Sonic hedgehog exerts distinct, stage-specific effects on tongue and taste papilla development

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Abstract

Taste papillae are ectodermal specializations that serve to house and distribute the taste buds and their renewing cell populations in specific locations on the tongue. We previously showed that Sonic hedgehog (Shh) has a major role in regulating the number and spatial pattern of fungiform taste papillae on embryonic rat tongue, during a specific period of papilla formation from the prepapilla placode. Now we have immunolocalized the Shh protein and the Patched receptor protein (Ptc), and have tested potential roles for Shh in formation of the tongue, emergence of papilla placodes, development of papilla number and size, and maintenance of papillae after morphogenesis is advanced. Cultures of entire embryonic mandible or tongues from gestational days 12 to 18 [gestational or embryonic days (E)12–E18] were used, in which tongues and papillae develop with native spatial, temporal, and molecular characteristics. The Shh signaling pathway was disrupted with addition of cyclopamine, jervine, or the 5E1 blocking antibody. Shh and Ptc proteins are diffuse in prelingual tissue and early tongue swellings, and are progressively restricted to papilla placodes and then to regions of developing papillae. Ptc encircles the dense Shh immunoproduct in papillae at various stages. When the Shh signal is disrupted in cultures of E12 mandible, tongue formation is completely prevented. At later stages of tongue culture initiation, Shh signal disruption alters development of tongue shape (E13) and results in a repatterned fungiform papilla distribution that does not respect normally papilla-free tongue regions (E13–E14). Only a few hours of Shh signal disruption can irreversibly alter number and location of fungiform papillae on anterior tongue and elicit papilla formation on the intermolar eminence. However, once papillae are well formed (E16–E18), Shh apparently does not have a clear role in papilla maintenance, nor does the tongue retain competency to add fungiform papillae in atypical locations. Our data not only provide evidence for inductive and morphogenetic roles for Shh in tongue and fungiform papilla formation, but also suggest that Shh functions to maintain the interpapilla space and papilla-free lingual regions. We propose a model for Shh function at high concentration to form and maintain papillae and, at low concentration, to activate between-papilla genes that maintain a papilla-free epithelium.

Keywords: Sonic hedgehog; Cyclopamine; Taste papillae; Tongue development; Patched receptor; Papilla patterning

Introduction

Sonic hedgehog (Shh) has important functions in formation of ectodermally derived specializations, such as teeth, hair, and feathers, which result from epithelial–mesenchymal interactions (Cobourne and Sharpe, 2003; Oro and Scott, 1998; Thesleff, 2003; Yu et al., 2002). In previous work, we hypothesized a role for Shh in morphogenesis of another set of ectodermal specializations, the discrete gustatory papillae that develop from epithelial–mesenchymal interactions in the developing tongue. The papillae are unique sensory compartments that encompass lingual epithelium and underlying con-
nective tissue, and the taste bud cells and their progenitors. Using whole, embryonic tongue organ cultures, we demonstrated that Shh can regulate taste papilla induction, and number and pattern during a narrow temporal window for papilla formation from placodes (Mistretta et al., 2003). In the current study, we have investigated roles for Shh across a broad range of developmental stages before, during, and after formation of the tongue, as well as in development of the taste papillae in embryonic rat. First, Shh protein was localized in early branchial arch and tongue tissue, and in well-formed tongues from mid- to late gestation. Second, we disrupted Shh signaling and now report distinctive roles for Shh at various periods encompassing formation of the tongue, development of papilla placodes, and papilla induction, morphogenesis, spatial distribution, and maintenance.

The oral portion of the rat tongue emerges from the floor of the mandible as a set of mesenchymal swellings, covered with epithelium [gestational or embryonic days (E)12–E13] (Mbiene et al., 1997; Mistretta, 1972, 1991). Two lateral lingual swellings form anteriorly on either side of the midline of branchial arch I, and a third medial swelling, termed the tuberculum impar, forms posteriorly and directly in front of the foramen caecum. The lateral swellings enlarge and meet, although an anterior midline separation, known as the median furrow or sulcus, remains. Posteriorly, the lateral swellings merge with the growing tuberculum impar. As the three tongue swellings grow and fuse, the oral tongue is formed (E13) and acquires a spatulate shape (E14). The tongue increases in length with extensive growth of anterior and posterior components (E15–E21). Furthermore, the oral tongue is progressively demarcated by the sulcus terminalis from the pharyngeal portion of the tongue, which essentially derives of the midline of branchial arch I, and a third medial swelling, termed the tuberculum impar, forms posteriorly and directly in front of the foramen caecum. The lateral swellings enlarge and meet, although an anterior midline separation, known as the median furrow or sulcus, remains. Posteriorly, the lateral swellings merge with the growing tuberculum impar. As the three tongue swellings grow and fuse, the oral tongue is formed (E13) and acquires a spatulate shape (E14). The tongue increases in length with extensive growth of anterior and posterior components (E15–E21). Furthermore, the oral tongue is progressively demarcated by the sulcus terminalis from the pharyngeal portion of the tongue, which essentially derives of the hypobranchial eminence. This sequence of rat tongue formation is similar in all respects to mouse and human tongue embryogenesis (Carlson, 1988; Kaufman, 1992; Paulson et al., 1985).

Whereas the lingual epithelium is histologically homogeneous at E13, by late E14, there are rows of placodal thickenings on the anterior tongue where fungiform papillae will develop and a swelling on the posterior tongue midline demarcates where the single circumvallate papilla will form, just anterior to the foramen caecum (Mbiene et al., 1997; Mistretta, 1972). The papillae are distinct and protrude from the dorsum of the tongue by E15. However, papilla growth and other aspects of morphogenesis proceed through the rest of gestation. Histologically defined, early taste buds are not seen until E20–E21, and taste bud development within the gustatory papillae is essentially a postnatal process (Hill, 2001; Mistretta, 1972, 1991).

The gradual yet distinctive stages from formation of the tongue itself, a major organ at the oral extreme of the gastrointestinal system, through differentiation of numerous cell types, including sensory receptors, provide a particularly fertile arena for discerning how ectodermal special-
morphogenesis include separable processes with distinctive requirements for Shh. A model is proposed for Shh in papilla formation and patterning based on high and low levels of the protein within papillae and between papillae, respectively.

Methods

Rat embryo and tongue dissection

Timed, pregnant Sprague–Dawley rats 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) was designated the day on which the dam was sperm-positive. E12 to E18 embryos were used, and all dissections were between 9 AM and 1 PM to insure consistency across litters.

Dams were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg body weight) which also anesthetizes the embryos. Anesthetized embryos were removed using aseptic techniques and placed in cold Earle balanced salt solution (EBSS) containing gentamicin sulfate (50 μg/ml), and buffered with 20 mM HEPES (pH 7.4), in a sterile Petri dish. Embryo heads were dissected and moved to fresh EBSS. For organ cultures, mandibles (E12) were dissected with the second branchial arch included, or whole tongues (E13–E18) were dissected away from the mandible. For whole tongue immunohistochemistry, tongues with a portion of the mandible (E13 through E18) were dissected away from the mandible. For whole tongue immunohistochemistry, tongues with a portion of the mandible (E13 through E18) were dissected and fixed (described below). For immunohistochemistry on intact embryo tissue sections (described below), entire heads were rapidly frozen in O.C.T. compound (Miles Scientific, Elkhart, IN).

Mandible and tongue organ cultures

E12 mandibles or E13–E18 tongues were cultured using a whole tongue organ culture system as described previously (Mbiene et al., 1997). Dissected mandibles or tongues were positioned with the dorsal surface upward on small squares of sterile Millipore HA filter (0.45-μm pore size) wetted with EBSS. Tongues and filter papers were then placed on stainless steel grids in standard organ culture dishes (Falcon 3037). Cultures were fed with a 1:1 mixture of Dulbecco’s modified Eagle medium and Ham’s nutrient F12 (DMEM/F12, GIBCO, Gaithersburg, MD), containing 1% fetal bovine serum, 50 μg/ml gentamicin sulfate, and 2% B27 culture supplement (GIBCO) (standard medium). The level of the medium was adjusted so that the cultures were maintained at the interface between the gas (5% CO2 in air) and liquid phases of the culture in a humidified incubator at 37°C (MacCallum, 1994). After 1–3 days, tongue cultures were removed and processed for light or scanning electron microscopy or submerged in O.C.T. compound and rapidly frozen.

