CHICKEN TASTE BUDS:

ANALYSIS OF THE STRUCTURE, NUMBER, DISTRIBUTION AND ORIGIN

by

PRASANGI IROSHA RAJAPAKSHA

(Under the Direction of HONGXIANG LIU)

ABSTRACT

Our recent findings demonstrated a previously unrecognized origin of taste bud cells from the underlying connective tissue that is primarily derived from neural crest (NC). The aim of the present study was to collect direct evidence for the migration and differentiation of NC cells to taste buds in chickens. We used immunohistochemistry to label chicken taste buds at the embryonic and post-hatching stages and found that α-Gustducin and Vimentin signals label distinct and overlapping populations of taste bud cells in all examined taste buds. Further, we developed an efficient method to label chicken taste buds with the antibodies in the oral epithelial sheets. Last, microinjection of DiI into neural tube was used to label NC cells and trace NC derived cells in taste buds. We found that DiI injection is not suitable for a long-term labeling of NC and a better option (quail-chicken chimera) is being adopted for further studies.

Index words: Chicken, taste buds, Vimentin, α-Gustducin, neural crest, and origin

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B.S, University of Peradeniya, Sri Lanka, 2014

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2016

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Dean of the Graduate School
The University of Georgia
August 2016

DEDICATION

Dedicated to,

My Mother and My Father

For making me who I am today, for being the role models of my life, for giving me wings of freedom to achieve my dreams, for being best motivators and advisers in my life

My Brother and My Friends

For not letting me give up when life was difficult, for the unconditional love and support, for bringing warmth of happiness and sounds of laughter to my life

ACKNOWLEDGEMENTS

I give thanks to Dr. Hongxiang Liu for giving me guidance and a novel research experience in my academic life, continuous support in completion of my degree and for the tremendous efforts in writing and editing the thesis.

I thank my advisory committee members Dr. Steven L Stice and Dr. Franklin West for their guidance, inputs and support in my research work.

I give thanks to the members in Dr. Liu's lab (Kristin Boggs, Nandakumar Venkatesan, Mohamed Ishan, Guiqian Chen, Brett Marshall, Xiushen Wang, Wenxin Yu, Zhonghou Wang, Xiaogang Cui) for their support in my work. Special thanks go to Dr. Nandakumar Venkatesan for his enormous support in doing the experiments side-by-side with me, drafting and editing the thesis for the completion on time. Also, thanks to the undergraduate student, Sanam Desai, for his assistance in conducting experiments.

I give thanks to Dr. Robert Beckstead and Mr. Jason Payne for their huge support by providing us advice and resources to start and continue this work; to Dr. Steven Stice and his lab members (Mr. Forrest Goodfellow and Mr. Raymond Swetenburg) for assistance in conducting experiments and manuscript editing; and to Dr. Luke Mortensen and Dr. Kayvan Tehrani for helping us with 2-photon microscopy, imaging analysis and editing manuscripts.

I give thanks to Dr. Robert Dove for his help and instructions in using the animal facility for chickens at ADS, UGA. I thank graduate coordinator Dr. Michael Azain,

department head Dr. Keith Bertrand and all other staff members in the Department of Animal and Dairy Science for their advice and support throughout this time.

I thank Dr. Muthugapatti Kandasamy at Biomedical Microscopy core, staff at Georgia electron Microscopy (Dr. John Shields, Ms. Mary Ad and Dr. Eric Formo) for their technical support. I would like to thank all the members in the Regenerative Bioscience Center (RBC) for their ideas and discussions on how to improve my research work throughout the studies.

I give thanks to Mr. Steven Fierros and other staff members at Cobb vantress Cleveland, GA hatchery for providing us animals for the project; to Dr. S. Tabata and Dr. F. Kawabata for providing us antibodies and their support in writing manuscripts; to Dr. Venkatesan Perumal for conducting the statistical analysis and help me with this project; Ms. Valerie Marcano for providing me all the journal articles for reference.

I would like to extend my thanks to my friends for their support and for giving me courage in every step. To my Athens family (Ms. Natasha Perumal, Mrs. Zahra Nawaz, Mr. Afaq Mohammed, Mr, Suganthan Amirthagunanathan, Mr.T.C Jayalath and Mrs. Samanthi Jayalth) for their continuous support.

Last but not the least my heartiest acknowledgment goes to my parents and my brother for guiding me to come this far, being the best support and reasons for my success.

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CHAPTER 1

INTRODUCTION

Taste buds have been described as deriving from local surrounding epithelium, which is in contrast to other sensory receptors that have a neuronal origin including neural crest (NC). Recent studies using transgenic mouse lines, *P0-Cre*, *Dermo1-Cre,* and *Vimentin-CreER*, to label NC cells and derivatives, indicate that the NC derived underlying connective tissue contributes to taste buds. However, we lack direct evidence for the migration and differentiation of NC cells to taste buds. The goal of the present study is to collect direct evidence about the NC derivation of taste bud cells in chickens.

Chickens were selected due to multiple reasons. First, chicken is a well-established experimental model with several advantages including the chimera with quail, easy manipulation of the eggs (*in ovo* studies), high availability and rapid development. Second, chickens have many taste buds, and previous studies have shown that Vimentin, an intermediate filament protein and mesenchymal cell marker, is expressed in a large subpopulation of taste bud cells. This supports that the underlying mesenchymal cells, primarily derived from NC, contribute to taste buds in chickens. Third, *in ovo* manipulated chicken embryos (including NC cell labeling) can survive in "cultures" long enough for tracing the labeled NC cells in early embryos until taste buds are formed at peri-hatching stages.

In order to demonstrate the contribution of NC cells to taste buds in chickens, the following studies were performed: (1) distribution of α-Gustducin and Vimentin in

premature and mature taste buds in chickens; (2) labeling and analysis of chicken taste buds with molecular markers in the oral epithelial sheets; (3) NC labeling and contribution to taste buds in chickens.

CHAPTER 2

LITERATURE REVIEW

TASTE SENSING IN CHICKENS

Prasangi Rajapaksha, Nandakumar Venkatesan, Hong-Xiang Liu. To be submitted to *The Journal of Animal Science*

Abstract

Taste is important in guiding nutritive choices and motivating feed intake. The sensory organs for taste are the taste buds that transduce chemical stimuli into neural signals. It has been reported that chicken has low taste bud number and thus low taste acuity. However, more recent studies indicate that chickens have a well-developed taste system and the reported number and distribution of taste buds may have been significantly underestimated. Chicken is the major species of animals in poultry industry. The implications of taste sensation on nutrient sensing, nutrient absorption and appetite are significant and our understanding of taste organ formation and the effects of the taste system on nutrition and feeding practices is important for finding better strategy in improving livestock production. In this review we focus on addressing the major findings in chicken taste biology including the basic information, development, maintenance, innervation of chicken taste buds; taste behaviors and potential impact of taste sensing on poultry industry.

Index words: chicken, taste bud, number, location, taste, poultry

Introduction

Taste sensation is conserved in animals and most species (e.g., mammals, amphibians, reptiles) have a well-developed taste system that is comprised of taste sensory organs, the innervating nerves and central nervous system. Although the implications of taste sensation on nutrient sensing, nutrient absorption and appetite are significant, our understanding of the effects of the taste system on nutrition and feeding practices relevant to farm animals including poultry, lags far behind. The role of taste sensing in regulating feed intake for improved animal productivity has been ignored, and presents significant opportunities to improve livestock production through better understanding of the physiological and molecular mechanisms. In this article, we will review the studies on the taste system in chickens, the major animal for meat and egg production in poultry industry.

Sensory organs for taste in chickens

The sensory organs for taste are taste buds that detect different types of taste stimuli and transduce the chemical signals into neural signals that are transmitted to the brain. Among different species, the distributions of taste buds vary. For example, mammalian taste buds are primarily located in the tongue and also observed in soft palate, epiglottis, pharynx, larynx, uvula (Höfer et al., 1996; Lalonde and Eglitis, 1961; Travers and Nicklas, 1990). Amphibians have large disc-like (~100 μm) taste organs on the tongue and palatal mucosa. In zebrafish, taste buds are found on the lips, jaws, and barbells. In reptiles, the taste buds are identified on the oral epithelium and the buccal floor (Doty, 2015).

The taste organ system in avian is one of the most prominent examples of a non-lingual taste system. Chicken taste buds are different from mammals in many aspects. Basic information about chicken taste bud structure, number, distribution, and development is available.

Taste bud structure, number, and distribution

Chicken taste buds, comprised of a cluster of specified fusiform cells, are ovoid ("egg" shaped) (Fig.2.1), which is in contrast to mammals whose taste buds are onion-like/bud-shaped. Unlike mammals, chickens do not have specialized structures like papillae (i.e., fungiform, foliate, circumvallate) to host the taste buds. The taste buds are embedded in the epithelium, and grouped in clusters that surround the salivary gland openings in a rosette pattern (Ganchrow and Ganchrow, 1985a).It has been reported that the ratio of

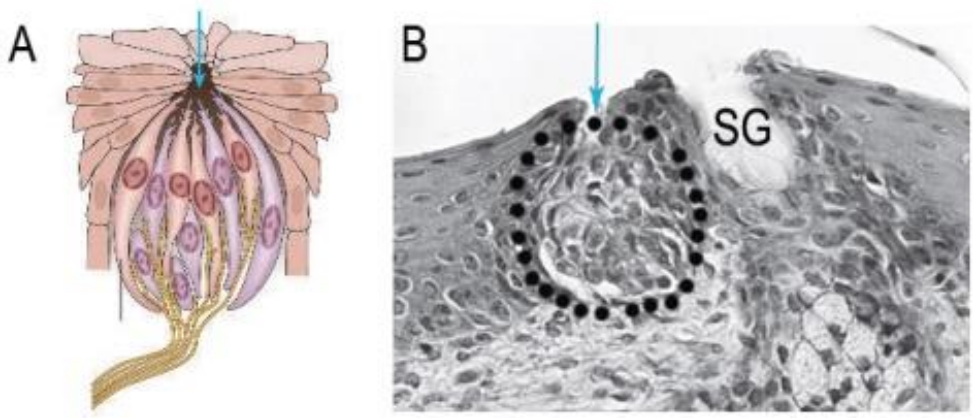

Fig. 2.1 Comparison of taste bud structure and shape in mammals (A) with that in chickens (B). **A:** a schematic diagram of a mammalian taste bud (mouse, human, rat), onion/bud shaped taste bud composed of different types of taste receptor cells. Receptor cells are innervated by gustatory nerves. Adapted and reproduced with permission from Macmillan Publishers Ltd: [Nature](Chandrashekar et al., 2006), copyright (2006). **B**: an ovoid-shaped chicken taste bud (outlined with black dots) located next to a salivary gland opening (SG). Adapted and reproduced with permission from John Wiley and Sons Inc.:[Animal Science Journal] (Kudo et al., 2008), copyright (2008). Blue arrows in A and B point to the taste pore.

salivary gland openings to taste buds in chickens is around 1:2.5, and this ratio does not change with ages (Ganchrow and Ganchrow, 1985a).

Initially, it was reported that chickens do not have taste buds (Lindenmaier and Kare, 1959) and later ~70 taste buds were found in the oral cavity (Berkhoudt, 1985). This number is low compared to mammals, e.g., rats (~1000 taste buds), humans (~10,000 taste buds (Doty, 2015) and cattle have about 15000-20000 taste buds (Davies et al., 1979). Further studies demonstrated that chickens have a higher number of taste buds that varies from 240-360 in average according to the breeds, e.g., broilers have more taste buds compared to the layer-type (Ganchrow and Ganchrow, 1985a; Kudo et al., 2008; Shiraishi et al., 2010).

Taste buds are primarily distributed in three regions in the oral cavity, i.e., palate (upper beak epithelium) (~69%) , base of the oral cavity (anterior mandibular gland region) (~29%) , and posterior ventrolateral regions of the keratinized anterior tongue and posterior region of the tongue (region posterior to the lingual spine) (~2%) (Ganchrow and Ganchrow, 1985a; Kudo et al., 2008) (Fig. 2.2). The proportions of taste buds in different regions vary with respect to different strains (Saito, 1966). Also, the total number of taste buds is similar in the young and adult chickens (Ganchrow and Ganchrow, 1987). The lower number of lingual taste buds suggests that tongue is not the primary organ for taste in chicken; it rather facilitates the food processing. The duck, another avian species, was reported to have similar distribution of taste buds in the palate and base of the oral cavity (Berkhoudt, 1976)

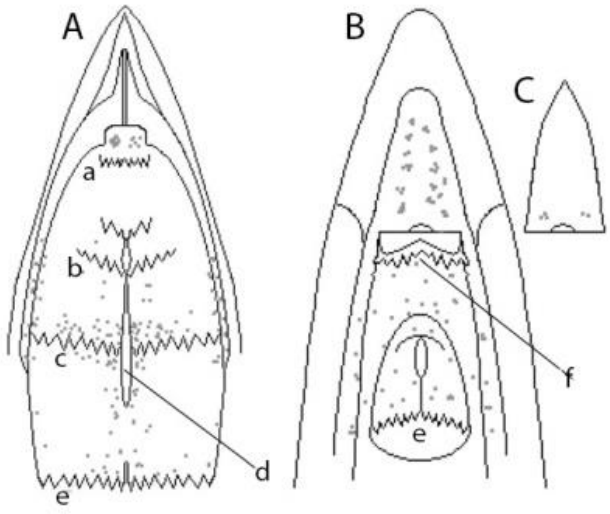

Fig. 2.2 Schematic diagrams illustrate the distribution of taste pores in the oral cavity of a post-hatching day 3 male chicken. Chicken taste buds are distributed in three tissue regions in the oral cavity, i.e., palate (A), base of the oral cavity (B, anterior and lateral region), posterior region of the tongue (B), and keratinized anterior tongue (C). Gray dots represent the taste pores of individual taste buds. Tissue marks are labeled as follows: a (lateral palatine), b (palatine papillae), c (papillae of the choanal opening), d (choanal opening), e (papillae of the pharynx), and f (lingual papillae). Adapted and reproduced with permission from John Wiley and Sons Inc.:[Animal Science Journal] (Kudo et al., 2008), copyright (2008).

In the oral epithelium, taste buds are located in the surround of salivary gland openings (diameter >20 μm). The current data on the number and distribution of taste buds have been obtained from the observation of taste pores (2-10 μm in diameter) (Ganchrow and Ganchrow, 1985a). It was also reported that some taste pores open to the salivary gland ductules instead of the surface of the oral epithelium (Roura et al., 2013). This indicates that the reported taste bud number and distribution in chickens may be underestimated by quantifying the visible taste pores on the surface of the oral tissue (Fig. 2.3).

Taste bud cell types & receptors

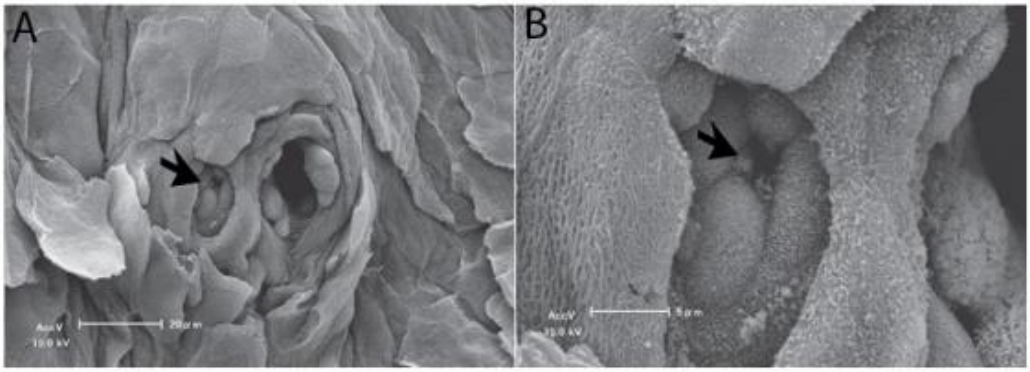

Fig. 2.3 Scanning electron photomicrographs of a chicken taste bud located next to a salivary gland opening. A single, shallow and large taste pore (arrow in A) is more easily seen in B (higher magnification image of the taste bud). Arrow in B points to a tubule of the taste bud. Adapted and reproduced with permission from John Wiley and Sons Inc.:[Animal Science Journal] (Kudo et al., 2008), copyright (2008). Scale bars: 20 µm in A, 5 µm in B.

Similar to mammals, the chicken taste bud cell population is heterogeneous in structure (Ganchrow and Ganchrow, 1985a; Kurosawa et al., 1983a). In mammals, there are four types of taste bud cells, type I cells (dark), type II cells (light) that are considered to be "receptor cells" , type III (intermediate) and type IV (basal) cells. In chickens, different taste bud cell types have been identified based on the studies using transmission electron microscopy. At least four taste bud cell types have been classified based on appearance, including basal cells, dark cells, light cells, and flattened/intermediate cells (Ganchrow et al., 1991). The dark cells, the most abundant cell type in chicken taste buds, have cytoplasmic extensions (similar to microvilli in mammalian taste bud cells) and its main function is in support of the taste bud (Ganchrow et al., 1998). In addition, dark cells have dense cytoplasm with scattered chromatin and less number of vesicles. The light cells/receptor cells, similar to type II taste bud cells in mammals, have less dense cytoplasm but has more number of vesicles compared to the dark cells. Both the

light cells and light cells synapse with the nerve fibers for signal transmission. The intermediate cells have the characteristics of both light and dark cells (Ganchrow et al., 1991). Basal cells, as the name indicates, are located in the basal region of the taste buds, have an irregular shaped nucleus, dense with particles and darker than the other cell types(Ganchrow et al., 1991).

