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Bone morphogenetic proteins and noggin: Inhibiting and inducing fungiform taste papilla development

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Abstract

Fungiform papillae are epithelial specializations that develop in a linear pattern on the anterior mammalian tongue and differentiate to eventually contain taste buds. Little is known about morphogenetic and pattern regulation of these crucial taste organs. We used embryonic rat tongue, organ cultures to test roles for bone morphogenetic proteins, BMP2, 4 and 7, and antagonists noggin and follistatin, in development of papillae from a stage before morphological initiation (E13) or from a stage after the pre-papilla placodes have formed (E14). BMPs and noggin proteins become progressively restricted to papilla locations during tongue development. In E13 cultures, exogenous BMPs or noggin induce increased numbers of fungiform papillae, in a concentration-dependent manner, compared to standard tongue cultures; BMPs, but not noggin, lead to a decreased tongue size at this stage. In E14 cultures, however, exogenous BMP2, 4 or 7 each inhibits papilla formation so that there is a decrease in papilla number. Noggin substantially increases number of papillae in E14 cultures. Using beads for a highly localized protein delivery, papillae are inhibited in the surround of BMP-soaked beads and induced in large clusters around noggin-soaked beads. Follistatin, presented in culture medium or by bead, does not alter papilla formation or number. In all fungiform papillae that form under various culture conditions, the molecular marker, sonic hedgehog, is within each papilla. However, the BMP inhibitory effect on papillae is not prevented by disrupting sonic hedgehog signaling through addition of cyclopamine to cultures. BMPs and noggin alter cell proliferation in tongue epithelium in opposite ways, demonstrated with Ki67 immunostaining. We propose that the BMPs and noggin, colocalized within papilla placodes and the fungiform papillae per se, have opposing inhibitory and activating or inducing roles in papilla development in linear patterns. We present a model for these effects.

Keywords: Taste papilla; Bone morphogenetic protein; Noggin; Follistatin; Pattern formation; Fungiform papilla; Sonic hedgehog signaling; Embryonic tongue culture; Ectodermal specialization; Cyclopamine

Introduction

During development of ectodermal specializations that arise in a pattern, for example feathers, whisker follicles, hair and teeth, differentiation of both organs and the tissues between organs is essential for acquiring a particular spatial distribution (Meinhardt and Gierer, 2000). The fungiform taste papilla organs are lingual epithelial specializations that emerge on the anterior tongue of the mammalian embryo in a pattern of longitudinal rows, bracketing a median furrow (Mistretta, 1998). After birth in the rodent, the taste buds differentiate in these lingual papillae, and therefore papillae are key determinants in peripheral taste receptor distribution (Mistretta, 1991). Little is known, however, about regulation of papilla development or patterning.

The embryonic rat tongue is initially apparent as three tissue swellings on the floor of the mandible at embryonic day 13 (E13) (Mistretta et al., 2003). At E14, a spatulate tongue is seen and papilla placodes have formed, distinctive groupings of columnar cells in the dorsal epithelium that are the first morphological sign of the taste papillae. By E15, the tongue has a distinctive topography and well-formed papillae have developed. These stages are equivalent to embryonic mouse tongue at about E11.5–12, 12.5–13.0 and 13.5, respectively (Kaufmann, 1992). In addition to fungiform papillae distributed on the anterior two thirds of the tongue, morphologically distinctive circumvallate papillae develop at the posterior

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border between oral and pharyngeal tongue, and foliate papillae on the posterior lateral tongue in mammals, and these also will house taste buds at later developmental stages (Mistretta, 1991).

Prior to papilla placode formation, the lingual epithelium at E13 in rat has a relatively homogeneous topography, histology and molecular phenotype. However, molecular markers acquire a gradually restricted distribution to placodes, and then to the papillae per se, from a diffuse distribution at E13 (Liu et al., 2004).

Some regulatory proteins have demonstrated roles in the differentiation, growth and patterning of fungiform papillae, including sonic hedgehog (Hall et al., 2003; Mistretta et al., 2003; Liu et al., 2004) and epidermal growth factor (Liu et al., 2005). However, compared to understanding of feather, hair and tooth development, there is sparse knowledge about determination of: the restriction of fungiform papillae to the anterior tongue; the pattern of fungiform papillae; or, the number and size of papillae. Further, direct inhibitors of papilla formation are not known.

An important group of regulatory molecules that may well direct papilla development are the bone morphogenetic proteins (BMPs). BMPs are a large subgroup of secreted factors of the TGFβ superfamily, with numerous developmental roles in regulation of cell proliferation, differentiation, apoptosis and migration (Balemans and Van Hul, 2002; Botchkarev and Sharov, 2004; Zhang and Li, 2005). BMPs are known to act in organ patterning; for example as inhibitors of feather placode formation, they contribute to spatial distribution of feather buds (Jung et al., 1998; Noramly and Morgan, 1998).

Three BMPs are obvious candidates for potential roles in taste papilla development. BMP2 and 4 have been implicated in papilla development through in situ expression analysis in embryonic mouse papillae (Bitgood and McMahon, 1995; Jung et al., 1999; Hall et al., 2003), and BMP2, 4 and 7 have known roles in development of neural, skeletal and epithelial tissues (Chen et al., 2004). Among the BMP antagonists are noggin and follistatin. Noggin binds BMP2, 4 and 7 with high affinity whereas follistatin binds activin with high affinity and BMPs with lower affinity (Balemans and Van Hul, 2002; Botchkarev, 2003). The inhibitory mechanism of noggin is different from that of follistatin, or another BMP antagonist, chordin (Iemura et al., 1998; Balemans and Van Hul, 2002).

Because mouse mutant models for BMP knockouts have multiple facial, organ and skeletal defects (Botchkarev and Sharov, 2004), an in vitro system is important for functional studies. BMP2 and 4 mutant mouse embryos die between E7.5 and 10.5, and BMP7 mutants die shortly after birth (Chen et al., 2004). We use a whole embryonic tongue culture developed in our laboratory to study tongue and taste papilla development (Mbiene et al., 1997). In organ cultures begun at varying embryo stages, the tongue can progress from three lingual swellings to a spatulate tongue with taste papilla placodes, or to a larger tongue with distinctive taste papillae. The tongue cultures manifest papilla formation with retention of spatial, temporal and molecular information that is similar to in vivo development (Nosrat et al., 2001; Mistretta et al., 2003; Liu et al., 2004).

In the present studies, embryonic rat tongue cultures were used to test hypotheses that BMP2, 4 and 7 have regulatory roles in fungiform papilla development and patterning, and that BMP antagonists, noggin and follistatin, would have counter effects to BMPs in papilla formation. Cultures were begun at E13, when the tongue is a set of three swellings with a topographically uniform epithelium, or at E14, when the spatulate tongue has roughly spaced, fungiform papilla placodes on the anterior tongue. BMP molecules and antagonists were added to tongue culture medium or applied via beads set into the tongue dorsum. We have demonstrated that BMPs have varying roles in papilla development at different embryonic stages; that BMPs seem to induce papillae from naive tongue epithelium but inhibit papilla formation from placodes; and, that noggin but not follistatin has opposing roles to BMPs. Furthermore, BMPs and noggin have different effects on cell proliferation in embryonic lingual epithelium. Whether fungiform papillae were increased or decreased in number in various experiments, all papillae retained the fungiform papilla marker protein, sonic hedgehog.

Materials and methods

Animals and embryonic tongue cultures

Timed pregnant rats were obtained from Charles River breeders. The morning when a vaginal plug was detected was termed embryonic day 0 (E0), and noon of the day of vaginal plug detection is E0.5. E13.0 to E15.0 embryos were used and all dissections were made between 9 AM and noon to minimize developmental variability across litters. Animal maintenance and use protocols were in compliance with approved institutional use and were according to guidelines of the National Institutes of Health.

The pregnant dam was deeply anesthetized with an intraperitoneal dose of sodium pentobarbital at 60 mg/kg body weight, which anesthetizes the embryos also. Embryos were removed, using aseptic technique, to cold Earl's balanced salt solution (EBSS), containing gentamicin sulfate (50 μg/ml) and buffered to pH 7.4 with 20 mM HEPES. Embryo heads were dissected, moved to fresh EBSS and tongues were dissected free from the mandible.

Tongues were cultured as previously described (Mbiene et al., 1997). E13 or E14 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 standard medium of 1:1 mixture of Dulbecco's modified Eagle's 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). The level of the medium was adjusted so that the cultures were maintained at the interface between the gas (5% CO_2 in air) and liquid phases of the culture, in a humidified incubator at 37°C (MacCallum, 1994). After 2 or 3 days, tongue cultures were removed and processed for scanning electron microscopy or whole tongue immunohistochemistry, or submerged in O.C.T. compound (Miles Scientific, Elkhart, IN) and rapidly frozen.

Reagents

To study roles of BMPs and BMP antagonists in papilla development, proteins were added to the standard medium for E13 and E14 tongue cultures. Recombinant BMP2 (0.03, 0.3, 1.5 μg/ml), BMP4 (0.03, 0.3, 1.0 μg/ml), BMP7 (0.05, 0.5, 1.5 μg/ml), noggin (1.0, 3.0, 10.0 μg/ml) or follistatin (0.25, 1.0, 4.0 μg/ml), all from R&D Systems (Minneapolis, MN), was added to reach final concentration in the culture medium. All were human recombinant proteins except noggin which was mouse.

To disrupt Shh signaling and learn whether BMPs still altered papilla development, 5 μM cyclopamine (CYCL) or jervine, steroidal plant alkaloids

known to specifically interrupt the Shh signaling process (Gaffield et al., 1986, 1999), was added to the standard culture medium. CYCL and jervine (gifts of William Gaffield) were prepared as 10 mM stock solutions in 100% ethanol and stored at 4°C. Before experiments, the alkaloids were added to the culture medium and were present for the duration of tongue cultures.