To study the role of the Shh signaling pathway in tongue formation and papilla development, cycloamine (CYCL) or jervine (JERV), steroidal plant alkaloids known to specifically interrupt the hedgehog signaling process (Gaffield et al., 1986; 1999), or a monoclonal Shh-blocking antibody, 5E1 (A-Shh, Developmental Hybridoma Bank, University of Iowa; Ericson et al., 1996) was added to the standard culture medium. Three control culture conditions were employed: standard culture medium (STAND) as described above; STAND with addition of solanidine (SOL), an alkaloid structurally similar to CYCL but essentially ineffective in disrupting Shh signaling pathway (Gaffield and Keeler, 1996; Quint et al., 2002); or addition of normal mouse immune globulin (IgG) to compensate for the dilution of medium when the 5E1 blocking antibody was employed. CYCL, JERV, and SOL were prepared as 10 mM stock solutions in 100% ethanol and stored at 4°C. Before experiments, alkaloids (5 or 10 μM) or IgG (20 or 40 μg/ml) were added to the culture medium and were present for the duration of tongue cultures.

Short-term disruption of Shh signaling

In experiments to determine whether short-term disruption of the Shh signal alters tongue or papilla development, E12 mandible cultures were exposed to CYCL or JERV for 4, 24, or 48 h. After each exposure period, cultures were rinsed three times and then maintained in fresh STAND for up to 3 days. E14 tongue cultures were exposed to CYCL for short periods (5 μM for 4, 8, or 12 h; or 10 μM for 1, 2, 4, or 8 h) followed by the fresh STAND without CYCL for up to 2 days.

To determine Shh immunoreactivity after the brief CYCL exposure, E14 tongue cultures were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4, at 4, 8, 12 or 48 h under STAND or 5 μM CYCL condition.

Whole and sectioned tongue immunohistochemistry

The goat polyclonal, affinity-purified antibody AF464 (R & D Systems, Minneapolis, MN) against the mouse Shh N-terminal peptide (aa 25–198) was used for whole and sectioned embryonic tongues, as well as mandible and tongue cultures, with an antigen retrieval procedure. Other rabbit (H-160) and goat (N-19) polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) against the amino terminus of Shh also were used with similar results. Slides treated with no primary antibody or with the same concentration of normal IgG were used as controls. Other investigators have reported data with these various Shh antibodies (e.g., Thayer et al., 2003; Van Den Brink et al., 2001; Watkins et al., 2003), and we have no indication of nonspecific staining with positive and negative controls in our experiments.
To localize Shh, 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. Endogenous peroxidase activity was blocked with 6% H2O2 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 (CTS015; R & D Systems). Nonspecific staining was blocked in PBS/MT (PBS with 2% skim milk powder and 0.1% Triton X-100). Tongues were incubated overnight at 4°C with the primary antibody in PBS/MT blocking solution at 1:100–250 dilutions. After rinsing (five times, 1 h each), tongues were incubated overnight at 4°C with a biotin-conjugated rabbit antigoat secondary antibody (Kirkgaard and Perry, Gaithersburg, MD) at 1:500 in blocking solution. Tongues were subsequently rinsed and incubated overnight at 4°C with peroxidase-conjugated streptavidin at 1:500 in blocking solution. Following thorough rinsing, five times at 1 h each in PBS/MT and two times at 1 h each in PBS with 0.1% Triton X-100 and 0.2% bovine serum albumin (Sigma, St. Louis, MO), tongues were preincubated in nickel-intensified DAB solution (Vector Laboratories, Burlingame, CA) without H2O2. Reactions were run in the same DAB solution with the addition of 0.0003% H2O2. Tongues were rinsed twice in PBS for 1 h each and stored in 4% buffered paraformaldehyde at 4°C for subsequent photography.

For Shh immunohistochemistry on tongue sections, dissected embryo heads were frozen in O.C.T. compound. Serial sagittal sections were cut at 12 μm, thaw-mounted onto subbed slides, and fixed at 4°C for 1.5 h in 4% paraformaldehyde in 0.1 M PBS, pH 7.4. After fixation, sections were rinsed in 0.1 M Tris buffer solution (pH 7.4). Endogenous peroxidase activity was blocked in 0.5% H2O2 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 (CTS015; R & D Systems). Nonspecific staining was blocked with 10% normal rabbit serum in PBS and 0.3% Triton X-100 (Sigma) 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 antigoat secondary antibody (Kirkgaard and Perry) at 1:250 in carrier solution at room temperature for 30 min. Sections were rinsed and incubated in HRP-labeled streptavidin (at 1:500 in 0.1 M PBS), and the HRP label was visualized with nickel-intensified DAB solution (Vector Laboratories). Reacted slides were dehydrated through alcohols, cleared in xylene, and coverslipped with Permount™ mounting medium (Fisher, Pittsburgh, PA).

Ptc was localized in whole embryonic tongues (E13–E18) with goat polyclonal antibodies (Santa Cruz Biotechnology) against the amino terminus (G-19, mouse origin) and the carboxy terminus (C-20, human origin), as well as mixtures of both antibodies, all with similar results. The Ptc antibodies have been widely used in several systems (e.g., Nakase et al., 2001; Thayer et al., 2003; Watkins et al., 2003). An antigen retrieval protocol was used similar to that for Shh immunolocalization, with overnight incubation in primary antibody at 1:25 dilution.

**Scanning electron microscopy**

Microdissected E12 to E18 mandibles, tongues, or tongue cultures were fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at room temperature. Tongues were then rinsed in buffer and subsequently postfixed in a sequence of aqueous 1% OsO4, 1% tannic acid, 1% OsO4, for 30 min each at room temperature. Tissues were then dehydrated through an ascending series of ethanol, and ethanol was displaced by three changes of hexamethyldisilazane (HMDS) for 45 min each. Residual HMDS was evaporated in a fume hood overnight. Tissues then were mounted on specimen stubs, lightly sputter-coated with gold/palladium, and analyzed with a scanning electron microscope.

**Papilla quantification, statistics, and image analysis**

Scanning electron micrographs of E13 whole tongue cultures at 100× and E14 at 75× original magnifications were used to count fungiform papillae in tongue cultures. Micrographs at 200× were used to measure the diameters of papillae on E13 control (STAND only or STAND with SOL) and Shh-perturbed tongue cultures (STAND with CYCL or anti-Shh). An area containing at least 40 fungiform papillae was chosen on the anterior part of the oral tongue to measure the diameter of every papilla enclosed within the area. For each papilla, the diameter was measured along the longest axis. Fungiform papillae were counted on 4 to 10 tongues in each experimental condition.

In E14 tongues cultured for 2 days with short exposures to CYCL, the number of fungiform papillae was quantified in different regions of the oral tongue—the anterior tongue half, extending from the distal border of the intermolar eminence to the tongue tip; the intermolar eminence (IE) area; and the areas just lateral to the intermolar eminence area (L. IE) (Fig. 1).

Diameter and number of fungiform papillae are presented as means ± standard error (SE). Statistical analyses were conducted with analysis of variance (ANOVA) followed by Duncan post hoc test. The significance level was set at $P \leq 0.05$.

