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Cyclopamine and jervine in embryonic rat tongue cultures demonstrate a role for Shh signaling in taste papilla development and patterning: fungiform papillae double in number and form in novel locations in dorsal lingual epithelium

Charlotte M. Mistretta,[a,*] Hong-Xiang Liu,[a] William Gaffield,[b] and Donald K. MacCallum[c]

[a] *Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, MI 48109, USA*
[b] *Western Regional Research Center, ARS, USDA, Albany, CA 94710, USA*
[c] *Department of Cell and Developmental Biology, Medical School, University of Michigan, Ann Arbor, MI 48109, USA*

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Abstract

From time of embryonic emergence, the gustatory papilla types on the mammalian tongue have stereotypic anterior and posterior tongue locations. Furthermore, on anterior tongue, the fungiform papillae are patterned in rows. Among the many molecules that have potential roles in regulating papilla location and pattern, Sonic hedgehog (Shh) has been localized within early tongue and developing papillae. We used an embryonic, tongue organ culture system that retains temporal, spatial, and molecular characteristics of in vivo taste papilla morphogenesis and patterning to study the role of Shh in taste papilla development. Tongues from gestational day 14 rat embryos, when papillae are just beginning to emerge on dorsal tongue, were maintained in organ culture for 2 days. The steroidal alkaloids, cyclopamine and jervine, that specifically disrupt the Shh signaling pathway, or a Shh-blocking antibody were added to the standard culture medium. Controls included tongues cultured in the standard medium alone, and with addition of solanidine, an alkaloid that resembles cyclopamine structurally but that does not disrupt Shh signaling. In cultures with cyclopamine, jervine, or blocking antibody, fungiform papilla numbers doubled on the dorsal tongue with a distribution that essentially eliminated inter-papilla regions, compared with tongues in standard medium or solanidine. In addition, fungiform papillae developed on posterior oral tongue, just in front of and beside the single circumvallate papilla, regions where fungiform papillae do not typically develop. The Shh protein was in all fungiform papillae in embryonic tongues, and tongue cultures with standard medium or cyclopamine, and was conspicuously localized in the basement membrane region of the papillae. Ptc protein had a similar distribution to Shh, although the immunoproduct was more diffuse. Fungiform papillae did not develop on pharyngeal or ventral tongue in cyclopamine and jervine cultures, or in the tongue midline furrow, nor was development of the single circumvallate papilla altered. The results demonstrate a prominent role for Shh in fungiform papilla induction and patterning and indicate differences in morphogenetic control of fungiform and circumvallate papilla development and numbers. Furthermore, a previously unknown, broad competence of dorsal lingual epithelium to form fungiform papillae on both anterior and posterior oral tongue is revealed.

Keywords: Taste papilla; Sonic hedgehog; Patched; Cyclopamine; Pattern formation; Tongue

Introduction

During development, repeated, localized interactions between embryonic epithelium and underlying mesenchyme result in formation of specialized appendages, including hair, feathers, and teeth (Callahan and Oro, 2001; Chuong et al., 2000; Oro and Scott, 1998). Less appreciated and studied are embryonic tissue interactions that contribute to formation of the precisely patterned, gustatory papillae on the tongue (Mistretta, 1998). Fungiform papillae on the rodent tongue are distributed in rows on the anterior oral tongue, but are absent on the posterior oral tongue in a broad region around the single, midline circumvallate papilla (Mistretta,

* Corresponding author. Fax: +1-734-647-2110.
E-mail address: chmist@umich.edu (C.M. Mistretta).

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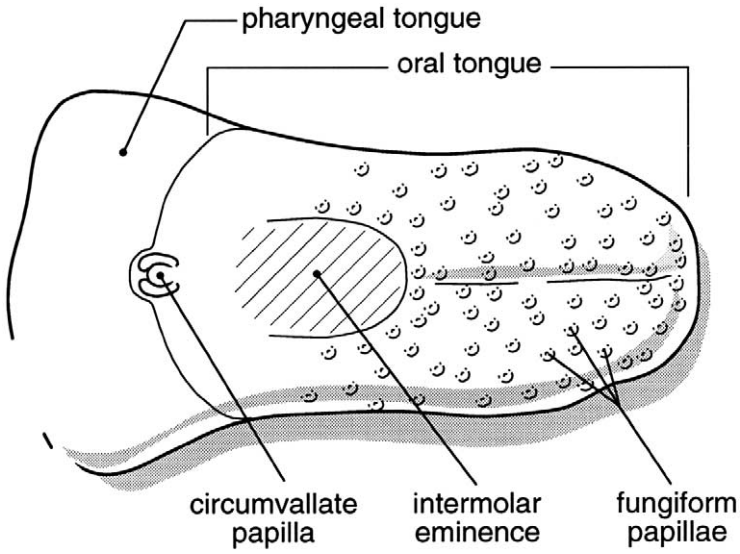


Fig. 1. Diagram of rat tongue to illustrate general boundaries of oral and pharyngeal tongue, and locations of fungiform and circumvallate papillae and the intermolar eminence. The embryonic, oral rat tongue originates from a set of mesenchymal swellings in the floor of the mouth, deriving from pharyngeal arch 1, whereas arches 2 and 3 are thought to contribute the broad tissue yoke that forms pharyngeal tongue (Kaufmann, 1992; Mistretta, 1991).

1972). This specialized distribution emerges early in tongue development (Mistretta, 1991) and is not dependent on an intact sensory innervation (Mbiene et al., 1997). In a continuing series of experiments to understand patterned papilla formation, we have studied the role of the vertebrate morphogen, Sonic hedgehog (Shh). Here, we use the steroidal alkaloids cyclopamine and jervine to disrupt the Shh signaling pathway, in addition to a Shh-blocking antibody, and demonstrate that Shh signal transduction has a major role in regulating papilla patterning and development.

The patterned distribution of different taste papilla types on the mammalian tongue suggests broad anterior and posterior specialization of oral tongue tissues and, on anterior tongue, an intricate patterning mechanism to position the fungiform papillae (Fig. 1). Because taste buds reside solely in gustatory papillae on the mammalian tongue, the papillae are necessary structures for lingual taste bud formation and development of the life-sustaining sensation of taste. Although potential neural regulation of papillae patterning has been studied with in vitro systems (Mbiene et al., 1997) and addressed with transgenic models (Mistretta et al., 1999; Nosrat et al., 1997; Zhang et al., 1997), little is known about the molecular signals that direct papilla emergence and morphogenesis (Mistretta, 1998).

Fungiform and circumvallate papillae develop between days E14 and E15 in rat (Mbiene et al., 1997; Mistretta, 1972). Although detailed descriptions are lacking, papilla development apparently begins with formation of epithelial placodes; epithelial–mesenchymal interactions follow to construct papillae that protrude from the surface of the tongue and are composed of an epithelium surrounding a mesenchymal tissue core (Farbman and Mbiene, 1991; Mistretta, 1991; Mistretta and Hill, 1995). From the time of appearance on the lingual dorsum at E15, the fungiform papillae are organized in distinct rows on the anterior tongue in front of the intermolar eminence, and a few papillae develop just lateral to the eminence (Mistretta, 1972; Mbiene et al., 1997). Therefore, similar to other epithelially derived specializations, such as tooth (Thesleff and Sharpe, 1997), feather (Jung et al., 1998), and hair (Oro and Scott, 1998), the fungiform papillae derive from epithelial–mesenchymal interactions and are arranged in a distinctive spatial pattern. Circumvallate papilla formation also occurs essentially over E14–E15 in the rat. Annular downgrowth of epithelial cells and an eventual clefting of the epithelium contribute to formation of a wall of epithelial cells surrounding a moat within which the central papilla lies (Mistretta, 1991). In contrast to an early induction and continued embryonic development of gustatory papillae, the collection of cells that histologically demarcate presumptive taste buds appears near the end of gestation, at E20 in apical fungiform or circumvallate papilla epithelium (Mistretta, 1972).

RNA messages of genes known to participate in organogenesis of various systems have been demonstrated in early fungiform papillae. These include: sonic hedgehog, *Shh* (Bitgood and McMahon, 1995; Hall et al., 1999a; Jung et al., 1999); bone morphogenic proteins, *Bmp 2, 4* (Bitgood and McMahon, 1995; Jung et al., 1999); fibroblast growth factor 8, *Fgf 8* (Jung et al., 1999); and Distaless-3, *Dlx 3* (Morasso et al., 1995). Of these molecules, Shh has multiple, demonstrated roles in processes that regulate development and patterning of various vertebrate organs (Christian, 2000; Hogan, 1999; McMahon, 2000), including epithelial specializations (Callahan and Oro, 2001; Wang et al., 2000). Shh mRNA has been described in some detail in mouse tongue and taste papillae (Bitgood and McMahon, 1995; Hall et al., 1999a; Jung et al., 1999). Expression of Shh

mRNA and the Shh receptor component, patched (Ptc), is initially diffuse in the early oral tongue, with subsequent, more restricted localization to developing fungiform and circumvallate papillae.

To determine whether Shh has a role in papilla development or is simply associated with these organs, we used the plant steroidal alkaloids cyclopamine and jervine, which selectively and potently disrupt signaling in the Shh pathway (Cooper et al., 1998; Incardona et al., 1998; Porter et al., 1996), to study effects on papilla morphogenesis and distribution in an embryonic tongue culture system. Cyclopamine and jervine are alkaloids of the *Veratrum* family that have a broad range of actions, from perturbing cholesterol metabolism to teratogenic effects (Binns et al., 1963; Cooper et al., 1998; Gaffield and Keeler, 1996; Incardona et al., 1998; Porter et al., 1996). The alkaloids act by altering the response of tissues to Shh, with proposed targets for disruption of signaling at the Shh receptor domains, Patched (Ptc) and Smoothened (Smo) (Cooper et al., 1998; Incardona et al., 1998; Incardona and Eaton, 2000; Taipale et al., 2000). Although the mechanism for cyclopamine action is not fully resolved, the effect is apparently downstream of Ptc and possibly involves alterations in the balance between active and inactive forms of Smo (Taipale et al., 2000).