Bead preparation and experiments

Affi-Gel blue agarose beads (100–200 μm, Biorad, Hercules, CA; #153-7302) were rinsed in phosphate-buffered saline (PBS) and soaked in dilutions of BMP2 (0.67 μg/μl), BMP4 (0.67 μg/μl) or noggin (3.0 μg/μl) at 4°C for 1 week. Beads soaked in PBS were used for controls. Soaked beads were placed with forceps in a specified region of the anterior tongue, and tongues were cultured according to experimental design. The contralateral side of the tongue, without a bead, also served as a control.

Whole and sectioned tongue immunohistochemistry

Antibodies against BMP2 (1:20, Santa Cruz Biotechnology, Santa Cruz, CA, SC6895), BMP4 (1:10–1:50, Vector Laboratories, Burlingame, CA, VP-B208), noggin (1:10, R&D Systems, Minneapolis, MN, AF719), Shh (1:100, R&D Systems, #AF464) and Ki67 (1:1000, Novocastra Laboratories, #NCL-Ki67p) were used with antigen retrieval procedures. For controls, the primary antibody was omitted or a similar concentration of normal IgG was used in place of the primary. In addition to procedural controls, specificity of antibodies is documented by the respective manufacturers in Western blots and/or direct ELISAs.

To immunolocalize protein in whole embryo tongues or tongue cultures, tissues 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. To begin reactions, 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 Universal Antigen Retrieval Agent (CTS015; R and D Systems, Minneapolis, MN). 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. After rinsing (5 times, 1 h each), tongues were incubated overnight at 4°C with a biotin-conjugated rabbit anti-goat secondary antibody (KPL, Gaithersburg, MD) at 1:250 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, 5 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 pre-incubated in nickel-intensified DAB solution (Vector Laboratories, Burlingame, CA) without H_2O_2. Reactions were done in the same DAB solution with the addition of 0.0003% 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 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 0.5 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% 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 (CTS015; R and D Systems, Minneapolis, MN). Nonspecific staining was blocked with 10% normal rabbit serum 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 in carrier solution (1% normal rabbit serum, 0.3% Triton X-100 in 0.1 M PBS). After rinsing in carrier solution, sections were placed in biotin-labeled, rabbit anti-goat or goat anti-rabbit secondary antibody (Kirkegaard and Perry, Gaithersburg, MD), at 1:250 in carrier solution at room temperature for 30 min. Sections were rinsed, incubated in HRP-labeled streptavidin (at 1:500 in carrier solution) 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 with Permount® mounting medium (Fisher, Fairlawn, NJ).

Scanning electron microscopy

Microdissected tongues or tongue cultures were fixed overnight in 4% paraformaldehyde/2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at room temperature. Tongues were then rinsed in buffer and subsequently post-fixed in a sequence of aqueous 1% OsO_4, 1% tannic acid, 1% OsO_4, 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 were mounted on specimen stubs, lightly sputter-coated with gold/palladium and analyzed with a scanning electron microscope.

Papilla definition, 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. All fungiform papillae on the entire tongue were counted. A papilla was defined as a rounded or ovoid structure, raised from the surface of the tongue. When papillae were essentially touching each other, or were in fused rows or clusters, a single papilla count was recorded when clear cellular boundaries were obvious in the fused group. Two investigators counted papillae in all tongues, across concentrations, for E13 BMP7 and noggin experiments, including control tongues, and for the E14 noggin experiment. Counts within an experimental group could differ by as much as 12% between investigators. However, conclusions about an increase or decrease across concentrations never differed, nor did assessment of statistical significance. Papillae were not counted in immunoreacted whole tongues because one positive immunoreactive "spot" does not always coincide with an entire papilla; and, some papillae can be too lightly stained for an accurate count.

To measure papilla size, the longest and shortest axis of each papilla was measured from an expanded region on a computer screen and averaged to calculate diameter. Within a tongue, size of papillae was measured from a region extending 250 μm back from the tip. To measure tongue area, used to calculate papilla density, we measured the length of the anterior tongue from the anterior-most point of the intermolar eminence to the extreme tip, and width from lateral tongue border to lateral border at the widest part.

For each experiment, two to four litter replicates were made. Fungiform papillae were counted on 16 to 33 tongues for each experiment for statistical analysis. Numbers of fungiform papillae are presented as means ± standard deviations. Statistical analyses were conducted with analysis of variance (ANOVA) followed by Tukey and Bonferroni post hoc tests. The significance level was set at $P \leq 0.05$.

Digital images were generated from scanning electron micrographs, immunostained whole tongues or slides. The images were then assembled into figures using Photoshop (Adobe Systems, Mountain View, CA).

Results

BMP 2, 4, and noggin distributions in embryonic tongue from E13 through E15

Scanning electron micrographs illustrate topographical features of native embryonic rat tongues (Fig. 1, top row). At E13, three lingual tissue masses are apparent and the epithelial surface is relatively homogeneous. By E14, the lingual swellings have merged, an intermolar eminence is apparent and papilla placodes have developed on the anterior tongue. At E15, fungiform papillae, formed from the E14 placodes, are distinct raised organs in linear rows on the tongue surface.

Whole tongue immunoreactions demonstrate an initial diffuse expression of BMP2 and 4 in the E13 tongue, which

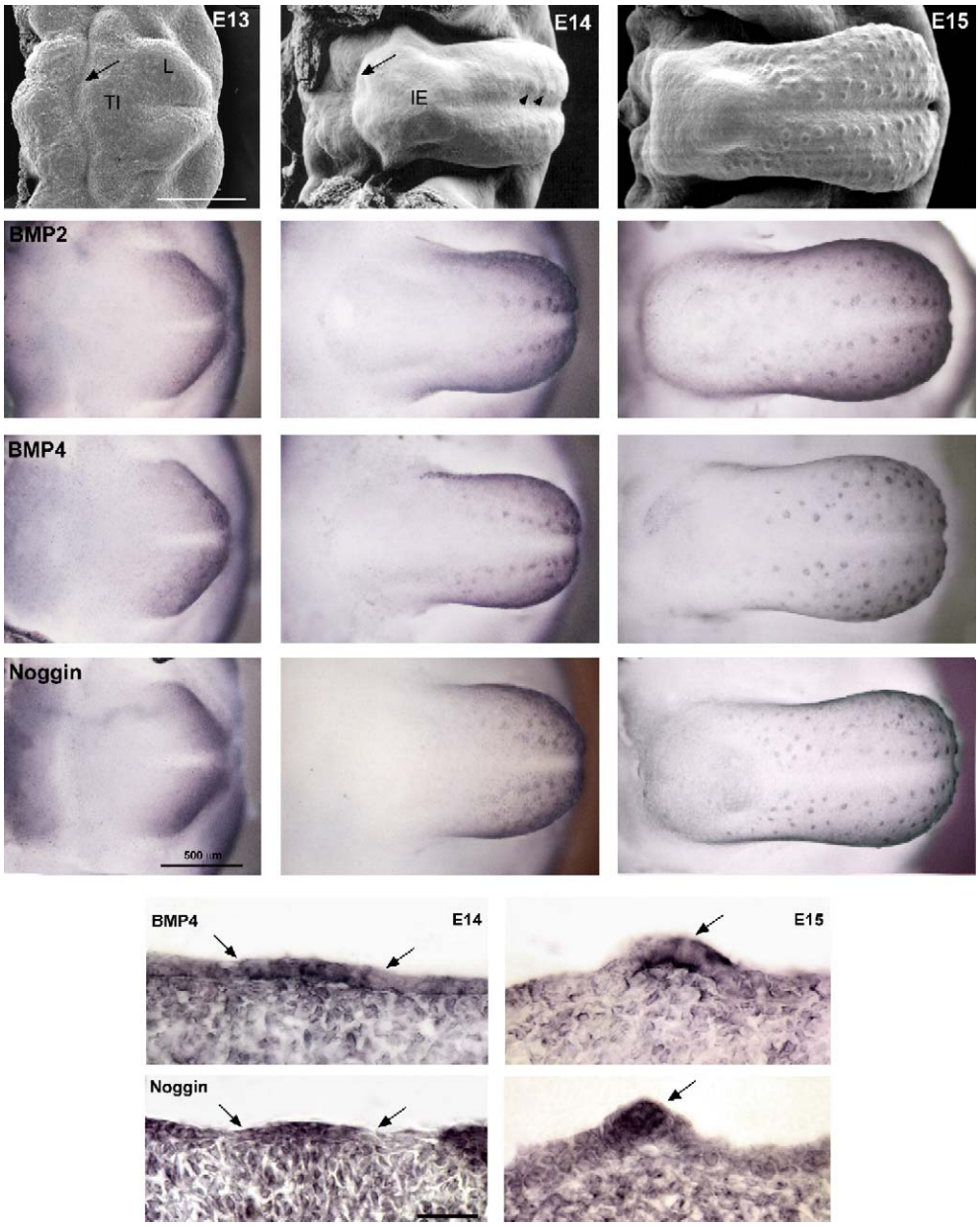


Fig. 1. Scanning electron, whole and sectioned tongue photomicrographs demonstrate distributions of BMP2, 4 and noggin in embryonic rat tongue. Top of plate, first row: Embryo tongues from E13, E14 and E15. The tongue at E13 is a set of three swellings: the tuberculum impar (TI) and lateral lingual swellings (L). An arrow demarcates the border between oral and pharyngeal tongue. At E14, the three lingual swellings have merged and the intermolar eminence (IE), a papilla-free region, is seen on the posterior oral tongue. Arrowheads point to two in a series of pre-papilla placodes, in a distribution that previews fungiform papilla formation. The tongue at E15 is well developed and the fungiform papillae are distributed in diagonal rows on the anterior oral tongue. Scale bar = 500 μm, for all 3 images. Rows 2, 3 and 4: Whole tongue immunoreactions at E13, 14 and 15 for BMP2, BMP4 and noggin. Each protein is in a diffuse distribution on the lateral lingual swellings at E13, restricted to pre-papilla placodes at E14, and confined within the fungiform papillae at E15. The median furrow that divides the two halves of the anterior tongue is free of these proteins and the intermolar eminence has no BMP immunoproduct. Bottom of plate: sagittal sections of E14 and E15 placodes and fungiform papillae immunoreacted for BMP4 (top) or noggin (bottom). Arrows delimit the extent of papilla placodes (E14) or point to fungiform papillae (E15). BMP4 is immunolocalized in the epithelium of placodes and papillae and is very dense in the basal lamina region of both structures. Noggin also is intense in the epithelium of placodes and the papilla apex, and has a more confined papilla distribution than BMP4 at E15. Both BMP4 and noggin have some immunodistribution in the mesenchyme under placodes at E14. Scale bar = 50 μm for all four sections.

is subsequently confined to discrete locations that correspond to fungiform papilla placodes in the E14 tongue (Fig. 1, rows 2 and 3). By E15, BMP2 and 4 are within each of the fungiform papillae that have differentiated from early placodes. Noggin immunoreactions demonstrate a similar progressive restriction, from an E13 diffuse distribution across the dorsal epithelium, to subsequent localization within papilla placodes at E14, and papillae at E15 (Fig. 1, row 4).