Special proteins, i.e., different taste receptors and ion channels that are localized in the cell membrane of different types of taste bud cells, are the mediating molecules for transducing different taste stimuli. Therefore, the taste receptor and ion channel gene expressions in taste bud cells are responsible for taste qualities. For example, in mammals taste quality is determined by taste receptor and channel gene expressions in different taste cell types, e.g., sweet by T1R2+T1R3 in type II, umami by T1R1+T1R3 in type II, bitter by T2Rs in type II, salt by ENaC in type I, sour by PCKD channels in type III cells (Chandrashekar et al., 2006).

Compared to mammals, chickens seem to have fewer taste receptor genes, e.g., the sweet taste receptors are missing and their bitter taste receptor repertoire is small, consisting of only 3 members (T2R1, T2R2 and T2R7), in contrast to humans (25), cows (11), and mice(35) (Go, 2006; Meyerhof, 2005). Further, chickens' umami taste is detected by the GPCR T1R family receptors (T1R1 and T1R3), but it lacks the sweet receptor T1R2 (Cheled-Shoval et al., 2015; Lagerström et al., 2006). Gustducin is a G-protein present in type II taste bud cells in mammals (Yang et al., 2000). However, α-Gustducin was identified in a large subset of chicken taste cells (Kudo et al., 2010b), which has been reported to be expressed in sweet and bitter sensing taste cells in mice, hamsters and rats (Boughter Jr et al., 1997; Cho et al., 1998; McLaughlin et al., 1992).

The expression of α-Gustducin in chicken taste bud cells suggests a signaling mechanism that is similar to mammals (Fig. 2.4). Furthermore, recent studies have demonstrated that G-protein-coupled receptor-120 mediates the response of taste cells to oleic and linoleic acid, and is regarded as one of the functional fat taste receptors in chickens (Sawamura et al., 2015)

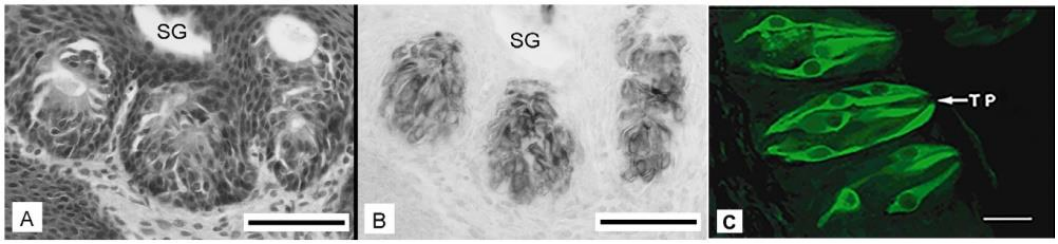

Fig. 2.4 Photomicrographs of taste buds in frozen sections immunoreacted with Gustdcuin, representing differences of Gustducin expressions between mouse and chicken. **A**: HE stained frozen tissue sections of the base of the oral cavity in a P3 male chicken. It illustrates the ovoid-shaped taste bud structures. **B**: Immunoreactivity of Gustducin in chicken taste buds. SG: salivary gland opening. Images in A and B were adapted and reproduced with permission from John Wiley and Sons Inc.: [Animal Science Journal] (Kudo et al., 2010b), copyright (2010). **C**: Expression of the Gustducin (green) in the spindle-shaped taste receptor cells of a mouse circumvallate papilla. TP: taste pore. Adapted and reproduced by permission from John Wiley and Sons Inc.: [Journal of Comparative Neurology], (Yang et al., 2000), copyright (2000). Scale bars: 50 μm (A, B) and 20 μm (C).

Development and renewal of taste buds

Developmental courses of formation

Chicken beaks and tongue, where taste buds are located, develop by embryonic day (E) 8, and taste buds emerge at E17 (Hamburger Hamilton stage 43) as a spherical shaped cluster of cells in the base of the epithelium (Fig. 2.5). From E17-18, the cell clusters further develop without a pore to penetrating the surface of the epithelium and the

number of taste buds increases at this stage. At E19, the taste bud cells elongate and form an ovoid-shaped structure, with a narrow and shallow taste pore penetrating the surface of the epithelium. At E19, the number of taste buds peaks and it has been reported to be consistent after hatch (Ganchrow and Ganchrow, 1987). Based on the quantitative analysis, the taste bud number in young chicks and adults, was similar and no age related effect on the taste bud development was observed, similar to mammals (Shin et al., 2011).

Compared to the rodents, chicken taste buds are functional early, i.e., immediately after hatch (Ganchrow et al., 1990). In humans taste buds begin to develop in 8-15 weeks of gestation (Witt and Reutter, 1996) and in primates taste buds starts develop by the end of first trimester (Zahm and Munger, 1983), but in rats (21 days gestation) and rabbits (30 days gestation) it starts at the end of the third trimester (Farbman, 1971). In rodents, taste bud differentiation and maturation happen postnatally. At birth (P0), the majority of taste buds do not have a taste pore and this taste pore development is completed in 2-3 weeks after birth (Hosley and Oakley, 1987).

Origin of taste bud cells in chickens

Origin of chicken taste buds is undefined. It has been described that the taste buds arise from the non-neuronal surrounding epithelial cells (Okubo et al., 2009; Stone et al., 1995). However, studies in mammals have revealed a dual origin of taste bud cells (Boggs et al., 2016; Liu et al., 2012), demonstrating a potential contribution of underlying connective tissue to the formation and renewal of taste buds in addition to that of surrounding epithelium.

In chickens, vimentin, an intermediate filament protein expressed in mesenchyme/connective tissue and neural precursors (Ganchrow, 2000), is also expressed in the majority of taste bud cells (Witt et al., 1999a; Witt et al., 2000). This supports the idea that a population of taste bud cells are from underlying connective tissue. It has been reported that in chickens, vimentin expression starts early as E17 and peaks at E19, and at the post-hatching stages the number of Vimentin^{+} cells reduced from 70% at E19 to 50% at post-hatching day 17 (P17) (Witt et al., 2000). In humans, vimentin is expressed in the taste bud primordium and later it is expressed in marginal epithelial cells (Witt and Kasper, 1999), which also indicates that connective tissue is a potential source of progenitors for taste bud formation and maintenance.

Distribution of vimentin^{+} cells in the taste buds indicates a potential contribution of the underlying connective tissue to the formation of taste bud cells but the exact cell lineage for chicken taste bud cells remains unclear (Witt, Reutter, Ganchrow, & Ganchrow, 2000). It has been reported that fibronectin, a specific component at the interface of epithelium and mesenchymal extracellular matrix is distributed in apical region and basal gemmal region of the taste cells. It suggests that fibronectin may play an important role in chemotactic mediated differentiation and migration of taste cells (Witt et al., 1999a).

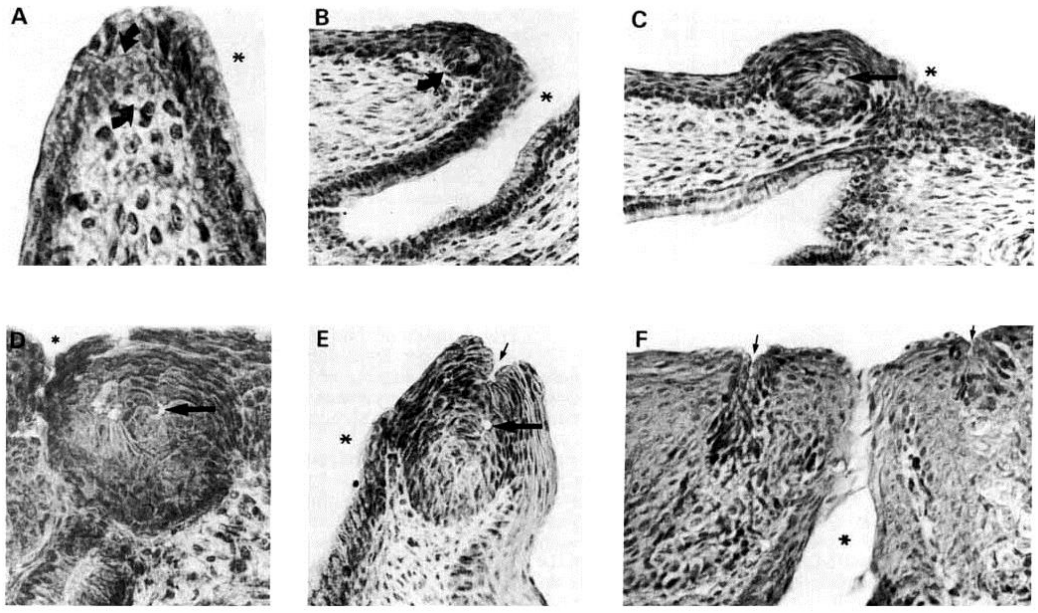

Fig. 2.5 Photomicrographs of chicken taste buds in the oral epithelium from E16-E20 illustrate the developmental progresses. A: Spherical shape chicken taste bud primordia at E16 (curved arrows point to the taste bud cells). B: A spherical taste bud primordia in the basal oral epithelium at E17. C: A representative image of a developed, spherical bud at E17 (black arrow pointed to the tubule). D: A spherical bud at E19 with tubules (arrow). E: A taste bud changed in to ovoid shape at E19 with a developing taste pore (small arrow). F: Taste buds located around a salivary gland duct opening. Small arrows point to the taste pores, and thick arrows point to the tubules. Asterisks mark the salivary gland ducts and openings. Adapted and reproduced by permission from John Wiley and Sons Inc.: [The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology] (Ganchrow and Ganchrow, 1987) copyright (2005).

Taste bud cell maintenance and renewal

Taste buds reside in the epithelium and the cells in taste buds have an epithelial feature, i.e., have a short life span and undergo continuous turnover. It has been suggested that, the cell turnover rate depends on the species, age and also the location of taste buds (Ganchrow et al., 1994; Ganchrow et al., 1993). In chickens, the average life span of taste bud cells is shorter compared to other vertebrates that have been reported so far. The life span of chicken taste bud cells in the base of the oral cavity is 3-4 days in

average (Ganchrow et al., 1994; Ganchrow et al., 1993), in contrast to 7-14 days in mammals (Beidler & Smallman, 1965; Farbman, 1980).

The high turnover rate of taste bud cells requires progenitor/stem cells for the rapid cell renewal in order to maintain the proper function of taste. The type IV or basal cells have been regarded as one of the stem cell niches. In addition, the "edge" cells that immediately surround taste buds are highly proliferating cells which is implicit for their function in the taste bud renewal. With regard to the origin of these progenitors, as we discussed earlier, both surrounding epithelium and underlying connective tissue have a contribution to taste buds (Witt et al., 2000).

Regulation of taste bud development

Taste organs are epithelial appendages that in general require epithelial-mesenchymal interactions and involvement of multiple signaling pathways. Sonic hedgehog (Shh) and bone morphogenetic proteins (BMP) signaling cascades have been identified in the development of avian tongue (Huang et al., 2001), where a small population of taste buds are located. The signaling mechanisms underlying chicken taste bud development are largely unknown. Knowledge about the regulation of taste organ development in rodents may give us perspectives and be beneficial for in-depth studies in chickens.

In rodents, numerous molecules have been identified in embryonic tongue and in developing taste papillae. For example, Molecular members of morphogen, growth and transcription factor families reported in embryonic fungiform papillae include: bone morphogenic proteins, Bmp 2,4 (Jung et al., 1999; Zhou et al., 2006); brain-derived neurotrophic factor, BDNF (Nosrat and Olson, 1995; Nosrat et al., 2001); Distaless-3, Dlx 3 (Morasso et al., 1995); fibroblast growth factor 8, Fgf 8 (Jung et al., 1999); insulin-

like growth factor binging proteins, IGFBPs (Suzuki et al., 2005); noggin (Zhou et al., 2006); patched receptor protein, Ptc (Hall et al., 1999; Mistretta et al., 2003); sonic hedgehog, Shh (Hall et al., 2003; Hall et al., 1999; Jung et al., 1999; Liu et al., 2004; Mistretta et al., 2003); Sox2 (Okubo et al., 2006); and, Wnt 6, 10a, 10b (Iwatsuki et al., 2007; Thirumangalathu et al., 2006). Direct demonstrations of regulatory roles for some of these molecules have been reported (Iwatsuki et al., 2007; Jung et al., 1999). Listed below are a couple of selected examples for a glance at the importance and complexity of molecular signaling pathways in regulating taste organ development.

Sonic hedgehog (Shh) signaling is important for taste organ formation and also regulation of cell fate determination at all stages (Miura et al., 2005). At early stages, disruption of Shh alters shape and growth of the tongue and they are upregulated in the taste placodes (Liu et al., 2013; Mistretta et al., 2003). Shh is important for the development of fungiform papillae and also responsible for the maintenance of interpapillary space via lateral inhibition process. The inhibition of Shh results in increase of fungiform papillae and ectopic presence of papillae in the intermolar eminence (Hall et al., 2003; Liu et al., 2004). In adult mice, Shh expression is limited to the basal cells of the taste buds, and it regulates the taste bud cell renewal and differentiation (Miura et al., 2003; Miura et al., 2001). Another signaling pathway, BMPs, functions differently from Shh; upregulation of BMP affects papillae development and its inhibition (blocking BMPRII receptor) advances papillae development in the anterior tongue region. Thorough understanding of the role of these signaling pathways and their interactions in the chickens will help to modify the taste sensing for improving the health and productivity.

Innervation of chicken taste buds and the role of CNS

In mammals, taste bud cells (type III, neuronal-like) have conventional synaptic contact with the gustatory neurons to transduce the chemical information to the central nervous system. Therefore type III cells called as “presynaptic cells” (Chaudary and Roper, 2010). The synapse-associated taste cells transmit electrochemical signals to the afferent nerve fibers of three different cranial nerve ganglia (VII, IX and X) and signals are transmitted to the taste sensory region in the hind brain (Doty, 2015; Scott, 2005). In vertebrates, branches of the facial nerve (VII), glossopharyngeal nerve (IX) and vagal nerves (X) are important for the transducing the gustatory information to the nervous system. The chorda tympani (branch of facial nerve) innervate the fungiform and anterior foliate taste buds in the anterior two-thirds of the tongue (Gentle, 1983, 1984).

Similar to mammals, chicken taste buds are innervated by the sensory nerve fibers for conveying the signals to the brain (Gentle, 1972). Chorda tympani (VII cranial nerve) innervates the taste buds in the anterior mandibular region of the oral cavity, which is supported by the electrophysiological studies to reveal that chorda tympani nerve responds to the taste stimuli (Gentle, 1972, 1983). The glossopharyngeal nerve is responsible for transducing the lingual gustatory information. No responses were observed in the trigeminal or facial nerves, via lingual taste stimulations (Kitchell et al., 1959).

The central nervous system is not well studied. In chickens, the hypothalamus region is important for the taste sensation in addition to controlling the tongue movement. Lesions in hypothalamic region result in gustatory hyposensitivity (i.e., fail to show aversive responses to quinine hydrochloride) (Gentle, 1975).

Studies have demonstrated that the maintenance of taste buds is nerve-dependent, which has been reported in mammals (Barlow et al., 1996; Barlow and Northcutt, 1997), in amphibians (Northcutt and Barlow, 1998).Taste bud loss is associated with neurological disorder (e.g., familial dysautonomia) and mechanical damages to gustatory nerves (Guth, 1971).

However, the role of innervation in the development of taste buds is not well defined. Two different hypotheses have been suggested including nerve-dependent and independent mechanisms. Nerve-dependent mechanism suggests that the innervation of the surrounding epithelial and receptor cells is necessary for the development of taste buds. Nerve independent mechanism suggests that initiation of taste bud formation occurs independently (Witt et al., 2000).

Behavioral responses of chickens to taste stimuli

Chickens respond to taste stimuli right after hatch, and newly hatched chicks respond to different taste stimuli and showed aversion/acceptance behavior for different tastants (Ganchrow et al., 1990; Gentle, 1972). In contrast to mammals that have five types of taste receptors, chickens have only four types of receptors (for sour, umami, salt and bitter) as the taste receptor T1R2 for sweet is lacking. Behavioral studies have identified the typical response to tastants including shaking the head, wiping the beak, and tongue/beak movements. In addition, the ability of signaling tastants to the brain has been analyzed with electroencephalogram (EEG) (Halpern, 1962).

Taste sensitivity of chickens

The sensitivity of taste in chickens positively correlates with the total number of taste buds, i.e., the more the taste buds, the more sensitive the bitter taste (Kudo et al., 2010a); (Miller Jr and Reedy Jr, 1990). Broiler-type males are more sensitive to taste stimuli than layer-type males because they have more taste buds (Ganchrow and Ganchrow, 1985a; Kudo et al., 2008). There has been a broad consensus that birds have a lower taste acuity compared to mammals due to their low taste bud numbers. However, emerging knowledge shows that birds have a well-developed taste system.

As discussed above, the distribution and number of chicken taste buds have been determined by observing taste pores with SEM (Ganchrow and Ganchrow, 1985a; Kudo et al., 2008) and the reported taste bud number and distribution in chickens may be underestimated because of the limitation of the technique. One of the limitations is that it based on the quantification of taste buds with taste pores opening to the surface epithelium. In reality, chickens (from P0 to adult) respond to chemical stimulants (e.g., hydrochloric acid, acetic acid) even at the low concentrations (Gentle, 1972). Although taste sensitivity cannot be quantified by behavioral responses, there exists a correlation between oral response and taste sensitivity (Gentle, 1972). It will be important to find an ideal method for evaluating the actual response to chemical stimuli.