We used these steroidal alkaloids in an organ culture system of embryonic rat tongue, in which we have previously shown that fungiform and circumvallate papillae develop in vitro in the absence of intact sensory innervation, in a spatially correct pattern and at temporally appropriate embryonic stages (Mbiene et al., 1997). Neurotrophins are expressed in the tongue cultures in distributions and temporal sequences that match in vivo embryonic expression (Nosrat et al., 2001). Furthermore, in vitro expression of Shh and Ptc mRNAs in taste papillae replicates their expression in the developing tongue in vivo (Hall et al., 1999b). The tongue organ culture, therefore, provides an ideal system for studying factors that regulate papilla induction, development, and morphogenesis in the absence of neural influences.

In this study, we report the development of an overabundance of fungiform papillae on anterior tongue, with a density that virtually eradicates interpapilla spacing, in embryonic rat tongues cultured with cyclopamine and jervine. Furthermore, fungiform papillae develop on posterior locations of the oral tongue, near the circumvallate papilla, where the fungiform papilla type is never observed in vivo. Use of a Shh-blocking antibody leads to similar effects. Shh and Ptc are immunolocalized in all fungiform papillae in tongue cultures, and furthermore, Shh is especially prominent in regions near the basement membrane of fungiform papillae in standard medium and cyclopamine-treated cultures. Results demonstrate a role for the Shh pathway in papilla induction, development, and patterning, and in a surprising finding, illustrate broad competence of dorsal lingual epithelium for fungiform papilla formation. An earlier report has appeared in abstract form (Mistretta et al., 2000).

Methods

Rat embryo and tongue dissection

Timed, pregnant Sprague Dawley rats were used and were obtained from Charles River breeders. Animal maintenance and use were in compliance with approved institutional animal care protocols and were according to National Institutes of Health guidelines. Embryonic day 0 (E0) designated the day on which the dam was sperm-positive. E14 embryos were used and all dissections were between 9AM and noon to insure consistency across litters. E14 was chosen because the tongue is still in early developmental stages, the three lingual swellings have fused, and the fungiform and circumvallate papillae are beginning their prepapilla emergence as just discernable tissue thickenings.

Dams were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital at 60 mg/kg body weight, which also anesthetizes the embryos. Dissections were made by using aseptic procedures. Anesthetized embryos were removed in groups of six and placed in cold Earle's balanced salt solution (EBSS) containing gentamicin sulfate (20 μg/ml), and buffered with 20 mM Hepes (pH 7.4), in a sterile petri dish. For tongue dissection, the embryo was decapitated, the head moved to fresh EBSS, and the entire tongue was microdissected from the mandible and placed in a petri dish with fresh cold EBSS.

Tongue organ cultures, steroidal alkaloids, and Shh-blocking antibody

Tongues were cultured by using a whole tongue organ culture system developed in our laboratory (Mbiene et al., 1997). Dissected tongues were positioned with dorsal surface upward on small pieces of sterile Millipore HA filters (0.45-μm pore size), wetted with EBSS. Tongues and filter papers were placed on stainless steel grids in standard organ culture dishes (B.D. Falcon Plastics). Cultures were fed with a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12; Life Technologies, Rockville, MD), containing 1% fetal bovine serum, 20 μg/ml gentamicin sulfate, and 2% B27 culture supplement (Gibco). Tongue cultures were maintained at the interface between gas (5% CO_2 in air) and liquid phases of the culture, in a humidified incubator at 37°C. The level of the medium was adjusted so that the tissue was wet but not covered with medium (MacCallum, 1994). After 2 days in culture, tongues were removed and processed for scanning electron or light microscopy.

To study the role of the Shh signaling pathway in papilla development and patterning on the tongue, we added steroidal plant alkaloids, known to interrupt the Shh signaling process, to the standard culture medium (Gaffield et al., 1986, 1999). Four culture conditions were used: standard culture medium (STAND), as described in the paragraph above with no added alkaloids; addition of one of two

alkaloids with known, selective, potent effects in disrupting Shh signaling, cyclopamine (CYCL) or jervine (JERV) at 5 or 10 μM; and addition of an alkaloid that is structurally similar to cyclopamine and jervine, but that does not disrupt Shh signaling, solanidine (SOLAN) at 5 or 10 μM. Cyclopamine, jervine, and solanidine were prepared in 10 mM stock solutions in 100% ethanol, stored at 4°C. Before experiments, alkaloids were added to the culture medium and were present for the duration of tongue cultures.

To disrupt Shh signaling in a second manner, we added 20 μg/ml of the Shh-blocking antibody 5E1 (Developmental Hybridoma Bank, University of Iowa) to the standard culture medium. Furthermore, to learn if addition of the Shh protein had any effect on papilla development, we added recombinant mouse sonic hedgehog amino-terminal peptide (Shh-N) (R&D Systems, #461-SH, Minneapolis, MN) to standard culture medium at concentrations of 10, 20, or 30 nM, and maintained cultures for 2 days.

Scanning electron and light microscopy

Scanning electron microscopy

After 2 days in culture, tongues were removed, rinsed briefly in EBSS, and fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.025 M cacodylate buffer (pH 7.3) for 6 h at 4°C. Tissues were then rinsed in buffer and subsequently postfixed in a sequence of aqueous 1% OsO_4, 1% tannic acid, 1% OsO_4, for 1 h each. Tongues were then dehydrated through an ascending series of alcohols, and displacement of alcohol was accomplished with three changes of hexamethyldisilazane (HMDS). Residual HMDS was evaporated in a fume hood overnight before mounting the tongues on specimen stubs for light sputter coating with gold/palladium. Scanning electron micrographs were scanned as digital images, and assembled into plates by transporting images to Adobe Photoshop.

Light microscopy

Tongue cultures for 1-μm sections were rinsed briefly in EBSS and then placed in a 50:50 mixture of 2.5% glutaraldehyde and 2% OsO_4 in buffered cacodylate (0.1 M Na cacodylate, pH 7.4, 4% sucrose, 2 mM $CaCl_2$) for 2 h at 4°C, and stained en bloc with 2% uranyl acetate (MacCallum et al., 1982). Tissues were then dehydrated in ethanol and propylene oxide and embedded in epoxy resin. Then, 1-μm sections were mounted on glass slides and stained with toluidine blue O, pH 9.0.

Papilla quantification

Scanning electron micrographs of whole tongues at 75 and 100× original magnification were used to count the number of fungiform papillae on control (standard medium and standard medium with solanidine) and Shh-perturbed tongue cultures (standard medium with cyclopamine or jervine). Papilla counts were made on 11 standard medium cultures, and on 6 solanidine, 13 cyclopamine, and 8 jervine-treated cultures at 5 and 10 μM concentrations. For each culture condition, embryos from 3 to 7 separate litters were used. Counts were made of all papillae on the entire tongue; of papillae on the anterior tongue in front of the most distal edge of the intermolar eminence; and of papillae on the posterior tongue, lateral to and behind the most distal edge of the intermolar eminence (Fig. 1). Numbers of papillae were analyzed statistically across the 4 control and experimental conditions with analysis of variance (ANOVA; $P \leq 0.05$ significance level). In addition to cultures for papilla counting, 9 other litters were used in 6 experimental series for qualitative analysis and histology.

Immunohistochemistry

A goat polyclonal antibody against the mouse Shh N-terminal peptide (R&D Systems, Minneapolis, MN) was used for sectioned and whole embryo tongues and tongue cultures, with an antigen retrieval procedure. Other rabbit (H-160) and goat (N-19) polyclonal antibodies against the amino terminus of Shh (Santa Cruz Biotechnology, Santa Cruz, CA) also were used with similar results.

For immunocytochemistry on tongue sections, dissected embryo heads were frozen in O.C.T. compound (Miles Scientific, Elkhart, IN). Serial sagittal sections were cut at 12 μm and fixed at 4°C for 1.5 h in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. After fixation, sections were rinsed in PBS. Endogenous peroxidase staining was blocked in 0.5% H_2O_2 in methanol. After rinsing in PBS, antigen retrieval was performed by heating at 92–95°C for 2–3 min in Universal antigen retrieval agent (R&D Systems, Minneapolis, MN). Nonspecific staining was blocked with 10% normal sera in PBS and 0.3% Triton X-100 (Sigma, St. Louis, MO) for 30 min, and then sections were incubated overnight at 4°C in primary antibody at 1:3000, in carrier solution (1% normal rabbit serum, 0.3% Triton X-100 in PBS). After rinsing in carrier solution, sections were placed in biotin-labeled, rabbit anti-goat secondary antibody (Kirkegaard and Perry, Gaithersburg, MD), at 1:250. Sections were rinsed and incubated in HRP-labeled streptavidin, and the HRP label was visualized with nickel-intensified DAB solution (Vector Laboratories, Burlingame, CA). Reacted slides were dehydrated through alcohols, cleared in xylene, and coverslipped.

Whole embryo tongues or tongue cultures were fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, at 4°C for 2 h. Tongues were transferred to 100% methanol and stored at −20°C. For immunocytochemistry, endogenous peroxidase activity was blocked with 6% H_2O_2 in methanol at room temperature for 5 h. Tongues were rehydrated into PBS, through a descending methanol series, at 4°C for 30 min each. Antigen retrieval was performed by heating at 92–95°C for 3–5 min, with the Universal antigen retrieval agent (R&D Systems, Minneapolis, MN). Nonspecific staining was blocked in PBS with 2% skim milk powder and 0.1%

