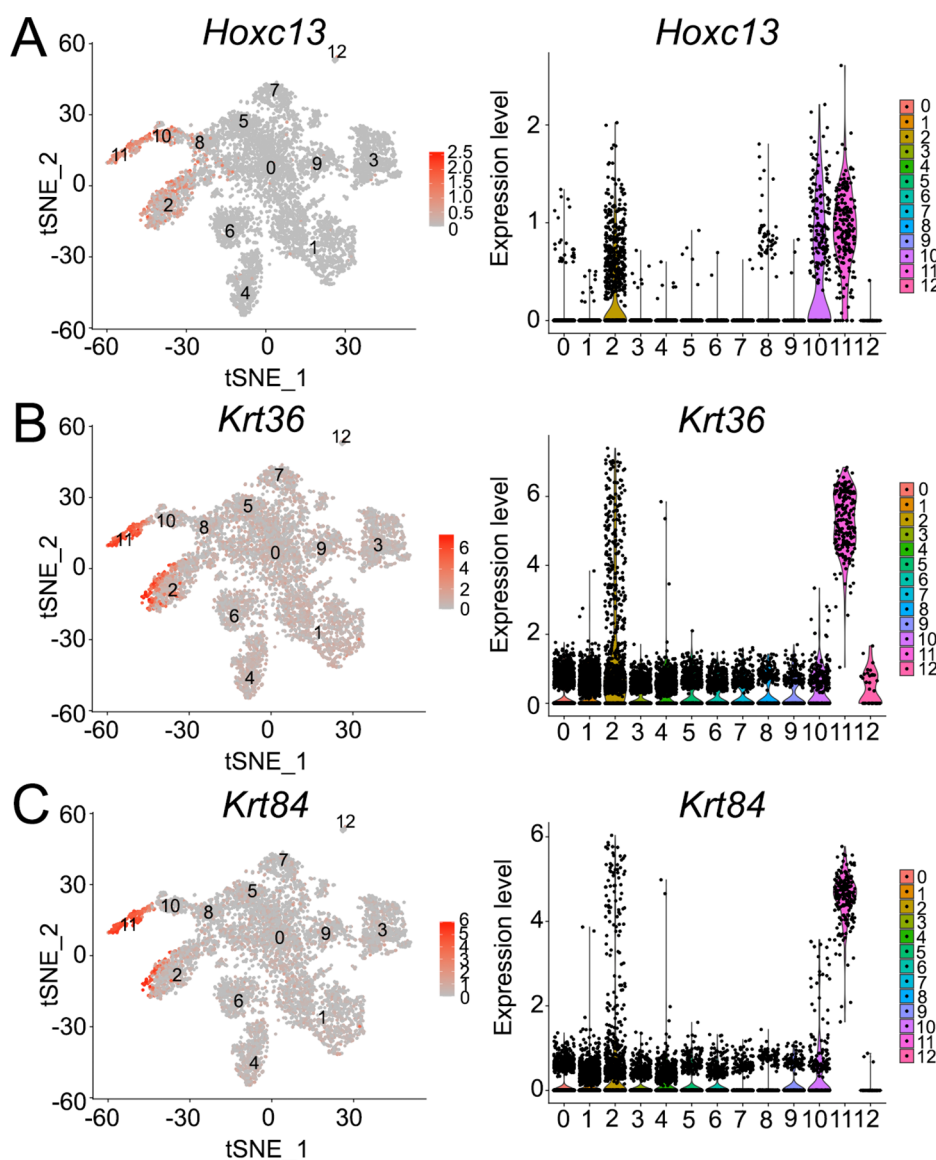


Figure 4. t-SNE maps and violin plots to illustrate expression ($\log(\text{UMIs count}/10\,000 + 1)$) of filiform papilla cell markers, *Hoxc13c* (A), *Krt36* (B), and *Krt84* (C) of distinct cell clusters in the scRNA-seq data set (7538 cells in total) from Schaum et al.²⁷ In the t-SNE maps, each dot represents an individual cell in the data set and the color gradients of dots represent the expression levels of the gene indicated in the title. In the violin plots, each dot represents an individual cell that expressed the gene indicated in the title.