To define the tissue location for BMPs and noggin, immunoreactions were made in sagittal tongue sections (Fig. 1, bottom panel). At E14 and E15, BMP4 immunoproduct is intense in the lingual epithelium corresponding to regions of

placodes (E14) or fungiform papillae (E15). At E14, BMP4 is especially intense in a thin line along the basement membrane of both placode and between-placode epithelium, and in cells of the placode epithelium (Fig. 1, BMP4, E14). More lightly immunoreacted cells are seen in epithelium between placodes and in the tongue mesenchyme. A very dense, basement membrane-associated, BMP4 immunoproduct is apparent in the fungiform papilla epithelium at E15 and more lightly stained cells are within the fungiform papilla itself, and in scattered cells of the tongue epithelium and mesenchyme (Fig. 1, E15).

Noggin immunoproduct is dense within cells of the papilla placodes and is weaker in the epithelium surrounding placodes and in underlying mesenchyme at E14 (Fig. 1, Noggin). Noggin is extremely dense within a highly restricted region of the apical papilla epithelium at E15. Thus, the BMPs and noggin co-localize in developing fungiform papillae. However, at E15 in the newly formed fungiform papillae, noggin is more restricted to cells in the papilla apex compared to BMP4.

The progressive localization of BMP and noggin protein distribution to fungiform papillae suggest roles in both placode and papilla formation. However, the diffuse distribution of BMPs and noggin across the relatively homogenous tongue epithelium at E13 versus restriction in papilla placodes at E14 and within papillae at E15, predicts that these proteins would have different developmental roles at E13 versus E14.

E13 cultures: BMP and noggin effects on tongue and papillae

To test for functional effects on papilla induction, development and patterning, we added BMPs or noggin to embryonic tongues cultured at E13, a time before placodes have formed in the anterior tongue epithelium.

BMP 2, 4 or 7 alters tongue size and shape, and increases fungiform papilla number and density

The E13 tongue dorsum has a homogeneous topography on which papilla placodes appear as small mounds at E14 and these form into obvious papillae at E15 (see Fig. 1, scanning micrographs). After 2–3 days in culture in standard medium, the E13 lingual swellings have merged and the tongue has acquired an intermolar eminence and distinctive fungiform papillae (Fig. 2, E13 and STAND, top).

When BMP2, 4 or 7 is added to the culture medium at E13, tongues are shorter by about 25%, more narrow by about 55%, and more pointed at the tip than tongues cultured in standard medium (Fig. 2, BMP2, BMP4, BMP7). Numerous, distinct fungiform papillae form on the anterior tongue with any of the exogenous BMPs. However, compared to standard tongue cultures, the number of fungiform papillae is increased significantly with addition of BMP2, 4 or 7 (*BMP2*, $F(3,15) = 4.4$, $P = 0.03$; *BMP4*, $F(3,32) = 6.7$, $P = 0.001$; *BMP7*, $F(3,18) = 13.1$, $P < 0.001$) (Fig. 2, graphs on right). These effects are concentration-dependent. With post hoc tests, it is apparent that at high concentrations (1–1.5 μg/ml), there is no further increase in papilla number. However, tongue topography is altered in BMP7 tongues at 1.5 μg/ml so that some papillae develop on regions of the intermolar eminence.

Because tongues are shorter and narrower in tongue cultures treated with BMP2, 4 or 7, tongue area is reduced while papilla numbers are increased. This yields an increased density of papillae. We calculated the change in papilla density for tongues cultured with BMP7, as an example for the three BMPs. Expressed as papillae per square mm, across concentrations from 0 to 1.5 μg/ml, density more than doubled from 375 to 837. It is important to emphasize, however, that the extent of this change in density in tongues cultured with BMP is not simply attributable to the smaller tongue size relative to standard tongue cultures, but rather that the actual number of papillae increased. In studies of papilla formation, counts of all papillae are essential to know how many organs form independently of tongue size consideration.

Noggin alters fungiform papilla size, number and distribution; tongue size is not altered

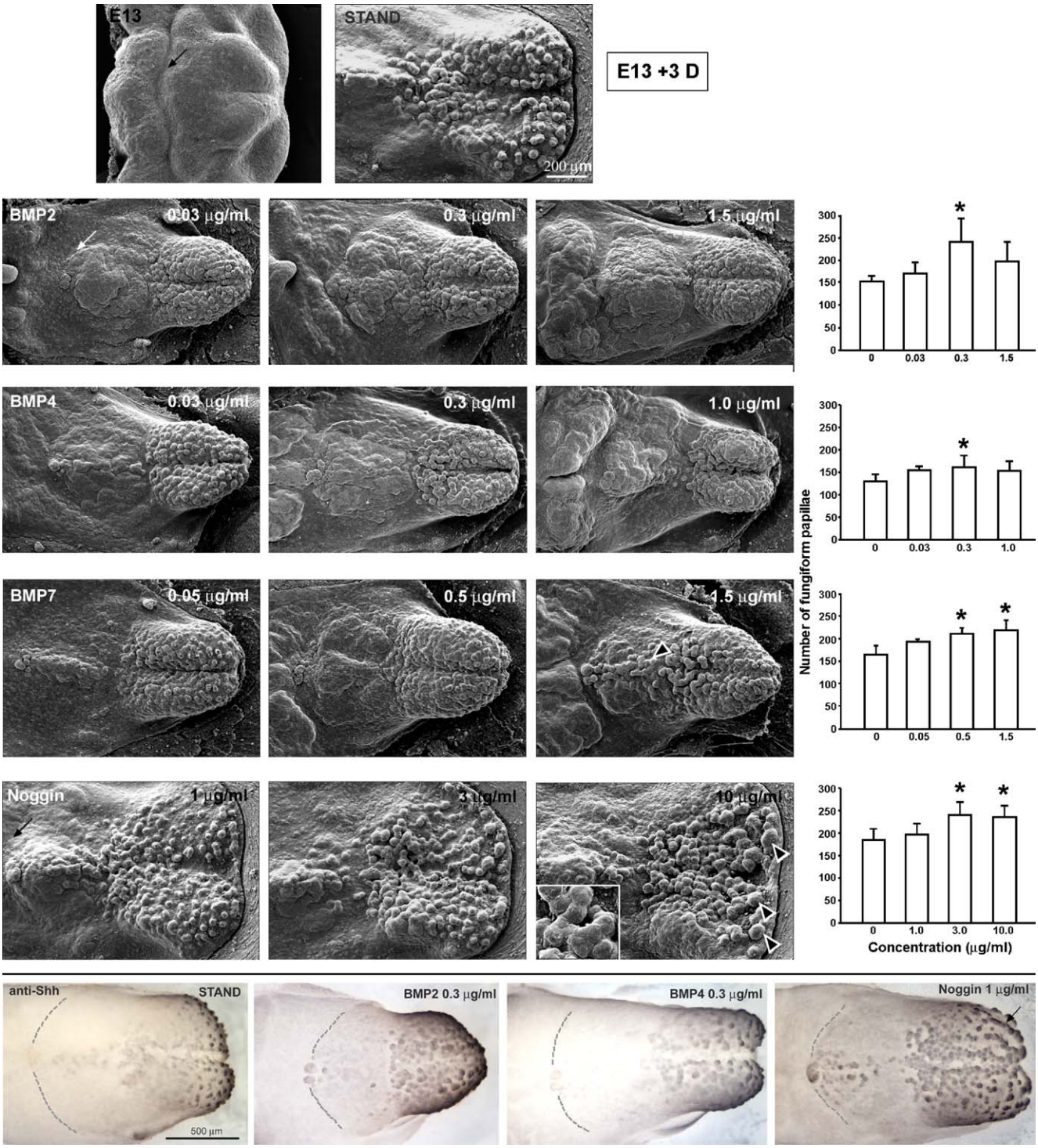
Observations that BMPs can affect fungiform distribution and number on the E13 tongue led to the prediction that the secreted inhibitor of BMP action, noggin, also would affect papilla development. When noggin is added to the medium in E13 cultures, tongue size and shape are not obviously altered in comparison to tongues in standard medium (Fig. 2, compare Noggin tongues with STAND tongue at top). This is very different from the BMP effect that led to smaller, more pointed tongues compared to the STAND condition.