There has not been an ideal method to easily visualize all chicken taste buds for acquiring an accurate number and distribution pattern. Molecular labeling in whole mount tissues provides an efficient way to obtain general information in many organs and tissues. Recently we have developed a simple method for labeling mouse taste buds in the intact tongue epithelial sheet (Venkatesan et al., 2015). This protocol, if applicable to

chicken gustatory tissues, will be significant in facilitating studies on the quantification of chicken taste buds and the association of bud numbers with feeding behaviors.

Taste quality of chickens

Identification of the five basic taste qualities (sweet, bitter, umami, sour, salty) has been accepted. Behavioral studies have been performed in chickens using some commonly used tastants, including sucrose, saccharine, quinine acid, sodium chloride, acetic acid, and hydrochloric acid. Newly hatched chicks were able to distinguish bitter and sour tastes, exhibiting aversive responses. Chickens' aversive responses to quinine chloride in a dose-dependent manner are similar to mammals but no significant response towards the 'sweet' stimuli probably due to the absence of T1R2 receptor (Ganchrow et al., 1990; Gentle, 1972). The oral responses are different for different stimuli (fructose, sucrose etc.) (Gentle and Harkin, 1979) and some stimuli are not perceived until specific concentration. So far, there has not been a standard method available to determine the taste responses. Different outcomes have been reported from different studies which could be attributed to the differences among the testing methods (Gentle, 1972; Gentle and Harkin, 1979). Therefore, establishing a standard for studying the oral responses when subjected to the different stimuli is important.

As described earlier, different taste receptors expressed in different taste bud cell types serve as mediating molecules for transducing different taste stimuli, and thus determine taste qualities. Bitter stimuli (e.g., quinine chloride) activates all three bitter receptors (Cheled-Shoval et al., 2014) for aversive responses (Gentle, 1972; Gentle and Harkin, 1979). Furthermore, chickens respond to fat via G-protein coupled receptor 120

(GPR120) and prefers corn oil which contains high amount of oleic and linoleic acid (Sawamura et al., 2015).

In chickens, the role of a specific cell type in determining taste quality is unclear. A recent report showed that Gustducin+ spindle-shaped cells isolated from taste buds respond to umami and bitter taste stimuli (Kudo et al., 2014), similar to Gustducin+ cells in mammals. Further studies on the differentiation of different taste cell types and the expression of different taste receptor genes that determine taste qualities will be significant for deepening our understanding of how taste qualities are determined in chickens.

Association of taste sensitivity and quality in chickens

In chickens, the taste sensitivity is different for different taste stimuli. For instance, chickens are more tolerant to 'sour' taste compared to mammals, but they are highly sensitive to 'bitter' taste although they have a lower number of bitter taste receptors (Hirose et al., 2015). It responds well to umami, composed of inosine-5'monophospahte and monopotassium L-glutamate rather than the single one, suggesting that the 'umami' taste synergism is highly conserved from birds to mammals (Yoshida et al., 2015). Embryonic and new born chicks are hypersensitive to water because water alone is considered to be a strong stimulus for avian, but there was no significant response when use egg fluid (Vince, 1977). However, they do not respond to sweet and salty taste stimuli until the tastants concentration is increased up to a high level (i.e., sucrose, 0-5N) (Ganchrow et al., 1990; Gentle, 1972).

Taste sensitivity for specific taste quality may be altered under certain conditions. Zinc-deficiency in chickens affects water intake and enhances bitter and salt taste stimuli

response, in contrast, zinc deficiency in humans and rats leads to taste loss (Catalanotto and Nanda, 1977; Henkin, 1984). However, no morphological changes in taste bud were associated with these deficiencies but when feed was supplemented with these minerals, there was a significant increase in response to taste stimuli (Gentle et al., 1981). Another example is that Vitamin A deficiency in chickens caused a decrease in response to taste stimuli (Gentle and Dewar, 1981), since Vitamin A is important for the maintenance of integrity of epithelial sheets. Similarly, in rats (Bernard et al., 1961) Vitamin A is deficiency leads to a significant decrease in response to NaCl and quinine chloride stimuli (Gentle and Dewar, 1981).

Impact of understanding taste bud formation and regulation on poultry industry

Chickens are one of the major livestock animals used for egg production and meat. Taste buds are the sensory organs that guides nutritive choices and motivates food intake, and thus have a direct impact on the productivity. Factors that influence the performance of livestock animals include health, growth, reproduction and the feed efficiency. Thorough knowledge about the taste bud formation/ regulation and taste response to different stimuli helps to improve the feed efficiency, thereby increasing the productivity. It has been reported that there exists a positive correlation between the number of taste buds and taste sensitivity (e.g., bitter taste). Further mechanistic studies on the taste bud development and taste receptor gene expressions will provide information about how taste sensitivity and quality are determined which will be beneficial for improving feed intake and performance.

Acknowledgements

This study was supported by the National Institutes of Health (grant number R01 DC012308 to HXL) and University of Georgia Start-up fund to HXL. The authors acknowledge John Wiley and sons. Inc., Macmillan Publishers Ltd., (Nature) for giving us the permission to reproduce figures from the journal articles.

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CHAPTER 3

DISTRIBUTION OF α-GUSTDUCIN AND VIMENTIN IN PREMATURE AND MATURE TASTE BUDS IN CHICKENS

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Abstract

The sensory organs for taste in chickens (*Gallus* sp.) are the taste buds in the oral epithelium of the palate, base of the oral cavity, and posterior tongue. Although there is no pan taste cell marker to label all the chicken taste bud cells, it has been reported that α-Gustducin and Vimentin each labels a subpopulation of taste bud cells. In the present study, we used both α-Gustducin and Vimentin to further characterize chicken taste buds at the embryonic and post- hatching stages (i.e. E17-P5). We found that both α-Gustducin and Vimentin label distinct and overlapping populations of taste bud cells. α-Gustducin immunoproducts were observed as early as E18 and consistently distributed in the taste bud primordium and mature taste buds at post-hatching stages. Vimentin immunoreactivity was sparse initially at the embryonic stages and apparent in the taste buds after hatch. In the post-hatch chickens, α-Gustducin and Vimentin signals were largely co-localized in the taste buds. A small subset of taste bud cells was labeled by either α-Gustducin or Vimentin. Importantly, each of the markers was observed in all the examined taste buds. Together our data suggest that the early onset of α-Gustducin in taste buds might be important for enabling chickens to respond to taste stimuli immediately after hatch and that the distinctive populations of taste bud cells labeled by different molecular markers, α-Gustducin or/and Vimentin, might represent different cells types, or different phases/stages of taste bud cells. Additionally, α-Gustducin and Vimentin can potentially become useful molecular markers for visualizing all the chicken taste buds in whole mount tissues.

Index words: α-Gustducin, Vimentin, taste bud, chicken, poultry, molecular marker, connective tissue, mesenchyme.

Introduction

Like mammals, chickens respond to taste stimuli (Ganchrow et al., 1990; Roura et al., 2013). Right after hatch, chickens exhibit aversive responses to bitter and sour taste (Ganchrow et al., 1990; Gentle, 1972; Hirose et al., 2015), and prefer umami and fat taste substances (Sawamura et al., 2015; Yoshida et al., 2015). In mammals and most species the sensory organ for taste is the taste bud and chicken taste buds are located in the epithelium of the oral cavity, i.e., palate, base of the oral cavity, and posterior region of the tongue (region posterior to the lingual spines) (Ganchrow and Ganchrow, 1985a; Kudo et al., 2008). The locations and structures have been identified by scanning electron microscopy (Ganchrow and Ganchrow, 1985a; Kudo et al., 2008) and histological analyses (Ganchrow and Ganchrow, 1987; Kudo et al., 2010b). However physically counting structures is time consuming and provides little information on source or type of cells that contribute to the chicken taste buds. While molecular markers for mammalian taste buds are available, e.g., K8 (Knapp et al., 1995; Mbiene and Roberts, 2003) and KCNQ (Wang et al., 2009), similar labeling for all chicken taste bud cells is lacking.

Vimentin, an intermediate filament that is expressed in the mesenchyme/connective tissue cells and neural precursors (Cochard and Paulin, 1984; Franke et al., 1978), was reported to label a significant population of chicken taste bud cells from the embryonic to the post-hatching stages (Witt et al., 1999a; Witt et al., 2000). Distribution of Vimentin^{+} cells in chicken taste buds suggest a potential contribution of underlying connective tissue to taste buds, which is consistent with our recent findings in mice (Boggs et al., 2016; Liu et al., 2012). More recently, Gustducin expression was found in mature chicken taste bud cells at post-hatching day 3, suggesting a similar signaling mechanism

as that in mammals (Kudo et al., 2010b). Questions arise (1) when does α-Gustducin starts expressing in the developing taste buds, for taste signal transduction; (2) whether α-Gustducin and Vimentin label the same or different populations of taste bud cells; (3) how Vimentin$^+$ and α-Gustducin$^+$ cells incorporate with each other for the taste bud formation. Clarification and comparison of different molecular markers in labeling chicken taste bud cells will provide new insight into how chicken taste buds develop and function.

Chicken taste bud primordia emerge at embryonic day 17 (E17) as clusters of spherical shaped cells and mature at E19 as ovoid shaped cell clusters that penetrate to the epithelium with a taste pore (Ganchrow and Ganchrow, 1987). However, we lack a clear understanding of fundamental issues about chicken taste bud formation: (1) what constitutes taste cell precursors and how the precursors differentiate in oral epithelium to taste cells, and (2) how the underlying mesenchyme, directly or indirectly, contribute to taste bud formation. Similar to mammals, chicken taste bud cell population is heterogeneous in structure and thus potentially also in function (Kurosawa et al., 1983b). According to ultrastructural appearance, at least four types of taste bud cells have been identified, i.e., basal cells, dark cells, light cells and flatten (intermediate) cells (Ganchrow et al., 1991; Ganchrow et al., 1998; Ganchrow et al., 1993). Molecular markers are not available for identifying the different taste bud cell types in chickens. Further studies on the distribution of molecular markers will be informative for understanding the mechanisms about the development and function of chicken taste bud cells.

In the present study, we define the distribution of α-Gustducin and Vimentin immunoreactivities in chicken taste buds at embryonic and post-hatching stages. We found that the α-Gustducin immunoreactivity emerged early in premature taste buds and were consistently distributed in a large population of mature taste bud cells. Brightly labeled Vimentin^{+} cells were not abundant in taste buds until after hatch. Both markers label distinct but overlapping taste bud cell populations and each of the markers was observed in all the taste buds. Our data suggest that (1) α-Gustducin and Vimentin can be useful molecular markers for visualizing *all* chicken taste buds, though not all the taste bud cells; (2) the emergence of α-Gustducin in premature taste buds enables chickens to respond to taste stimuli immediately after hatch, and (3) similar to mouse, different sources of progenitor cells contribute to chicken taste buds as evident by the two overlapping populations identified. The findings provide novel information about the development and function of chicken taste buds.

Materials and Methods

Animals and tissue collection

The 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.

Fertilized eggs and newly hatched (P0) male chicks were obtained from the Cobb-Vantress., Inc, Cleveland Hatchery, Georgia. The chicks (P0) were housed until P5 (Day 5) in the animal facility with brood temperature maintained at 35 ^{o}C and room temperature at 30 ^{o}C and continuously monitored. Animals were maintained with food (starter feed) and water *ad libitum* and in 12-12 hr dark-light cycle. For tissues at

embryonic day 17, 18 and 19 (E17, E18, and E19), the fertilized eggs were incubated in a standard egg incubator at 37.7 °C and 50-60% humidity.

Tissues from the palate, base of the oral cavity and posterior tongue were collected at E17, E18, E19, P0, P1, P3 and P5 stages. For the E17-E19 tissues, timely incubated eggs were cracked and embryos were collected into 0.1 M PBS solution. P0-P5 chickens were euthanized by decapitation. Tissues were dissected and fixed in 4% paraformaldehyde (PFA) for ~4 hr at room temperature. The tissues were briefly rinsed in 0.1 M PBS followed by cryoprotection with 30% sucrose at 4 °C for ~48 hr. The tissues were trimmed under dissecting microscope to include regions that contain taste buds, embedded in OCT compound (Tissue Tek) at a sagittal orientation and rapidly frozen. Serial and neighboring sections were cut at 6-15 µm in thickness, mounted onto gelatin-coated glass slides and processed for different analyses as below.

Histological analysis for identification of chicken taste bud structure

The 6 µm-thick frozen sections from E17, E18, E19 and P0 tissues were used for the analysis. In brief, slides were air dried at room temperature for 1 hr and rehydrated in 0.1 M PBS. Then slides were rinsed in running tap water (2 mins) and then dipped in each of the following solutions: Mayer's Hematoxylin (2 mins), Acid EtOH, Ammonia water and Eosin Y (30 s); rinsed in running tap water (2 mins). Then sections were sequentially dehydrated in 70% EtOH, 95% EtOH and 100% EtOH, followed by sequential immersion in Xylene and mounted with Permount ™ mounting medium (Sp 15-500, Fisher Scientific)**.** The mounted slides were air dried in a fume hood and examined under microscope**.**

Immunohistochemistry

The primary antibodies used were: α-Gustducin (1:500, serum of rabbit immunized with chicken α-Gustducin, generated by Dr. Shoji Tabata Lab) (Kudo et al., 2010b); Epcam (epithelial cell adhesion molecule markers) (1:200, MBS2027145, Mybioresource, Inc); Vimentin (1:200, abcam 28028, Vim3B4, Abcam, Cambridge, MA) and. Secondary antibodies were: Alexa Fluor 647 conjugated donkey anti-rabbit secondary antibody (1:500, Code: 711-605-152; Jackson Immuno Research Laboratories, Inc), Alexa Fluor 488 conjugated donkey anti-mouse (1:500, Code: 715-545-150, Jackson Immuno Research Laboratories, Inc)

Frozen sections of the base of the oral cavity tissue at E17-P5, and palate at E19 and P0 were used for immunohistochemistry. In brief, sections were air dried for 1 hr at room temperature and rehydrated in 0.1 M PBS. Non-specific staining was blocked using 10% normal donkey serum in 0.1 M PBS containing 0.3% Triton X-100 (PBS-X) for 30 min at room temperature. Then the sections were incubated with primary antibody in 1% normal donkey serum in PBS-X overnight at 4°C. Following rinses in 0.1 M PBS (10 min x 3) the sections were incubated with AF 488 (for Vimentin) and AF 647 (for α-Gustducin & Epcam) 1% NDS in PBS-X for 1 hr at room temperature. Then sections were rinsed with PBS and counterstained with DAPI (200 ng/ml in PBS) for 10 min, rinsed in 0.1 M PBS, air dried and cover slipped with ProLong® Diamond antifade mounting medium (P3697, ThermoFisher Scientific). In the negative control slide, primary antibody treatment was omitted or replaced with normal serum/IgG.

Photography and image analysis

The Hematoxylin & Eosin stained sections were examined under Zeiss AX10 light microscope. The α-Gustducin and Vimentin immunoreactivity was thoroughly examined using a fluorescence light microscope (EVOS FL, Life technologies). To further analyze the co-localization of α-Gustducin and Vimentin immunoproducts, single plane laser-scanning confocal images were taken using Zeiss LSM 710 microscope. Representative photomicrographs were assembled and edited using Adobe Photoshop CC 2015 software.

Results

Distribution of α-Gustducin and Vimentin in early taste buds in chicken embryos

Based on the previous report that chicken taste buds emerge at E17 and mature by E19, we examined the immunoreactivity of α-Gustducin and Vimentin in E17-19 chicken embryos. At E17 taste bud structures were identified in the H & E stained sections. However, α-Gustducin and Vimentin immunoproducts were not detected in the neighbor sections of those with taste bud structures (data not shown). At E18, α-Gustducin signals were observed in the specified cell clusters in the epithelium of the base of the oral cavity (Fig. 3.1A). Vimentin immunoreactivity was also observed, although sparse, in the α-Gustducin^{+} cell cluster region (white dotted outlines, Fig. 3.1A) and underlying mesenchyme (arrows, Fig. 3.1A). In the E19 embryos, α-Gustducin^{+} cell clusters were apparent and larger in size (Fig. 3.1B, C) compared to E18. Vimentin signals were seen but still sparse in the α-Gustducin^{+} cell cluster region in addition to the distribution of labels in the underlying mesenchyme. Double labeled cells with both α-Gustducin and Vimentin immunoproducts were observed infrequently (Figure 3.1B, arrowhead).

Of note, no signals were detected in the specified cell clusters (presumably taste buds) (white dotted outlines, Fig. 3.1D) when primary antibodies were omitted. However, bright immunofluorescence was observed in some cells scattered in both epithelium and mesenchyme (asterisks, Fig. 3.1A-C). The same pattern (solid labeling of the whole cell) was also seen in the negative control sections without using primary antibodies (asterisks, Fig. 3.1D), which suggests the labeling was non-specific.

α-Gustducin and Vimentin were largely co-localized in mature taste buds after hatch

At post hatching day 0 (P0), the α-Gustducin immunoproducts were apparent in the cluster of specified cells in the epithelium of base of the oral cavity (white dotted outlines, Fig. 3.2A), palate (white dotted outlines, Fig. 3.2B) and posterior region of the tongue (white dotted outlines, Fig.3.2C). Compared to embryonic stages (E18-E19), Vimentin immunoproducts were bright and seen in all α-Gustducin+ cell clusters, presumably taste buds. In addition to labeling a subset of cells in the taste buds, Vimentin signals were also distributed extensively in the underlying connective tissues. Importantly, Vimentin signals were not seen in the epithelium outside of the taste buds. Within the specified cell clusters of the oral epithelium, the co-localization of α-Gustducin and Vimentin immunoproducts were detected in a large proportion of taste bud cells (arrowheads, Fig. 3.2A-C). Also, singly labeled α-Gustducin$^+$ or Vimentin$^+$ cells were seen (open arrowheads, Fig. 3.2A-B).