To further confirm that *ACE2* is not expressed in taste bud cells, a third independent scRNA-seq data set (52 taste bud cells in total)²⁸ was analyzed. As shown in Figure 3H, most taste bud cells from distinct types (*Gad1*⁺ type III, *Gustducin*⁺ type II, *T1r3*⁺ sweet and umami type II, and manually selected type III) and taste bud progenitors (*Lgr5*⁺) did not express *ACE2* (Figure 3H). Out of the 52 analyzed cells, only 8% showed robust *ACE2* transcripts (higher than 160 FPKM), and all of them were type III cells. Collectively, *ACE2* is not enriched in most taste bud cells or the surrounding taste bud progenitors and taste papilla epithelial cells.

To further characterize the identity of *ACE2*-expressing cells, we examined the genes that have been reported to be exclusively expressed in the nongustatory filiform papillae of the dorsal surface of the tongue. *Hoxc13*⁺ cells, distributed in the basal region of filiform papillae,³¹ were enriched in 3 out of 13 cell clusters (Nos. 2, 10, and 11). It is noteworthy that *Hoxc13*⁺ and *ACE2*⁺ cell enrichment overlapped in two (Nos.

2 and 11) of the cell clusters (Figure 4A). An additional two genes (*Krt36* and *Krt84*) that are expressed in filiform papillae were enriched in the same cell clusters as *Hoxc13* and *ACE2*. Jointly, our data suggest that a proportion of *ACE2*⁺ cells are distributed in the epithelium at the basal region of filiform papillae.

Potential for Distinct Tropism of Multiple Viruses in the Tongue Epithelium. To understand the relevance of the distribution of viral receptor expression in oral epithelium and the vulnerability of taste sensation to infectious diseases, we performed a head-to-head comparison of the frequency and the abundance of key viral entry factors for SARS-CoV-2, HCoV-229E, influenza virus, and MERS-CoV45 across all 13 cell clusters identified in the first scRNA-seq data set.²⁷ The two most *TMPRSS2*-enriched cell clusters (Nos. 2 and 11) were also *ACE2*-enriched, although the proportion of *TMPRSS2*⁺ cells was generally low. The HCoV-229E's entry factor Anpep³² shared one cell-enriched cluster (No. 4) with

ACE2. However, the *St3gal4*-enriched clusters (essential for influenza virus entry) were totally separate from those for *ACE2*. The expression of *Dpp4*, which is essential for MERS-CoV infection,³² is very low in cell clusters throughout the tongue epithelium (Figure 5).

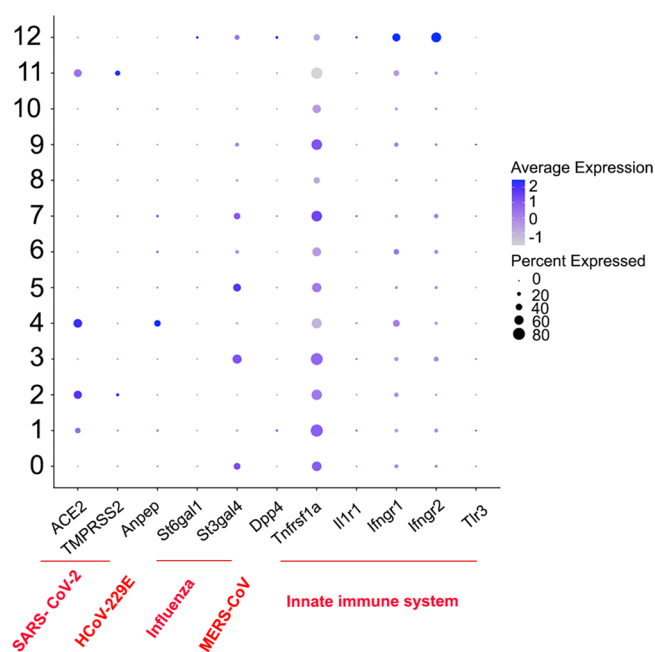


Figure 5. Dot plot to illustrate gene expression ($\log(\text{UMIs count}/10\,000 + 1)$) across the 13 cell clusters in the scRNA-seq data set (7538 cell in total) from Schaum et al.²⁷ Genes related to SARS-CoV-2, HCoV-229E, influenza, MERS-CoV, and the innate immune system are included. The size of the dots represents the proportion of gene-expressing cells, and the color intensity of the dots represents the average level of the gene expression.