Distinct fungiform papillae form on the E13 tongue cultured with exogenous noggin and the total number of

Fig. 2. Concentration-dependent effects of BMPs and Noggin on initial fungiform papilla formation. E13 + 3 day tongue cultures with standard medium (STAND), or with BMP2, 4, 7 or noggin added to the medium: scanning electron micrographs (top panel) and immunoreactions for Shh protein (bottom panel). Top panel, First row: E13 embryo tongue (left, repeated from Fig. 1) and E13 + 3 day tongue culture in standard medium (right) to demonstrate growth of tongue and development of fungiform papillae on the anterior tongue in culture. Subsequent rows: Tongue cultures with increasing concentrations of BMP2, 4, 7 or noggin, with graphs at far right to quantify papilla number as a function of protein concentration. Asterisks (*) indicate concentrations at which papilla numbers are significantly greater than in standard culture medium (0 concentration). To quantify papillae, counts were only made from scanning electron micrographs, not from immunoreactions. For tongues cultured in BMP2, 4 or 7, tongue length is reduced by about 25% compared to tongues in standard medium. For tongues in noggin, there is no reduction in size. For all BMPs and Noggin, papilla numbers increase with increasing concentration of exogenous protein. In cultures with added BMP7 at the highest concentration, a few papillae develop on the intermolar eminence region (see arrowhead), usually a papilla-free area. With exogenous Noggin, not only are papilla numbers increased on tongue cultures but also, papillae near the tip of the tongue are often very large (see arrowheads in 10 μg/ml photo) and can be fused in clusters or strings of papillae (see inset). Scale bar in STAND tongue culture micrograph at top applies to all culture images. Bottom Panel: Immunohistochemistry for Shh in whole tongues cultured in standard medium (STAND) or with added BMP2, 4 or Noggin. A dashed line on each image indicates the border between oral and pharyngeal tongue. Shh protein, a fungiform papilla marker, is within each fungiform papilla. On the tongue cultured with noggin, fused rows of papillae are seen (arrow). Scale bar applies to all four images.

papillae is increased compared to tongues in STAND medium (Noggin, $F(3,20) = 31.8$, $P < 0.001$) (Fig. 2, Noggin graph on right). The increase in number is concentration-dependent; however, at higher noggin concentrations, many fungiform papillae lose a separate identity and merge with each other to form clusters of fused papillae (Fig. 2, Noggin, inset, fused fungiform papillae).

On all E13 tongue cultures treated with exogenous noggin, we noted very large fungiform papillae on the tongue tip at 3.0 and 10.0 μg/ml (Fig. 2, Noggin, arrowheads). We quantified diameter of the largest discrete papillae in an area that extended 250 μm back from the tongue tip. For papillae in three tongue cultures with 10 μg/ml noggin, average diameter of the largest papillae was 78 μm (SD = 6.4, range = 70 to 90 μm). For papillae in three cultures in standard medium, average diameter of largest papillae was 42 μm (SD = 3.8, range = 37 to 51). Therefore, unusually large papillae emerged on E13 tongue tips in cultures with exogenous noggin,



suggesting an imbalance in a BMP effect that can constrain papilla size.

As noted, tongue size is not altered in tongue cultures treated with noggin, but number of fungiform papillae increases as a function of concentration. Density of papillae therefore is increased, across concentrations, from 344 (standard medium) to 414 papillae per square mm (in 10 μg/ml noggin). It is important to note that this increase of about 20% is attributable solely to the increased number of fungiform papillae on tongue cultures.

Results with E13 cultures demonstrate that adding BMPs or noggin at this stage prevents formation of the inter-papilla spacing, and directs epithelium to a fungiform papilla differentiation program at the expense of papilla-free epithelium. In contrast to BMPs, noggin affects a substantial alteration in formed papilla numbers, size of papillae and papilla fusion, without altering basic tongue growth. Thus, the effects of BMPs versus noggin are distinctly different at E13.

Shh expression retained in all fungiform papillae in E13 tongues exposed to BMP or noggin

To learn whether the additional fungiform papillae that form in the presence of added BMPs or noggin retain the papilla marker, sonic hedgehog (Shh) (Liu et al., 2004), E13 tongue cultures were immunoreacted to identify Shh protein distribution. Shh immunoproduct was apparent in fungiform papillae with standard medium, with exogenous BMP2 or 4, or with noggin (Fig. 2, bottom panel), all in a distribution similar to that of the morphologically identified fungiform papillae in scanning electron micrographs (top panel). With exogenous noggin, fused papillae in longitudinal rows are striking (Fig. 2 bottom, Noggin, arrow).

The Shh immunoproduct in cultures exposed to BMPs or noggin indicates that the additional papillae formed in E13 tongue cultures retain molecular as well as morphological integrity. Furthermore, it is clear that exogenous BMP or noggin does not down-regulate Shh expression in fungiform papillae.

E14 tongue cultures: BMP, noggin and follistatin effects

To test for functional effects of BMPs and noggin on papilla development and patterning from the stage of placode appearance, we added BMPs, noggin or follistatin to embryonic tongues cultured at E14 (see Fig. 1, E14 scanning micrograph).

BMP2, BMP4 or BMP7 reduces fungiform papilla number and increases fungiform papilla spacing on anterior tongue

After 2 days in culture, the E14 tongue has acquired a spatulate shape and distinctive fungiform papillae have formed on the anterior tongue from the characteristic placodes of E14 (Fig. 3, STAND tongues in left column). When exogenous BMP 2, 4 or 7 is added to E14 culture medium, tongue shape generally is similar to that in standard medium although the tip is somewhat more pointed (Fig. 3, BMP2, 4, 7).

In contrast to effects of exogenous BMP seen with E13 cultures, the total number of fungiform papillae is significantly reduced compared to tongues cultured in standard medium (*BMP2*, $F(3,21) = 7.8$, $P = 0.001$; *BMP4*, $F(2,24) = 21.7$, $P < 0.001$; *BMP7*, $F(2,15) = 13.4$, $P = 0.001$) (Fig. 3, graphs). The reduction in papillae is concentration-dependent; however, beyond a maximum dilution for any of the BMPs, there is no incremental increase in extent of papilla reduction (post hoc tests indicate no difference between fungiform papilla numbers at two highest concentrations). Maximum reductions are at about 30% less than control papilla numbers.

Because tongue size is not altered in cultures treated with BMP2, 4 or 7, whereas papilla number is decreased, there is a decrease in density of papillae as a function of BMP concentration. We calculated the change in papilla density (papillae per square mm) for tongues cultured with BMP7, as an example for three BMPs. Across concentrations from 0 to 1.5 μg/ml, density decreased by about 30%. It is important to emphasize, however, that this change in density of papillae relates solely to altered numbers of fungiform papillae while tongue size in culture is not affected.

Results with E14 cultures demonstrate that at the crucial stage of forming fungiform papillae from pre-papilla placodes, BMP2, 4 and 7 can play an inhibitory role in preventing differentiation of papillae. This effect is different from that in cultures initiated at E13.

Combining exogenous BMP2, 4 and 7 does not produce an additive effect on fungiform papilla reduction in tongue cultures

To test whether a combination of 'inhibitory' concentrations of BMP2, 4 and 7 would reduce papilla number to a greater extent than any BMP alone, the BMPs were combined and added to E14 tongue cultures to reach final concentrations of 0.3 μg/ml BMP2; 0.3 μg/ml BMP4; and, 0.5 μg/ml BMP7. Whereas each BMP alone reduced fungiform papillae by as much as 30% compared to tongues in standard medium ($F(4,22) = 14.2$, $P < 0.001$), a cocktail of all three did not further reduce papilla number (Fig. 3 bottom) (with ANOVA and post-tests, papilla number with BMP2 + 4 + 7 is different from that of tongues in standard medium but not different from that for BMP2, or 4 or 7).

Because effects are not additive, this suggests that the BMPs are not acting via independent signaling pathways that could each regulate papilla formation separately and lead to a combinatorial effect. Nor are the different BMPs acting on specific, distinct subsets of fungiform papillae that would be altered in particular ways by each BMP.

Noggin, but not follistatin, increases fungiform papilla number, size and distribution on anterior tongue

Noggin. When the BMP antagonist noggin is added to standard medium of E14 tongue cultures, the number of fungiform papillae increases in a concentration-dependent manner compared to standard tongues (*Noggin*, $F(3,32) = 13.5$, $P < 0.001$) (Fig. 4, top, STAND and Noggin tongues, and graph). Concentrations above 10 μg/ml were not studied, but effects suggest that even at this high dilution, the papilla response has not yet plateaued with a 60% increase in papilla number compared to standard tongues.