At later stages (P1-P5), intense α-Gustducin and Vimentin immunoproducts were consistently distributed in all the specified cell clusters in the base of the oral cavity (Fig. 3.3). There was no obvious change in the proportion of α-Gustducin$^+$ cells. Vimentin$^+$

cells in the taste bud were frequently seen and the signals were largely co-localized with α-Gustducin (arrowheads, Fig. 3.3A-C) in the taste bud like structures (white dotted outlines, Fig. 3.3A-C). A subpopulation of taste bud cells was labeled by either one of the markers (open arrowheads, Figure 3A-B).

Noteworthy, although neither α-Gustducin nor Vimentin labels all the taste bud cells, their immunoproducts were detected in all the examined taste buds at post-hatching stages.

Vimentin$^+$ taste bud cells acquire epithelial cell phenotype

Double labeling of Vimentin and epithelial cell adhesion molecule (Epcam) was performed to examine whether the Vimentin$^+$ cells in the taste buds are also epithelial-like in the base of the oral cavity at P0 and P3 (Fig. 3.4). Epcam immunoreactivity was distributed in the epithelium (Fig.3. 4A-B), including taste bud like structures in which Vimentin immunoreactivity was seen (Fig. 3.4A-B). Although Vimentin and Epcam immunoproducts were in the different cellular compartments, i.e., cytoplasmic and membrane respectively, colocalization of these signals in the taste bud cells were obvious. No Vimentin$^+$ cells were found outside of the taste bud like structures in the adjacent/surrounding epithelium.

Discussion

Chicken taste buds, located in the oral epithelium in the palate, base of the oral cavity, and keratinized anterior and posterior region of the tongue (Ganchrow and Ganchrow, 1985a; Kudo et al., 2008), emerge at the late embryonic stage (early embryonic day 17) (Ganchrow and Ganchrow, 1987). The development course can be divided into two phases according to the features of the structure: (1) initial stage where cluster of

spherical taste buds are just formed, and (2) later stage where they develop into ovoid-shaped clusters of elongated cells (Ganchrow et al., 1995; Ganchrow and Ganchrow, 1987). Taste buds are identified in clusters adjacent to the salivary gland opening and close to the epithelium (Ganchrow and Ganchrow, 1985a; Kudo et al., 2008). In the present study, we detected the α-Gustducin immunoreactivity in a significant population of taste bud cells as early as E18 in the immature taste buds and through the post-hatching stages in the mature taste buds. These results suggest an important role of α-Gustducin in mediating taste signals. Vimentin, a marker that labels the underlying connective tissues of the oral cavity (Witt et al., 1999a; Witt et al., 2000), was detected in a large population of α-Gustducin^{+} taste bud cells indicating a contribution of connective tissue to taste buds in chicken. Distinct but overlapping taste bud cell populations labeled by α-Gustducin and Vimentin explain explicit different origins/types/functions of taste bud cells.

α -Gustducin expression in premature taste buds suggests the possible function of taste bud cells at early stages

Gustducin is a G-protein that plays a key role in signaling taste transduction (Lagerström et al., 2006; McLaughlin et al., 1992). In rat, taste bud cells that express gustducin have a long life span compared to other cell types (Cho et al., 1998). In mammals, it is expressed in the type II taste bud cells that are differentiated postnatally and comprise 20-30% of the taste receptor cells for sweet, bitter and umami taste (McLaughlin et al., 1992; Zhang et al., 2008). α-Gustducin is explicitly expressed in the mature chicken taste buds at P3 (Kudo et al., 2010b). In the present study, we detected the α-Gustducin immunoreactivity in immature taste buds as early as E18. As mentioned

earlier, chickens respond to bitter stimuli right after hatch (Ganchrow et al., 1990). The expression of gustducin may be the mark of the early onset of differentiated cell type that enables chicken to respond to gustatory stimuli even before hatch (Vince, 1977). Also, α-Gustducin^{+} cells, although not quantified, comprise a significant population (conservatively greater than 50%) of taste bud cells indicating the importance of GPCR signaling mechanism for taste transduction in chickens. Kudo et al. reported that there were high expressions of α-Gustducin in the isolated chicken taste bud-like subset (Kudo et al., 2014) and the findings were identical with the present study.

Vimentin^{+} cells in taste buds indicate a contribution of connective tissue to taste buds

Our recent studies using transgenic mouse lines have demonstrated a novel progenitor source of taste bud cells, i.e., from underlying connective tissue (Boggs et al., 2016), in addition to the conventional concept that taste bud cells are from the surrounding epithelium (Okubo et al., 2009; Stone and Finger, 1994; Stone et al., 1995). The distribution of Vimentin^{+} cells in chicken taste buds identified in the present study and previous reports (Witt et al., 1999a; Witt et al., 2000) supports the idea about the dual origin of taste buds from both underlying connective tissue and surrounding epithelium. In human, Vimentin is also expressed in the taste bud primordia and at later stages is restricted to the marginal cells of the bud (Witt and Kasper, 1999), which adds another species for the taste bud origin from underlying connective tissue. Taken together, here we propose that, in contrast to the hypothesis of Witt et al (Witt et al., 1999a; Witt and Kasper, 1999; Witt et al., 2000) "the mechanisms of taste bud differentiation from source tissues may differ among vertebrates of different taxa", there may be a common link

among vertebrate species, at least in human, mouse and chicken if not all, connective tissue cells contribute to the formation and renewal of taste buds.

Differences do exist among these species. The relative proportion of taste bud cells retaining Vimentin expression and proportion arising from the Vimentin$^+$ connective tissue cells appear to differ. The turn-over cycle (or life span) of taste bud cells are different in different species, e.g., 10-12 days in average in mouse (Farbman, 1980) vs. 3-4 days in chicken (Ganchrow et al., 1994; Ganchrow et al., 1993), which might be the cause of different proportions of Vimentin$^+$ taste bud cells. It is reasonable to speculate that chicken connective tissue cells retain Vimentin expression more easily while they acquire taste and epithelial cell phenotype during the rapid migration and differentiation to taste bud cells than mammals. Indeed, the Vimentin$^+$ cells in the chicken taste buds were also labeled with epithelial cell marker indicating a mesenchymal-epithelial transition. Regarding the difference of proportions of taste bud cells from the Vimentin$^+$ connective tissue cells between mouse (40-20%) (Boggs et al., 2016) and chicken (70-50%) (Witt et al., 2000), it is too early to have a rational explanation about the mechanisms under the mesenchymal epithelial transition (Witt et al., 1999a; Witt et al., 2000). Further studies on how the interactions between the underlying connective tissue and the surrounding epithelium are regulated will be important for better understanding how taste buds are formed and renewed.

Distinct and overlapping distribution of α-Gustducin and Vimentin labeling indicate different features of taste bud cells

It has been reported that α-Gustducin and Vimentin each labels a large subpopulation of taste bud cells in chickens (Kudo et al., 2010b; Witt et al., 1999a; Witt et al., 2000).

However, this is the first study using both markers to examine the distribution of labeled taste bud cells in early immature and later the mature taste buds. We found distinct and overlapping distribution of α-Gustducin and Vimentin in taste bud cells provide novel information about the taste bud formation and function. First, both α-Gustducin and Vimentin immunoreactivity were distributed in all the examined taste buds, although without labeling all the taste bud *cells*, which indicate that they can be the potential molecular markers for taste bud visualization and quantification if a protocol is available in whole mount tissues. Second, these two markers labeled distinct, though overlapping, populations of taste bud cells. The α-Gustducin^{+}, Vimentin^{+}, and α-Gustducin^{+} Vimentin^{+} taste bud cells may represent different taste cell types or taste cells at different phase of development (maturity). Four different cell types (dark, light, intermediate and basal cells) have been identified in chickens (Ganchrow et al., 1991; Ganchrow et al., 1993). So far there are no molecular markers available to identify the cell types. As mentioned earlier, in mammals Gustducin is expressed in the type II taste bud cells (Yang et al., 2000) that are important for the transduction of sweet, umami and bitter (Chandrashekar et al., 2006; Chandrashekar et al., 2000). Further studies are needed to identify whether the large population of chicken taste bud cells labeled by α-Gustducin are also type II cells.

Taken together our data suggest that α-Gustducin and Vimentin signals were co-localized in a large population of taste bud cells, and a small subset of taste bud cells was labeled by either α-Gustducin or Vimentin. Importantly, each marker was observed in all the examined taste buds. We propose that early onset of α-Gustducin in taste buds is important for enabling chickens to respond to taste stimuli immediately after hatch and

initiate trans-differentiation of Vimentin^{+} mesenchymal cells to taste bud cells in the epithelium. Different taste bud cell populations labeled by different molecular markers might represent different cells types, or different phases/stages of taste bud cells.

Acknowledgements

We give thanks to the staff at Cobb Vantress Cleaveland Hatchery, GA for providing the chickens and eggs. This study was supported by the National Institutes of Health (grant number R01 DC012308 to HXL) and University of Georgia Start-up fund to HXL.

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Figures

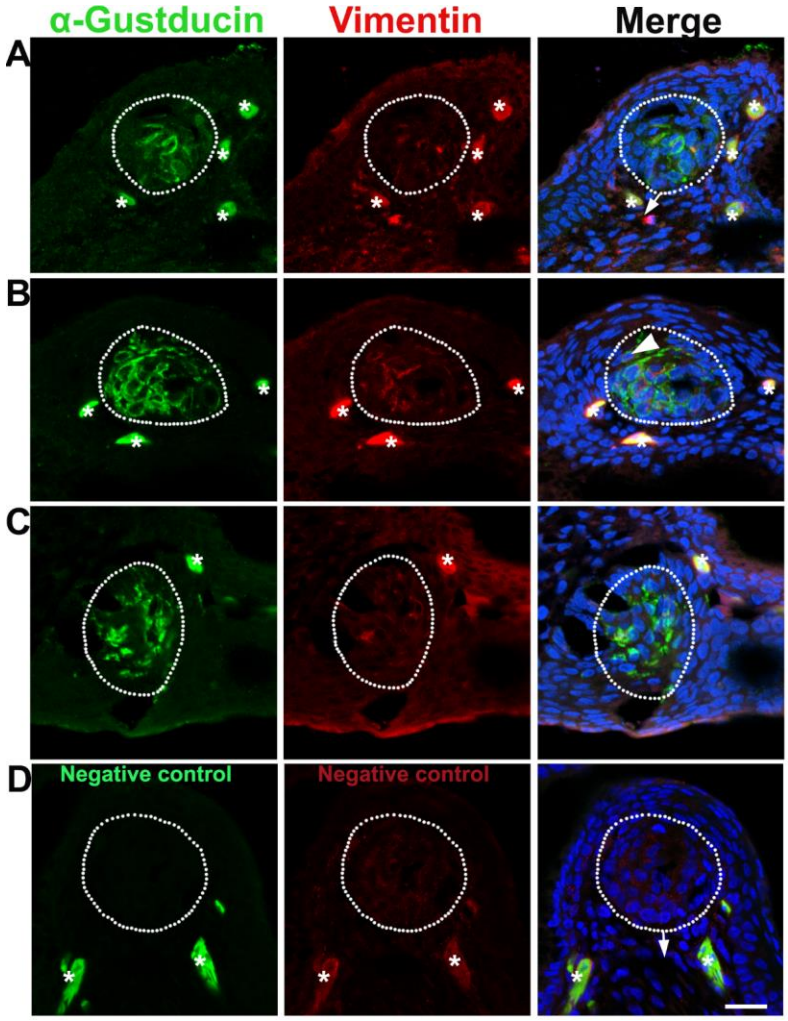

Fig. 3.1 Single plane laser-scanning confocal photomicrographs illustrate the distribution of α-Gustducin (green) and Vimentin (red) immunoreactivity in the oral tissue of chicken at E18 (A), E19 (B-C) in the base of the oral cavity (A, B) and palate (C). Sections of base of the oral cavity without adding primary antibodies were used as negative control (D, E19). Sections were counterstained with DAPI (blue) to stain the nuclei of cells. White dots outline the specified cell clusters where taste buds develop. Arrows point to the connective tissue. Arrowhead in B points to a cell double labeled with both α-Gustducin (green) and Vimentin (red). Asterisks mark the nonspecifically labeled cells. Scale bars: 20μm for all images.

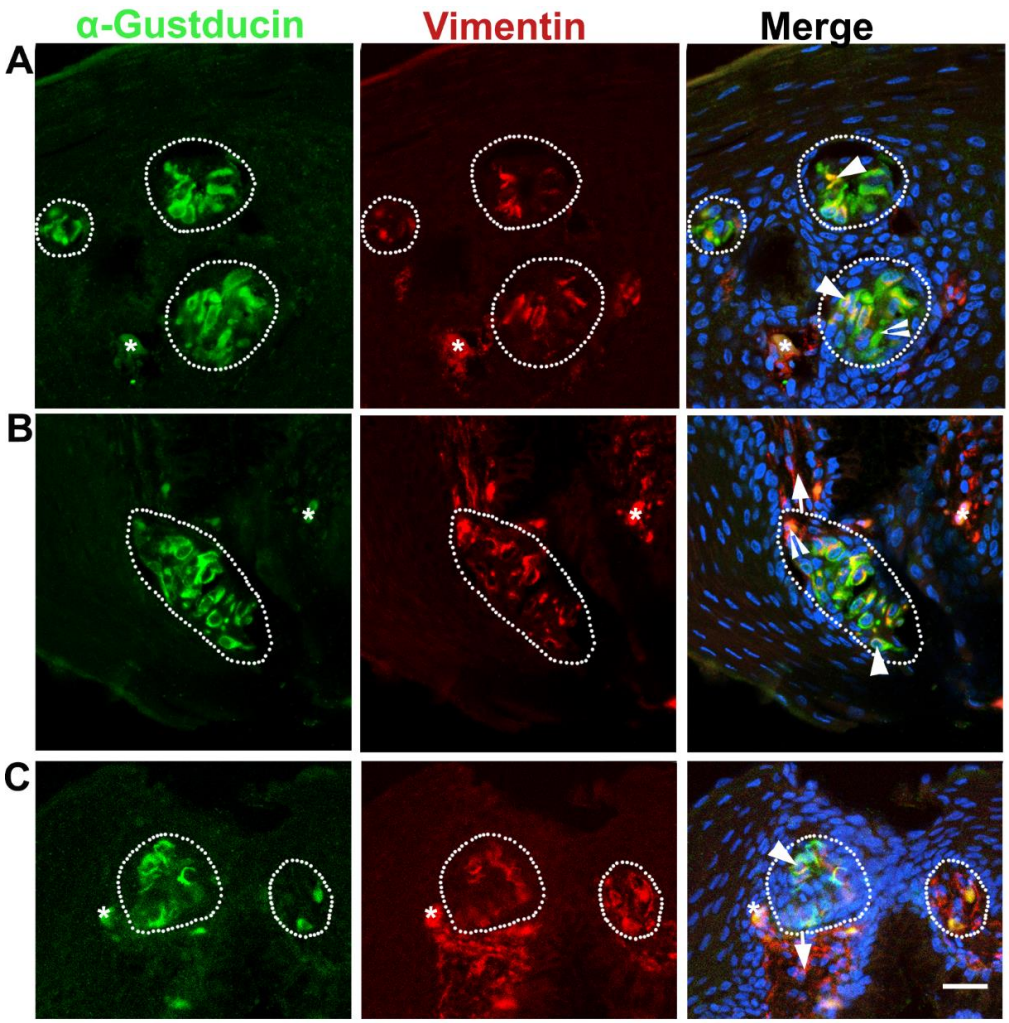

Fig. 3.2 Distribution of α-Gustducin (green) and Vimentin (red) immunoreactivity in the oral tissues of a P0 chicken, e.g., base of the oral cavity (A), palate (B) and posterior region of the tongue (C). White dots outline the specified cell clusters, presumably taste buds. Arrows point to the connective tissue. Arrowheads in A-C point to some of the cells double labeled with α-Gustducin (green) and Vimentin (red). Open arrow heads in A-B point to singly labeled α-Gustducin^{+} or Vimentin^{+} cells. Asterisks in A and C mark the cells with nonspecific labelling. Scale bar: 20μm for all images (single plane laser-scanning confocal).