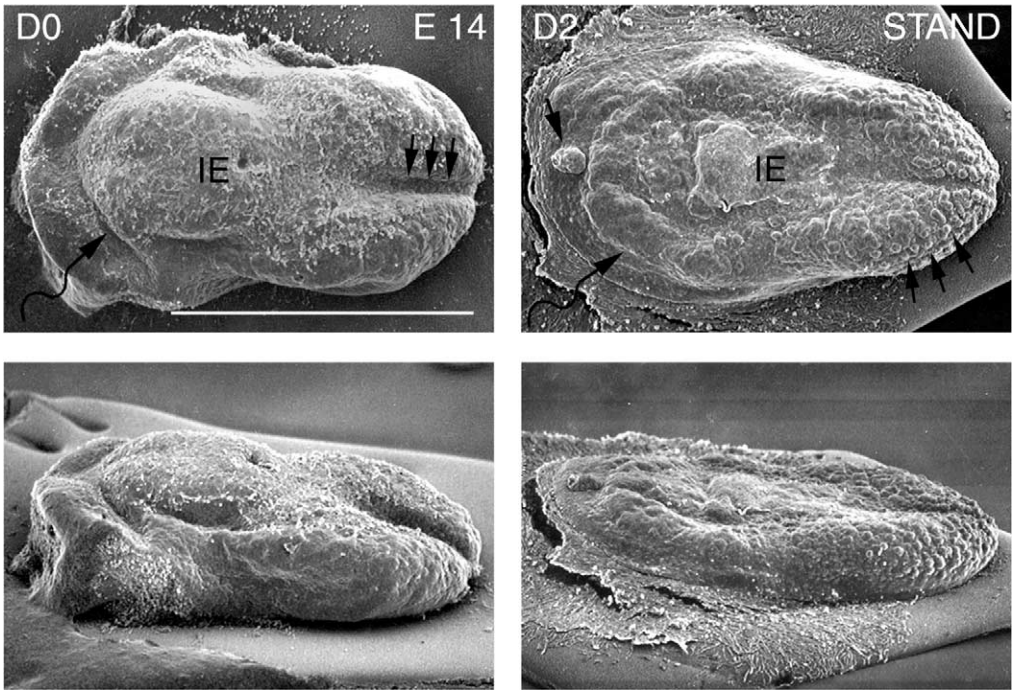


Fig. 2. Scanning electron micrographs of embryonic rat tongue in organ culture. D0: The embryonic tongue placed in culture on gestational day 14 (E14) has a relatively homogeneous dorsal surface; fungiform and circumvallate papillae are not apparent. The border between oral and pharyngeal tongue is noted with a curved arrow. The intermolar eminence (IE) and anterior median furrow (arrows) are labeled. D2: After 2 days in culture in standard medium (STAND), multiple fungiform papillae have formed on the anterior tongue (arrows) and the circumvallate papilla has emerged at the border of posterior oral tongue (single arrow). The intermolar eminence and median furrow remain apparent. The intermolar eminence often exhibits a sheet of desquamating cells after 2 days in culture. Scale bar, 1.0 mm.

Triton X-100 (Sigma, St. Louis, MO). Tongues were incubated overnight at 4°C with the primary antibody in blocking solution at 1:100 dilution. After rinsing, tongues were incubated overnight at 4°C with a biotin-conjugated rabbit anti-goat secondary antibody (Kirkegaard and Perry, Gaithersburg, MD) at 1:500 in blocking solution. Then, tongues were rinsed and incubated overnight at 4°C with peroxidase-conjugated streptavidin at 1:500 in blocking solution. Reactions were done in nickel-intensified DAB solution (Vector Laboratories, Burlingame, CA) with 0.003% H_2O_2. Tongues were rinsed twice in PBS for 1 h each and stored in 4% buffered paraformaldehyde at 4°C for subsequent photography.

For Ptc whole tongue and tongue culture immunohistochemistry, goat polyclonal antibodies (Santa Cruz Biotechnology) against the amino terminus (G-19, patched of mouse origin) and the carboxy terminus (C-20, patched of human origin) and mixtures of both antibodies were used with identical results. An antigen retrieval protocol was used similar to that for Shh immunolocalization, with overnight incubation in primary antibody at 1:25 dilution.

Digital images were generated from slides or scanned from original scanning electron micrographs and assembled into figures by using Photoshop (Adobe Systems, Mountain View, CA).

Results

Papilla development in E14 tongue organ cultures

The dissected embryonic rat tongue at E14 has a spatulate shape, but regions of earlier, posterior and anterior, lateral tongue swellings remain clear (Fig. 2, D0, E14). Neither fungiform nor circumvallate papillae are observed at this stage with scanning electron microscopy. However, after 2 days in culture with standard medium, a well-formed circumvallate as well as fungiform papillae are present in tongue locations that mimic in vivo development (Fig. 2, D2, STAND). As observed on the E16 tongue in vivo, fungiform papillae are not seen posterior to the intermolar eminence, or in the area in front of the circumvallate papilla; nor are they numerous lateral to the intermolar eminence.

Papilla development in E14 tongues cultured with cyclopamine

E14 tongues cultured for 2 days in the presence of exogenous cyclopamine have retained the typical spatulate shape of tongues maintained in standard medium, and both circumvallate and fungiform papillae have developed (Fig. 3, STAND & CYCL). In contrast to tongues in standard medium, tongues with exogenous cyclopamine exhibit in-

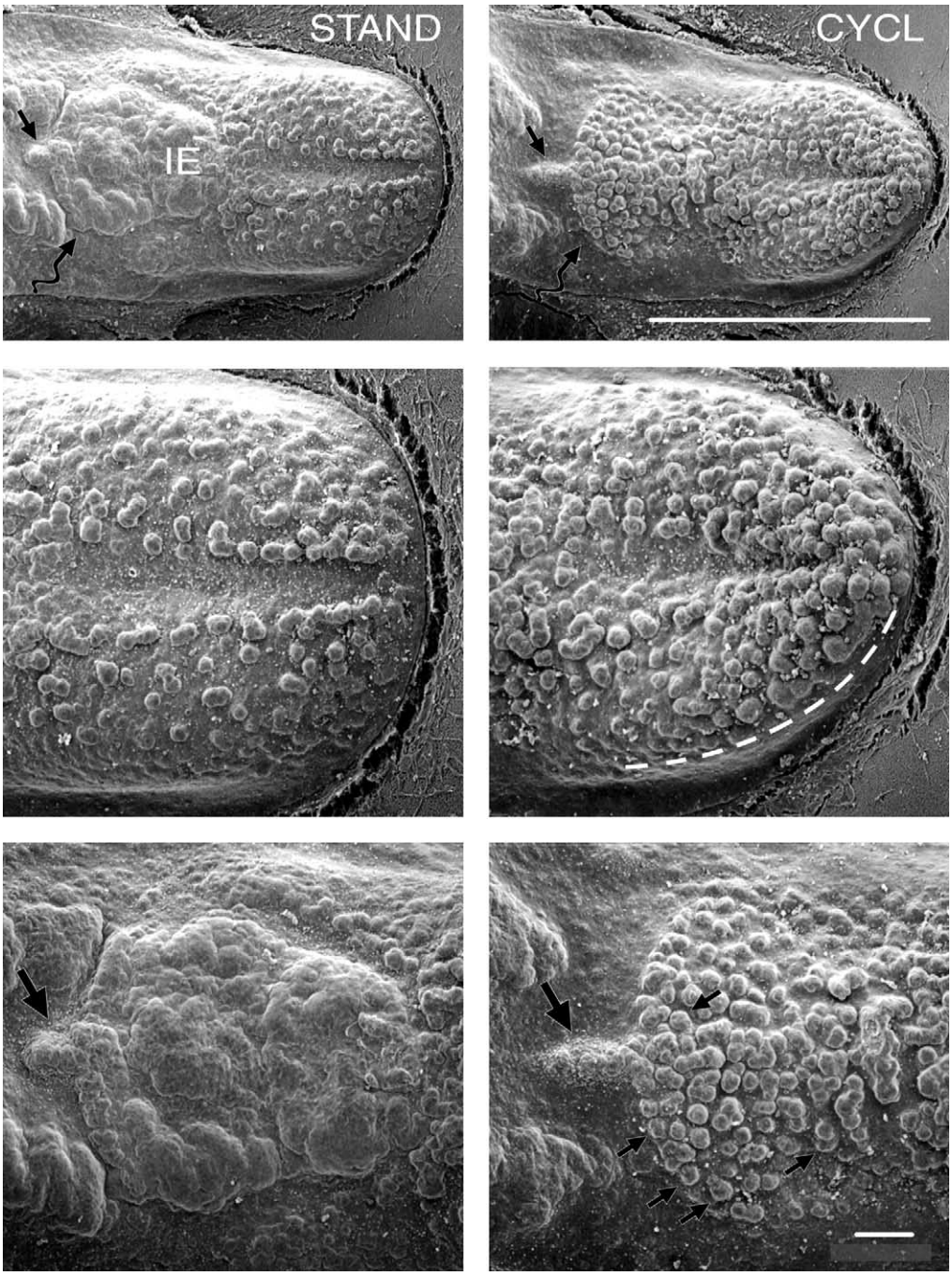


Fig. 3. Scanning electron micrographs of E14 tongues maintained in culture for 2 days in standard medium (STAND, left column) or standard medium containing 5 μM cyclopamine (CYCL, right column). The border between oral and pharyngeal tongue is marked with a curved arrow, and the single circumvallate papilla on the posterior tongue is noted with a straight arrow in the top and bottom micrographs. Compared to the tongue in STAND culture, CYCL addition results in a very dense distribution of fungiform papillae on anterior tongue (middle panels). However, the border between dorsal and ventral tongue epithelium is retained in tongues cultured with CYCL (dashed white line) and papillae form on the lingual dorsum only. Examination of posterior tongues (bottom micrographs) demonstrates that with CYCL multiple fungiform papillae (examples noted with small arrows) also develop in front of and beside the single circumvallate papilla (larger arrow). These are regions where fungiform papillae do not typically form (compare with STAND tongue). Scale bar for top micrographs, 1.0 mm. Bar at bottom right, 100 μm, for middle and bottom micrographs.

creased numbers of fungiform papillae closely distributed on anterior tongue and lateral to the intermolar eminence (Fig. 3, CYCL). Although fungiform papillae are very dense on the anterior tongue, it is apparent that borders between dorsal and ventral tongue epithelium are respected (Fig. 3, CYCL, anterior tongue) with no modification of ventral lingual epithelial surface.