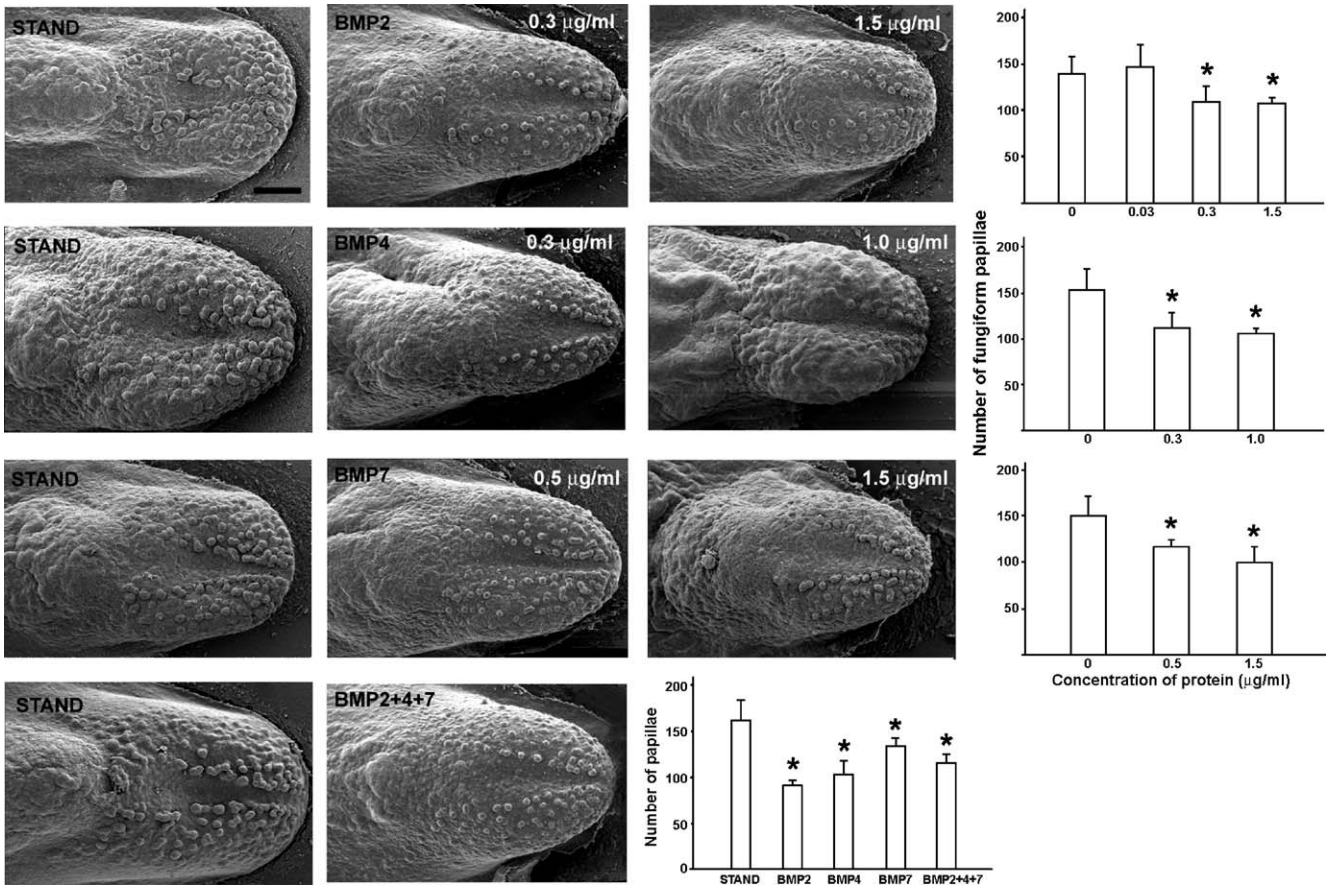


Fig. 3. After placodes have developed, BMP inhibits papilla formation. Scanning electron micrographs of E14 tongues cultured for two days in standard medium (STAND, left figure for each row) or with added BMPs. Graphs on the far right of each row present number of fungiform papillae as a function of protein concentration. Asterisks denote a significant difference from the STAND culture condition (0 concentration). With exogenous BMP2, 4 or 7, number of papillae is reduced on the tongue, in a concentration-dependent manner. When BMP2 + 4 + 7 were added to tongue cultures in a cocktail (bottom row), there was no difference in papilla numbers between any of the BMP groups alone or the BMP cocktail group. Each group was significantly reduced compared to tongues in standard medium. Scale bar in first image = 200 μm, applies to all images.

The effect of noggin is in fungiform papilla regions only, on the anterior two-thirds of the tongue; extra papillae do not form in typically nonpapilla epithelium on posterior tongue, for example in the intermolar eminence. General tongue size and shape are not compromised with exogenous noggin in cultures.

Tongue size is not altered in the presence of exogenous noggin, as noted above, but number of fungiform papillae increases as a function of concentration. Density of papillae therefore is increased, across concentrations, by about 40% in tongues cultured with exogenous noggin compared to standard medium. As in E13 tongue cultures, the increase is statistically significant but again, is attributable solely to the increased number of fungiform papillae on tongue cultures that are not altered in overall size by exogenous noggin.

Our results demonstrate that exogenous noggin can oppose a BMP action that inhibits fungiform papilla formation in the inter-papilla space on the anterior tongue and that the inter-papilla epithelium is competent to form fungiform papillae.

Follistatin. Follistatin, another, structurally distinct, molecule that antagonizes BMP signaling, also was studied in E14 cultures. In contrast to the effects of noggin on fungiform papilla number, exogenous follistatin across a range of concentrations did not alter papilla number or distribution (*Follistatin*, $F(3,22) = 0.3$, $P = 0.82$) (Fig. 4, STAND, Follistatin tongues and graph).

Shh protein, a fungiform papilla marker and regulatory molecule, is retained in all fungiform papillae after BMP or noggin addition to E14 cultures

Because Shh is a marker for developing and formed fungiform papillae, and Shh signaling has a crucial role in regulating fungiform papilla number and pattern (Mistretta et al., 2003; Liu et al., 2004), we used immunoreactions to localize the Shh protein in E14 tongues cultured with exogenous BMP4 or noggin. Whether fungiform papilla numbers are decreased after BMP4 addition to the culture medium, or increased after noggin addition to the medium, Shh protein remains localized in each papilla (Fig. 4, bottom panel, STAND, BMP4, Noggin tongues).

This demonstrates that alterations to BMP signaling, by adding BMPs or antagonizing BMP action, do not eradicate Shh protein expression and localization in fungiform papillae.

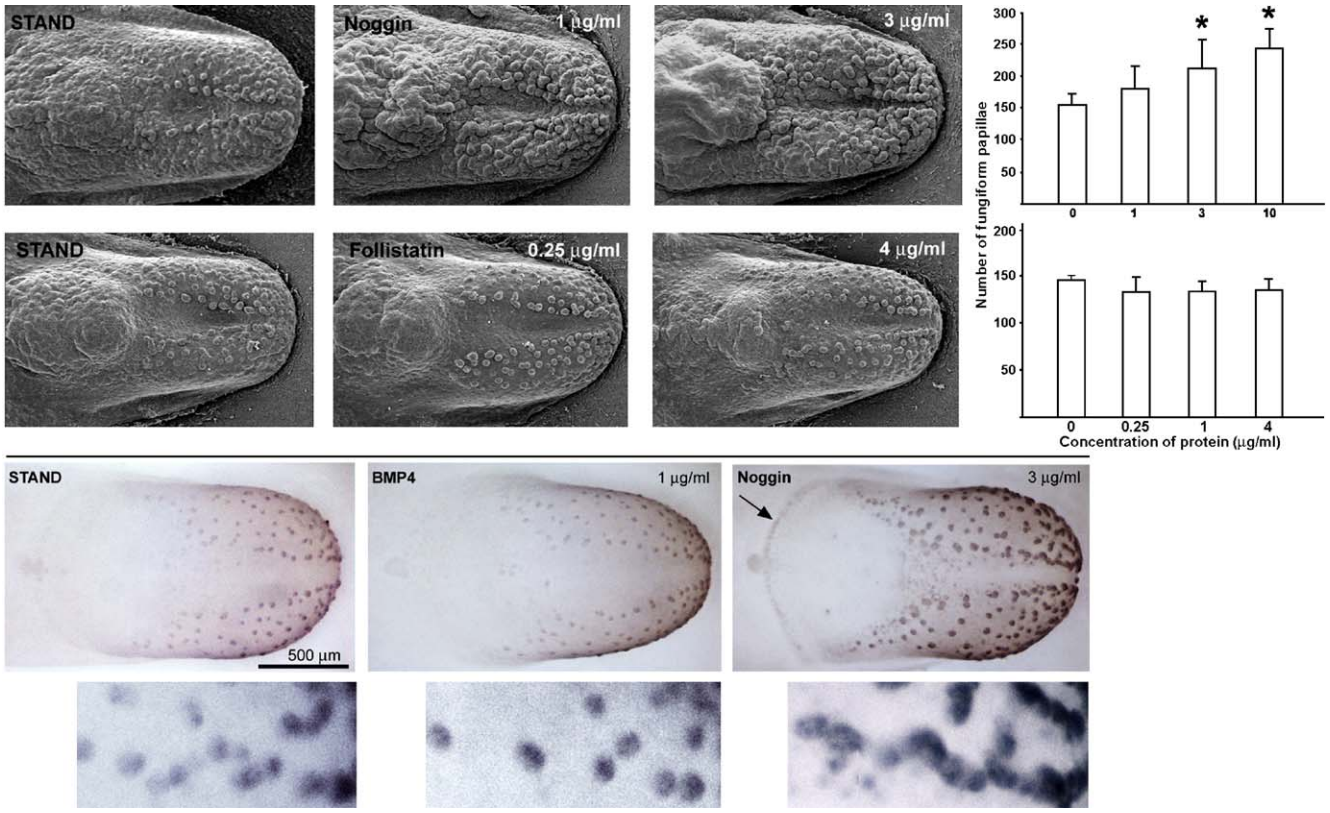


Fig. 4. Top panel: Noggin, not follistatin, effects a large increase in papilla number. Scanning electron micrographs of E14 + 2 day cultures in standard medium (STAND), or with added noggin or follistatin. When noggin is added to E14 tongue cultures, there is a significant increase in number of fungiform papillae, whereas with follistatin there is no difference in number (graphs at right; asterisks denote significance from 0 concentration). To quantify papillae, counts were only made from scanning electron micrographs, not from immunoreactions. Bottom panel: Shh immunoreactions of E14 + 2 day cultures demonstrate that the Shh papilla marker is within each fungiform papilla in STAND, BMP4 or Noggin tongue cultures. For tongues with exogenous Noggin, there is a distinct Shh-immunopositive border demarcating oral and pharyngeal tongue (arrow). Higher power images of immunoreacted tongues demonstrate fused rows of papillae in cultures with exogenous Noggin compared to STAND or BMP tongues (bottom).

Therefore, Shh protein expression within fungiform papillae is not directly dependent on BMP signaling.

Papilla fusion. At high noggin concentrations, fungiform papillae not only are produced in excess numbers, but also merge in linear fusions (Fig. 4, bottom, Noggin). Sonic hedgehog immunoreactions demonstrate papillae in long, linear chains that bracket the median furrow and are not observed in standard tongue cultures or with exogenous BMP (Fig. 4, STAND, BMP4, Noggin tongues and higher power images).

These fusions presumably occur because the BMP function that maintains an inter-papilla space is opposed by excess noggin. In addition, they suggest a papilla-inducing role for noggin. In cultures begun at E13, papilla fusions also are typically observed (Fig. 2, Noggin). In fused papillae, Shh immunoproduct is very intense compared to that in STAND or BMP4 tongues. Furthermore, Shh is apparent in the individual elements that constitute merged papillae in noggin-treated cultures (Fig. 4, Noggin higher power image). A posterior tongue, boundary expression of Shh is also observed, demarcating oral and pharyngeal tongue (Fig. 4, Noggin tongue, arrow). Overall an up-regulation of Shh immunoproduct in noggin-treated tongues is indicated.

Localized application of BMPs or noggin affects surrounding fungiform papilla formation

Although addition of exogenous protein to the standard medium of E14 tongue cultures demonstrated clear effects of BMPs and noggin in papilla development, the full extent of these effects can be muted by the extent of protein access to tissues. Therefore, we used beads to deliver purified proteins to a highly restricted region of the tongue dorsum so that bead release mimics a concentration gradient from a point source in vivo. Our predictions were that if BMPs inhibit papilla formation from placodes at E14, then the BMP bead surround should be papilla-free; on the other hand, the noggin bead surround should have excess fungiform papillae.