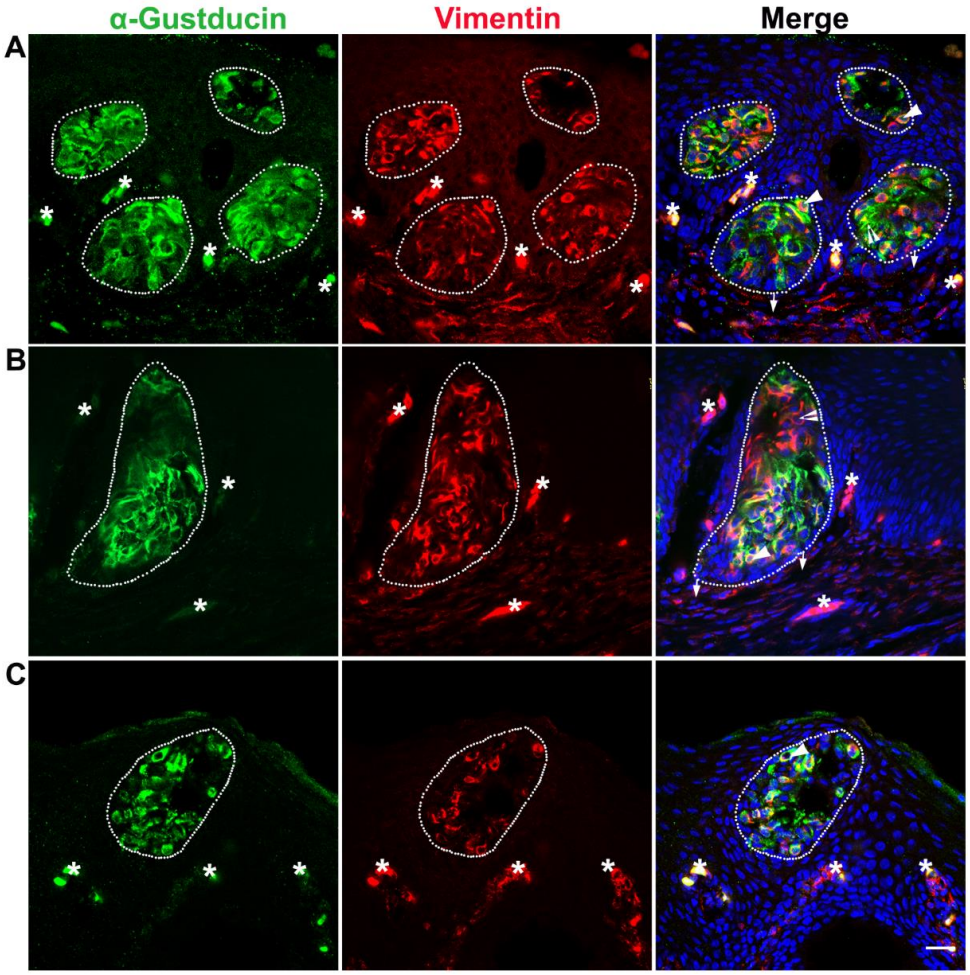

Fig. 3.3 Overlapping but distinct distribution of α-Gustducin (green) and Vimentin (red) immunoreactivity in the taste buds in the base of the oral cavity at P1 (A), P3 (B), and P5(C). White dots outline the specified taste bud cell clusters. Arrows point to the connective tissue. Arrowheads point to some of the cells double labeled with both α-Gustducin (green) and Vimentin (red). Open arrow heads in A-B point to singly labeled α-Gustducin^{+} or Vimentin^{+} cells. Asterisks mark the nonspecifically labeled cells. Scale bars: 20μm for all images (single plane laser-scanning confocal).

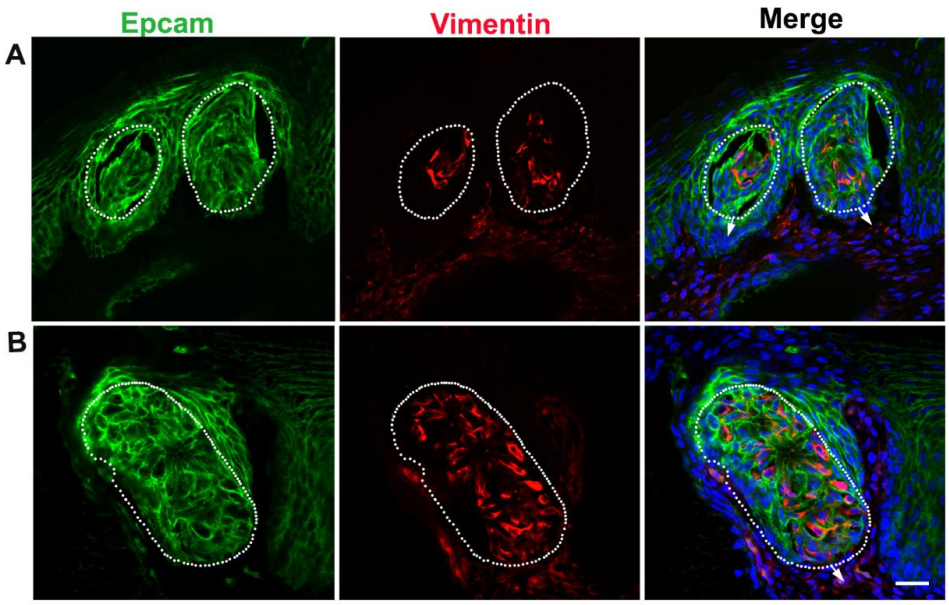

Fig. 3.4 Distribution of Epcam (green) and Vimentin (red) immunoreactivity in the taste buds in the sections of oral tissues from the base of the oral cavity of P0 (A) and P3 (B). Vimentin^{+} cells in the taste buds were also Epcam^{+}. White dots outline the specified taste bud cell clusters. Arrows point to the connective tissue. Vimentin and Epcam immunoproducts were localized in the different cellular compartments, i.e., cytoplasmic and membrane respectively, but the colocalization of signals in the taste bud cells was obvious. Scale bar: 20μm for all images (single plane laser-scanning confocal).

CHAPTER 4

LABELING AND ANALYSIS OF CHICKEN TASTE BUDS WITH MOLECULAR MARKERS IN THE ORAL EPITHELIAL SHEETS

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Abstract

The sensory organs for taste in chickens are the taste buds located in the oral cavity, ~240-360 in total based on scanning electron microscopy (SEM) and histological analysis. So far, there has not been an easy way to visualize all taste buds in chickens. In the present study we report a highly efficient method for labeling all chicken taste buds in oral epithelial sheets using molecular markers Vimentin and α-Gustducin. Incubation with sub-epithelially injected proteases, followed by immediate fixation with paraformaldehyde, enabled us to peel an intact epithelial sheet while maintaining the shape and tissue integrity. Immunoreactivity against Vimentin and α-Gustducin in the peeled epithelial sheets was bright and labeled taste buds were easy to identify under a light microscope, which provides a simple method for analyzing their distribution pattern and number. Further, we observed more taste buds labeled with molecular markers than those identified with SEM. Compared to females, male chickens have a higher number of both taste buds and bud clusters which change over stages after hatch. For the first time, we present the 3D structures of chicken taste buds with 2-photon microscopy and found big barrel-shaped taste buds in addition to the ovoid ones. We believe that our protocol for labeling chicken taste buds with molecular markers, and the new information about differences between genders and among stages, will facilitate future mechanistic studies on chicken taste bud development and its association with feeding behaviors.

Index words: chicken, taste buds, epithelium, Vimentin, Gustducin, poultry

Introduction

In chickens, taste buds that transduce chemical stimuli into neural signals are the sensory organs for taste which guide nutritive choices and motivate feed intake. Chicken taste buds are different from mammals in several aspects. For example, chicken taste buds are ovoid, not "bud"-shaped, and appear mostly near the openings of the salivary glands. About 69% of the taste buds are located in the epithelium of the palate, while 29% are in the base of the oral cavity (anterior mandibular gland region) (Doty, 2015; Ganchrow and Ganchrow, 1985; Kudo et al., 2008). Unlike mammals in which taste buds are primarily located in the tongue, only around 2% of chicken taste buds are distributed in the posterior region of the tongue (Ganchrow and Ganchrow, 1985; Kudo et al., 2008).

Taste bud number is a vital factor and positively correlates with the sensitivity of taste in chicken; e.g., the more the taste buds, the more sensitive they are to bitter taste (Kudo et al., 2010a). It has been reported that chicken taste buds develop at late embryonic (E) and early post-hatching (P) stages, and that taste bud number remains constant after E19 (Ganchrow and Ganchrow, 1987). The total number of taste buds falls into a wide range averaging around 240 - 360, depending on the breed and use; e.g., broiler type males have more taste buds than the males from female lines (Ganchrow and Ganchrow, 1985; Kudo et al., 2008).

Previously, the distribution and number of chicken taste buds have been determined by quantifying taste pores using scanning electron microscopy (SEM) (Ganchrow and Ganchrow, 1985; Nishimura and Tabata, 2008), which is time-consuming and can only identify mature taste buds with a taste pore open to the surface of oral epithelium (Ganchrow and Ganchrow, 1985; Ganchrow and Ganchrow, 1987). There has not been

an ideal method to easily visualize all chicken taste buds for acquiring an accurate number and distribution pattern. Molecular labeling of whole mount tissues provides an efficient way to obtain general information from many organs and tissues. Recently we have developed a simple method for labeling mouse taste buds in the intact tongue epithelial sheet which is easy to quantify the taste buds (Venkatesan et al., 2015). This protocol, if applicable to chicken gustatory tissues, will be significant in facilitating studies on the number of chicken taste buds that is associated with taste sensitivity and thus feeding behaviors.

In chickens, molecular markers have been used to label taste buds on tissue sections (Kudo et al., 2010b; Witt et al., 1999; Witt et al., 2000). For instance, previous studies have demonstrated that Vimentin and α-Gustducin each labels a subpopulation of chicken taste buds (Kudo et al., 2010b; Witt et al., 2000). Our recent data (unpublished) using both markers showed that Vimentin and α-Gustducin label a largely overlapping population of taste bud cells. Although each marker does not label all cells in a taste bud, the signals were observed in all the taste buds examined in post-hatch chickens. Thus, α-Gustducin and Vimentin are potentially useful molecular markers for visualizing all chicken taste buds in whole mount tissues.

In the present study we tested a protocol for peeling intact oral epithelial sheets in post-hatch chickens. Taste bud labeling with the molecular markers Vimentin and α-Gustducin was successful in the peeled epithelial sheets of the base of oral cavity and palate where taste buds are primarily located. We found that the number of taste buds labeled with molecular markers was much higher than that obtained with conventional method, i.e., SEM. The difference in taste bud numbers between male and female

chickens exist from immediately post-hatch through the examined post-hatching stages until P8. Further, taste bud numbers in male chickens continues to change after hatch, which provides a temporal window for mechanistic studies on nutrient sensing. Moreover, we used 2-photon microscopy and report the various 3D structures of chicken taste buds. Our findings are important for further studies on the character of chicken taste buds that are essential in guiding nutritive choices and motivating feed intake.

Material and Methods

Animal and tissue collection

The use of animals throughout the study 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.

The newly hatched Cobb500 (P0) male (from female line) and female (from male line) chicks were received from the Cobb-Vantress., Inc, Cleveland Hatchery, Georgia. The chicks were housed in separate cages in an animal facility at Department of Animal Science, University of Georgia, until P8 (8 days). The brooder temperature was ~35 °C and room temperature was maintained at 30 °C with food (starter feed) and water *ad libitum* for the animals in a 12-12 hr light-dark cycle.

P0, P1, P3, P4, P5 and P8 chicks were euthanized by decapitation. The oral tissues in the palate and base of the oral cavity with the tongue were dissected and processed for different analyses as below.

Oral epithelial sheet peeling

The palate and base of the oral cavity were dissected and briefly rinsed in 0.1 M PBS. An enzyme mixture of Collagenase A (1 mg/ml, Cat# 10103578001, Roche Diagnostics)

and Dispase II (2.5 mg/ml, Cat# 04942078001, Roche Diagnostics) was injected (6 ml in total) into the sub-epithelial space of the palate, the base of the oral cavity floor and the posterior region of the tongue, followed by an incubation at 37 °C for 2 hr. Following enzymatic tissue digestion, the tissues were immediately fixed in 4% paraformaldehyde (PFA) for 1 hr at room temperature followed by brief rinse in 0.1 M phosphate buffered saline (PBS). The soft tissue regions containing taste buds were dissected from the beaks and epithelial sheets of the palate, base of the oral cavity and posterior tongue were peeled off from the underlying connective tissue. After thorough rinsing in 0.1 M PBS, the epithelial sheets were processed for taste bud labeling using immunohistochemistry.

Vimentin and α-Gustducin immunohistochemistry in epithelial sheets

Peeled epithelial sheets were evaluated under a stereomicroscope. Intact sheets from different stages (P0, P1, P3, P4, P5, P8) were selected (n=3-7, for each stage and gender) for further processing. Epithelial sheets were rinsed in 0.1 M PBS; non-specific staining was blocked with 10% normal donkey serum (NDS) (Cat# D9663-10ml, Sigma-Aldrich) and 10% Bovine Serum Albumin Fraction V (Cat#15260-037, ThermoFisher Scientific) in 0.1 M PBS containing 0.3% Triton-X100 (PBS-X) overnight at 4 °C. Sheets were then incubated with primary antibodies against Vimentin (1:200, abcam 28028; Vim3B4, mouse monoclonal antibody, Abcam, Cambridge, MA) and α-Gustducin (1:250, serum of rabbit immunized with chicken α-Gustducin , generated by Dr. Shoji Tabata Lab) (Kudo et al., 2010b) in 1% normal donkey serum, 1% BSA in PBS-X for 72 hr at 4 °C. Following rinses in 0.1 M PBS, epithelial sheets were incubated with Alexa Fluor 488 conjugated donkey anti-mouse (1:500, Code: 715-545-150, Jackson Immuno Research Laboratories, Inc) and Alexa Fluor 647 conjugated donkey anti-rabbit secondary antibody

(1:500, Code: 711-605-152; Jackson Immuno Research Laboratories, Inc) in 1% NDS in PBS-X overnight at 4 °C. The epithelial sheet was rinsed in 0.1 M PBS and photomicrographed using SZX2-ILLT Olympus stereomicroscope with CellSens software (Olympus, Life sciences). Z-projection confocal images were taken under LSM710 laser confocal microscope using ZEN 2012 software in the Biomedical Microscopy Core (BMC) at the University of Georgia for the analysis of labeled taste buds at the cellular level.

Scanning electron microscopy (SEM)

The palate and base of the oral cavity from P3 chickens were dissected and fixed in SEM fixative containing 4% PFA, 2.5% Glutaraldehyde in 0.1 M PBS over 48 hr at room temperature. The samples were trimmed and thoroughly rinsed in 0.1 M PBS and processed further in series of 1% osmium tetroxide (OsO_4), 1% tannic acid and 1% OsO_4 aqueous solutions for 1 hr on ice. The tissues were dehydrated sequentially with gradient ethanol (35%, 50%, 70%, 95% and 100%) three times at each concentration for 2 hr each. Specimens were dried completely using a critical point dryer (Autosamdri-814 Critical Point Dryer, Tousimis Research Corporation, Rockville, MD, USA). The samples were mounted onto SEM stub, sputter coated with gold and photomicrographs were taken using Zeiss 1450EP scanning electron microscope (Carl Zeiss MicroImaging, Inc., NY, and Oxford Instruments X-Ray Technology, Inc., CA).

2-photon microscopy and 3-D image reconstruction of chicken taste buds

The P3 stage base of the oral cavity and the palate epithelial sheets immunoreacted against Vimentin and α-Gustducin were used for the 3D imaging process. The 3D images were acquired with a home-built two photon microscope. A 1550 nm, 370

femtosecond pulsed fiber laser (Calmar Cazadero) with wavelength of 1550 nm and repetition rate of 10 MHz was used. The beam was frequency doubled using a second harmonic generation (SHG) crystal (Newlight photonics) to produce a 775 nm beam that was used for 2-photon excitation of the sample. The beam power was modulated using a Pockels cell (Conoptics) and the beam was scanned over the sample by a resonant-galvanometer (fast axis – slow axis) scanner (Sutter instruments RESSCAN-GEN). A 60x Olympus (LUMFLN60x) water immersion objective with NA of 1.1 was used for imaging. Z-scanning was performed using an X-Y-Z stage from Sutter Instruments (MPC-200). Emitted signal from the sample was separated into two channels. A 690/40 nm and a 520/50 nm filters were used for collection of signal from Alexa Fluor 647 and Alexa Fluor 488 respectively. Photon multiplier tubes (PMT) from Hamamatsu were used for collection of the signal. A transimpedance amplifier (Edmund Optics 59-178) was used for each channels to convert current output of PMTs to an amplified voltage. National Instruments DAQ cards and FPGA module were used for control and synchronization of the system, and digitization of the detected signal. A Matlab-based open-source software ScanImage (Pologruto et al., 2003) was employed to control the microscope. More information on the laser and optical setup can be found in the recent reports (Mortensen et al., 2015; Tehrani et al., 2016).

Quantification and statistical analysis

Quantitative analysis was performed for the total number of taste buds, taste bud clusters, and taste bud number per cluster in the base of the oral cavity and palate of the male and female chickens at different stages, i.e., P0, P1, and P3 (n=6-7) and P4, P5, and P8 stages (n=3) by one investigator to maintain consistency and avoid bias. Vimentin

and/or α-Gustducin immunoreactivity was used to visualize taste buds in the oral epithelial sheets. All the quantification was carried out manually by the same investigator for the consistency among groups using the photomicrographs from Olympus stereomicroscope. The quantification data was represented as means ± standard deviation (X±SD; n=3-7). Two way analysis of variance (ANOVA) followed by‘t’ test was performed to test the statistical significance of differences between males and females. One way analysis of variance (ANOVA), followed by Post Hoc Turkey HSD Tests, was used to test the significance between the examined stages. All statistical analysis was performed using SPSS Statistics v.20 software.

Results

Oral epithelial sheet peeling and taste bud labeling

To efficiently label all taste buds, oral epithelial sheets from the palate, the base of the oral cavity, and posterior region of the tongue in post-hatch chickens were peeled with modifications to our recently reported method for mouse tongue (Venkatesan et al., 2015). The major steps are shown in Fig. 4.1 using the base of the oral cavity as an example. In brief, dissected beaks (Fig. 4.1A) were given a sub-epithelial injection of proteases into the oral tissues (Fig. 4.1B). Following the incubation with injected proteases for 2 hr at 37 ^{o}C, immediate fixation of the tissues in 4% PFA for 1 hr at room temperature was helpful to maintain the shape and integrity of the epithelial sheets (Fig. 4.1C). Salivary gland openings in the peeled epithelium (asterisk, Fig. 4.1C inset) were seen as holes under a stereomicroscope, and surrounded by dark structures where taste buds are primarily located (Fig 4.1C).