Digital images were generated from scanning electron micrographs, immunostained whole tongues and/or mandibles, or slides. The images were then assembled into
 dense in discrete, irregular patches on the anterior tongue where papilla placodes are forming. Some low level Shh may be present on the intermolar eminence representing the forming circumvallate papilla (black arrow). A curved white arrow demarcates oral from pharyngeal tongue. Shh immunoproduct (on right) is and intense Shh immunoproduct is present bilaterally in developing tooth buds (t). At E13 (SEM), the two lateral lingual swellings of the anterior embryonic tongue are prominent and separated by a deep median furrow (mf). The anterior tongue is defined as the region distal to the median furrow and beside the intermolar eminence (IE area). The IE is a papilla-free area during development and in adult. The posterior oral tongue incorporates the IE, the circumvallate papilla (black arrow), and lateral regions to the IE (L. IE). The curved white arrow at the edge of the posterior tongue serves to demarcate the boundary of the oral from pharyngeal tongue. Scale bar: 1 mm.

Figures using Photoshop (Adobe Systems, Mountain View, CA).

### Results

**Embryonic tongue and taste papilla development, and Shh protein localization proceed in direct association**

To localize Shh protein in native rat embryonic tongue and papillae during development and morphogenesis, scanning electron micrographs and whole tongue immunohistochemistry were compared from E12 to E18 (Fig. 2). With scanning microscopy, at E12, two lateral swellings that will form the anteriormost regions of the embryonic tongue are apparent on the floor of the mandibular portion of branchial arch I (Fig. 2, SEM E12). By E13, the set of three swellings (lateral lingual swellings and tuberculum impar) that will form the oral tongue is obvious and well demarcated from the pharyngeal tongue by a deep groove separating components of branchial arches I and II (SEM E13). Fungiform papilla placodes become apparent at E14, and both fungiform and circumvallate papillae are recognized as discrete structures between E14 and E15 (SEM E14, E15). The intermolar eminence, which has formed at the site of the tuberculum impar, is large and conspicuously free of papillae, and the median furrow broadly separates anterior tongue halves. Fungiform and circumvallate papillae are well formed by E16 and remain in stereotypic locations at E18 (SEM E16, E18). It is important to note that the fungiform papillae initially form (E14) and progressively develop in rows on anterior oral tongue.

Localization of Shh protein in the embryonic tongue indicates an early, initially diffuse distribution that becomes progressively restricted to papilla taste organs (Fig. 2, Shh immunohistochemistry). At E12, before tongue formation, diffuse Shh immunoreactivity is seen in swellings on the mandible and as an intense immunoproduct in developing tooth germs bilaterally. At E13, even after considerable growth of the lower jaw and formation of the early tongue, a diffuse Shh distribution remains on the anterior part of the lateral lingual swellings. However, accompanying and corresponding with the emergence of fungiform papilla placodes, by E14, there are large, intense, and irregular patches of Shh immunoproduct on anterior oral tongue on either side of the median furrow and beside the intermolar eminence.

Fig. 1. Scanning electron micrograph of E14 tongue maintained in culture in standard medium for 2 days to illustrate tongue regions used for quantifying fungiform papillae. Fungiform papillae (examples at arrowheads) form on the anterior tongue on either side of a broad median furrow (mf). The anterior tongue is defined as the region distal to the edge of the intermolar eminence (IE area). The IE is a papilla-free area during development and in adult. The posterior oral tongue incorporates the IE, the circumvallate papilla (black arrow), and lateral regions to the IE (L. IE). The curved white arrow at the edge of the posterior tongue serves to demarcate the boundary of the oral from pharyngeal tongue. Scale bar: 1 mm.
Subsequently, from E15 to E18, Shh immunoproduct is restricted to the well formed but still developing fungiform papillae. Similarly, on the posterior tongue, Shh is observed in the region of the forming circumvallate papilla at E15 and then is tightly restricted to the single circumvallate papilla at E16 and E18.

From E14 to E18, Shh protein is absent from the median furrow. The intermolar eminence has low levels of Shh immunoproduct. No apparent Shh immunoreactivity is observed at any stage in the pharyngeal tongue.

Sections through embryonic tongue confirm the whole mount observations and demonstrate an initial uniform distribution of Shh immunoproduct in the epithelium of the anterior tongue swellings at E13 (Fig. 3, Sagittal tongue and Anterior, E13). The Shh protein signal then becomes intense and restricted to broad patches in the lingual epithelium that correspond to placodes of the forming fungiform papillae at E14. At E15 and E16, Shh protein is highly restricted and most concentrated in the apical epithelium of the forming fungiform papillae. At these stages, Shh signal also is
diffusely present in the mesenchyme immediately subjacent to the fungiform epithelium.

On the posterior part of the tongue, there are patches of Shh immunoprodut at E13, and a distinctive line of Shh is in the basement membrane region of the fungiform papillae at E14 (Fig. 3, Posterior). At E15 and E16, Shh is in posterior epithelium, although the immunoprodut is weaker than in fungiform papillae on the anterior tongue. In the developing circumvallate papillae, distinct Shh immunoreactivity is first observed at E14 in basement membrane regions of epithelium where the circumvallate papilla is developing (Fig. 3, Circumvallate). At E15 and E16, Shh is present throughout the depth of the epithelium of the forming papilla and appears to be distributed pericellularly in the E16 circumvallate papilla.

In summary, localization of the Shh protein is diffuse and homogeneous in the earliest anterior lingual epithelium and progressively becomes concentrated in developing papilla placodes and taste papillae, the fungiform and circumvallate. Accompanying fungiform papilla formation is an
apparent mesenchymal localization of Shh, just under apical basement membrane regions of the fungiform.

**Ptc protein surrounds Shh immunoproduct**

The distribution of Ptc receptor protein coincides with Shh, from a diffuse localization in anterior tongue swellings at E13 to progressive restriction within developing placodes and taste papillae from E14 to E18 (Fig. 4). However, the Ptc immunoproduct is always more diffuse in nature and encircles the more intense Shh protein signal in fungiform papillae at E15 and E16 (compare Shh and Ptc immunoproduct in fungiform papillae at higher magnification, Fig. 4).

![Fig. 4. Immunohistochemistry for Ptc in E13–E18 whole tongues. Insets at far right of E14–E18 illustrate the distribution of Ptc immunoproduct within papilla placodes and fungiform papillae at higher magnification, and two insets at E15 and E16 also show the distribution of Shh protein (Shh) in fungiform papillae to compare with Ptc. At E13, Ptc immunoproduct is diffusely distributed in the anterior tongue swellings and, by E14, is localized to broad patches on the anterior oral tongue. The broad median furrow is free of Ptc protein. Some weak immunoproduct may be apparent on the posterior tongue in the intermolar eminence region. At E15 and E16, Ptc is restricted to fungiform papillae and the forming circumvallate papilla. Ptc protein is more diffuse and encircles fungiform papillae in contrast to the dense Shh protein within the papillae (see insets for Shh compared with Ptc distribution at higher magnification). At E18, Ptc immunoproduct remains in the well-formed circumvallate papilla and is punctate within fungiform papillae on the tongue tip. Scale bars for whole tongues: 1 mm; bar for E15 tongue also applies to E13 and E14. Scale bar in bottom right panel: 100 μm, applies to all fungiform papilla insets at far right.](image-url)
The embryonic tongue does not form when Shh signaling is disrupted

To study Shh regulation of embryonic tongue formation, E12 mandibles, including the first and second branchial arches (see Fig. 2, E12), were dissected and cultured for 1–3 days under control conditions in standard medium (STAND) or with addition of normal immune globulin (IgG, 20 μg/ml), or with 5 μM CYCL or Shh-blocking antibody (A-Shh, 20 μg/ml) to disrupt Shh signaling. In STAND, the lateral lingual swellings (L) are clearly observed in branchial arch I after 1 day in culture, and after 2 days, the swellings are fused together and the oral tongue body (T) has developed. After 3 days in culture with STAND only or addition of normal IgG, a distinctive tongue forms (T). Shh is diffusely distributed in the anterior tongue of STAND cultures (STAND, Shh-ir). With CYCL, tongue formation is blocked in all cultures (CYCL, +2 days; +3 days, arrow points to region where tongue would form in STAND). No apparent Shh immunoreactivity is seen in the floor of the mandible with addition of CYCL (CYCL, Shh-ir). With A-Shh, tongue development is inhibited in about 30% of the cultures (not illustrated), but the tongues that do develop in 71% of the cultures are smaller and narrower (A-Shh, T) compared to those formed in STAND or IgG cultures (see Table 1). Scale bar: 500 μm, applies to all panels.