Not only are fungiform papillae more numerous on the

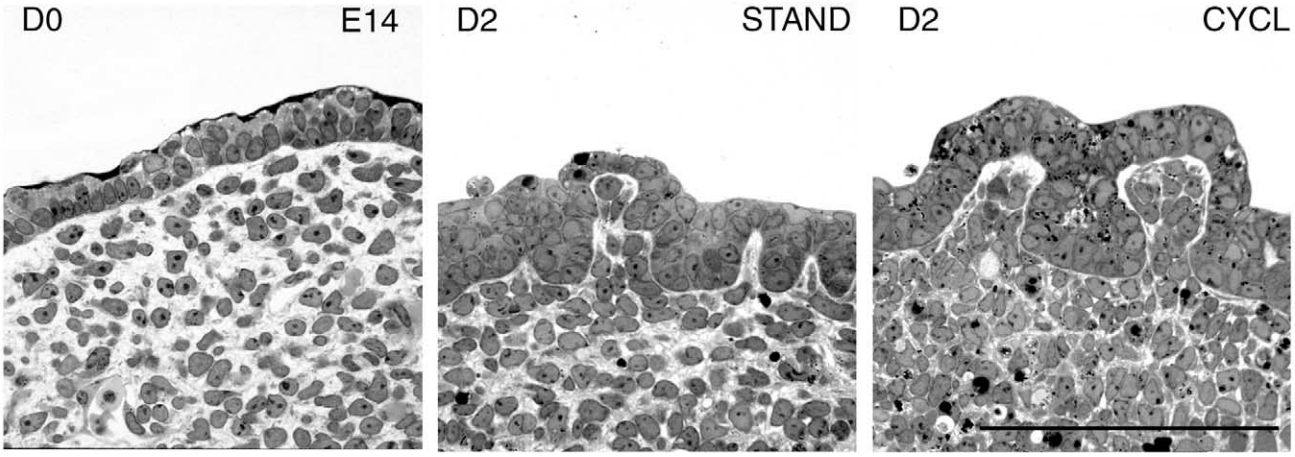


Fig. 4. Light micrographs of dorsal tongue epithelium from E14 tongue and tongue cultures. D0: At time of culture the E14 anterior, lingual epithelium is multilayered, without obvious papilla development. STAND: After 2 days in culture with standard medium, distinct fungiform papillae are seen. CYCL: Fungiform papillae in tongues cultured with cyclopamine, although more numerous, have similar histological features to those in STAND tongues. Scale bar, 100 μm.

anterior tongue in cultures with cyclopamine, but also, fungiform papillae are numerous on the posterior dorsal tongue in front of the circumvallate papilla (Fig. 3, CYCL). This is a region where fungiform papillae never form in vivo (Mistretta, 1972, 1991). These posterior fungiform papillae have not displaced the single midline circumvallate papilla, which develops in the usual location regardless of the culture condition. Furthermore, the newly located fungiform papillae do not encroach on pharyngeal tongue mucosa but remain solely in an oral, albeit posterior, tongue location.

Although the fungiform papillae are very closely spaced on anterior tongue, histological integrity of papilla epithelium and mesenchymal core is maintained (Fig. 4). A multilayered epithelium, covering a mesenchymal cell core, characterizes papillae from standard and cyclopamine culture conditions.

Shh and Ptc proteins in tongue cultures

To learn whether the Shh protein is found in newly formed fungiform papillae after cyclopamine treatment, we used immunohistochemistry to localize Shh in whole and sectioned tongues and tongue cultures. In the E14 embryo tongue, Shh is dense in discrete, irregular patches on the anterior tongue where fungiform papillae will form (Fig. 5, E14, D0). On the E16 embryo tongue, there is dense Shh in fungiform papillae, and in the circumvallate papilla, but no Shh reaction product is seen between papillae (Fig. 5, E16). The Shh protein in fungiform papillae appears in a pericellular distribution with intense staining near the basement membrane under the apical papilla epithelium (Fig. 5, E16, fp). In tongue cultures begun at E14 and maintained for 2 days in standard medium (Fig. 5, D2, STAND), Shh is restricted to each fungiform papilla and the circumvallate papilla, and is not seen elsewhere on the tongue. Within the fungiform papilla, Shh is distributed pericellularly in apical epithelial cells and is abundant near the basement membrane (Fig. 5, D2, STAND, fp).

With cyclopamine in the culture medium, Shh immunoproduct is very dense in all fungiform papillae, on anterior and posterior tongue (Fig. 5, D2, CYCL and D2, CYCL, post). In STAND and CYCL sections immunoreacted on the same slide under identical conditions, CYCL tongue cultures have a much more abundant Shh product than STAND tongues (Fig. 5, compare D2 STAND, fp and D2 CYCL, fp). Within the fungiform papilla in CYCL tongue cultures, Shh is very intense in the region of the basement membrane beneath apical epithelial cells, and there appears to be some Shh protein in the mesenchyme in the apical part of the papilla core (Fig. 5, D2, CYCL, fp).

Because Ptc regulates the activity of the Shh signal (Ingham, 1998; Ingham and McMahon, 2001; Johnson and Scott, 1998), it was important to confirm that Ptc was present in newly formed fungiform papillae after cyclopamine treatment in tongue cultures. Ptc detection would be expected near sources of Shh, in responding cells. In the E14 embryo, Ptc is localized to broad, prepapilla patches on anterior tongue (Fig. 6, E14, D0). The wide median furrow and intermolar eminence are free of Ptc immunoproduct. In E14 tongues cultured for 2 days in standard medium, Ptc is within each fungiform papilla and in the most posterior aspect of the circumvallate papilla (Fig. 6, D2, STAND). The distribution of the Ptc immunoproduct in prepapillae at E14, and in papillae after 2 days in culture, is more diffuse and less intense than Shh (see Fig. 5, E14 and STAND).

E14 tongues cultured with cyclopamine exhibit Ptc in each papilla on anterior and posterior tongue (Fig. 6, D2, CYCL). Ptc also is localized within the circumvallate papilla. The pattern of Ptc immunoproduct directly corresponds to that of Shh (compare Fig. 6, D2, CYCL with Fig. 5, D2 CYCL, whole tongue immunoreactions in dorsal view). Insets of fungiform prepapillae and papillae in Fig. 6

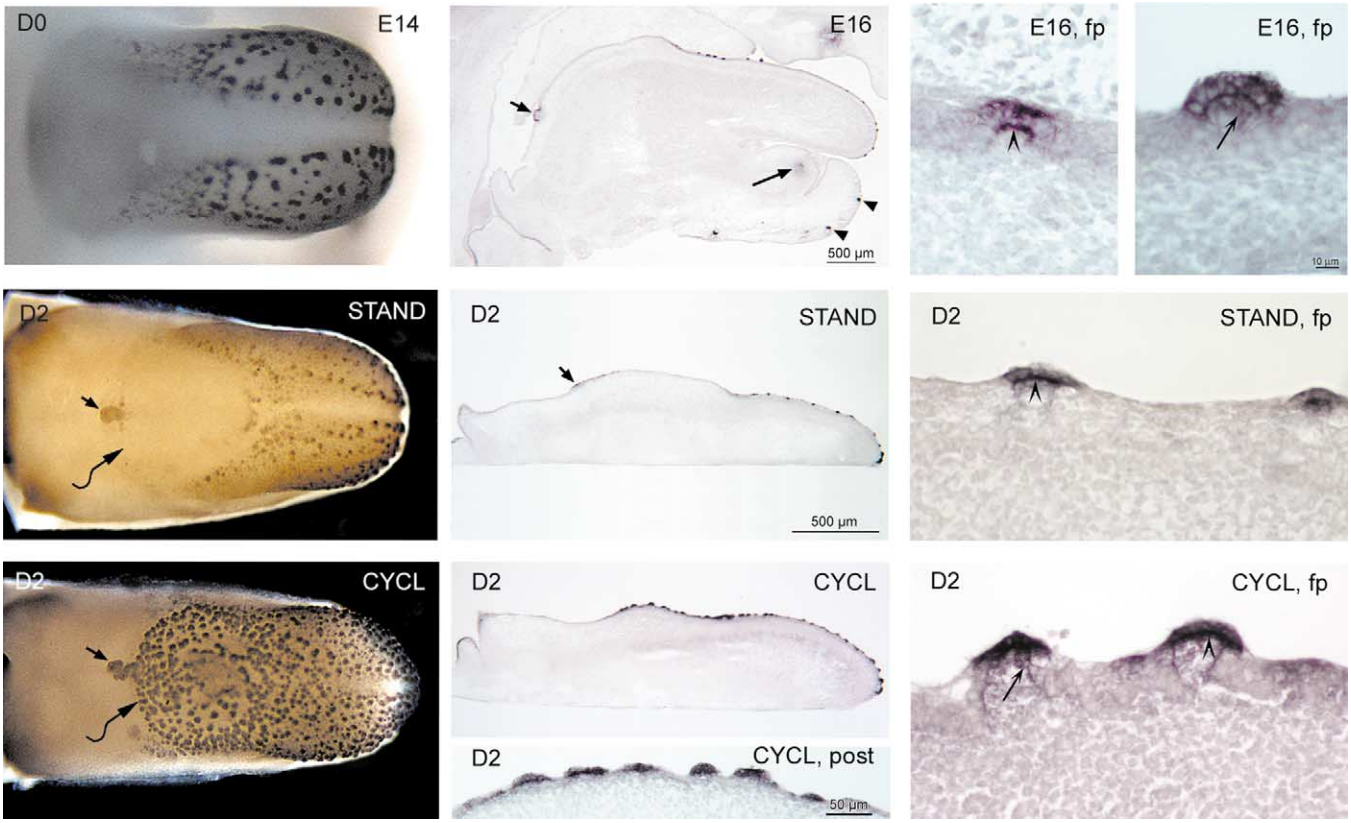


Fig. 5. Immunohistochemistry to demonstrate Shh protein in whole embryo tongues and whole tongue cultures, and in sectioned tongues and cultures. (Top row) The E14 whole tongue at time of culture (D0, E14) shown at left is compared with a sagittal section of the entire E16 tongue seated on the mandible (E16), and two E16 fungiform papillae shown in section at right (E16, fp). At E14, Shh protein is in broad patches where fungiform papillae will form on the anterior tongue. Although not visible on this photomicrograph due to tongue curvature, protein also is faintly seen where the circumvallate papilla is forming on the posterior tongue. In the E16 tongue, Shh is in all fungiform papillae and the circumvallate (short arrow, back of tongue). In this section, a long arrow indicates Shh in a tooth bud, and two arrowheads point to whisker follicles. In the E16 fungiform papillae at right, Shh is dense at the basement membrane region of the apical papilla (open arrowhead) and some immunoproduct also is observed in the mesenchyme just under the apical papilla epithelium (arrow). (Middle row) The E14 tongue in culture after 2 days (D2) with standard medium (STAND), shown in a whole tongue immunoreaction at left; in complete sagittal section at center; and, two fungiform papillae are at higher magnification at right. In the whole tongue and sagittal section, it is apparent that after 2 days in standard medium, Shh is in all fungiform papillae and in the single circumvallate papilla (short arrow). A curved arrow in the whole tongue photograph at left demarcates the border of oral from pharyngeal tongue. Within fungiform papillae (D2, STAND, fp), Shh is intensely located in the basement region (open arrowhead). (Bottom row) The E14 tongue in culture after 2 days (D2) with cyclopamine (CYCL), shown in a whole tongue immunoreaction at left (curved arrow demarcates the border of oral from pharyngeal tongue); in complete sagittal section and higher magnification of posterior tongue, at center, and, two fungiform papillae at higher magnification, at right. In the whole tongue and sagittal section, it is apparent that after 2 days in CYCL, Shh is in each of the multiple fungiform papillae and in the single circumvallate papilla (short arrow). Shh protein also is intense in all of the fungiform papillae that from in novel locations on the posterior tongue (CYCL, post). Within fungiform papillae (D2, CYCL, fp), Shh is very dense in the basement region (open arrowhead) and some Shh also is in mesenchymal cells of the papillae (arrow).

demonstrate a diffuse Ptc immunoproduct and suggest a somewhat peripapilla distribution relative to Shh, which we plan to investigate in detail with sections of tongues and tongue cultures.