With beads soaked in BMP2, 4 or 7, placed in tongues cultured in standard medium, fungiform papilla formation was prevented in the lingual epithelium surrounding the bead, demonstrated with scanning microscopy or with Shh immunoreactions (Fig. 5, left panel, PBS, BMP2, BMP4, BMP7). Beyond the local region of bead release, and on the contralateral side of the tongue, fungiform papillae developed. By comparing the bead surround area to contralateral tongue, we found that formation of 11–16 fungiform papillae was “inhibited”. For reasons not fully understood, more papillae were inhibited anterior to bead location than posterior.

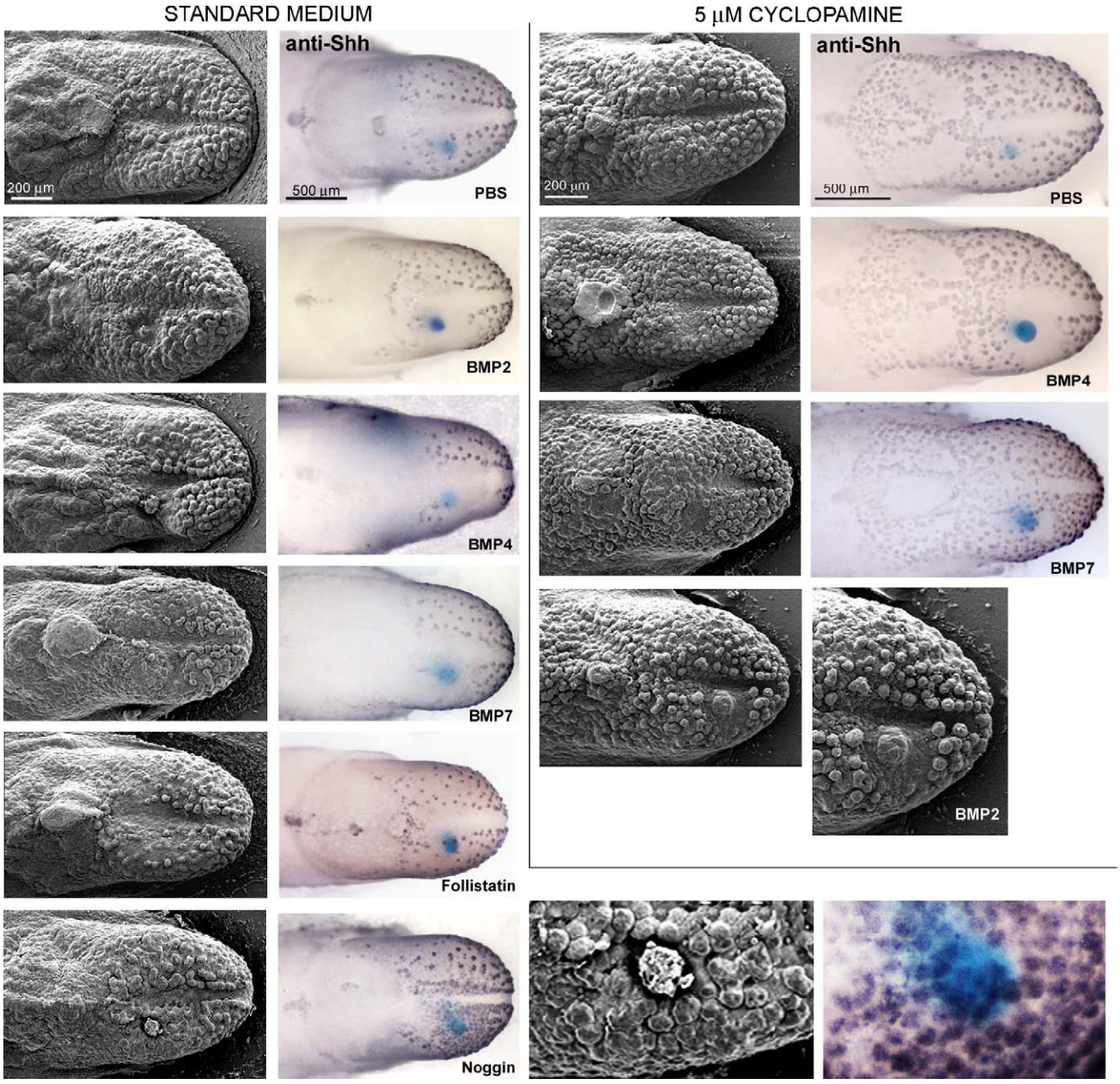


Fig. 5. Effects of localized presentation of BMPs and Noggin, and interactions with Shh signaling. E14 + 2 day tongue cultures in standard medium (left panel) or in medium with cyclopamine (right panel), with blue beads soaked in PBS, BMPs, Noggin or Follistatin placed in one side of the anterior tongue. For each panel, data from scanning electron micrographs and whole tongue immunoreactions for Shh protein are presented. Left panel: in the surround of beads soaked in BMP2, 4 or 7, fungiform papillae do not develop, in comparison to the contralateral tongue or tongues with PBS beads. About 16 papillae are inhibited in the BMP bead surrounds. With follistatin beads, there is no effect on papillae in the bead surround, compared to contralateral tongue. In contrast, multiple papillae form in the surround of beads soaked in Noggin (see inset). Insets at the bottom right of this panel emphasize the nature of fungiform papilla formation in clusters (scanning electron micrograph, Noggin bead, left) and the multiple, Shh-positive dots in a comparable area from the immunoreacted tongue (whole tongue immunoreaction, Noggin bead, right). The scale bars in top images apply to all images in the left panel. Right panel: tongues cultured in cyclopamine, to interfere with Shh signaling, have increased numbers of fungiform papillae on the anterior tongue compared to control cultures, and papillae on the intermolar eminence. When BMP4, 7 or 2 beads were inserted in the tongue, papilla formation in the bead surround was inhibited compared to the contralateral tongue or to cultures with a PBS bead. The scale bars at top apply to all images in the right panel, except bottom right.

Implanted beads soaked in follistatin had no effect on fungiform papilla distribution and were comparable to PBS beads (Fig. 5, left panel, Follistatin). However, in striking contrast to BMP or follistatin beads, around noggin-soaked beads, fungiform papillae formed at high density (Fig. 5, left panel, Noggin tongue). The papillae were large and sometimes fused (Fig. 5, Noggin, SEM and inset), and there were multiple points of Shh expression around noggin beads (Fig. 5, anti-Shh, Noggin tongue and inset). The Shh expression “dots”, about 100 in the bead surround, presumably indicate individual papillae within a larger fused cluster.

The point source application of BMP2, 4 or 7 demonstrates a potent inhibitory effect that declines with distance from the

bead. In contrast, noggin can induce formation of fused papillae with very large numbers of Shh-positive centers.

Shh signaling disruption and BMP inhibition of papilla formation

To learn whether Shh signal disruption would interfere with BMP effects in preventing fungiform papilla formation, E14 tongues were cultured with added cyclopamine or jervine. Cyclopamine and jervine are steroidal alkaloids that specifically disrupt hedgehog signaling and radically alter fungiform papilla patterning in embryonic tongue cultures (Mistretta et al., 2003; Liu et al., 2004).

After beads soaked in PBS, or in BMP 2, 4 or 7 were placed in dorsal tongue tissue, tongue cultures were maintained for 2 days with cyclopamine or jervine in the medium. As a result of Shh signal disruption, fungiform papillae form in increased numbers on anterior tongue and in large numbers on atypical posterior tongue locations in cultures with cyclopamine or jervine (Fig. 5, right panel, PBS bead; note that only cyclopamine tongues are illustrated), as previously reported (Mistretta et al., 2003). However, in the lingual tissue immediately surrounding BMP4, 2 or 7 impregnated beads, no papillae formed (Fig. 5, right panel, BMP4, 2 and 7).

The area of the BMP bead surround that was free of fungiform papillae could accommodate up to about 16 fungiform papillae (Fig. 5, right panel, BMP4 and 2 beads). This illustrates the potency of BMP in inhibiting fungiform papilla formation, even with addition of cyclopamine to tongue cultures. Yet beyond the apparent reach of bead diffusion, papillae developed in a distribution comparable to the contralateral tongue.

Our results demonstrate that BMPs can restrict fungiform papilla formation even in the absence of intact Shh signaling, and again suggest the independence of these pathways.

Cell proliferation effects of BMP4 and noggin

Cell proliferation of lingual tissue in the vicinity of PBS, BMP4 or noggin beads was assessed with Ki67 immunoreactions. Lingual tissue near PBS beads had numerous proliferating cells in the inter-papilla epithelium and in mesenchyme of E14 cultures (Fig. 6, PBS bead). Within the raised fungiform papilla per se, there was evidence of reduced cell proliferation (Fig. 6, PBS, arrows).