Table 1
Quantification of tongue formation in E12 mandible cultures maintained for 3 days under different control (STAND, IgG) and experimental (CYCL, A-Shh) conditions

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>Total cultures</th>
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<tr>
<td></td>
<td>n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAND</td>
<td>25</td>
<td>25</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>IgG 20 μg/ml</td>
<td>6</td>
<td>6</td>
<td>100</td>
<td>121</td>
</tr>
<tr>
<td>CYCL 5 μM</td>
<td>18</td>
<td>2a</td>
<td>11</td>
<td>65</td>
</tr>
<tr>
<td>CYCL 10 μM</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A-Shh 20 μg/ml</td>
<td>14</td>
<td>10b</td>
<td>71</td>
<td>100</td>
</tr>
<tr>
<td>A-Shh 40 μg/ml</td>
<td>8</td>
<td>6b</td>
<td>75</td>
<td>102</td>
</tr>
</tbody>
</table>

a In these two cultures, only tissue masses formed, without a tongue shape or topography.
b Formed tongues were much more narrow than those in STAND and IgG control groups (see Fig. 5).
tongue formation (Fig. 5, +2 or +3 days, CYCL). We note in Table 1 that a tongue “rudiment” formed in 2 of 18 of the E12 mandibles cultured with 5 μM CYCL; however, these were tissue masses rather than distinguishable tongues. No detectable Shh protein was seen in the tongue-free mandible of CYCL cultures (Fig. 5, +3 days, CYCL, Shh-ir).

In an apparently less robust disruption of Shh signaling with the 5E1 anti-Shh-blocking antibody (20 or 40 μg/ml), an atypical tongue formed in about 70–75% of E12 mandible cultures (Fig. 5, A-Shh; Table 1). However, the very narrow shape of these tongues was markedly different from those in STAND cultures; they were reduced in width by about 40% compared to STAND tongues (Table 1). In 25–30% of these cultures, there was no tongue.

In summary, when the Shh signaling pathway is disrupted with CYCL, the tongue formation that would normally proceed from the floor of the mandible in culture is abrogated.

To investigate the potential reversibility of Shh signal disruption during tongue development and determine the time required to achieve effective signal disruption, E12 mandible cultures were exposed to CYCL or JERV for 4, 24, or 48 h followed by fresh STAND for up to 3 days in culture (Fig. 6). Cultures with STAND medium only, refreshed at the corresponding time points, were used as controls.

In STAND cultures, a distinguishable tongue was formed reliably as described above, with all medium changes (Fig. 6, STAND 4, 24, and 48 h). A short disruption of Shh signaling for 4 h with 5 or 10 μM CYCL or JERV did not block tongue formation, although tongues were about 10% shorter than those in STAND (Fig. 6, CYCL, JERV, 4 h). In contrast, 24 or 48 h of exposure to 5 μM CYCL was equally effective in preventing tongue development, although the culture medium was returned to STAND for the remaining 1 or 2 days (Fig. 6, CYCL 24, 48 h). Therefore, 24 h of CYCL exposure is sufficient to irreversibly prevent tongue formation from the E12 mandible. We did not investigate Shh disruption periods between 4 and 24 h.

Roles for Shh signaling in early tongue formation and development, and emergence of the patterned distribution of taste papillae

To define the period when Shh is active in early tongue morphogenesis and in taste papilla formation, tongues were cultured for 2 or 3 days started at E13 when the tongue is a set of discrete tissue swellings and gustatory papillae have not yet appeared, and at E14 when the tongue is well formed and papilla placodes make their initial morphological appearance. Both E13 and E14 are therefore crucial periods for tongue growth and morphogenesis, and for papilla induction and patterning. CYCL or A-Shh was added to STAND culture medium to disrupt Shh signaling, and SOL was used as a control.

Fig. 6. Scanning electron micrographs of E12 mandible cultures with different exposure periods to STAND, CYCL, or JERV for 4, 24, or 48 h, all followed by fresh STAND for up to 3 days in culture. In cultures with STAND medium only, changed at the corresponding time points, a distinguishable tongue was formed reliably (STAND, 4, 24, and 48 h; T). A distinctive but somewhat smaller tongue developed in the cultures with 5 μM or 10 μM CYCL or JERV for 4 h (CYCL, JERV, 4 h; T). However, no tongue developed with either 24- or 48-h exposure to 5 μM CYCL (CYCL 24, 48 h; arrow indicates where tongue would form in STAND). Scale bar: 500 μm, applies to all panels.
Tongue cultures from E13

In culture, the three lingual swellings that represent the E13 tongue have intrinsic signals to direct: tongue morphogenesis and growth, fungiform papilla formation in rows with timing equivalent to in vivo development, and circumvallate papilla development. Thus, E13 tongues grow and acquire a more spatulate form with 2 or 3 days in culture (Fig. 7A, STAND, SOL). However, in STAND and SOL cultures, the anterior tongue halves retain a somewhat bifurcate shape, comparable to the E15 embryo tongue (see Fig. 2). Scanning electron micrographs demonstrate that dorsal surface specializations including numerous fungiform papillae, the single posterior circumvallate papilla, and the intermolar eminence all develop in culture with appropriate spatial distributions. Fungiform papillae develop on either side of the midline which is characterized by a wide median furrow.

In contrast, E13 tongues cultured in CYCL or A-Shh acquire a pointed tip and lack a discernable median furrow and the bifurcate anterior halves that characterize tongues in control cultures (Fig. 7A, CYCL, A-Shh). Numerous, crowded fungiform papillae form on the anterior tongue, distributed across or encroaching upon the median furrow. Furthermore, fungiform papillae also are seen on the posterior oral tongue anterior to the circumvallate papilla. The latter is a region where fungiform papillae never form in vivo or in STAND and SOL cultures. Thus, in cultures with disrupted Shh signaling, the intermolar eminence is virtually eliminated.

Fungiform papillae were counted in control and experimental culture conditions. In both CYCL and A-Shh cultures, number of papillae was about double that in cultures with STAND or SOL (Fig. 7B, F(3,20) = 74.6). Furthermore, the diameter of fungiform papillae in CYCL cultures, but not in A-Shh cultures, was larger than in cultures with STAND or SOL (Fig. 7C, F(4,38) = 15.5). We tested whether an even higher concentration of CYCL (10 μM vs. 5 μM) would elicit larger papilla dimensions; however, the increased concentration did not affect papilla size or distribution (Fig. 7C).

The distribution of Shh protein in the superabundant papillae was studied in E13 tongues cultured with CYCL. Shh was within and restricted to each of the multiple fungiform papillae and to the circumvallate papilla, which formed in culture with CYCL (Fig. 8). The Shh immunoproduct was much more abundant in CYCL cultures than in STAND tongues within the apical epithelium of all of the large anterior and posteriorly located fungiform papillae, and in the mesenchyme of the papillae core. In STAND

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**Fig. 7.** Scanning electron micrographs (A) and histograms of number (B) and diameter (C) of fungiform papillae in E13 + 2- or 3-day cultures under control (STAND, SOL) or Shh signal disruption (CYCL, A-Shh) conditions. In A, the entire (left column) or anterior right quadrant of the oral tongue at higher magnification (right column) is presented. Control tongues in STAND and with addition of SOL (10 μM) have multiple fungiform papillae on the anterior tongue on either side of the median furrow, and a single circumvallate papilla forms on posterior tongue. The curved white arrow demarcates the oral tongue border. In contrast, when Shh signaling is interrupted with 5 or 10 μM cyclopamine (CYCL) or A-Shh (20 μg/ml), the tongue cultures have a pointed tip, and the median furrow is obscured. In CYCL cultures, fungiform papillae form on anterior and posterior tongue locations and are increased in both size and number (B and C; ** = different from other conditions at P ≤ 0.01). In tongues cultured with A-Shh, fungiform papillae are increased in number (B; ** = different from other conditions at P ≤ 0.01) but not in size (C), compared to those in STAND or SOL cultures. Scale bar in A for left column: 1.0 mm; right column: 100 μm.
cultures, Shh immunoproduct was lacking or very weak in the broad interpapilla spaces.