Other steroidal alkaloids, anti-Shh blocking antibody, and Shh protein as positive and negative controls for cyclopamine effect

Two other steroidal alkaloids were used to further evaluate a role for Shh in papilla development. Based on holoprosencephaly induction observed in hamster embryos (Gaffield and Keeler, 1996), jervine would be predictably more or equally potent in blocking Shh signaling, compared with cyclopamine, but solanidine would be much less potent. At 5 μM concentrations, papillae and tongues cultured in solanidine were indistinguishable from those in standard medium (Fig. 7, STAND and SOLAN), whereas jervine had effects on papilla density and localization similar to those of cyclopamine (Fig. 7, CYCL and JERV). Notably, fungiform papillae were numerous in posterior, oral tongue regions in front of the circumvallate papilla in jervine-treated tongue cultures, but were absent from this area in solanidine tongues. Interestingly, the addition of fungiform papillae on posterior tongue as a result of either cyclopamine or jervine treatment served to accentuate demarcation of the oral from pharyngeal tongue in organ cultures (Fig. 7, CYCL and JERV).

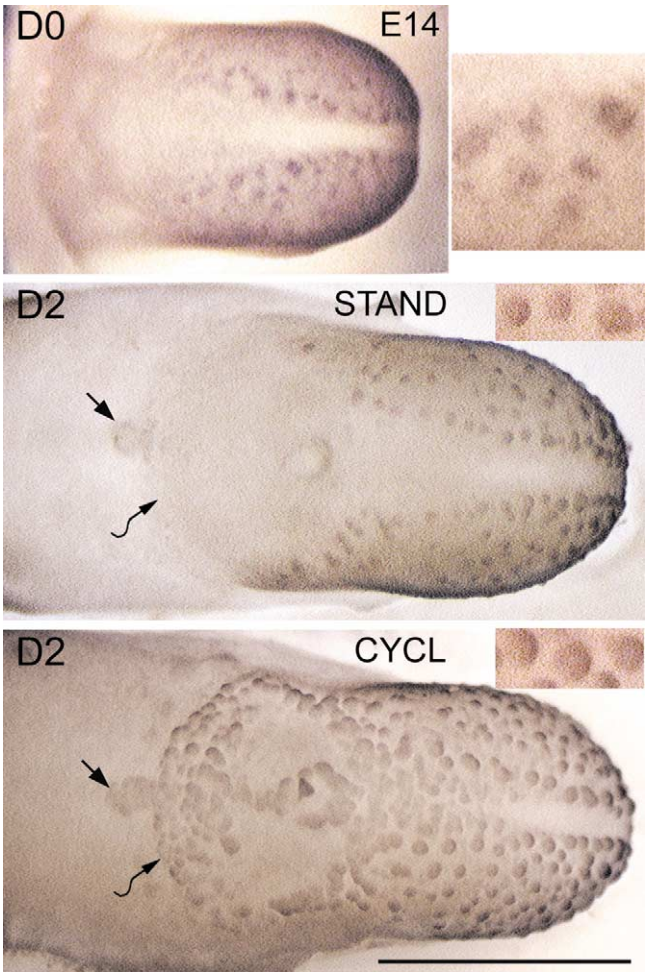


Fig. 6. Immunohistochemistry to demonstrate Ptc protein in whole embryo tongues and whole tongue cultures. In the E14 embryo tongue at time of culture (D0, E14) the Ptc immunoproduct is localized to broad patches where fungiform papillae will develop on the anterior tongue. The inset at right illustrates the diffuse nature of the Ptc protein within the prepapilla patches. The broad median furrow of the tongue is clear of Ptc protein. After 2 days in culture with standard medium (D2, STAND). Ptc is in each fungiform papilla that formed on anterior tongue, and is in the most posterior aspect of the circumvallate papilla (straight arrow; a curved arrow demarcates the border of oral from pharyngeal tongue). With cyclopamine in the culture medium (D2, CYCL) Ptc is within each fungiform papilla, including the increased numbers on anterior tongue and those on posterior oral tongue in front of the circumvallate papilla. This distribution of Ptc directly matches that of Shh in the Day 2, CYCL tongue culture shown in Fig. 5. Insets of papillae at higher magnification suggest that Ptc protein localizes more toward the edge of the fungiform papillae. Ptc immunoproduct also is in the circumvallate papilla (D2, CYCL, straight arrow; a curved arrow demarcates the border of oral from pharyngeal tongue). The scale bar, 500 μm, applies to all three tongues.

To evaluate disruption of Shh signaling on papilla development via another approach, a blocking antibody to Shh was added to tongue cultures. Blocking Shh also results in an increase in fungiform papillae on anterior tongue and a new posterior tongue distribution, directly comparable to results with cyclopamine (Fig. 7, CYCL and A-Shh). As with cyclopamine, the border with pharyngeal tongue is respected in tongues cultured with anti-Shh, but papillae are crowded on posterior oral tongue in front of the circumvallate papilla. On the other hand, addition of recombinant mouse sonic hedgehog peptide (Shh-N) at concentrations of 10–30 nM had no effect on papilla number or morphology (data not shown).

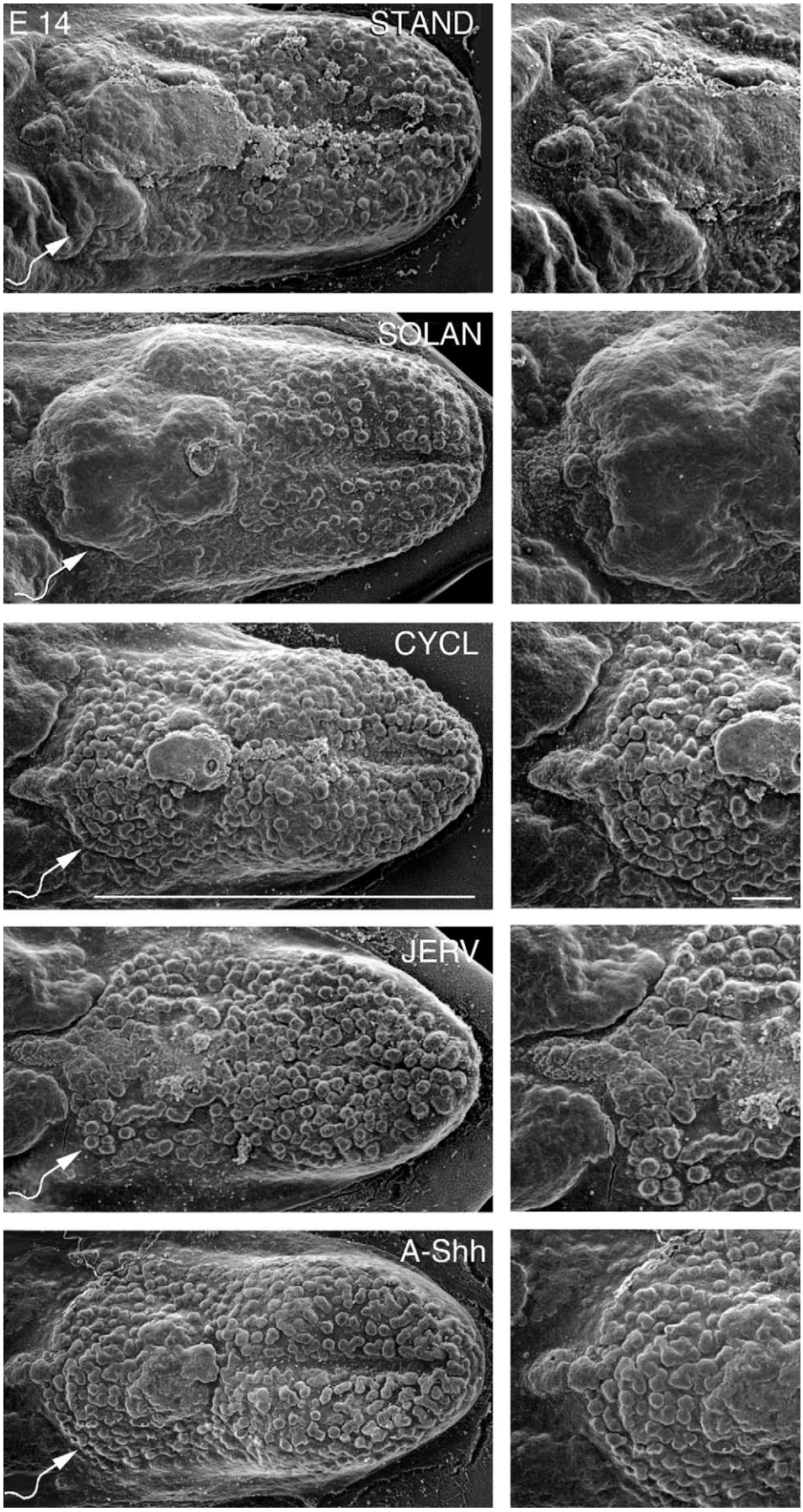
Increasing concentrations of the alkaloids solanidine, cyclopamine, and jervine from 5 to 10 μM did not alter their respective effects on papilla development and distribution. In tongues cultured with 10 μM solanidine, papilla spacing on anterior tongue was maintained similarly to that on standard medium tongues (Fig. 8, STAND and SOLAN), and no fungiform papillae formed on posterior tongue. Tongues treated with cyclopamine and jervine at 10 μM exhibited a dorsal anterior tongue that was almost completely occupied by fungiform papillae (Fig. 8 JERV; tongue cultures with 10 μM CYCL are not illustrated but are similar in all aspects to JERV cultures), and papillae were closely spaced in front of the midline circumvallate papilla.