Given that a strong innate immune response was activated by SARS-CoV-2 infection³³ and that the resulting inflammation that may affect taste bud homeostasis,^{2,34} the response of the innate immune system of the host may be involved in taste loss in COVID-19 patients. Strikingly, tumor necrosis factor receptor 1 gene, *Tnfrsf1a*, displays abundant expression across all cell clusters. Moreover, the expression of IFN receptor genes *Ifngr1* and *Ifngr2* (but not the Il1 receptor gene *Il1r1*) was detected broadly in the tongue epithelium. Expression of *Tlr3*, which is essential for the recognition of viral pathogen and activation of the immune system, was very low (Figure 5).

DISCUSSION

COVID-19, caused by the infection of SARS-CoV-2,³⁵ is highly contagious and often life-threatening due to severe acute respiratory syndrome and the failure of other organs.^{36,37} The entry of the virus into cells is through the obligatory receptor *ACE2*^{18–22} and the protease *TMPRSS2*.²⁹ Several studies showed that COVID-19 viral infections occur through multiple potential routes including respiratory, oral, and contact.^{38–40} The symptoms vary among patients including sore throat, cough, fever, difficulty breathing, and loss of taste and smell.³⁶ In this report, we provide a new understanding of loss of taste in COVID-19 patients and a potential correlation of the susceptibility with the gestational stages. We acknowledge that mouse *ACE2* is, unlike humans, not susceptible to SARS-CoV-

2;²⁶ however, given that mice and humans share similar gene expression patterns, *ACE2* expression in mice may still provide insights into how pathological changes develop in humans.

Taste Loss in COVID-19 Patients Is Likely Not Due to an Initial Direct Viral Infection in Taste Bud Cells. A matter of particular interest is that taste loss, often together with smell loss, is one of the common symptoms of COVID-19, even when there are no other apparent symptoms.^{16,41} Recently reported data showed that *ACE2* is enriched in tongue epithelial cells in humans,²³ and *ACE2* mRNA is detected in both the gustatory and nongustatory tongue epithelium in mice.²⁵ However, it remains unclear what specific types of cells highly express *ACE2* and where they are localized in the tongue epithelium.

Taste buds are primarily located in the tongue, and lingual taste buds reside in the epithelium of the taste papillae. The three types of taste papillae (fungiform, foliate, and circumvallate) are stereotypically distributed on the tongue, i.e., fungiform located among the nongustatory filiform papillae on the anterior 2/3 oral tongue, foliate on the two lateral edges of the posterior oral tongue, and circumvallate papillae (8–12 in humans and 1 in rodents) in the border between the oral and pharyngeal tongue.⁴² Mammalian taste bud maintenance and homeostasis require the surrounding progenitor cells to continuously renew the differentiated taste bud cells (types I, II, and III) for intact taste function.⁶ Multiple lineages of taste bud cell progenitors have been reported including *Krt14*,⁴³ *Lgr5*,⁴⁴ *Lgr6*,⁴⁵ *SOX2*,⁴⁶ and *Gli1*⁴⁷ epithelial cells that surround taste buds.

To dissect the heterogeneity of gustatory epithelial cells, rapidly developing scRNA-seq techniques provide a powerful tool. In this report, the mining of two independent sets of data from a single cell RNA-Seq of anterior tongue epithelium provides insights into the molecular heterogeneity of these cells (Tables S1 and S2). Our results indicate that *ACE2* is expressed in a subpopulation of tongue epithelial cells. The *ACE2*⁺ cells are enriched in 4 out of 13 clusters of these tongue epithelial cells. Two of the four clusters highly express markers (*Hoxc13*, *Krt36*, and *Krt84*) that are restricted to a subpopulation of epithelial cells in the basal region of filiform (nongustatory) papillae.^{31,48} However, *ACE2*⁺ cell-enriched clusters do not frequently overlap with those clusters in which markers are expressed in taste bud cells and the surrounding taste papilla epithelial cells, i.e., *Krt8*,⁴⁹ *Lgr6*,⁴⁵ and *Gli1*.⁴⁷ Furthermore, scRNA-seq data of taste bud cells²⁸ confirm that most taste bud cells have no or extremely low expression level of *ACE2*. Expression of *ACE2* was detected in a small proportion of type III taste cells, which is consistent with mRNA detection in taste buds using RT-PCR.²⁵ Collectively, these scRNA-seq data provide a more precise information than the previously reported²⁴ regarding *ACE2*⁺ cell types and enriched tissue compartments. The enrichment of *ACE2* in nongustatory papillae but not taste buds indicates that taste loss in COVID-19 patients is not, at least initially, primarily due to a direct viral infection of taste bud cells.