**Tongue cultures from E14**

E14 tongues cultured for 2 days with different steroidal alkaloids or the 5E1 Shh-blocking antibody were previously studied (Mistretta et al., 2003). For a complete developmental sequence and direct comparison with earlier and later tongue cultures, we replicated the E14 cultures and provide a brief summary of these results here. Importantly, we also extended previous findings by short exposures to Shh signal-blocking agents during culture.

The dissected embryonic rat tongue at E14 has a spatulate shape, and fungiform papilla placodes have emerged as small surface eminences at this stage (see Fig. 2, SEM). Shh immunoproduct is very dense in irregular but discrete patches on the anterior tongue (Fig. 2, Shh immunohistochemistry). After 12 and 24 h in culture with STAND, large fungiform papillae form on either side of the median furrow, and the circumvallate papilla develops on posterior tongue (Fig. 9, STAND, +12 and +24 h). After 48 h in culture, the distribution of fungiform papillae mimics in vivo development, and a well-formed circumvallate papilla is on the posterior tongue (Fig. 9, STAND, +48 h). Notably, fungiform papillae are not seen on the intermolar eminence or in the area in front of the circumvallate papilla, nor are they numerous lateral to the intermolar eminence. This time series demonstrates that there is a gradual and progressive process of papilla development in vitro, as in embryonic development.

In culture with CYCL or anti-Shh, the tongues retain a typical spatulate shape similar to cultures in STAND, and taste papillae develop (Fig. 9, CYCL, A-Shh). Notably, in 12-h cultures, CYCL tongues are directly comparable to STAND tongues. However, in CYCL tongues cultured for 24 h, fungiform papillae appear crowded, and in CYCL tongues cultured for 48 h, there is a dramatic effect of Shh signal disruption. In contrast to tongues in STAND, tongues with 48-h CYCL or A-Shh exhibit increased numbers of fungiform papillae not only on the anterior tongue, but also on the posterior oral tongue in front of the circumvallate papilla where fungiform papillae never form in vivo. The median furrow and pharyngeal tongue remain papilla-free. Also, a single circumvallate papilla is sustained on posterior tongue.

Shh protein is immunolocalized in all fungiform papillae and in the circumvallate papilla not only in STAND, but also in all newly emerged papillae in CYCL and A-Shh tongue cultures, and is not seen elsewhere on the tongue (Fig. 9, Shh-ir). Within the fungiform papillae, Shh is intense in apical epithelial cells and is especially abundant near the basement.

Fig. 8. Photomicrographs of E13 + 2-day cultures analyzed with Shh immunoreactivity in whole tongue cultures (top panels), sagittal and coronal sections at low magnification (middle panels), and sagittal sections of papillae at high magnification (bottom panels). In STAND cultures, Shh immunoproduct is in fungiform papillae on either side of the midline in the anterior tongue culture and in the circumvallate papilla (arrow). The median furrow is devoid of Shh. Shh is intense in the elevated epithelium in the fungiform papillae with no or very weak signals seen in interpapilla spaces. In CYCL tongue cultures, Shh is intense in the enlarged fungiform papillae and developing circumvallate (arrow). The median furrow is obscured by multiple fungiform papillae. Shh is very intense in the apical papilla epithelium and is seen in underlying mesenchyme (arrow in panel at bottom). There is apparently little Shh-free space between papillae. Scale bar for top panels: 1.0 mm; middle: 400 μm; bottom: 25 μm.
Fig. 9. Scanning electron micrographs and whole tongue or sectioned tongue Shh immunoreactions (Shh-ir) of E14 cultures in STAND, 5 μM CYCL or 20 μg/ml A-Shh medium. Cultures were terminated after 12 (top row), 24 (second row), or 48 h (third row and all others). In STAND and CYCL cultures maintained for 12 h, multiple fungiform papillae form on the anterior tongue (curved white arrow demarcates oral from pharyngeal tongue). In cultures kept for 24 h, the fungiform papillae protrude more from the tongue, and the circumvallate papilla is obvious in the midline at the border of posterior oral tongue (black arrow). After 48 h, fungiform and circumvallate papillae are more distinct on STAND tongues, but on CYCL tongues, the fungiform papillae are more numerous on anterior tongue and notably have formed also across posterior regions. Shh immunoproduct is in the single circumvallate papilla and each fungiform papilla (STAND, Shh-ir) including those that form in novel locations on the posterior tongue in culture with 5 μM CYCL or A-Shh (CYCL and A-Shh, Shh-ir). Within fungiform papilla sections, Shh is localized in the epithelial cells and is intense in the basement membrane region (bottom, sagittal sections, Shh-ir). Some Shh immunoproduct is also seen in the mesenchymal core of papillae. In CYCL cultures, the high-power micrograph at bottom right illustrates that some Shh is in the basal cell region between fungiform papillae (arrows). With added A-Shh (A-Shh, SEM and -ir), fungiform papillae form on both anterior and posterior oral tongue as in CYCL cultures. The median furrow and pharyngeal tongue remain papilla-free. Scale bars: 1.0 mm for whole tongue images; 400 μm for sagittal tongue sections; 25 μm for sections at high power on the bottom.
membrane. There appears to be some Shh protein in the mesenchyme within the apical part of the papilla core and between fungiform papillae in CYCL cultures (Fig. 9, CYCL, Shh-ir, bottom).

**Shh roles in fungiform papillae formation and patterns: short and long periods of signal disruption**

We studied specific timing effects and competence of lingual epithelium in responding to Shh by testing whether a short period of Shh signal disruption would lead to the massive increase in numbers of fungiform papillae and to papilla formation on posterior tongue that is seen in cultures maintained in CYCL over 2 days. E14 tongue cultures were exposed to CYCL for periods of a few to several hours, followed with a culture period up to 48 h in STAND. Tongues were cultured in medium with 5 μM CYCL for 4, 8, or 12 h, or with 10 μM CYCL for 1, 2, 4, or 8 h. Cultures with STAND alone or with added CYCL for the entire 48-h culture period were used as negative and positive controls.