In epithelium and mesenchyme on top of BMP4 soaked beads, Ki67 immunoproduct was much reduced, whereas the nearby tissue had numerous proliferating cells (Fig. 6, BMP4). In contrast, cell proliferation was increased in tissue on top of noggin beads (Fig. 6, Noggin), compared to tissue near either PBS or BMP4 beads, and the epithelium near noggin beads was generally thicker compared to that near PBS beads. The large,

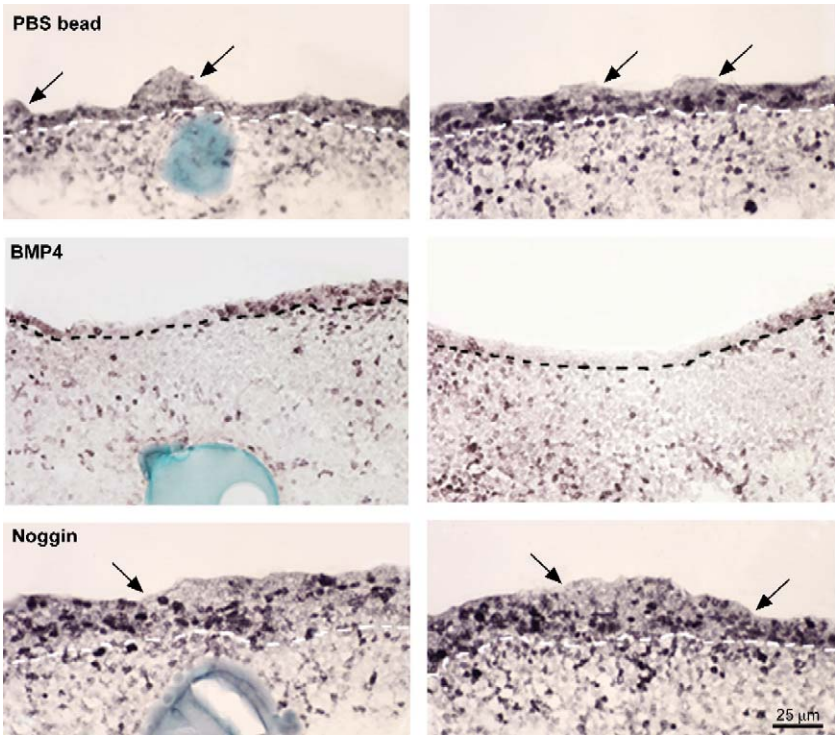


Fig. 6. BMP and Noggin: opposing effects on proliferation in lingual epithelium. Sections through tongue cultures maintained in standard medium, with beads soaked in PBS, BMP4 or Noggin inserted into anterior tongue, and immunoreacted for Ki67 to label proliferating cells. Sections on the left are directly through the bead region (portion of beads seen in blue) and on the right are near the bead region. In all images, a white or black dashed line is used to demarcate the epithelium. Fungiform papillae (arrows) in sections at or near PBS beads have fewer proliferating cells compared to the inter-papilla epithelium. Near BMP4 beads papillae do not form, and the lingual epithelium in thinner than that near PBS beads seen in images above. In the BMP4 bead surround, there are virtually no proliferating epithelial cells. Near Noggin beads the epithelium is much thicker than near PBS beads and multiple Ki67-positive cells are seen. In addition, the large, fused papilla complexes observed in noggin cultures are evident (bracketed by arrows).

fused papillae near noggin beads had evidence of numerous proliferating cells (Fig. 6, Noggin, arrows).

These data suggest that BMPs are inhibiting fungiform papillae through functions that include reduced cell proliferation and perhaps increased cell death (although our data cannot directly address the latter possibility). In contrast, noggin apparently triggers increased cell proliferation and differentiation, and may thereby contribute to production of large, fused papilla clusters in cultures.

Discussion

In a detailed study to discern roles for BMPs and BMP antagonists in taste papilla development, we show that BMP2, 4 and 7, known to function in feather, hair and tooth development (Jung et al., 1998; Noramly and Morgan, 1998; Botchkarev et al., 1999; Bardot et al., 2004; Wang et al., 2004), have stage-specific roles in fungiform papilla formation and patterning. At a stage before papilla placode formation, exogenous BMPs increase the number of fungiform papillae that form in embryonic tongue culture. However, when added to cultures at a stage just after papilla placodes have formed, the BMPs inhibit fungiform papilla formation. The BMP inhibitory effect on fungiform papilla formation is not prevented by disrupting Shh signaling. In contrast, noggin, a BMP antagonist, can apparently act as an "activator" to induce papillae. When added to cultures at stages before or after placode formation, noggin results in substantial increases in papilla number and in fused papilla formations. Another structurally different BMP antagonist, follistatin, does not affect papilla development. BMPs and noggin alter cell proliferation in embryonic tongue epithelium and papillae, in opposing ways. We propose that the BMPs and noggin, co-localized within fungiform papilla placodes and the papillae per se, have balanced roles that maintain stereotypic papilla numbers in linear patterns. The BMPs can have a powerful inhibitory effect that must be neutralized by antagonists for papilla development to proceed appropriately.

BMPs promote extra fungiform papilla formation at early stages, but inhibit papilla development from placodes at later stages

The progressive localization of BMP and noggin protein distribution to fungiform papillae suggests roles in placode and papilla formation. However, the diffuse distribution of BMPs and noggin protein across the relatively homogenous tongue epithelium at E13 versus the pronounced restriction in papilla placodes at E14 and within papillae at E15, predicts that these proteins would have different roles in papilla development at E13 versus E14. A diffuse distribution of BMP2 and 4 mRNAs in embryonic mouse tongue and subsequent localization to fungiform papillae has been noted previously (Jung et al., 1999). Other in situ data refuted an initial diffuse distribution for BMP4 (Hall et al., 2003). However, as predicted by our results on protein expression, we indeed found distinctly different effects of exogenous BMPs in cultures begun at E13 versus E14.

At E13, when the tongue epithelium is histologically and topographically homogeneous, and BMPs are diffusely distributed in anterior tongue epithelium, exogenous BMP2, 4 or 7 stimulates production of additional fungiform papillae on the anterior tongue. This contrasts with BMP effects at later stages, which are inhibitory for papilla development. In tongue cultures begun at E14, BMPs are localized within broad patches corresponding to the pre-papilla placodes in the epithelium. At this stage, BMP2, 4 or 7 inhibits production of fungiform papillae, so that papilla number is reduced by about 30%. The effects are concentration-dependent and reach a plateau at high protein dilutions.

BMPs also have distinctive and opposing effects at different stages in development of the pancreas, and can induce a pancreas identity early, but repress pancreas identity in favor of liver at later stages (Kumar and Melton, 2003). In chick, BMP2 beads applied to skin at early stages of development (stages 17–22) promote feather formation (Scaal et al., 2002) and BMP7 also is necessary for feather placode formation (Harris et al., 2004). However, BMP overexpression at later stages (around stage 29) suppresses feather bud formation (Jung et al., 1998; Noramly and Morgan, 1998; Bardot et al., 2004). Thus, although there is not a single, comparable developmental study to address effects of three BMP molecules at two very different stages of organogenesis, as in our current report, it is clear from synthesizing the literature that BMP effects are stage-specific in other organ systems.

The BMP inhibition of feather bud formation is effective over several cell diameters (Bardot et al., 2004). In our results from BMP4 bead placement, it is apparent that BMP can act over a large area to totally prevent papilla formation, encompassing a region where about 16 fungiform papillae form in the presence of control beads. Similarly, BMP4 overexpression resulted in large areas without feather buds in chick skin (Bardot et al., 2004). Other inhibitory roles for BMPs in organogenesis include the suppression of hair follicle development in embryonic mouse skin culture (Botchkarev et al., 1999). Exogenous BMP4 inhibits branching in submandibular salivary gland development in vitro, whereas BMP7 increases number of terminal buds (Hoffman et al., 2002).

BMP2, 4 and 7 have quantitatively similar effects on fungiform papilla inhibition. The molecular and inhibitory actions in feather formation also were similar for these BMPs (Patel et al., 1999). We tested the hypothesis that BMP2, 4 and 7 could have additive or synergistic effects in inhibiting papilla formation by adding all three BMPs at concentrations that were effective alone to E14 tongue cultures. The combined effect was not greater than that of any individual BMP. This could be attributed to a ceiling for BMP effects at this stage. The data suggest that these BMPs do not regulate aspects of papilla development separately nor are there subsets of papillae that are distinctly altered by BMPs.

Our data provide a direct demonstration for BMP effects at two distinct stages of taste papilla development. Before placodes even appear on the forming tongue, exogenous BMP2, 4 or 7 can effect an increased number of fungiform

papillae. This indicates that BMPs are key molecules in pathways that act to form papilla placodes and subsequent papillae on the developing tongue. Prior studies in our laboratory have shown that all anterior dorsal tongue epithelium, other than that of the midline furrow, is competent to form papillae (Mistretta et al., 2003; Liu et al., 2004). At early developmental stages, exogenous BMPs at high concentrations may over-ride the native sequestration of BMPs into the papilla placodes and thereby prevent interactions to shape papilla and inter-papilla fates in the tongue. However, once papilla placodes have formed on the spatulate tongue, BMPs inhibit papilla development so that substantially reduced numbers of fungiform papillae form. High concentrations of BMPs in vitro prevent adoption of a papilla fate for many placodes that already are on the way to papilla differentiation. The inhibitory BMP effect can mediate the spacing that must arise to establish between-papilla distances and regulate papilla pattern.

The BMP antagonist noggin, but not follistatin, stimulates production of supernumerary fungiform papillae at E13 and at E14

There are various secreted BMP antagonists that belong to structurally and functionally distinct protein families and can prevent ligand binding to BMP receptors (reviewed in Botchkarev, 2003). These antagonists include noggin and follistatin. Noggin binds BMP2 and 4 with an affinity that is 10–15 times higher than BMP receptors and also binds BMP7 with somewhat lower affinity. Noggin blocks BMP activity by binding ligand epitopes for both type I and II BMP receptors (Chen et al., 2004). On the other hand, follistatin binds activins, another family within the TGF-β superfamily, with high affinity. Activin effects on tissues are distinct from those of BMPs. Follistatin also can bind BMP2, 4 and 7 but with lower affinity than for activin (Balemans and Van Hul, 2002). We tested effects of both noggin and follistatin in fungiform papilla development.

With addition of noggin to culture medium, numbers of fungiform papillae are substantially increased relative to tongues in standard medium, by about 60%, in E13 or E14 cultures. Each additional papilla contains the fungiform papilla marker, Shh. With noggin bead placement in E14 cultures, very large, sometimes fused papillae formed in the bead vicinity. Thus, exogenous noggin apparently biases the lingual epithelium away from an inter-papilla fate and toward a papilla fate. Neutralization of the inhibitory activity of BMP2, 4 and 7 by noggin may be an important mechanism in development of papillae from placodes. In similar results, noggin-soaked beads in embryonic mouse skin cultures induced hair follicles at four times that in controls (Botchkarev et al., 1999). In contrast, BMP4 beads eliminated hair follicle formation.