We hypothesize that taste loss in COVID-19 patients may result indirectly from multiple potential causes. First, local and/or systematic immune responses to SARS-CoV-2-induced inflammation may disrupt taste bud homeostasis.⁶ It has been recently reported that deletion of *TNF α* prevents taste bud loss caused by the chronic inflammation.⁵⁰ Thus, abundant expression of *Tnfrsf1a*, the gene encoding tumor necrosis factor receptor 1 (TNFR1), in single-cell transcriptomic

analyses of anterior mouse tongue epithelium suggests that TNF signaling may be a mediator in taste loss during SARS-CoV-2 infection. In addition, systemic IFNs can trigger IFN-mediated signaling cascades in taste buds at posterior tongues.³⁴ Detection of both *Ifngr1* and *Ifngr2* expression in our single cell-level analysis of anterior tongue epithelium indicate the involvement of IFN-mediated signaling in regulating fungiform taste bud homeostasis in the anterior tongue. Second, infections of SARS-CoV-2 in the nervous system may damage the innervation of taste buds which leads to taste bud degeneration.⁵¹ Finally, if *ACE2* expression in taste buds can be activated by IFN⁴⁰ after the virus first enters into a host as recently reported in the olfactory epithelium in humans,⁴⁰ then there is a possibility that SARS-CoV-2 in the blood and saliva⁵² may infect taste bud cells via the subsequently expressed *ACE2* in these cells. This possibility requires further experimental testing.

Fetuses May Have Developmental-Stage-Dependent Susceptibilities to the Infection of SARS-CoV-2. The available pandemic data shows that elderly people are more susceptible to SARS-CoV-2 infection and are more likely to have a poor prognosis, including death.⁵³ A recent report showed that the *ACE2* protein level in some organs, e.g., olfactory epithelium, increases with age,⁵⁴ thus providing a possible explanation as to why the severity and mortality rate are higher in older people. Though young people are also susceptible to infection when exposed to the virus, the symptoms are mostly mild.⁵⁵ The youngest COVID-19 patient reported was only 4 weeks old.⁵⁶

To understand whether fetuses are also susceptible to COVID-19 through maternal transmission, thorough examinations on *ACE2* expression in the body of fetus and placenta are needed. It has been reported that *ACE2* is highly expressed in early ovum-phase human embryos (2- and 4-cell stages), and the expression level drops afterward.⁵⁷ Furthermore, the presence of *ACE2* in the urine of 23 week old human fetuses suggests the expression in the body.⁵⁸ In mouse pancreas, *ACE2* is detected in embryos and peaked at E16.5.⁵⁹ In the present study, we analyzed the expression of *ACE2* receptor and coreceptor *TMPRSS2* in different tissue compartments of the oral cavity in mouse embryos and newborns. Although the transcripts of *TMPRSS2* for SARS-CoV-2 entry were detected in the lingual and palatal epithelium of both embryos and newborns, those of the receptor *ACE2* were only detected in newborns, not in embryos at examined developmental stages (E12.5 and E14.5). Together, these findings indicate that the susceptibility of fetuses to SARS-CoV-2 infections is likely to be stage-dependent, i.e., a high level immediately after fertilization and during late fetal stages. Given that *ACE2* is highly expressed in the fetal part of placenta (labyrinth and basal zones)⁶⁰ and the female reproductive tract including uterus and vagina,⁶¹ the potential risk of maternal–fetal transmission exists before and during birth, although SARS-CoV-2 infections were not found in neonates of infected pregnant mothers in a recent report.⁶²

Different Viral Infections Transmit through Distinct Cell Targets in the Tongue Epithelium. It is known that various viral infections affect taste function to different extents, e.g., at distinct incidence and severity levels of taste dysfunction.⁶ Taste loss occurs more frequently in COVID-19 patients¹² than in other patients with influenza-like symptoms.¹² The underlying mechanisms in these infectious disease conditions remain elusive at the cellular level. In this

study, scRNA-seq profiling of anterior tongue epithelial cells that are heterogeneous structurally, molecularly, and functionally revealed that molecules for various viruses' entries into cells are enriched in different cell clusters. We hypothesize that different viral infections may cause impairments of different subpopulations of tongue epithelial cells and may trigger various immune responses leading to distinct taste deficiencies. To better understand how taste bud cells and/or progenitors are affected in various disorders, further experimental studies are needed. The available data from our analysis and others²⁷ will be useful in these future studies.