To better preserve the tissues for the long process of immunoreaction, the peeled epithelial sheets were further fixed in 4% PFA for 2 hr and then the eligibility of taste bud labeling was tested with immunoreaction against Vimentin. The clustered structures surrounding salivary gland openings were labeled with Vimentin immunoreactivity. The Vimentin signals were distributed in the anterior mandibular region of the base of the oral cavity in the taste bud locating areas previously reported (Fig. 4.1D). In addition, distribution of labeled taste buds were seen in an extention to the edge of the base of the oral cavity. Individual cell clusters labeled by Vimentin immunoproducts were easily identifiable and distributed in clusters in a rosette-like pattern.

The taste bud labeling was also successful in the palate epithelial sheets (described below in details). However, immunoreactivity of Vimentin was not observed on the region posterior to the anterior lingual spines of the tongue (data not shown) although it has been reported that taste bud pores were observed there with SEM.

Distribution and structure of taste buds labeled with Vimentin and α-Gustducin immunoreactivity in the base of the oral cavity

It has been reported that α-Gustducin is specifically expressed in chicken taste buds (Kudo, 2014; Kudo, 2010). Both molecular markers, Vimentin and α-Gustducin were used for labeling taste buds in epithelial sheets of the base of the oral cavity in P0 to P8 chickens. The distribution and structure of labeled taste buds were analyzed at organ and cellular levels (Fig. 4.2). The immunoreactivities of Vimentin and α-Gustducin were distributed in the same population of taste buds in the epithelial sheet (Fig. 4.2A). To confirm whether the fluorescent "dots" under the stereomicroscope were individual taste buds and could be used for further quantitative analysis, Vimentin and α-Gustducin

labeling was further verified by laser-scanning (Fig. 4.2B) and 2-photon (Fig. 4.2C) confocal microscopy. The Z-projection of laser-scanning confocal photomicrographs and 3-D reconstructed 2-photon images of a cluster of taste buds from an epithelial sheet illustrated a bud cluster with the rosette-like arrangement of individual taste buds, which were separate from each other (Fig. 4.2B-C). The number of taste buds obtained from the Z-projection and 3-D images was identical to that obtained from images taken under the light stereomicroscope. Vimentin and α-Gustducin signals were distributed in every detected taste bud, each labeling a significant subpopulation of taste bud cells that were largely overlapping. The 3D images also show an egg-shaped structure for individual taste buds (Fig. 4.2C).

Quantitative analysis of Vimentin labeled taste buds in the base of the oral cavity

As mentioned above, taste buds were frequently clustered around salivary gland openings in a rosette-like pattern. The total number of individual taste buds and taste bud clusters in the base of the oral cavity of male and female chickens at different stages (P0-P8) were quantified using Vimentin immunoreactivity (Fig. 4.3A, B).

Overall, the total number of taste buds in males was statistically different than in females (Fig. 4.3A, two-way ANOVA, ($F(5,49) = 2.61$, $P=0.0358$). t-tests showed that the taste bud number in the base of the oral cavity of male chickens was higher than in females at P0 ($P=0.005$), P1 ($P=0.004$), P3 ($P<0.001$), and P5 ($P=0.002$).

Among post-hatch stages, the differences were statistically significant in male (one-way ANOVA, $F(5, 28) =4.069$, $P=0.002$). However, in females the discrepancies of total taste bud numbers among stages were small and statistically insignificant (one-way ANOVA, $F(5, 21) =1.42$, $P=0.223$). In males, taste bud number increased in the young

chicks and reached their peak at P3 (190 at P0 vs 260 at P3), and then returned to a relatively low level at later stages (P4-P8). Post-hoc Tukey HSD tests followed by one-way ANOVA showed that P3 male chickens had a significantly higher number of total taste buds than P4 (P=0.034) and P8 (P=0.007).

Similar to the number of total taste buds, the number of bud clusters (Fig. 4.3B) between the males and females were also significantly different and higher in males than in females (two-way ANOVA, (F (5,49) = 3.08, P=0.017). The differences between males and females were statistically significant at P0, P1, P3, & P5 stages (t- tests, P=0.019, P=0.015, P=0.000, P=0.011 respectively). Also, statistically significant changes over stages were observed in males (One-way ANOVA, F (5, 28) =6.057, P=0.001), but not in females (One-way ANOVA, F (5, 21) =1.732, P=0.171). The number of bud clusters in male chickens reached the highest level (60) at P3, which was significantly higher than that at P0 (P=0.045), P1 (P=0.025), P4 (P=0.021), and P8 (P=0.000).

The number of taste buds in each cluster in the base of the oral cavity of male and female chickens at different stages (P0-P8) was also analyzed using Vimentin immunoreactivity (Fig. 4.3C, D). In males, the taste buds per cluster varied from 1-14 with 4 buds/cluster being the most prevalent (Fig. 4.3C). In contrast, the females had a smaller range of taste buds per cluster, i.e., 1-9 and prevailed at 3 buds/cluster (Fig. 4.3D).

Taste bud labeling with Vimentin immunoreactivity in the palate

Epithelial sheets of the palates were peeled using the same procedures as those from the base of the oral cavity (Fig. 4.1). However, peeling of the palate epithelial sheets was more challenging, likely due to the smaller sub-epithelial space, lower holding capacity

of the proteases solution and abundant protruded palatine spines. Nonetheless, it was still possible to obtain intact epithelial sheets (Fig. 4.4A_1, brightfield) for taste bud labeling with Vimentin (Fig. 4.4A_2) and α-Gustducin (image not shown).

In the palate epithelial sheet, the taste buds labeled with Vimentin were distributed in a unique pattern (Fig. 4.4A_2). Similar to the locations described in the previous reports (Ganchrow and Ganchrow, 1985; Kudo et al., 2008), Vimentin signals were mainly located in three tissue regions: (1) anterior to the lateral palatine wrinkles (maxillary gland region) (mgr, Fig. 4.4$A_{2\text{-}4}$), (2) lateral to the nasopalatal region (palatine papilla region) (ppr, Fig. 4.4$A_{2,\ 5\text{-}6}$), and (3) poster region adjacent to the choanal opening (pr, Fig. 4.4$A_{2,\ 7\text{-}8}$). In the maxillary gland opening region of the palate, two large clusters of taste buds were brightly labeled, each comprised of multiple taste buds (Fig. 4.4$A_{3\text{-}4}$). In the palatine papilla region, two lines of taste bud clusters were observed on both lateral sides and a scattered distribution of taste buds was observed in the medial areas (Fig. 4.4$A_{5\text{-}6}$). In the posterior palate, mostly scattered taste buds were observed (Fig. 4.4$A_{7\text{-}8}$). 2-photon 3-D reconstructed images of taste bud cluster shows ovoid shaped buds forming rosette pattern similar, to the base of the oral cavity in the palatine papilla region (Fig. 4.4B) and elongated tube-shaped buds in the posterior region (Fig. 4.4C). Taste bud structure varies within the palate epithelium. The total taste bud number quantified with Vimentin immunoreactivity in the palate epithelial sheets was 588± 19 on average at P3 (n=3).

Analysis of taste buds with the traditional method-scanning electron microscopy

Taste buds labeled with Vimentin and α-Gustducin are significantly higher in number compared to those reported using SEM (~90 in the base of oral cavity, ~218 in the palate)

(Ganchrow and Ganchrow, 1985; Kudo et al., 2008). To verify whether the differences were due to the variation between strains of chickens, we used scanning electron microscopy to quantify the total number of taste pores from higher magnification images of base of the oral cavity and the palate in the same strain of chickens at P3 (Fig.4.5). The total number of readily identifiable taste pores was 74 (n=2) in base of the oral cavity and 185 (n=2) in the palate, which was consistent with previously reported (Ganchrow, 1985; Ganchrow, 1987).

In the base of the oral cavity (Fig. 4.5A) taste buds with pores (arrows, Fig. 4.5B) were identified surrounding salivary gland openings (asterisk, Fig. 4.5B). Salivary gland openings were deep with a diameter >20 µm, while the diameter of the taste pore shallow (<12 µm). The number of taste pores in a cluster varied from 1 to 5. Taste buds with a typical pore were also observed in the absence of salivary gland opening (open arrowheads, Fig. 4.5C).

In the palate, the number of taste pores in a cluster was lower, varying from 1 to 3 (Fig. 4.5D). Compared to the base of the oral cavity, taste buds with a typical pore were more frequently observed in the absence of a salivary gland opening in the palate (open arrow heads, Fig. 4.5E, F). Taste bud-like protruded structures without an obvious taste pore were also seen (arrow heads, Fig. 4.5F). Specified cell clusters (dotted outlines, Fig. 4.5G) were also seen surrounding salivary gland openings (asterisks, Fig. 4.5G), but individual taste pores were not obvious.

Discussion

Chicken taste buds have been identified with scanning electron microscopy and histological analysis. Molecular labeling has been limited to tissue sections. There has not been an ideal method for visualizing all chicken taste buds in whole mount tissues using molecular markers. In the present study, we developed an efficient method to label chicken taste buds in the oral epithelial sheets using traditional antibody labeling against Vimentin and α-Gustducin. With this new method, we identified a higher number of taste buds in oral tissues compared to previous reports and, we present the 3-D structure (ovoid- and tube-shaped of chicken taste buds obtained from the immunoreacted epithelial sheets using 2-photon microscopy.

Taste bud labeling in the oral epithelial sheet with molecular markers efficiently reveals the distribution and number of chicken taste buds

Structural and molecular analyses of whole mount tissues are efficient for evaluating the phenotypes and alterations of many organs and tissues. However, the gustatory epithelium has a strong permeability barrier that makes it difficult to label mature taste buds in whole tissues. Recently, we reported a protocol describing adult murine tongue epithelial sheet peeling following incubation with intralingually injected proteases and immediate 4% PFA fixation. This enables us to remove the permeability barrier in the basal region and reliably label the taste buds in the tongue epithelium with the *in situ* shape maintained (Venkatesan et al., 2015). In the present study, we adapted this technique and optimized the protocol specifically for chicken oral tissues. We found that intact epithelial sheets can be obtained through sub-epithelially injected proteases, although a 1.5-2 hr incubation was required for chicken oral tissues compared to a 30

minute incubation for peeling mouse tongue epithelium. This is probably due to the small sub-epithelial space and the unique structure of chicken oral tissues including multiple thick and long spines.

Taste bud labeling using specific antibodies against Vimentin and α-Gustducin was successful in peeled epithelial sheets of palate and the base of oral cavity where chicken taste buds are primarily located. The labeled taste buds were easy to identify under a light microscope, providing a highly efficient method to analyze the distribution pattern and number of taste buds in the epithelial sheets. Moreover, the immunoreacted epithelial sheets were eligible for further examinations of taste bud structure at the cellular level using laser-scanning and 2-photon confocal microscopy. 3-D reconstructed imaging analysis indicated that taste bud structures were different in different regions of the oral cavity. Chicken taste buds have been described and accepted as ovoid-shaped. In the present study, both ovoid- and tube-shaped taste buds were observed in the palate. We believe this method can be used to label taste buds and inter-bud epithelium not only with antibodies but also with probes for *in situ* hybridization. Paired with traditional techniques, this method is efficient for phenotypical analysis of chicken gustatory tissues, and thus will facilitate the studies on the role of taste buds in regulating feed intake and on the formation and regulation of chicken taste buds.

Our findings about taste buds using molecular markers labeling bring new information about the sensory organs for taste in chickens. First, we found that the reported number of chicken taste buds has been significantly underestimated in the previous reports. We observed around ~588 taste buds in the palate and ~260 in the base of the oral cavity in P3 male chickens, which is much higher than the 218 (palate) and 91

(base of the oral cavity) taste buds reported with SEM (Ganchrow and Ganchrow, 1985). The taste buds were clustered in a rosette-like pattern the bud number in each cluster was found to be higher than earlier report; e.g., up to 14 in males and 9 in females in the base of the oral cavity. Second, we found that taste buds are distributed more broadly in the oral cavity than earlier reported, extending further to the lateral side of the tissue in the base of oral cavity. Third, 3-D reconstructed imaging analysis indicated that taste bud structures were different in different regions of the oral cavity. Chicken taste buds have been described and accepted as ovoid-shaped. In the present study, both ovoid- and tube-shaped taste buds were observed in the palate.

There has been a broad consensus that birds have a lower taste acuity than mammals due to their low taste bud numbers (Gentle, 1972; Roura et al., 2013). However, emerging knowledge shows that birds have a well-developed taste system (Doty, 2015). Our findings about the high number of taste buds in chickens indicate a larger than expected impact of taste on the feeding behaviors in birds.

Significant differences in taste bud and cluster number in both genders and among stages

It has been reported that the total taste bud numbers vary among different breeds; e.g., taste bud numbers in White Leghorn layer-line males , Rhode Island Red layer-line males, and broiler-line males are different at P5 (Kudo et al., 2010a). In the present study we found a significant difference between genders in the numbers of taste buds and bud clusters from the same strain. For example, males have more taste buds than females from hatch, with a higher number of taste bud clusters and taste buds per cluster. Also,

the prevailing cluster size in males is higher, 4 buds/cluster versus 3 buds/cluster in females.

Another interesting difference is that in male chickens, the numbers of taste buds and bud clusters change with age from P0-P8. We found that taste bud number in males continued to increase after hatch, peaks at P3 and then return to a lower level at later stages. In contrast, in female chickens taste bud numbers in the base of oral cavity were more stable among post-hatching stages. In previous studies analyzing taste bud numbers, P0, P50-60 and adult chickens have been used and differences were not found among these stages in Anak strain broiler chicks (Ganchrow and Ganchrow, 1985; Ganchrow and Ganchrow, 1987). Our finding of the continuing development of taste buds in the newly hatched male chickens provides a time window for us to modify taste sensing in early hatched chickens.

Although the mechanisms underlying the differences between males and females is not clear, there are many possible candidates for future study including sex hormones and growth factors. A significant difference between males and females was observed from P0, which makes it reasonable to speculate that the difference exists during the initial development of taste buds in late embryos. Taste bud number is an important factor that determines taste sensitivity (Kudo et al., 2010) and is thus associated with feeding behavior. Further studies on how taste bud formation is regulated will be beneficial for improving chickens' healthful food choices and intake, thus, improving animal health and productivity.

Acknowledgements

We give thanks to the staff at Cobb Vantress Cleaveland Hatchery, GA for providing the chicks. We give thanks to Dr. Venkatesan Perumal for conducting the statistical analysis. This study was supported by the National Institutes of Health (grant number R01 DC012308 to HXL) and University of Georgia Start-up fund to HXL.

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Figures

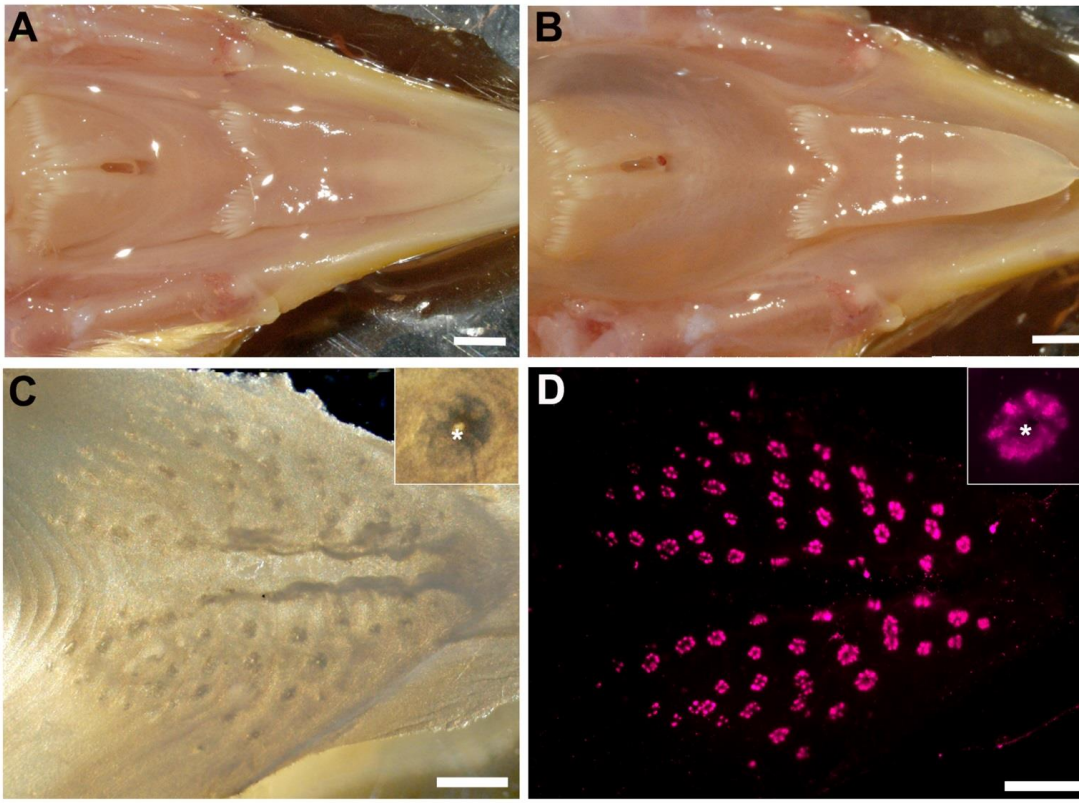

Fig. 4.1. Representative photomicrographs of the base of the oral cavity in P3 chickens to illustrate the major steps in the protocol for taste bud labeling in the peeled oral epithelial sheets. A: A lower beak to show the base of the oral cavity dissected with the tongue kept *in situ*. B: Swollen tissues of the base of the oral cavity and posterior tongue after the sub-epithelial injection of proteinases. C: Bright-field image of an epithelial sheet from the base of the oral cavity. D: Photomicrograph of an epithelial sheet taken under a fluorescence stereomicroscope after immunoreaction against Vimentin. Purple signals show the immunoreactivity of Vimentin in taste buds. Asterisks in the insets in C and D mark a salivary gland opening. Scale bars: 2 mm for A and B; 1 mm for C and D.