Only 4 h of 5 μM CYCL exposure resulted in formation of increased numbers (almost double) of fungiform papillae on the anterior tongue as well as more distinctive fungiform papillae on the tongue area lateral to the intermolar eminence (Figs. 10A,B). In contrast, fungiform papillae on the intermolar eminence region did not form in significant numbers

![Fig. 10. Scanning electron micrographs (A) and histograms of fungiform papilla numbers (B, C) in E14 + 2-day tongue cultures with different exposure times to CYCL (B: 5 μM CYCL for 4, 8, or 12 h; C: 10 μM CYCL for 1, 2, 4, and 8 h). After exposure to CYCL, cultures were rinsed thoroughly and then maintained in fresh standard medium with no CYCL for up to 48 h. Cultures with STAND alone (0 h) or with added CYCL for the entire 48-h culture period were taken as negative and positive controls. Multiple fungiform papillae form on the anterior tongue in the cultures with standard medium alone (A: 0 h, 5 and 10 μM CYCL). There are no fungiform papillae on the intermolar eminence (IE), but several papillae are seen in areas lateral to the intermolar eminence (L. IE). A single circumvallate papilla is at the border of posterior oral tongue (arrow). With 5 μM CYCL, 4 h of exposure results in an increased number of fungiform papillae on anterior tongue and L. IE area (A; and B: significant differences at 4 h). Exposure to CYCL for 8 h results in some additional fungiform papillae in the IE area in front of the circumvallate papilla (A and B). The effect of 12 h of CYCL exposure is equivalent to that in cultures exposed to CYCL for the entire culture period (48 h), with numerous fungiform papillae in the whole oral tongue (anterior tongue half, IE, and L. IE area in the posterior oral tongue) (A and B). With 10 μM CYCL, only 1-h exposure results in a significant increase in the numbers of fungiform papillae on the anterior tongue and L. IE area (A and C). Four hours of 10 μM CYCL exposure fully alters papilla number and pattern, compared to CYCL exposure for 48 h; fungiform papillae are increased on the IE area as well as anterior tongue and L. IE area (A and C). Looking at differences in total papilla number in histograms at far right of B and C, it is apparent that only 1 h of CYCL exposure, with subsequent return to STAND, has substantial effects on fungiform papillae distribution. However, papilla formation on the IE is not at maximum level until 4 (10 μM) or 12 (5 μM) h in CYCL. Scale bar in A = 1.0 mm. ANOVA demonstrates a significant change across all the groups in number of fungiform papillae on the anterior and posterior (IE and L. IE) areas of the tongue cultures (P values ≤ 0.01). Arrows in B and C indicate time at which there is a significant difference from 0 h but no difference from 48 h. (continued)
until Shh signaling was disrupted for at least 12 h. The 12-h CYCL exposure was about equivalent to continuous exposure for 48 h in effects on papilla number and pattern.

With a higher concentration of CYCL (10 μM), it was apparent that disrupting Shh signaling for only 1 h, with a return to STAND, was sufficient to substantially increase the numbers of fungiform papillae on the anterior tongue as well as on the area lateral to the intermolar eminence (Figs. 10A,C). On the intermolar eminence per se, however, fungiform papillae formed in significant numbers after 4 h of Shh signal perturbation with 10 μM CYCL. Notably, effects of brief CYCL exposures on papilla formation were irreversible in subsequent STAND medium.

In summary, only a few hours of Shh signal disruption followed with STAND culture conditions are sufficient to double fungiform papilla number and induce papilla formation on the intermolar eminence. The effect of Shh signaling disruption is long lasting. This is apparent by contrasting papilla number after 24 h in continuous CYCL (Fig. 9, CYCL, E14 +24 h, there are not yet multiple new fungiform papillae) and at 48 h after a brief, 4-h exposure to CYCL. The significant disruption period is shorter with 10 μM compared to 5 μM CYCL, and at either concentration, a longer signal interruption period is necessary to induce papilla formation on the intermolar eminence, compared to the interpapilla regions on the anterior tongue.

**Roles for Shh signaling in later embryonic tongues after papillae are well formed**

**Tongue cultures from E16 and E18**

E16 and E18 embryonic tongues exhibit well-formed fungiform papillae arranged stereotypically on the anterior tongue and a distinct, single circumvallate papilla on the posterior (see Fig. 2, SEM). When these tongues were cultured for 2 days in STAND, CYCL, or A-Shh, no differences were observed across groups, in fungiform papilla number or pattern, or in dorsal tongue topography (Fig. 11). The absence of effects of Shh disruption at later tongue and papilla stages suggests that, once papilla morphology and placement are advanced, Shh signaling does not have a direct role in papilla maintenance. Furthermore, the competence of the intermolar eminence to form fungiform papillae apparently is not retained in later development.

**Discussion**

We have shown that the entire embryonic rat tongue will progress through developmental stages in organ culture, beginning with an initial E12 mandible dissection before tongue formation has commenced, and will be maintained as late as an E18 dissection when tongue shape and topography are advanced. Importantly, highly specialized spatial and temporal information is retained in the tongue cultures, demonstrated by development of the fungiform and circumvallate gustatory papillae in locations and at embryonic stages that coincide with in vivo, embryonic development. Immunolocalization of Shh and Ptc demonstrate early diffuse distributions that become progressively restricted in taste papilla placodes and then within the papillae per se, both in the embryo and in vitro.

Furthermore, from our experiments, it is now apparent that Shh not only is present, but also is a major morphogen both in formation of the tongue organ itself and in directing fungiform papilla formation and the stereotypic anterior tongue patterning of these papillae in rows. Interruption of Shh signaling prevents in vitro tongue formation from E12 mandible cultures. In later development, disrupting Shh signaling for only 1 h, at the time of active papilla morphogenesis, alters papilla numbers on the tongue. The effect of this short disruption is irreversible. However, once papillae

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**Fig. 11.** Scanning electron micrographs of E16 (left) and E18 (right) tongues cultured for 2 days in STAND or 5 μM CYCL. Micrographs of the tongue tip (right) and the circumvallate papilla (left) present regions of the whole tongue cultures at higher magnification. In STAND cultures of E16 and E18 tongues, well-formed fungiform papillae are distributed stereotypically on the anterior, and a single circumvallate papilla remains on the posterior tongue. With addition of 5 μM CYCL for the entire culture period, no apparent difference is observed in papilla size, number, or pattern compared with STAND cultures. Scale bars: 1.0 mm for whole tongue images.
are well formed and the tongue is morphologically advanced, Shh apparently does not have an obvious role in papilla maintenance, nor does the tongue retain competence to add fungiform papillae in atypical locations.

Our work distinguishes tongue formation and various stages of gustatory papilla development as separable processes, with finite periods of developmental competencies, and demonstrates that Shh has distinctive roles in formation of the tongue versus formation and patterning of the taste papillae. Use of the whole embryonic tongue culture system, in which tongues and papillae undergo morphogenesis and retain molecular signatures characteristic of native embryos (Mbiene et al., 1997; Nosrat et al., 2001), has made possible our studies of Shh regulatory roles throughout tongue and papilla development. Whereas Shh mutant rodents have major facial defects that include cyclopia, various oral malformations, and even absence of a tongue (Chiang et al., 1996; Incardona and Roelink, 2000), the in vitro tongue culture system provides a major advance for determining molecular pathways that control tongue and taste papilla development.

Shh has a major role in forming the embryonic tongue from branchial arch I tissue components

There are few published reports of in vitro systems that support lingual development. Tongue formation and development reportedly progress in cultures of mandibular processes from mouse embryos initiated at 9–10 days of gestation (Chai et al., 1994; Kronmiller et al., 1991; Slavkin et al., 1989). However, in these reports, the focus has been on morphogenesis of cartilage and teeth in the mandible. Therefore, little is known about tongue development or regulatory factors.

Because tongue formation from our E12 mandible cultures was blocked consistently with cyclopamine, a major role for Shh in tongue formation is demonstrated. Whereas a narrow, tongue-like structure developed in 70–75% of cultures with A-Shh, differences in extent of signaling block with A-Shh and cyclopamine have been noted in other systems (Lamm et al., 2002) and attributed to the more potent signaling disruption with cyclopamine acting at the Patched-Smoothened receptor interface (Chen et al., 2002; Incardona et al., 1998; Taipale et al., 2000). The elimination of tongue formation with cyclopamine treatment is similar to the complete block of adenohypophyseal development in zebrafish embryo with cyclopamine (Sbrogna et al., 2003) and indeed to early reports of lambs born without tongues from ewes that had grazed on cyclopamine-containing grasses (Keeler, 1984).