We are aware of the seemingly different expression levels of some genes between our study and literature reports. *Il1r1*, the gene encoding interleukin 1 receptor type 1, and *Tlr3*, the gene encoding Toll-like receptor 3, were detected at a low level in the scRNA-seq data set of anterior mouse tongue epithelium. However, detection of proteins IL1- β and IL1-RI receptor has been reported in taste buds in rats.⁶³ Moreover, multiple TLRs were detected in taste buds from posterior tongues using RT-PCR.⁶⁴ These discrepancies may be due, in part, to the difference of innate immune system components between the anterior vs posterior tongue. Furthermore, loss-of-function analysis of one or more innate immune components under different conditions will be beneficial for understanding the effects of inflammation on taste bud maintenance and function.

Overall, our results show that different viral infections may affect distinct cell targets in the tongue epithelium. We identified the basal region of epithelial cells in nongustatory filiform papillae as the prime targets for SARS-CoV-2 infection in tongue epithelium. We revealed that *ACE2* was detected in newborns, but little to no expression was found in mouse fetal oral tissues. Thus, the results in mouse tissues suggest when applied across species and organs that taste loss in COVID-19 patients is likely not caused by an initial direct infection of taste bud cells by SARS-CoV-2, that fetal susceptibility to SARS-CoV-2 is stage-dependent during prenatal development, and that risks of maternal–fetal transmission exist.

METHODS

Animals. Animal use was approved by The University of Georgia Institutional Animal Care and Use Committee and was in compliance with the National Institutes of Health Guidelines for care and use of animals in research.

Timed pregnant and newborn mice (C57BL/6J, The Jackson Laboratory, stock no. 000664,) were used to collect tissues for the in-house bulk RNA-Seq. Noon (12 pm) on the day in which a vaginal plug was detected was designated embryonic day (E)0.5. The day when the pups were born was noted as postnatal day (P)1.

Transcriptomic Profiling of Oral Tissues (Bulk RNA-Seq) in Mouse Embryos and Newborns. Pregnant mice at E12.5 and E14.5 were euthanized with CO₂, followed by cervical dislocation, and newborn (P1) mouse decapitation. Tongues and soft palates were dissected. To separate the epithelium and underlying mesenchyme, E12.5 tissues were incubated with 2.5 mg/mL dispase II (no. 04942078001, Roche Diagnostics); a 1 mg/mL collagenase A (no. 10103578001, Roche Diagnostics) was added for the E14.5 tissues. In the P1 tissues, the enzyme mixture was injected into the subepithelial space. After enzyme incubation at 37 °C for 30 min, the epithelium and mesenchyme of tongue and soft palate were separated for RNA extraction using Trizol and RNeasy Plus kits (Qiagen). RNA Quality was assessed on an

Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Each group of tissues included 3 biological replicates and each sample of the replicate contained up to 10 (E12.5), 3 (E14.5), and 1 (P1) tongues or soft palates for sufficient RNA.

cDNA libraries were prepared with Kapa Stranded mRNA-seq kit (KAPA Biosystems, Wilmington, MA). Library quality and quantity were measured by Fragment Analyzer Automated CE (Advanced Analytical, Evry Cedex, France) and Qubit (Thermo Fisher) systems, respectively. Libraries were subsequently subjected to 2×75 base pair (bp) paired-end sequencing on a NextSeq 500 system (Illumina). All samples were pooled and sequenced on one lane. The RNA quality check, library preparation, and sequencing were conducted at the University of Georgia Genomics and Bioinformatics Core (Athens, GA). The raw RNA-Seq data of each sample was aligned to mouse reference genome (GRCm37.1) via STAR,⁶⁵ and the transcripts were quantified via StringTie.⁶⁶ Gene expression was calculated in FPKM and presented as FPKM ($X \pm SE$, $n = 3$). Raw read counts were quantified by HTSeq and subsequently analyzed for differentially expressed genes (DEGs) between stages via DESeq2.⁶⁷ Significance was declared when the adjusted P value was less than 0.05.