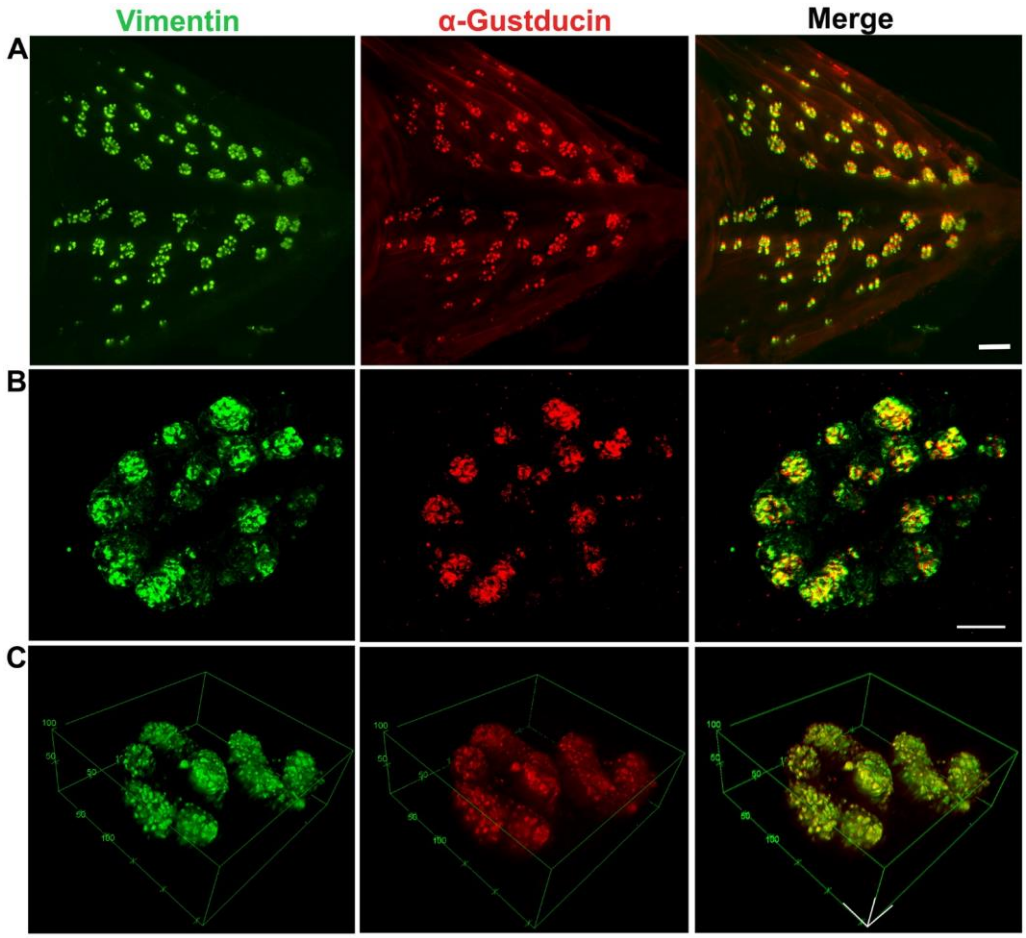

Fig. 4.2 Photomicrographs illustrate the distribution of Vimentin (green) and α-Gustducin (red) immunoreactivity in the taste buds in an epithelial sheet from the base of the oral cavity in a P3 male chicken. A: Photomicrographs taken under a fluorescent stereomicroscope show the overlapping distribution of Vimentin (green) and α-Gustducin (red) immunoreactivities in the taste buds. B: Laser-scanning confocal photomicrographs (Z projection) were taken at a high magnification to show the individual taste buds in a cluster. Signals of Vimentin and α-Gustducin were seen in all taste buds and largely overlapped in the taste bud cells. Taste buds were arranged in a rosette pattern. C: 3-D images taken under a 2-photon microscopy illustrate the separated individual cell clusters and ovoid-shaped buds surrounding a salivary gland opening. Scale bar: 500 μm for A, 25 μm for B, 50 μm for C.

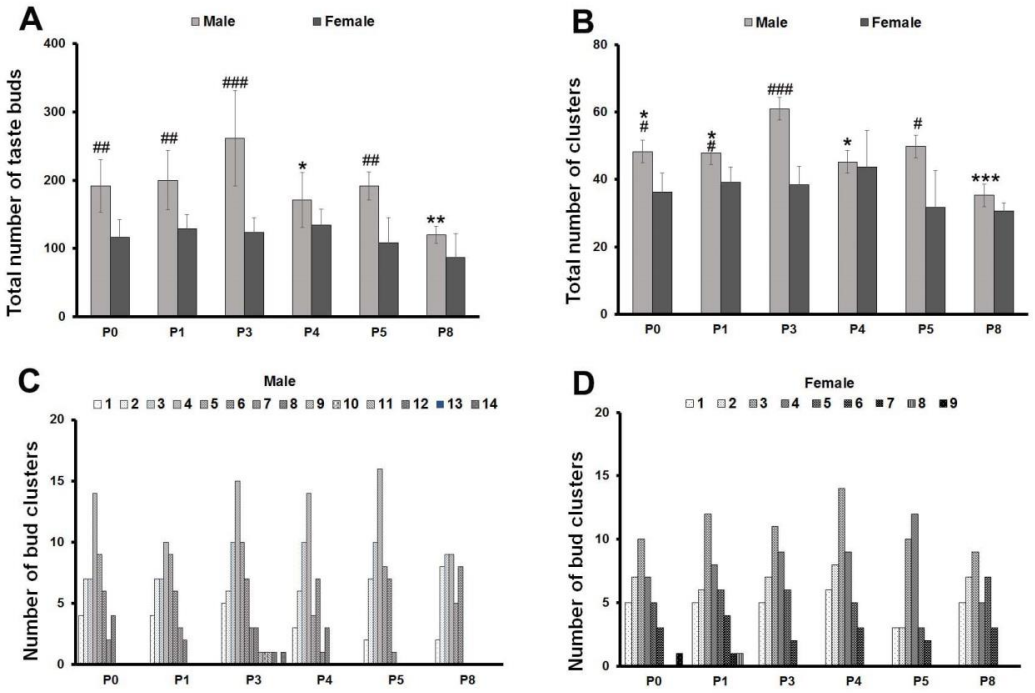

Fig. 4.3 Quantitative analysis of total taste bud number, total number of bud clusters, and taste bud number per cluster in male and female chickens at post-hatching day 0, 1, 3, 4, 5 and 8. The data were obtained from the epithelial sheets of the base of the oral cavity immunoreacted against Vimentin. A: Histograms represents the average ($\overline{x}$ ±SD, n=3-7) of total individual taste bud number in males (gray bars) and females (black bars). B: Histograms show the average ($\overline{x}$ ±SD, n=3-7) of number of taste bud clusters at each stage in males (gray bars) and females (black bars) C and D: The histograms shows the variation of taste bud number within a taste bud cluster (male 1-14 and females 1-9), illustrating most abundant cluster types in males (i.e., 4 taste buds in a cluster) (C) and females (i.e., 3 taste buds in a cluster) (D). $^{\#}$ *P≤0.05,* $^{\#\#}$ *P≤0.01,* $^{\#\#\#}$ *P≤0.001* compared to female group at the same stage, * *P≤0.05,* ** *P≤0.01,* *** *P≤0.001* compared to P3 male chicken group.

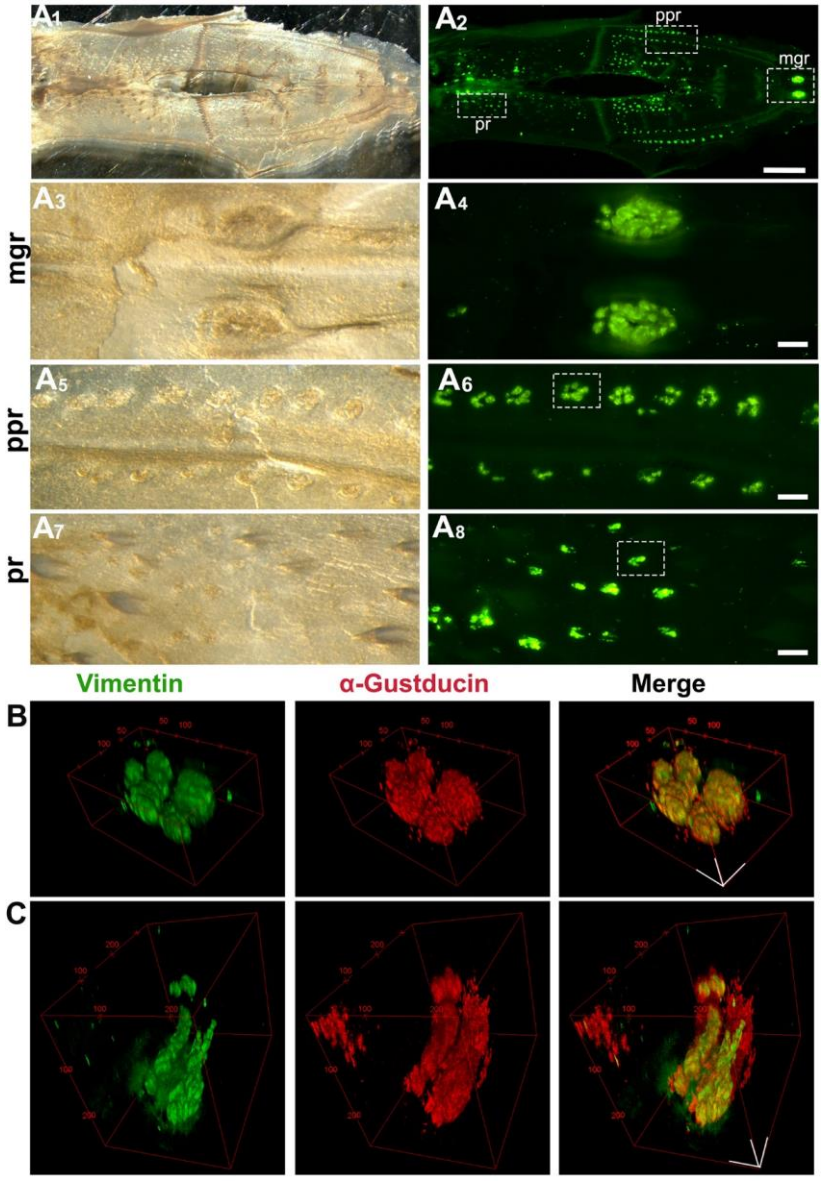

Fig. 4.4 Photomicrographs of a palate epithelial sheet from a P3 male chicken, labeled with Vimentin immunoreactivity (green) and α-Gustducin (red) to illustrate the distribution pattern of taste buds. A: Bright field ($A_{1, 3, 5, 7}$) and corresponding fluorescent images ($A_{2, 4, 6, 8}$) of the palate labelled with Vimentin (green) represents a unique pattern of distribution. Squares with dashed lines mark the maxillary gland region (mgr), palatine papillae region (ppr) and posterior region (pr) of the palate for the higher magnification images in $A_{3\text{-}4}$, $A_{5\text{-}6}$, $A_{7\text{-}8}$ respectively. Scale bars: 2 mm for A_1-A_2, 200 μm for A_3-A_8. B and C: 2 photon 3-D reconstructed images of a taste bud cluster showing ovoid-shaped (B) and tube-shaped (C) taste buds in the palatine papilla region and posterior region. . Scale bars: 50 μm.

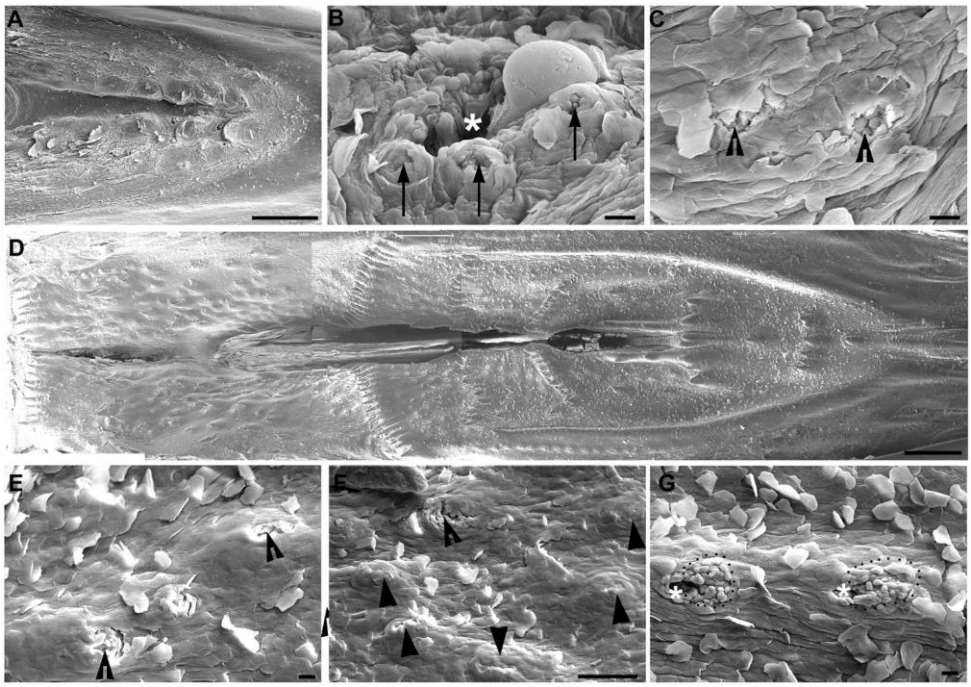

Fig. 4.5 Scanning electron photomicrographs from the base of the oral cavity and palate in a P3 male chicken. A: Low magnification of the base of the oral cavity showing the salivary gland openings and taste bud distribution (scale bar: 1 mm). B: A taste bud cluster showing 3 taste buds (arrows) located around a salivary gland opening (asterisks). C: Representative images of taste buds (open arrowheads) which were not close to a salivary gland opening. D: Low magnification image of a palate showing the topography of the oral surface. E-F: taste buds (open arrowheads) located in the absence of salivary gland opening in the palate. Solid arrowheads in F point to protrusions that were probably developing taste buds without an obvious taste pore. G: Representative images of taste buds (dotted outlines) surrounding the salivary gland opening (asterisks) in the palate. Open arrowheads point to taste pores in the absence of salivary gland. Scale bars: 1 mm for A and D; 20 μm for B-C, E & G; 100 μm for F.

CHAPTER 5

NEURAL CREST LABELING AND CONTRIBUTION TO TASTE BUDS IN CHICKENS

Prasangi Rajapaksha, Jason Payne, Brett Marshell, Sanam Desai, Forrest Goodfellow, Zhonghou Wang, Steven Stice, Robert Beckstead, Hong-Xiang Liu. To be submitted to the *Animal and Dairy Science Reports*

Abstract

Taste buds, the sensory organs that transduce gustatory stimuli into neural signals, have been described as arising solely from the surrounding local epithelium. In contrast, our recent findings using multiple mouse models strongly suggest that taste bud cells have a dual origin, i.e., from both the surrounding epithelium and underlying connective tissue that is primarily derived from neural crest (NC). However, there is no direct evidence in demonstrating the migration and differentiation of NC cells into taste bud cells. The present study was aimed at labeling NC in chickens and tracking the migration of NC cells to the taste buds. We found that microinjection of cell tracker CM DiI into the neural tube specifically labeled the neural tube including the dorsal part where NC cells are generated. Also the delaminated cells which were labeled with DiI were positive for the NC cell marker Pax7. Unfortunately, the fluorescent dye was undetectable 3 days after DiI injection, possibly because the DiI in the labeled cells was diluted during the rapid cell divisions in the developing chicken embryos. Our data suggest that DiI labeling is not suitable for the long term labeling of NC cells until taste buds develop. Further studies are ongoing in developing a technique for a permanent labeling of NC cells in chickens, e.g., quail-chicken chimera.

Index words: chicken, neural crest, taste bud, DiI, Pax7,

Introduction

Sensory receptors (e.g., thermo- and mechanoreceptors), as parts of the peripheral nervous system, are known to arise from neurogenic ectoderm that includes the neural tube, neural crest (NC) or ectodermal placodes (Dupin et al., 2006; Kirby et al., 1983; Le Douarin, 1984; Lindsay et al., 1985). Thus, receptor organs, in general, have neural progenitors that migrate and differentiate locally to specific receptors. In contrast, taste bud cells have been described as arising solely from the local epithelium (Okubo et al., 2009; Stone et al., 1995). However, multiple lines of evidence suggest different tissue source(s) could contribute to taste buds. Furthermore, our studies using mouse models to map the cell fate of NC demonstrated that labeled cells, potentially NC derived cells, were abundantly distributed in both early and mature taste buds (Boggs et al., 2016; Liu et al., 2012). The data strongly suggest a potential origin of taste bud cells from NC (Boggs et al., 2016). However, we lack direct evidence for the migration and differentiation of NC cells to taste buds. Tracing NC cell lineage in mouse embryo cultures, from early embryos (NC cells production phase) to birth (when taste buds are formed), is extremely challenging. An alternative animal model is needed for directly tracing the NC cell migration.