Development of the mandible itself with cyclopamine or A-Shh was not noticeably different from standard cultures. A role for Shh in lower jaw formation has been reported, but signal disruption with the alkaloid jervine was needed at very early mouse embryo stages (E9.5) to discern an effect, which could not be demonstrated at E10.5 (ten Berge et al., 2001). We found that a 24-h exposure to cyclopamine, with return to standard culture conditions for 2–3 days, was sufficient to fully block tongue formation from the E12 mandible. On the other hand, a short 4-h exposure to cyclopamine or jervine did not block tongue development in vitro. This narrows the window for a key period of Shh action in development of the lingual organ in vitro. Although the early steps of tongue formation remain poorly understood, we have shown a clear and previously unknown dependence of tongue development on Shh signaling. This establishes Shh as a crucial morphogen in tongue formation. Since this paper was submitted, descriptions of mutant mice with disrupted Shh signaling have demonstrated that, whereas initial formation of branchial arches is normal, many neural crest-derived tissues are eliminated in craniofacial structures (Jeong et al., 2004). For example, the tongue does not form in Wnt1-Cre;SmoCre embryos, and no prospective tongue muscle cells are detected. This finding implicates a cranial neural crest contribution in initial tongue development and also reinforces our demonstration that cyclopamine in E12 cultures prevents tongue formation.

Shh signaling affects tongue shape after lingual swellings have merged

Whereas cyclopamine disruption of Shh signaling at E12 prevents tongue formation from branchial arch tissues, in cultures from E13 with cyclopamine or A-Shh, the lingual swellings will continue to coalesce and form a more advanced tongue. However, these tongues are pointed and the median furrow is essentially obliterated with papilla overgrowth. In addition, the papilla-free nature of the intermolar eminence region is breached with formation of numerous fungiform papillae. These new data add the tongue to several other organs such as pancreas and spleen (Hebrok, 2003), prostate (Freestone et al., 2003), pituitary (Treier et al., 2001), and eye (Zhang and Yang, 2001) in which Shh signaling has distinctive roles in initial development and subsequent differentiation of functional tissue regions.

By E14, Shh signaling apparently no longer affects tongue shape in culture. Thus, once the lingual swellings that comprise initial tongue elements have merged and grown to form a spatulate tongue, intrinsic molecular signals can direct tongue development in vitro and disrupting the Shh pathway no longer prevents the process.

Shh signaling has a role in establishing taste papilla pattern from tissues of the early lingual swellings

In the E13 tongue, a merged set of three lingual swellings, Shh is immunolocalized strongly to the more distal portions of the anterior lingual swellings and weakly in posterior tubercular impar. The Ptc protein overlaps Shh. Therefore, a molecular boundary is apparent that demarcates anterior oral tongue, where fungiform papillae will develop, from the tuberculum impar which will give rise to fungiform-free
regions on the intermolar eminence of the posterior tongue. This implicates Shh in establishment of the anterior tongue as a fungiform papilla-permissive tissue region and suggests that other molecular signals may interact with Shh to effectively inhibit Shh expression in the papilla-nonpermissive tuberculum impar.

When the E13 tongue is cultured, tongue morphogenesis and growth proceed, fungiform papillae form on the anterior tongue, and the single circumvallate papilla develops on posterior tongue. With Shh signal disruption in vitro, the fungiform papillae develop in increased numbers compared to control conditions and are larger in diameter. In very different organ systems, extrapancreatic buds form in embryonic chick pancreas anlage (Kim and Melton, 1998), and increased numbers of ductal tips develop in ventral prostate epithelium from mouse (Freestone et al., 2003), when cyclopamine is used to disrupt Shh signaling. Polyductly is observed on anterior and posterior limbs in mutant mice with vastly down-regulated Shh transcription and translation (Krebs et al., 2003). Thus, in developing taste papillae, pancreas, prostate, and digits, disrupting Shh can effect multiplication of organ structures and thereby alter spatial patterns.

The increased taste papillae that form with Shh disruption seemingly overgrow and obliterate the median furrow of anterior tongue, and develop in large numbers on the intermolar eminence. Therefore, the typical fungiform papilla pattern is totally altered, and a normally papilla-free region incorporates papillae. This suggests that Shh not only functions in maintaining papilla and interpapilla regions on anterior tongue, but also signals more distantly to interact with other molecules in maintaining a papilla-free intermolar eminence. Finally, a competence of the embryonic intermolar eminence to permit papilla formation is revealed.

Shh signaling regulates papilla number and location on embryonic tongue at stages from placode to papilla formation; brief periods of signal disruption irreversibly alter papilla distribution

Our data demonstrate that an initial uniform distribution of Shh in the anterior tongue at E13 progresses to a highly localized and intense protein distribution in the fungiform papilla placodes at E14. In mouse urogenital system, also, there is an early, continuous distribution of Shh expression in urogenital sinus epithelium that becomes more localized in a subset of epithelial cells which evaginate into mesenchyme and form prostate buds (Lamm et al., 2002); this progressive localization accompanies functional roles for Shh in bud formation. Associated with the restriction of Shh within taste papilla placodes is also a clear requirement for Shh in numerous aspects of papillagenesis. Whereas Shh signaling disruption no longer alters in vitro tongue morphogenesis at this stage (E14), fungiform papilla formation, patterning, numbers, and location are radically affected by Shh signal interruption. Our results indicate that the Shh pathway has a role in preventing papilla formation in the interpapilla spaces on anterior tongue, the intermolar eminence, and on posterior oral tongue. We present a model at the end of the Discussion that suggests that Shh acts differently at high and low concentrations to induce and sustain papillae or activate interpapilla gene complexes that suppress papilla formation, respectively.

The temporal requirements for effectiveness of cyclopamine in culture are striking: only 1 h of Shh signal disruption is sufficient to alter the interpapilla lingual epithelium so that fungiform papilla numbers are increased on anterior tongue. Effects of this brief exposure are not reversed by rinsing and continuing cultures through 2 days in standard medium only. Therefore, once initiated, the molecular programs for fungiform papilla formation progress, although the source of Shh signal disturbance is removed. However, to effect fungiform papilla development on the posterior intermolar eminence structure, 4 h of cyclopamine exposure is required (and again, the brief exposure effect is not reversible in standard medium). This longer period to alter the nonpermissive papilla program of the intermolar eminence suggests that the molecular signals are working within an epithelium which is less susceptible to signaling pathway disruptions that can ultimately reveal an underlying competence for papillagenesis. At a lower concentration of cyclopamine, longer exposure periods are needed to disrupt fungiform papilla patterns on anterior tongue and intermolar eminence. However, effective exposures are still on the order of hours and are not reversible with return to standard medium conditions. Such brief cyclopamine exposure periods are not effective in preventing tongue formation from the E12 mandible. Together, our data reinforce the different roles for Shh in tongue formation, in fungiform papilla formation and patterning, or in sustaining areas of epithelium that usually do not reveal a competence to support papillae.

It is noteworthy that whereas Shh signaling has varied and crucial roles in fungiform papilla development, an obvious effect on the single circumvallate papilla is not apparent. The single midline papilla forms at the border of oral and pharyngeal tongue in culture with cyclopamine, jervine, or anti-Shh. Our data suggest, however, that there are differences in shape of the circumvallate when Shh signaling is interrupted, and we are pursuing specific study of this papilla, which develops in tissue from a different lingual swelling than the fungiform papillae.

Shh does not alter fungiform papilla number or pattern once papillae are morphologically advanced

As the tongue epithelium and taste papillae are progressively specified, competence of interpapilla and nonpapilla epithelium to respond to Shh disruption is lost. Whereas Shh remains highly localized within papillae and immunolocalization of Ptc in the fungiform papilla surround at E16 and E18 indicates that the Shh signal is active, at these stages,
there is no effect from cyclopamine treatment on papilla number or distribution in tongue cultures. Neither differentiated fungiform papillae, interpapilla epithelia, nor non-papillary tongue epithelium of the intermolar eminence is responsive to disruption of the endogenous Shh signal. These data suggest that, once papilla development is advanced, Shh signaling does not have a direct role in the maintenance of fungiform papilla patterns. Similarly, in later stages of feather development, Shh signaling is active but no longer affects the interfollicle ectoderm (Morgan et al., 1998). Shh activity and the ability of epidermal cells to respond to Shh also are temporally regulated in hair follicle cell development and cycling (Oro and Higgins, 2003).