The median furrow of the tongue forms initially as the lateral lingual swellings merge in early tongue morphogenesis and it is normally devoid of fungiform papillae. Similarly, in embryonic tongue cultures, the median furrow did not support fungiform papilla development either with standard medium or with 5 and 10 μM solanidine, cyclopamine, or jervine conditions (Fig. 8). Even as interpapilla spacing was totally lost on the anterior of cyclopamine and jervine-treated tongues, the tongue median remained papilla-free.

Fungiform papilla numbers doubled in cyclopamine-treated tongue cultures

To quantify effects of the steroidal alkaloids on fungiform papilla number, all papillae were counted on the entire cultured tongue and on anterior and posterior tongue regions separately (Fig. 9). There were significant differences in papillae number across culture conditions, for the entire tongue [ANOVA: $F(3,27) = 69.1$; $P < 0.001$], and for anterior [ANOVA: $F(3,27) = 28.9$; $P < 0.001$] and posterior [ANOVA: $F(3,27) = 29.6$; $P < 0.001$] regions. The number of papillae in standard medium vs solanidine-treated, or in cyclopamine vs jervine-treated, tongue cultures were not different from each other ($P > 0.05$). On the other hand, papilla numbers in either standard or solanidine tongues were significantly different from either cyclopamine- or jervine-treated tongues ($P < 0.05$).

Papilla number doubled on tongues cultured with cyclopamine or jervine, compared with those in standard medium or solanidine-containing medium (Fig. 9). Because the tongue size did not change with any alkaloid treatment compared with standard medium, there was a true increase in fungiform papilla density. When papillae were quantified on posterior tongue regions lateral to the intermolar eminence (a region that normally has spare numbers of fungiform papillae), and just in front of


E 14
STAND
SOLAN
CYCL
JERV
A-Shh

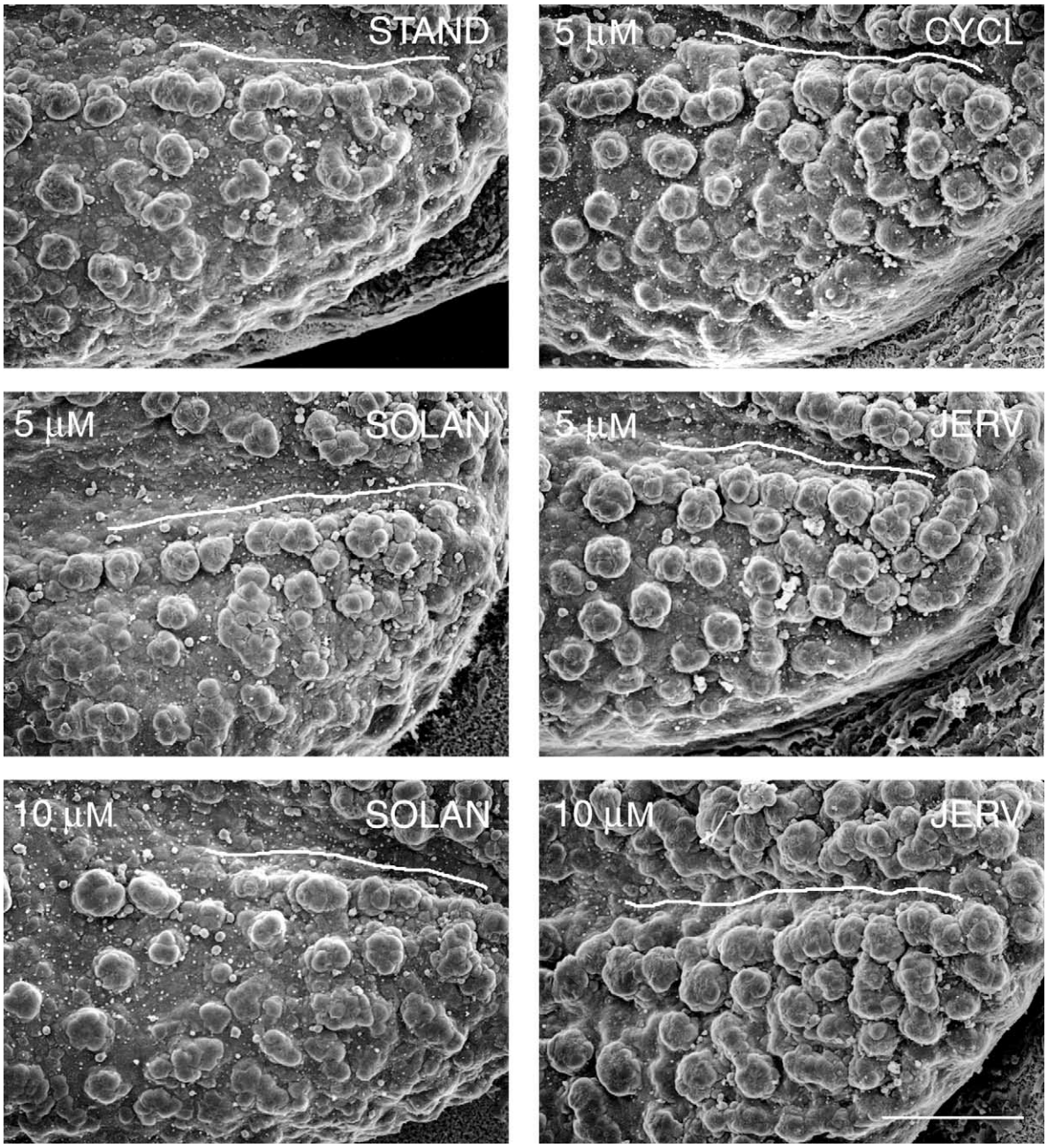


Fig. 8. High power scanning electron micrographs of E14 tongue tips after 2 days in culture with standard medium (STAND), 5 μM cyclopamine (CYCL), and 5 or 10 μM solanidine (SOLAN) and jervine (JERV). Note that even when fungiform papillae formation occurs over virtually all of the anterior tongue in CYCL and JERV conditions, the median furrow (white line demarcates bottom of furrow) does not support papilla development. Scale bar, 100 μm.

and beside the circumvallate papilla (where fungiform papillae do not usually form), the number in cyclopamine- and jervine-treated cultures was increased by about threefold.

Fungiform and circumvallate papilla surface epithelium

The surface structure of fungiform papillae had a lobulated appearance in standard medium and in all alkaloid

Fig. 7. Scanning electron micrographs of E14 tongues cultured for 2 days in different steroidal alkaloids, and with a blocking antibody to Shh. A curved arrow demarcates the border between oral and pharyngeal tongue in each low power micrograph (left column). The circumvallate papilla and surround are presented at slightly higher magnification (right column). Tongues in both standard medium (STAND) and with addition of 5 μM solanidine (SOLAN) have multiple fungiform papillae on the anterior tongue and a single circumvallate papilla on posterior tongue. In comparison, tongues cultured with 5 μM cyclopamine (CYCL) or jervine (JERV) have an increased density of fungiform papillae on the anterior tongue, and numerous fungiform papillae on the posterior tongue near the circumvallate papilla (right column). Tongues cultured with an antibody to Shh (A-Shh) are similar in all aspects to cultures with cyclopamine or jervine. Scale bar for left column, 1.0 mm; for right column, 100 μm.

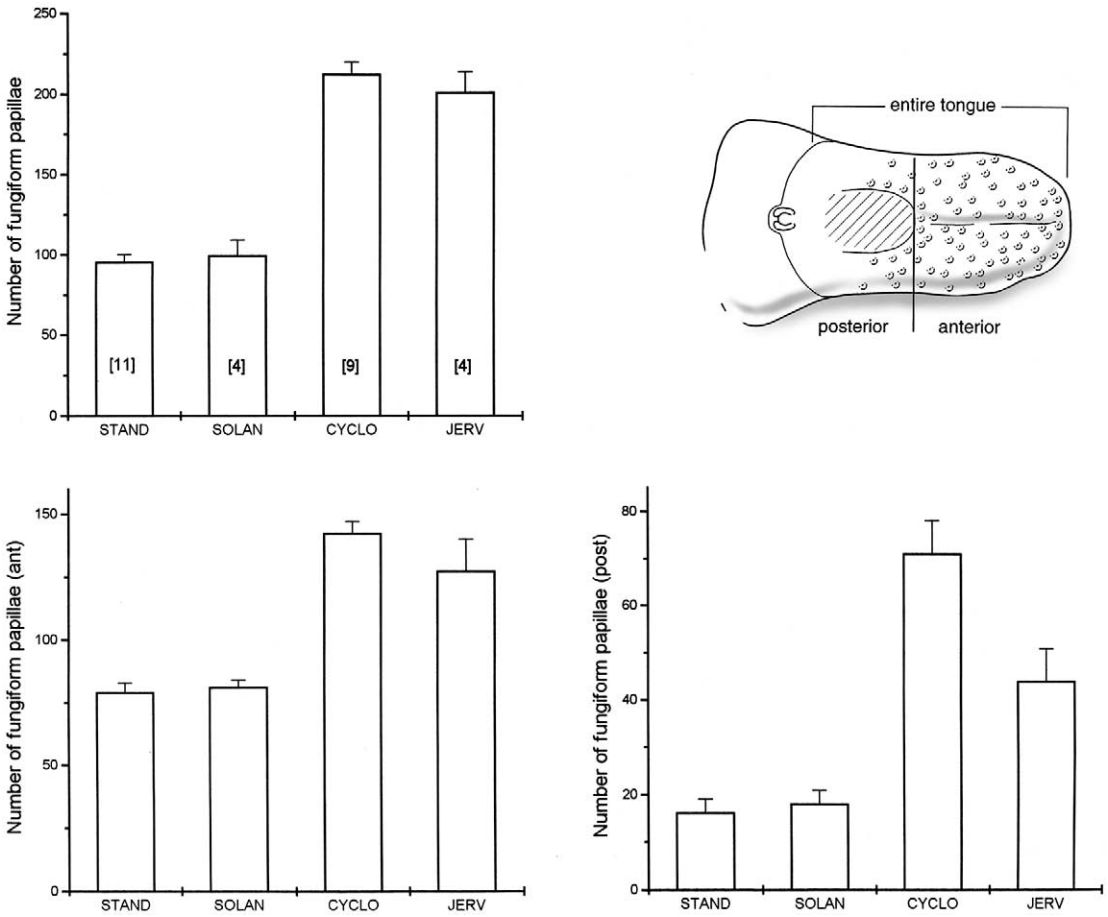


Fig. 9. Histograms of mean number of fungiform papillae (with standard error bars; number of tongues analyzed in brackets) on the entire tongue (top left) or on anterior or posterior tongue only (bottom graphs), in standard medium, or in 5μM solanidine, cyclopamine or jervine culture conditions. The diagram at top right indicates regions for anterior and posterior tongue counts. Number of papillae essentially doubled on the entire tongue in cultures with steroidal alkaloids that disrupt the Shh signaling pathway. For entire, anterior, or posterior tongue counts, there was a significant difference across groups ($P < 0.001$). There were no differences between standard and solandine, or between cyclopamine and jervine-treated tongues. However, either the standard or the solandine tongues were significantly different from either the cyclopamine or jervine-treated tongues.

culture conditions (Fig. 8). There is an impression of proliferative activity and an increase in size in papillae from cyclopamine- and jervine-treated tongues. However, in all tongues, there was a substantial range in papilla size and any systematic attempt at quantification would have to incorporate more than a surface diameter measurement from scanning micrographs.