All raw sequencing data and processed data were deposited in Gene Expression Omnibus (GSE151205).

Analysis of Public Data from Single-Cell Transcriptomic Profiling (scRNA-seq) of Adult Mouse Tongue Epithelium. Three independent data sets were used. Two sets of tongue scRNA-seq data were downloaded from *Tabula Muris*,²⁷ including one that was based on microfluid platform and contained 7538 adult anterior tongue epithelial cells (for unique molecular identifiers (UMIs)) and another based on the FACS method and contained 1432 tongue epithelial cells (for reads). Genes detected in less than three cells were filtered out. Cells in which less than two hundred genes were detected were removed.

The third set of data (SRP094673) contained 52 available cells: 47 circumvallate taste bud cells from different types (11 *Gad1*⁺ cells, 10 *Gustducin*⁺ cells, 17 type III cells, and 9 *Tas1r3*⁺ cells) and 5 taste bud cell progenitors (*Lgr5*⁺).²⁸ The raw sequencing data for each cell from Sukumaran et al.²⁸ was aligned to a mouse reference genome using STAR,⁶⁵ and the transcripts of genes were quantified using RSEM.⁶⁸ Gene expression was calculated in FPKM.

Analysis of scRNA-seq data sets were performed with *R* package *Seurat* (V3.1.5).⁶⁹ The expression matrix from Schaum et al.²⁷ underwent logistic transformation and scaling. Clusters of cells were identified by unbiased machine learning algorithms. Principal component analysis (PCA) was applied for dimension reduction, and t-Distributed Stochastic Neighbor Embedding (t-SNE) was used to visualize data at low dimensions.

Data Visualization. Visualization of bulk RNA-Seq data was conducted using *R* package *ggpubr*. For scRNA-seq analyses, t-SNE maps, heatmaps, violin plots, and dot plots were implemented using the built-in functions of *R* package *Seurat* (V3.1.5).⁶⁹

■ ASSOCIATED CONTENT

SI Supporting Information

Table for The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acspsci.0c00062>.

Top 10 significant genes for each cluster (13 clusters in total) identified in data set from Schaum et al. (7538 cells in total); top 10 significant marker genes for each cluster (6 clusters in total) identified in data set from Schaum et al. (1432 cells in total)²⁷ (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Hong-Xiang Liu – Regenerative Bioscience Center and Department of Animal and Dairy Science, College of Agricultural and Environmental Sciences, University of Georgia, Athens, Georgia 30602, United States; orcid.org/0000-0001-5515-9869; Phone: 1-706-542 7048; Email: lhx@uga.edu; Fax: 1-706-583 0274

Authors

Zhonghou Wang – Regenerative Bioscience Center and Department of Animal and Dairy Science, College of Agricultural and Environmental Sciences, University of Georgia, Athens, Georgia 30602, United States

Jingqi Zhou – Department of Genetics, Franklin College of Arts and Sciences, University of Georgia, Athens, Georgia 30602, United States

Brett Marshall – Regenerative Bioscience Center and Department of Animal and Dairy Science, College of Agricultural and Environmental Sciences, University of Georgia, Athens, Georgia 30602, United States

Romdhane Rekaya – Department of Animal and Dairy Science, College of Agricultural and Environmental Sciences and Institute of Bioinformatics, University of Georgia, Athens, Georgia 30602, United States

Kaixiong Ye – Institute of Bioinformatics and Department of Genetics, Franklin College of Arts and Sciences, University of Georgia, Athens, Georgia 30602, United States

Complete contact information is available at:

<https://pubs.acs.org/doi/10.1021/acspsci.0c00062>

Author Contributions

H.X.L. and R.R. designed the experiments for bulk RNA-Seq. H.X.L., Z.W., K.Y., and J.Q. designed the strategy for data analysis. H.X.L. and B.M. performed tissue harvest. Z.W. analyzed bulk-RNA seq data. Z.W. and J.Q. analyzed the scRNA-seq data. Z.W. performed data visualization and final figure assembling. Z.W. and H.X.L. wrote the manuscript. All authors read and approved the final manuscript.

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Notes

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