Chickens (*Gallus* sp.) as a research model are widely used for the studies in genetics, genomics and, developmental biology due to multiple beneficial aspects, e.g., convenience of *in ovo* manipulation of the embryos, high availability and rapid development (Hughes, 1955; Odani et al., 2009). *In ovo* manipulation of chicken embryos has facilitated lineage tracing studies that include NC cell fate mapping ((Giovannone et al., 2015; Le Lièvre and Le Douarin, 1975; Selleck and Bronner-Fraser, 1996). Similar to

the mammals, chickens have many taste buds in the oral cavity and respond to taste stimuli (Ganchrow and Ganchrow, 1985b; Ganchrow and Ganchrow, 1987; Ganchrow et al., 1990; Gentle, 1972). Recently, we have developed an efficient method to visualize all chicken taste buds in the oral epithelial sheets with molecular markers (Vimentin and α-Gustducin). Importantly, it has been reported that Vimentin, an intermediate filament protein and mesenchymal cell marker, is expressed in a sub population of taste bud cells in chickens (Witt et al., 1999b; Witt et al., 2000). This supports that the underlying mesenchymal cells; primarily derived from NC, contribute to taste buds in chickens. Therefore, chickens can serve as an ideal model to collect direct evidence for the NC migration and differentiation to taste buds (Witt et al., 1999b; Witt et al., 2000).

The aim of the present study was to demonstrate the contribution of NC cells to taste buds in chickens. NC cells were labeled with Cell tracker^{TM} CM DiI fluorescent dye and the labeled cells were positive for the NC cell markers Pax7 (paired box transcription factor 7). However, the DiI signals were faded in 3 days suggesting this method is not suitable for a long-term labeling of the NC cells. Further studies using another method, e.g., quail-chicken chimera, are ongoing for a permanent labeling of NC cells.

Materials and Methods

Animals

The use of animals throughout the study 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.

The fresh eggs from the broiler strain, for the study was obtained from the Department of Poultry Science, University of Georgia. The fresh eggs stored at 10-15 °C,

were kept at room temperature overnight prior to the use and incubated in a standard egg incubator at 37.7 °C and 50-60% humidity for 26-27 hr.

Preparation of the eggs for dye injection

The eggs incubated for ~26 hr were taken from the incubator and prepared for the injection. In brief, eggs were cleaned with 70% ethanol, marked on the top of the egg and 1.5 ml of the thin albumin was removed from the narrow end of the egg using a needle (18-20G). The hole was immediately closed using hot glue. Marked area was taped with transparent tape (i.e. scotch tape). Then a window at ~2 cm in diameter was open on the taped area on the top of the egg for injection. The embryos were staged with Indian ink injected underneath embryo. The embryos at 3-6 somite stages were chosen for the study.

DiI labeling of neural tube and neural crest

The stock solution of CellTracker ™ CM- DiI (Cat# C7000, ThermoFisher Scientific) (50 µg dissolved in 10 µl of the 100% ethanol) was diluted at 1:10 in the diluent (0.1 M PBS and 5% Glucose mixed at a ratio of 1:4 and added gentamicin, stored at 4 °C) (Li et al., 2008).The working DiI solution was loaded into micropipettes made from glass capillary tubes (diameter-1 mm, length-10 cm, Cat# B100-75-10, Sutter instruments, Novato, CA, USA). The DiI was injected in to the neural groove using microinjector (Picospritzer III - Intracellular Microinjection Dispense Systems, Parker Hannifin Precision Fluidics Division, Hollis, NH, USA) with train of 10-12 pulses. Then penicillin (1:1000 dilution) was added to avoid any contamination, followed by the closing of the window by gluing a coverslip. Eggs were incubated in the standard egg incubator (37.7 °C and 50-60% humidity) until specific time points (i.e., 3 hr, 6 hr, and 1-3 days) for tissue collections.

Collection and evaluation of embryos after DiI injection

The embryos were collected at 3 hr, 6 hr, 1 day, 2 days and 3 days post injection. The collected embryos were fixed in 4% paraformaldehyde overnight at 4 °C. Then embryos were briefly rinsed in 0.1 M PBS and, those were photographed under the Olympus stereomicroscope to evaluate the labeling of the neural tube. Embryos with strong labeling were selected for cryoprotection with 30% sucrose at 4 °C for ~48 hr. Next, embryos were trimmed under dissecting microscope, embedded and oriented in OCT compound (Tissue Tek) for transverse sections and rapidly frozen. Neighboring sections were cut at 8-10 µm in thickness, mounted onto glass slides and processed for immunohistochemistry as below.

Immunohistochemistry

The primary antibody used was: Pax7 (1:50, AB 528428, mouse monoclonal, DSHB, University of Iowa, USA). Secondary antibody was: Alexa Fluor 647 conjugated donkey anti-mouse secondary antibody (1:500; Cat#A21447, Life technologies). Frozen sections of the embryos were used for immunohistochemistry. In brief, sections were air dried for 1 hr at room temperature and rehydrated in 0.1 M PBS. Non-specific staining was blocked using 10% normal donkey serum in 0.1 M PBS containing 0.3% Triton X-100 (PBS-X) for 30 min at room temperature. Then frozen sections were incubated with primary antibody (Pax7) in 1% normal donkey serum in PBS-X overnight at 4 °C. Following rinses in 0.1 M PBS (10 min x 3) the sections were incubated with secondary antibody AF 647 in 1% NDS in PBS-X for 1 hr at room temperature. Then sections were rinsed with 0.1 M PBS and counterstained with DAPI (200 ng/ml in PBS) for 10 min, rinsed in 0.1 M PBS, air dried and cover slipped with ProLong® Diamond antifade

mounting medium (P3697, ThermoFisher Scientific). The Pax7 immunoreactivity was thoroughly examined using a fluorescence light microscope (EVOS FL, Life technologies).

Results

Neural folds were labeled with microinjection of DiI to the neural tube in chicken embryos

The analysis on the whole mount chicken embryos at different time points showed that DiI labeling was restricted to the neural tube. The embryos were well developed suggesting that *in ovo* manipulation did not affect the development of the embryos. At 3 hr and 6 hr after DiI injection embryos were developed up to 8 and 10-somite stages respectively (Fig. 5.1A_1, 5.1B_1). At 8-somite stage the neural tube, almost fully closed in the cranial region, was specifically and brightly labeled (Fig. 5.1A_2). At 10-somite stage neural tube was fully closed and DiI labeling was extended to trunk and cranial regions (Fig. 5.1B_2). At both stages, the whole neural tube and the neural folds were labeled with DiI (Red). Immunoreactivity of Pax7 (green), a cell marker for pre-migratory and early migrating NC cells, was seen in the cells in the neural fold region and co-localized with DiI (Fig 5.1$A_{3\text{-}5}$, 5.1$B_{3\text{-}5}$).

Delamination and migration of DiI labeled neural crest cells

At 1 day (23-somite stage) post-injection of DiI, the whole neural tube from rostral to the caudal end was labeled with DiI. The cranial region was more brightly labeled with DiI compared to the trunk region of the embryo (Fig 5.2A_2). Furthermore, streams of DiI labeled cells, presumably migrating NC cells that were delaminated from the neural fold, were observed (asterisk, Fig. 5.2A_2). The immunoreactivity of Pax7 was observed in the

lateral NC cell region in addition to the neural folds compared to the group at 3 hr and 6 hr after DiI injection.

DiI labels were reduced and undetectable after 3 days post-injection

At 2 days after DiI injection the embryos were well developed with divided brain regions. DiI labeling was diminished in the trunk region, while the labels were sustained in some of the developing brain regions (Fig. 5.2B_2). At 3 days post-injection the embryos were further developed and DiI labeling was undetectable in all the examined embryos (n=3) (Fig. 5.2C_2).

Discussion

The chicken has been widely used as a research model for studies in developmental biology, genetics (gene knock out, knock-in), and transplantation procedures because it is convenient to conduct the *in ovo* manipulations. Neural crest (NC) labeling has been well established using electroporation, lipophilic dye injection and chimera surgeries (i.e. chicken-quail, chicken-chicken) (Griswold and Lwigale, 2012; Itasaki et al., 1999; Odani et al., 2009). Among these techniques the injection of cell tracker dyes such as DiI and DiO has been widely used for the NC labeling in chickens and other species (i.e. mammals) (Li et al., 2008), not only for short-term but also for long-term tracing applications. DiI is a lipophilic, carbocyanine membrane dye, which can easily diffuse through the cell membrane (lipid bilayer) and stain the whole cell structures. The Dye passes over to daughter cells during the cell divisions, but they do not pass from one cell to another through the cell contact. Therefore, in the present study we selected a DiI derivative, Cell Tracker CM-DiI which is more water soluble (avoid precipitation) than regular DiI and retains well in the cells.

The present study is focusing on the identification of NC contribution to taste buds. It was important to label the NC cells before the closure of the tube and before cells delaminate, hence we selected the 3-5 somite stage for the dye injection. We found that DiI is useful in labeling the neural folds where NC cells are generated. Also, streams of DiI labeled cells, presumably migrating NC cells, were observed in the tissue regions lateral to the neural tube. These delaminated cells were confirmed with a commonly used NC cell marker, paired box transcription factor 7 (Pax7), in labeling the pre-migratory and early migrating NC cells (Basch et al., 2006; Chi and Epstein, 2002).

However, DiI labeling was not detected at 3 days post-injection probably because chicken embryos grow rapidly, and thus DiI in the cells was diluted during the rapid cell proliferations. Chicken taste buds emerge at embryonic day 17 (E17) and become mature by E19. Therefore, to trace NC cells in the taste buds, the dye labeling needs to be sustained at least until E17. The loss of DiI signals in 3 days after injection suggests that this method is not suitable for a long-term labeling of NC cells for studying NC contribution to taste buds in chickens. Alternative techniques such as microinjection of reporter gene constructs combined with electroporation and chimera surgeries (Griswold and Lwigale, 2012; Itasaki et al., 1999; Le Douarin, 1980; Odani et al., 2009) would provide a permanent labeling of NC cells for characterizing NC cell migration and differentiation to taste bud cells.

Acknowledgements

This study was supported by the National Institutes of Health (grant number R01 DC012308 to HXL) and University of Georgia Start-up fund to HXL.

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Figures

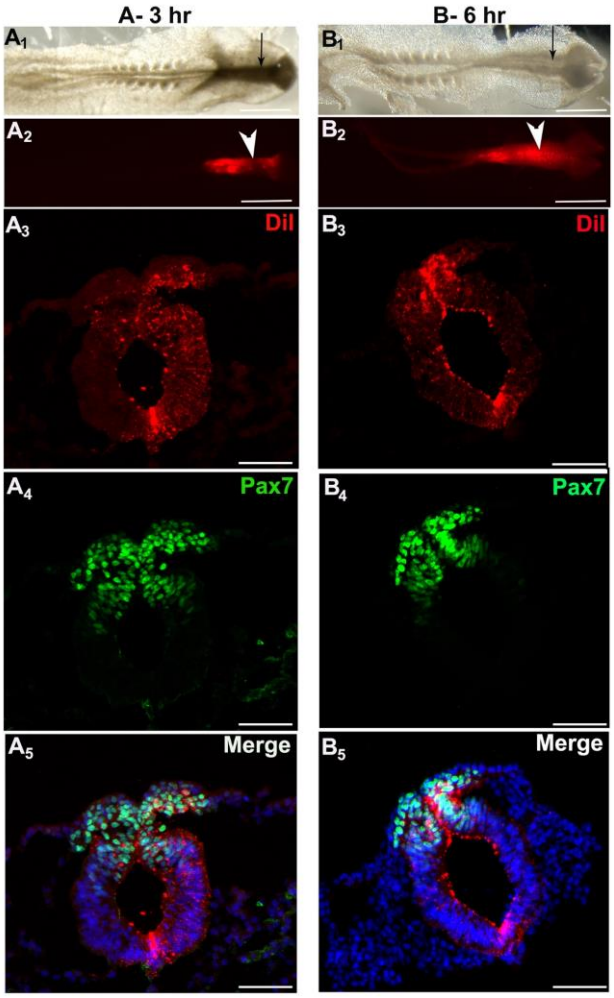

Fig. 5.1 Light microscopic photographs of CM DiI labeled chicken embryos at 3 hr (A) and 6 hr (B) post-injection and frozen tissue sections immunoreacted with Pax7. A_1-A_2: Whole chicken embryo images taken after 3 hr of the DiI injection. DiI (red) labeled the cranial region of the neural tube. A_3-A_5: images of a transverse frozen section of an 8-somite embryo labeled with DiI (red) (A_3) and Pax7 immunoreactivity (green) (A_4). The DiI labeled cells in the dorsal neural fold were $Pax7^+$ (A_5). B_1-B_2: bright field (B_1) and fluorescent (B_2) images of a whole chicken embryo at 6 hr after DiI injection. The embryo developed to 10-somite stage, DiI (red) labeled the neural tube and extended to the developing brain and trunk regions (arrowhead marks the neural tube). B_3-B_5: images of a transverse frozen section of a 10-somite embryo that labeled with DiI (red) (B_3) and Pax7 immunoreactivity (green) (B_4). The co-localization of DiI and Pax7 signals were observed in the neural fold region (B_5). The black arrows in A_1 and B_1 represent the levels of sections for $A_{3\text{-}5}$ and $B_{3\text{-}5}$ respectively. White arrowheads in A_2 and B_2 point to the neural tube. Scale bars: 20 µm in A_1-A_2 and B_1-B_2, 200 µm in A_3-A_5

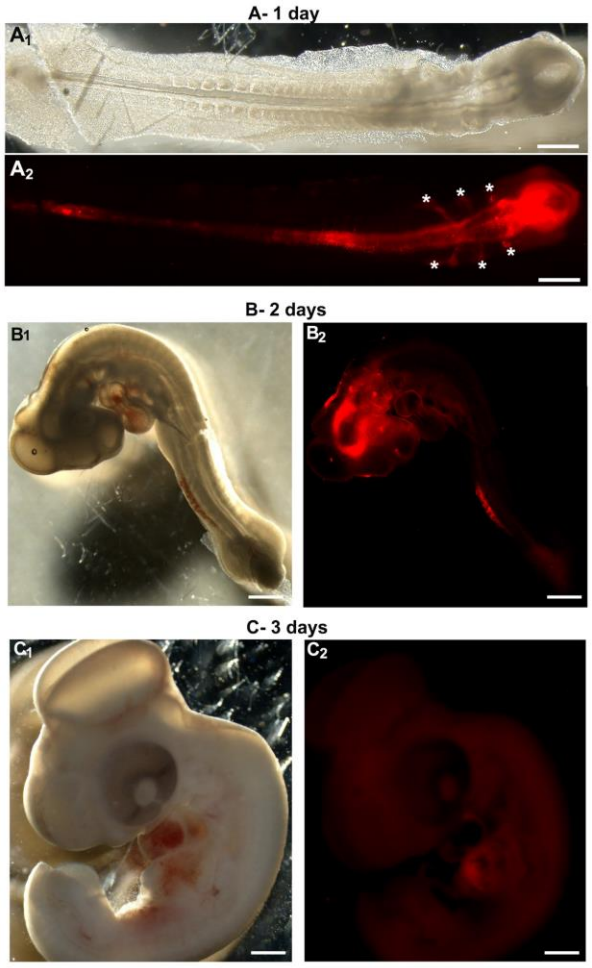

Fig. 5.2 Photomicrographs of DiI labeled chicken embryos at 1 (A), 2 (B) and 3 (C) days post-injection. A_1-A_2: bright field (A_1) and fluorescent (A_2) light microscopic images of a whole chicken embryo at 1 day after DiI injection. The embryo developed to 23-somite stage. DiI (red) labeling extended from rostral to the caudal end of the neural tube. Migrating cell streams were visible (asterisks mark the DiI labeled cell streams). B_1-B_2: photomicrographs of an embryo at 2 days after DiI injection. Bright DiI labeling was seen in the head region. C_1-C_2: At 3 days after DiI injection, DiI signals were not observed in the examined chicken embryos. Scale bars: 500 µm (A_1-A_2), 1 mm (B-C).

CHAPTER 6

SUMMARY

In the present study, we used (1) two molecular markers to label chicken taste buds at the embryonic and post-hatching stages, and (2) microinjection of DiI to label neural crest for demonstrating its contribution to taste buds. We found that α-Gustducin and Vimentin signals were seen in all examined taste buds, labeling distinct and overlapping population of the taste bud cells. The α-Gustducin signals was detected in premature taste buds in late embryos which suggests its important role in enabling chickens to respond to taste stimuli immediately after hatch. Taste bud labeling in the peeled oral epithelial sheets was established and provides a highly efficient method for analyzing the distribution pattern and total number of chicken taste buds. The analysis showed that chickens have far more taste buds than previously reported, >800 labeled with molecular markers vs ~300 with SEM. The total number of taste buds in male chickens is significantly higher than that in females and continue to change after hatch. DiI microinjection into neural tube is useful for a short-term labeling of neural crest but the labels were not sustained long enough to analyze the potential cell fate of neural crest in taste buds, suggesting the need for alternative techniques. These findings are highly significant and facilitate further mechanistic studies on chicken taste bud development and its association with feeding behaviors.