The circumvallate papilla appeared as an assembly of large and small lobes and surface elevations in all culture conditions (Fig. 10) and was very distinct from the surrounding epithelium. In contrast to alkaloid effects on fungiform papillae, multiple circumvallate papillae were not induced in tongue cultures with exogenous cyclopamine or jervine.

Discussion

Observations of Shh mRNA in embryonic rodent tongue have implicated this morphogen in gustatory papilla development (Bitgood and McMahon, 1995; Hall et al., 1999a; Jung et al., 1999). However, the role of Shh signaling in papilla morphogenesis and patterning has not been experimentally tested. Using the steroidal alkaloids, cyclopamine and jervine, and a blocking antibody to disrupt the Shh signaling pathway in embryonic rat tongue organ cultures, we provide direct evidence that Shh is involved in several aspects of fungiform papilla development.

One, Shh signaling plays a role in papilla induction and morphogenesis because disrupting the Shh pathway with either steroidal alkaloids or a blocking antibody resulted in doubling the number of fungiform papillae, compared with control conditions. Two, Shh participates in regulating the pattern of fungiform papillae because with Shh signal perturbation, papilla pattern on the anterior tongue was essentially obliterated by the formation of new papillae in interpapilla territories. Three, disruption of Shh demonstrates a previously unknown, wide competence of dorsal lingual epithelium to form papillae, because large numbers of mor-

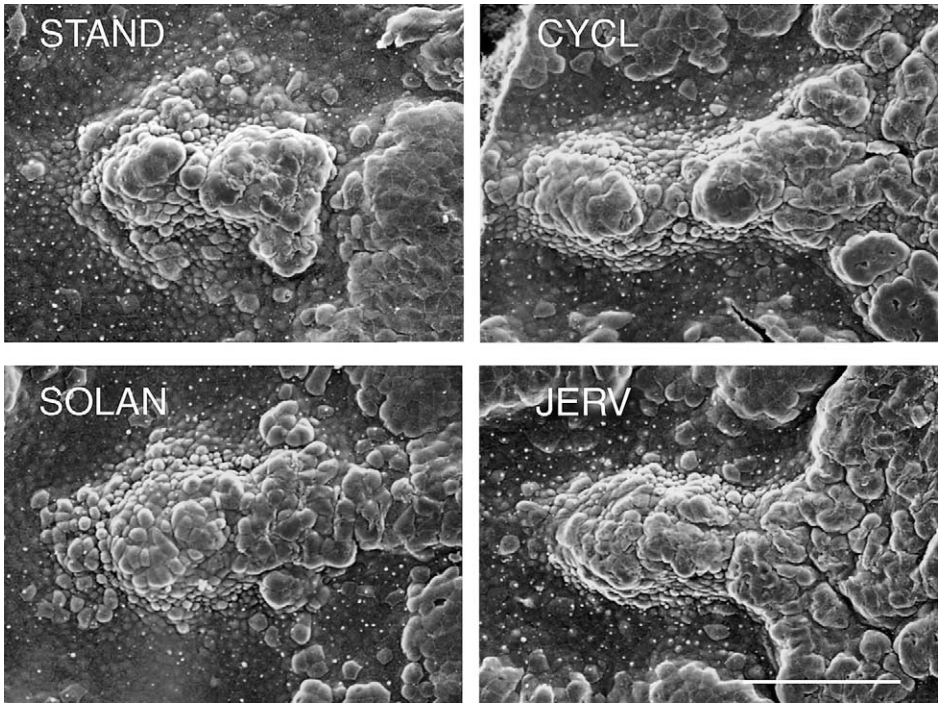


Fig. 10. High power scanning electron micrographs of the single circumvallate papilla in E14 tongues after 2 days in culture, with standard medium or supplemented with solanidine, cyclopamine, or jervine. In each culture condition, the single circumvallate papilla is an intricate assembly of large and small lobes. The photomicrographs illustrate the full range of observed papilla structures. Circumvallate papilla formation was not obviously altered by any steroidal alkaloid treatments. Scale bar, 100 μm.

phologically unequivocal fungiform papillae formed in novel posterior locations on the oral tongue; these were in front of and beside the circumvallate papilla, where fungiform papillae do not develop in vivo. Four, Shh is involved to different extents in regulating fungiform and circumvallate papilla formation and location, because development of the circumvallate papilla as a single, midline, posterior tongue structure was not altered by use of the steroidal alkaloids.

It might seem counterintuitive that steroidal alkaloids, interfering with a morphogen that is present in gustatory papillae, in fact result in an increased number of papillae in embryonic tongue cultures. However, the use of a function-blocking antibody that yields an identical and confirming result clearly establishes a role for the Shh pathway in fungiform papilla development. Use of cyclopamine in studies of developing chick neural tube (Cooper et al., 1998; Incardona et al., 1998), chick stomach and pancreas (Kim and Melton, 1998), mouse gastric epithelium (Van den Brink et al., 2001), and rodent whisker pad (Chiang et al., 1999) all demonstrate disruption of organogenesis. Although in some instances cyclopamine treatment prevents organ formation (e.g., whisker follicles; Chiang et al., 1999), in others alkaloid treatment results in epithelial proliferation (e.g., gastric glands; Van den Brink et al., 2001), increased thickness and multiple epithelial folds (stomach endoderm; Kim and Melton, 1998), and ectopic pancreatic buds (Kim and Melton, 1998). As a consequence of these latter observations, some investigators suggest that cyclopamine has revealed a proposed role for Shh as a "negative regulator" of differentiation in certain tissues and organs.

Whereas Shh functions in regulating gustatory papilla numbers and location in the E14 rat tongue, preliminary work reported from our laboratory demonstrates very different roles for Shh in papilla development at different embryonic stages (Liu et al., 2001). In cultures initiated at E13 and E14, cyclopamine treatment alters papilla size and/or distribution, as well as tongue shape at E13. However, from E16 to E18, cyclopamine does not effect numbers or distribution of fungiform papillae in tongue cultures. Furthermore, in mandible cultures from E12, the embryonic tongue that normally forms after 2 days is completely absent with use of cyclopamine (Liu et al., 2002). Thus, Shh has different actions in papilla formation and maintenance at different stages of rodent tongue development.

The Shh and Ptc proteins are in fungiform papillae in standard tongue cultures and are in supernumerary papillae after perturbing the Shh signaling pathway

The importance of immunohistochemical localization to clarify proposed morphogen roles for Shh has been highlighted in recent work (Gritli-Linde et al., 2001; Koyama et al., 2001; Van den Brink et al., 2001). There were no previous reports to locate either Shh or the Ptc receptor protein in embryonic papillae. We have demonstrated that

Shh is broadly distributed in prepapilla patches of lingual epithelium at E14, is abundant in a restricted region of the apical fungiform papillae in the rat tongue at E16, and is in a similar location in embryonic tongues cultured for 2 days from E14. The Ptc protein also is immunolocalized in the embryo in prepapillae and in well-formed papillae, and is in all papillae in cultured tongues. Therefore, Shh and Ptc in embryonic tongue cultures are distributed similarly to that observed in vivo at a comparable stage.

Importantly, Shh and Ptc also are concentrated in apical portions of all newly formed fungiform papillae after cyclopamine treatment. This further confirms our morphological characterization of these new papillae as *fungiform papillae*, with the demonstration of protein signatures that are present in all developing fungiform papillae in vivo. Additionally, a direct role for Shh in papilla formation is reinforced.

Shh protein is concentrated in the basement membrane region of developing papillae and is in the apical mesenchymal tissue

Immunolocalization of Shh in fungiform papillae, in vivo and in standard medium and cyclopamine-treated tongue cultures in vitro, demonstrates intense immunoreactivity in the basement membrane region of the apical papilla and extending along lateral papilla walls. Similar, strong Shh signal in the basement membrane region has been reported in developing mouse tooth (Gritli-Linde et al., 2001). Concentrated Shh at the interface of epithelial and mesenchymal tissues in papillae suggests possible roles in morphogenetic exchanges between tissue compartments important for shaping the papilla (Gritli-Linde et al., 2001; Koyama et al., 2001).

Our study further demonstrates some Shh accumulation in papilla mesenchyme just under the apical epithelium, again similar to reports in developing mouse tooth (Gritli-Linde et al., 2001). In bovine tooth germs, Koyama et al. (2001) immunolocalized Shh protein in the dental mesenchyme, in the absence of Shh mRNA. They proposed that there is diffusion of Shh from identified production sites in the epithelial stratum intermedium into the mesenchyme, with subsequent paracrine actions of the morphogen.

A mesenchymal location of Shh in fungiform papillae under the apical epithelium positions the protein for direct morphogenic effects and participation in signaling cascades within the mesenchyme of developing papillae. Further, this is a prime location for interactions with growing nerves (Trousse et al., 2001) that will eventually participate in taste bud development. More precise delineations of Shh location at different stages of tongue development should help to clarify its roles in papilla formation and innervation.

Shh signaling in taste papilla development

The work presented here is an extension of our demonstration, using a whole tongue organ culture system, that fungiform papillae form in a patterned array in the absence of target innervation (Mbiene et al., 1997). Here, we demonstrate that papillogenesis is amenable to manipulation in this same organ culture system and is radically altered when Shh signaling is perturbed. How might Shh function in fungiform papilla development?