**How Shh might regulate papilla formation and pattern**

From experiments with E12 mandible through E18 tongue cultures, it is clear that Shh has different roles in different contexts of tongue and taste papilla development. In several models of organogenesis, Shh effects various cell responses with different concentration thresholds, and there are demonstrations that signaling can be short and long range, and direct and indirect (Christian, 2000; Ingham and McMahon, 2001). Our experiments suggest different modes for Shh signaling in different stages of tongue and papilla formation and patterning (Fig. 12).

In early establishment of the rodent tongue from three lingual swellings, there are molecular territories that preview future functional regions of the tongue. At E13, the anterior, lateral lingual swellings that will differentiate to lingual tissue supporting fungiform papillae have a relatively uniform distribution of Shh and associated Ptc; in contrast, the more posterior tuberculum impar that will differentiate to the intermolar eminence that does not support papillae has only a very low Shh expression (Fig. 12, summary diagrams). Higher levels of Shh acting on nearby tissue may contribute to differentiation of anterior gustatory epithelium, whereas low levels of Shh may indirectly contribute to maintaining a papilla-free intermolar eminence.

In development of papillae per se, we provide several lines of evidence for involvement of Shh in papilla formation. First, there is a close relationship between the pattern of Shh expression and of fungiform papillae during development. In early establishment of the rodent tongue from three lingual swellings, there are molecular territories that preview future functional regions of the tongue. At E13, the anterior, lateral lingual swellings that will differentiate to lingual tissue supporting fungiform papillae have a relatively uniform distribution of Shh and associated Ptc; in contrast, the more posterior tuberculum impar that will differentiate to the intermolar eminence that does not support papillae has only a very low Shh expression (Fig. 12, summary diagrams). Higher levels of Shh acting on nearby tissue may contribute to differentiation of anterior gustatory epithelium, whereas low levels of Shh may indirectly contribute to maintaining a papilla-free intermolar eminence.

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In development of papillae per se, we provide several lines of evidence for involvement of Shh in papilla formation. First, there is a close relationship between the pattern of Shh expression and of fungiform papillae during development. In early establishment of the rodent tongue from three lingual swellings, there are molecular territories that preview future functional regions of the tongue. At E13, the anterior, lateral lingual swellings that will differentiate to lingual tissue supporting fungiform papillae have a relatively uniform distribution of Shh and associated Ptc; in contrast, the more posterior tuberculum impar that will differentiate to the intermolar eminence that does not support papillae has only a very low Shh expression (Fig. 12, summary diagrams). Higher levels of Shh acting on nearby tissue may contribute to differentiation of anterior gustatory epithelium, whereas low levels of Shh may indirectly contribute to maintaining a papilla-free intermolar eminence.
embryonic development. From the diffuse Shh distribution in the early tongue, there is a progressive restriction on anterior tongue in papilla placodes that establishes rows of Shh/no Shh (Fig. 12, diagrams, E14). This placodal localization demarcates intense Shh within placodes with an Shh-free interplacodal surround. The intense Shh in prepapilla placodes by definition precedes formation of papillae, consistent with an essential role for Shh in papilla formation. Subsequently, high intensity Shh is maintained within the developing fungiform papillae, and there is a papilla surround with much reduced Shh. Because Ptc immunoreactivity surrounds Shh immunoproduct in fungiform papillae, the Shh signal apparently emanates symmetrically from its source in papilla cells. Furthermore, there is a close relationship between the pattern of Shh expression and of fungiform papillae in E13 and E14 tongue cultures. Shh is intensely localized in every fungiform papilla, including those formed in increased numbers and atypical posterior locations on tongues cultured with cyclopamine or Shh antibody. In previous work, we demonstrated that the Ptc protein is also associated with every fungiform papilla in cultures with cyclopamine (Mistretta et al., 2003). Collectively, our data suggest that Shh has inductive and morphogenetic roles in fungiform papilla formation and also functions to maintain the interpapilla space.

We propose that, within papilla placodes, high levels of Shh interact with a putative fp (fungiform papilla) gene or gene complex to direct formation of fungiform papillae (Fig. 12, model). Cells surrounding the placode or early papilla respond to lower levels of Shh by activating a no-fungiform papilla pathway and maintaining transcription of a putative xfp (no-fungiform papilla) gene or gene complex. xfp could act on cell neighbors to antagonize or suppress fp gene activity, so that a papilla-free surround develops and interpapilla spacing is maintained. Lower levels of Shh on the posterior tongue could also function to activate and maintain a no-papilla signaling pathway.

Thus, Shh at high levels in fungiform papillae is required for the continuing morphogenesis of papillae from placodes and at low levels in the surround for the suppression of fungiform papillae. If cyclopamine disrupts the Shh pathway, existing papilla placodes and developing fungiform papillae remain and the interpapilla activation of xfp by low levels of Shh is lost so that new papillae emerge in interpapilla spaces (or on posterior tongue); an essentially uniform field of papillae results. This proposition suggests indirect and concentration-dependent effects of Shh in supporting a pattern of fungiform papillae, as in the roles for hedgehog in generating rows of larval denticles in Drosophila (see Ingham and McMahon, 2001). In Drosophila, cells in furrows that are neighbors to hedgehog-expressing rows respond to the hedgehog signal by secreting wingless. The wingless protein can act on neighboring cells to suppress dentine formation. With interruption of hedgehog activity, a uniform lawn of denticles develops because specific gene expression domains are disrupted. Similarly, in drosophila segment boundary (Hatini and DiNardo, 2001; Larsen et al., 2003; Lawrence and Struhl, 1996) and ommatidia (Dominguez, 1999) development, hedgehog acts on neighboring and distant cells to establish signaling compartments that regulate spatial patterns.

After fungiform papilla formation is advanced, Shh remains intense within papillae, especially in the basement membrane region, and disrupting Shh signaling does not alter papillae or their distribution (Fig. 12, summary diagrams, E16, E18). Thus, the lingual epithelium must have lost its competence to alter differentiation programs in response to Shh signal alterations.

What are candidates for the xfp gene(s) between fungiform papillae? Roles for Gli1, 2, and 3 in activator and repressor forms to mediate Shh signaling are various and differ across organ systems (Krebs et al., 2003; Litingtung et al., 2002; Ruiz i Altaba et al., 2003). Information on Gli proteins in tongue is sparse (Hall et al., 1999), but a potential between-papilla role for Gli proteins should be investigated. Other obvious molecules to consider for roles in interpapilla signaling include those in the Bmp, Fgf, and Wnt families (Capdevila and Belmonte, 1999; Christian, 2000; Hoffman et al., 2002). Data in progress from our laboratory demonstrate that EGF and EGFr have distributions in embryonic tongue that contrast with those of Shh and Ptc and that exogenous EGF in cultures results in a dose-dependent decrease in fungiform papillae and increase in interpapilla space on anterior tongue (Liu and Mistretta, 2004). EGF in culture apparently increases cell proliferation between but not within fungiform papillae and may play a role in maintaining interpapilla epithelium and spacing.

In summary we demonstrated that Shh has varying roles in regulating tongue formation; tongue shape; emergence of fungiform papillae from placodes; number, size, and pattern of fungiform papillae; and localization of fungiform papillae on the tongue. The graded distribution of Shh protein and concentration-dependent effects in tongue and papilla formation support a proposed role of Shh as a morphogen, in addition to indirect roles in sustaining papilla-free lingual epithelium. Only a few hours of Shh signal disruption can irreversibly alter number and distribution of fungiform papillae on anterior tongue and elicit papilla formation on the intermolar eminence. Not only are crucial roles for Shh signaling demonstrated, but also distinctive tissues of the tongue are clearly highlighted as papilla-competent and papilla-incompetent regions, and the developmental times are identified during which normal competency of these regions can be altered.

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References