Shh signaling is complex and not completely understood (Incardona and Eaton, 2000; Incardona and Roelink, 2000; Ingham and McMahon, 2001; McMahon, 2000; Martin et al., 2001; Murone et al., 1999a,b; Ruiz i Altaba, 1999). The Shh protein interacts with two transmembrane receptor components: Ptc, the ligand binding protein and Smo, the signaling protein. Ptc regulates Smo activity and inhibits constitutive signaling of Smo (Chuang and Komberg, 2000; Denef et al., 2000; Kalderon, 2001; Stone et al., 1996). However, when Shh binds to Ptc, Smo is derepressed by an unknown mechanism, and signals activation of transcription factors in the Gli family (Denef et al., 2000; Ingham, 1998). Different domains of Smo are required for Ptc interaction and Gli activation (Murone et al., 1999b).

During embryonic development, Shh–Gli function can affect cell, tissue and organ patterns by altering proliferation, differentiation, and survival of cells (Ruiz i Altaba, 1999). Shh can signal locally at high concentration and influence neighboring cells, or over a longer range at lower concentrations by direct action or by inducing secondary signals (Christian, 2000; Gritli-Linde et al., 2001; Johnson and Scott, 1998; Roelink et al., 1995; Strigini and Cohen, 1997). Normal development depends on mechanisms to limit the effective range of the Shh signal, and high levels of Ptc and Hedgehog-interacting protein (HIP) impede spread of the hedgehog protein (Chen and Struhl, 1996; Zeng et al., 2001). In cells where Ptc is absent, Shh diffuses further and induces target gene expression at a longer distance (Johnson and Scott, 1998).

Recent studies present evidence for a freely diffusible, biologically active form of Shh that can mediate long range signaling (Zeng et al., 2001). With the immunohistochemical demonstration of Shh in various target tissues, a direct effect of the protein in morphogenesis at long ranges is suggested (Gritli-Linde et al., 2001). Thus, Shh in the embryo can initially be a long range signal, that becomes progressively sequestered as it activates receptor components in its immediate surroundings, and is thereby restricted to signal at short range. Gradients of morphogen provide opportunities for actions on nearby cells responsive to high concentrations and actions on more distant targets responsive to lower protein concentrations (Goetz et al., 2002; Gurdon and Bourillot, 2001; Kodjabachian, 2001). We added Shh-N to cultures of E14 tongue and observed no effects on papilla development. However, this latter result is difficult to interpret because the potency of Shh-N may have

been inadequate to trigger a response based on recent observations that other forms of Shh can have 30 times the specific activity of Shh-N (Goetz et al., 2002).

The mechanisms responsible for transforming the dorsal lingual surface into a field covered by fungiform papillae after Shh signal disruption are unclear and not directly addressed by the present paper. However, results in our laboratory demonstrate that Shh is diffusely distributed when the rat tongue first forms, then is localized in broad patches with no protein in between, and finally is restricted to apical papilla regions as the papillae form and mature. This is similar to mRNA expression patterns in developing tongues (Bitgood and McMahon, 1995; Hall et al., 1999a; Jung et al., 1999).

We speculate that the early tongue is primed for fungiform papilla development by diffuse Shh that is subsequently altered to a prepapilla pattern as other molecules repress Shh gene activity in interpapilla tissue. Shh is thus restricted to prepapilla zones where it functions both in papilla development and in preservation of the interpapilla spaces. Once sequestered in developing fungiform papillae, a concentration gradient of the protein is established. This gradient opens possibilities for Shh to signal to multiple target cells, some within the papilla that would respond to high concentrations and some in tissue surrounding papillae that would respond to lower morphogen concentrations and thereby repress papilla-forming pathways. With perturbation of Shh signaling, new fungiform papillae could form as the Shh pathway is derepressed in interpapilla regions, and/or as transcription factors in the Shh pathway signal without constraints. It is very likely that Shh acts through secondary signals in at least some of its roles in papilla development. This outline of Shh action is similar in broad principles to actions of Shh in models from Drosophila ommatidium development (Dominguez, 1999) and digit patterning in chick limb (Drossopoulou et al., 2000), in which the protein has sequential long and short range functions, acting directly and through other molecules.

When cyclopamine is used to interfere with Shh signaling, there is a wide spectrum of reported morphogenetic effects which range from blocking organ differentiation to formation of redundant structures and overproduction of cells (Chiang et al., 1999; Cooper et al., 1998; Incardona et al., 1998; Kim and Melton, 1998; Van den Brink et al., 2001). Although specifics of cyclopamine site of action are not resolved (Cooper et al., 1998; Incardona et al., 1998, 2000; Martin et al., 2001; Taipale et al., 2000), it is apparent that disruption of Shh signaling will allow various elements in the Shh–Gli pathway to act without usual constraints. In other systems, Shh overexpression or loss of Ptc function can lead, for example, to polydactyly (Drossopoulou et al., 2000; Lewis et al., 1999) and multiple epidermal proliferations throughout the skin surface (Oro et al., 1997). These outcomes resemble the observed increase in fungiform papillae in tongue cultures, presumably after interactions between the Ptc and Smo receptor components are compromised by the action of cyclopamine.

In addition to the Shh–Gli pathway, numerous other molecules and signaling pathways (Capdevila and Belmonte, 1999; Oro and Scott, 1998) will presumably be active in taste papilla development and patterning. Furthermore, the fungiform papillae may well secrete molecules that serve as inhibitory signals to maintain interpapilla epithelium. Other pathways simply have not been studied. For example, the Bmp family has demonstrated roles in feather, limb, and digit development (Drossopoulou et al., 2000; Hogan, 1999; Jung et al., 1998), is expressed within forming fungiform papillae (Jung et al., 1999), and therefore, might well participate in papilla patterning.

Lingual epithelium: regional tissue competencies to form gustatory papillae

There are several, distinct epithelial–mesenchymal interactive programs that operate to form the dorsal lingual mucosa. In rat, one set of interactions forms about 180 precisely spaced, mushroom-shaped, fungiform papillae. In the adult rat, these papillae have one taste bud each, innervated by the chorda tympani nerve (reviewed in Mistretta, 1991). In contrast, a single circumvallate papilla forms in the posterior midline as a result of a different set of epithelial–mesenchymal interactions. This papilla contains hundreds of taste buds, innervated by the glossopharyngeal nerve. The foliate papillae present another unique papillary structure with numerous epithelial folds, a few hundred taste buds, and innervation by both chorda tympani and glossopharyngeal nerves. Much later in development, a complex series of epithelial–mesenchymal interactions takes place thousands of times across the dorsal tongue to arrange precisely columnated keratinocytes that form the nongustatory filiform papillae.

By perturbing the Shh pathway and analyzing subsequent papilla development, we have begun to learn about regional tissue competencies of the dorsal tongue epithelium. On anterior tongue, although fungiform papilla numbers double and essentially eliminate interpapilla epithelial spaces after cyclopamine treatment, fungiform papillae never develop in the anterior tongue midline or median furrow tissue. This midline region is not incorporated in the initial lateral lingual swellings that form the embryonic anterior tongue (Kaufman, 1992); nor are Shh, Ptc mRNA, or other morphogens expressed in the midline region in forming rodent tongue (Hall et al., 1999a; Jung et al., 1999); nor is this midline zone innervated during tongue development (Mbiene and Mistretta, 1997). Thus, we propose that this region, representing the line of fusion between lateral lingual swellings, is essentially nontongue tissue that possibly retains aspects of tissue from the floor of the mouth.

Inappropriately located papillae induced by disrupting Shh signal pathways also are never observed in ventral tongue, or in the pharyngeal tongue. Therefore, by E14 in

rat, these tissues are molecularly different from the dorsal epithelium on oral tongue, and apparently are not competent to sustain fungiform papilla development. Data on distribution of Shh signaling molecules in ventral or pharyngeal tongue are not available, but will be important for a complete understanding of tongue and papilla morphogenesis. As in limb bud development where dorsal and ventral fields are molecularly determined in early stages (Riddle et al., 1993), broad zones of dorsal and ventral tongue competency apparently are established in the early embryo.

However, a surprising and novel result of steroidal alkaloid disruption of the Shh pathway in rat tongue cultures was the appearance of densely distributed fungiform papillae on the lateral portions of posterior oral tongue and directly in front and beside the circumvallate papilla. These are areas where fungiform papillae are not observed in embryonic tongue in vivo. These posterior tongue fields, innervated by the glossopharyngeal nerve, have been assumed to have different tissue and molecular characteristics from that of the anterior tongue mucosa, which is innervated by the combined chorda tympani/lingual nerve (Mistretta and Hill, 1995). The pattern of innervation not withstanding, dorsal epithelium of the posterior oral tongue clearly is competent to form fungiform papillae.

Our results also demonstrate that the formation of additional fungiform papillae on both anterior and posterior tongue can be induced as late as E14, when we began the organ cultures for our experiments. So the molecular program to initiate and continue fungiform papilla development in interpapilla and extrapapilla epithelium is retained and rapidly executed in the presence of steroidal alkaloids. This is reminiscent of feather development, where epithelia retain the competency and ability to form feathers in the "between-feather" epithelium, even after feather primordia form and continuing until the early feather bud stage (Jung et al., 1998).

Development of the single circumvallate papilla, in the midline at the boundary of oral and pharyngeal tongue, apparently is not affected by cyclopamine or Shh function-blocking antibodies. Therefore, the role and/or timing of the Shh signaling pathway in regulation of circumvallate papilla development contrasts with that in fungiform papillae. The Shh and Ptc proteins are within the circumvallate papilla of E14 tongues cultured for two days, and are not in posterior tongue tissue surrounding the circumvallate papilla. Differences in cyclopamine effects on circumvallate and fungiform papillae raise the intriguing possibility that completely different differentiation programs (involving the Shh pathway) may operate in formation of these gustatory organs.

Conclusion

Lingual gustatory papillae are a required antecedent to development of the life-sustaining sensory organs, the taste buds. We have shown that development and patterning of one type of gustatory papilla, the fungiform, depends on the Shh signaling pathway. Thus, the taste papillae join other ectodermally derived organs, including hair, feathers, and teeth in demonstrated, developmental dependency on Shh. Furthermore, by perturbing Shh signaling, we have found a much greater competence than previously thought, for most of the embryonic dorsal tongue mucosa to form fungiform papillae. The circumvallate papilla, in contrast, is apparently less dependent on Shh for patterning. Whereas deletion of Shh signaling in the developing embryo results in complex malformations of the face that can include complete absence of a tongue (Incardona and Roelink, 2000), the embryonic tongue organ culture system offers numerous approaches for continuing study of Shh actions in taste development.

Acknowledgments

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