Although with exogenous noggin, the fungiform papillae are increased in number on the anterior tongue to an extent that essentially eradicates the inter-papilla space, no papillae form on the intermolar eminence. Nor is Shh expression induced on the intermolar eminence with added noggin. The intermolar eminence *is competent* to support fungiform papillae as demonstrated when cyclopamine is used to disrupt Shh signaling in tongue cultures (Mistretta et al., 2003). Because BMP immunoproduct is not seen in the intermolar eminence, we propose that noggin cannot act in the absence of BMPs. This is supported by results from noggin overexpression in chick where no noggin effects were found outside of the BMP expression zones of the feather fields (Noramly and Morgan, 1998). Our data also indicate that BMP signaling is not essential for establishing or maintaining the intermolar eminence as a papilla-free region.

In contrast to noggin, there were no effects on fungiform papilla development found with exogenous follistatin. Very different effects of noggin and follistatin also have been reported in neural progenitor cell development in chick (Liem et al., 2000). Furthermore, for antagonizing BMP effects in ameloblast differentiation in rodent incisors, follistatin and noggin have very different effects (Wang et al., 2004). Wang et al. (2004) refer to the different molecular actions of noggin and follistatin, indicating that the latter does not limit BMP access to its receptors as noggin does (Iemura et al., 1998). Other differences between noggin and follistatin, cited above, may well account for varying functional effects.

Papilla fusions with exogenous noggin

Noggin not only increases the number of fungiform papillae in tongue culture, but also, the supernumerary papillae fuse to adjacent fungiform papillae within rows. With noggin bead placement in anterior tongue, large papilla clusters develop in bead proximity and a remarkable superabundance of Shh immunopositive spots is seen around the bead. It seems that the larger papillae observed with scanning microscopy are clusters of smaller papillae, each labeled with Shh in the papilla center. This is similar to chick embryo skin when forced expression of noggin led to fusion of several feather buds into a single large region expressing placodal molecular markers (Noramly and Morgan, 1998). Another BMP antagonist, Drm/Gremlin, also induces fusion of feather buds when overexpressed in chick embryo skin (Bardot et al., 2004). In a study of gap junction proteins in embryonic mouse tongue, some alteration in Shh-positive "spots" was noted after noggin bead placement in cultures (Kim et al., 2005). The dramatic effect that we see, similar to results in feather formation, demonstrates a potent action of noggin in fungiform papilla induction.

The large number of Shh-positive foci that form in the noggin bead vicinity suggests that noggin can indeed induce a papilla molecular phenotype and also can up-regulate Shh expression in the embryonic tongue. In the hair follicle cycle, Shh is up-regulated after noggin treatment (Botchkarev et al., 2001). A role for noggin in neutralizing BMPs and as an upstream modulator of Shh is proposed for hair follicle growth. On the other hand, the distribution of Shh in epithelium of enlarged or fused fungiform papillae could be simply the consequence of the phenotype induced, not the specific up-

regulation of genes by noggin, as discussed for feather patterning by Bardot et al. (2004).

Stage-specific effects for BMPs, not noggin

As discussed above, the effects of BMPs are to increase the number of fungiform papillae in tongue cultures begun at E13, but to decrease papillae in E14 cultures. Furthermore, tongue size and shape are altered at E13 but not at E14. On the other hand, effects of noggin are to increase fungiform papilla numbers in both E13 and E14 cultures, while tongue size and shape are not altered relative to standard tongue culture conditions.

In considering BMP effects on E13 tongue size, it is important to note that, in cultures started at E13, the tongue is still a set of somewhat discrete tissue swellings (Fig. 1, SEMs; Liu et al., 2004). By E14 the spatulate tongue presents with structural integrity without obvious separate components. The E13 tongue, then, is likely to be more vulnerable to morphological disruption than the tongue at E14. In our previous study of Shh signaling in papilla development from E12 through E18, we reported that Shh signal disruption at E13 alters tongue size and shape compared to standard tongue cultures, whereas no such tongue alterations occur with E14 cultures (Liu et al., 2004). The current report adds to our previous publications to show that Shh and BMPs can affect formation of the tongue itself, whereas noggin and follistatin do not.

The differing effects of BMPs on papilla number at E13 and E14, while the antagonist noggin increases papillae beyond numbers that form in standard tongue culture at both stages, require further research to understand mechanisms. As discussed above, proliferative effects for BMPs in early tissues (Scaal et al., 2002; Amthor et al., 1999) versus anti-proliferative or apoptotic effects at later stages (Botchkarev, 2003) are reported in other systems. But it is not clear how both BMPs and their antagonist, noggin, would lead to increased papilla numbers at E13.

In E13 cultures, the tongue begins with an immature, relatively homogeneous epithelium that will progress to form papilla placodes and papillae during 2 days in culture. Furthermore, while papilla formation is progressing in the context of exogenous signals, endogenous BMPs and noggin are redistributing from a diffuse distribution to foci that constitute the papilla placodes and then, papillae (Fig. 1). There is, therefore, a very complex situation that obtains in papilla formation in cultures. For E14 cultures, the epithelium contains pre-papilla placodes in which BMPs and noggin are co-localized. The starting point relative to papilla formation is entirely different from that at E13. Thus, it is likely that pre-papilla and papilla placode cells in E13 versus E14 tongue epithelium will have different response thresholds to various factors and that the shift in a given molecular balance after adding exogenous factors will be very different at these two developmental stages.

In other developmental studies, BMP reportedly can repress its own transcription and on the other hand, can up-regulate expression of antagonists (Jung et al., 1998; Amthor et al., 1999; Patel et al., 1999). The stage-specific and cell context effects of these and other aspects of BMP and noggin signaling are consistently noted in the literature (Botchkarev, 2003). Our E13 BMP data are not at odds with data on very early feather bud formation (Scaal et al., 2002; Harris et al., 2004). However, how the BMPs, noggin and downstream effectors are interacting in the E13 versus E14 tongue simply is not known. The possibility that noggin can act independently to some extent, not just as an antagonist to BMP, cannot be excluded. Proposing a model to speak to these stage differences would be highly speculative at this point; but the data are reliable and we are pursuing experiments to understand BMP and noggin effects on other molecules at E13.

BMPs, noggin and Shh signaling

We know that Shh protein (Mistretta et al., 2003), and BMP2, 4 and 7 and noggin all are localized within both the prepapilla placodes and formed fungiform papillae in rodent embryos. Hall et al. (2003) noted the colocalization of BMP4 and Shh mRNAs in developing mouse fungiform papillae and suggested there may be interactions. In the present experiments, the molecular marker, Shh, is expressed in all fungiform papillae that remain after exogenous BMP in tongue cultures and is in all supernumerary papillae that form with noggin. Thus, BMPs do not eliminate Shh protein expression, whereas there is some indication that Shh expression in tongue and fungiform papillae may be up-regulated after exogenous noggin.

Disrupting the Shh signaling pathway with the alkaloids cyclopamine or jervine, which leads to doubling of fungiform papilla number, does not prevent the inhibition of papilla formation by BMPs in tongue culture. This suggests that BMP signaling is not dependent on intact Shh signaling in inhibiting papilla formation. These signaling pathways can function independently, at least in some aspects.

In chick embryo, Shh and BMP signaling have antagonistic roles in regulating neural progenitor fate in the neural tube (Liem et al., 2000). The BMPs apparently act downstream to Shh, but on the other hand BMPs can alter Shh action. Zhang et al. (2000) reported that excess BMP4 can repress Shh expression in developing tooth epithelium. In our tongue cultures, both Shh expression and fungiform papillae are inhibited in the surround of BMP-soaked beads. It is not clear whether Shh is reduced because fungiform papillae are not present, or fungiform papillae are inhibited because Shh is repressed. Our data certainly encompass a potential for interactions among Shh, BMPs and noggin, which all are within developing fungiform placodes and papillae, but clarifying the nature of these integrative roles requires further study.

Cell proliferation and BMP, noggin effects

In the surround of beads implanted in E14 tongues, we found reduced epithelial thickness and proliferation with BMP4, and much increased epithelial thickness and cell proliferation with noggin. The difference in epithelial thickness between cultures in exogenous BMP or noggin, and in relation to controls, is profound. In embryonic lung epithelium, BMP4 overexpression also inhibits epithelial cell proliferation and in fact promotes cell death (Bellusci et al., 1996). The surround of beads soaked in BMP4 had a dramatic reduction in thickness of embryonic mouse epidermis compared with controls, whereas with noggin

beads there was an increase in epidermal thickness and cell proliferation (Botchkarev et al., 1999); the treatment with BMP beads was accompanied by an increase in apoptotic cells in developing hair follicles. In contrast, in transgenic mice that overexpress noggin, superabundant crypts formed in the intestine relative to wild type animals, and Ki67 positive cells were numerous in these extra-invaginations (Haramis et al., 2004). Local expression of noggin can prevent BMP-induced apoptosis in other systems (Botchkarev, 2003). We have not evaluated cell death in fungiform papillae and epithelium in the surround of BMP4 or noggin beads. Noggin may directly stimulate proliferation in lingual epithelium and prevent BMP-induced apoptosis.

Summary

The first morphological indication of the fungiform papilla site is a collection of epithelial cells that makes a local thickening of the epithelium, called the placode (Mistretta, 1998). Because a molecular restriction in BMP and noggin distributions takes place as the lingual epithelium differentiates to acquire the pre-papilla placodes, from E13–E14, it is probable that these molecules participate in establishing the initial papilla pattern. Our demonstration that exogenous BMP in E13 cultures leads to an increased number of papillae, compared to tongues in standard culture, reinforces the idea of BMP as an initiator of papilla patterning.

As papilla development progresses, BMP and noggin co-localize within the pre-papilla placodes and fungiform papillae. The highest concentrations of BMP and noggin are within placodes or within fungiform papillae; between papillae, protein immunoproducts are much weaker. In contrast to E13 cultures, in cultures begun at a stage after the pre-papilla placodes have appeared on the tongue (E14), exogenous BMP prevents formation of the native number of papilla.

Our E14 data are consistent with a model in which BMPs inhibit fungiform papilla formation in inter-papilla epithelium while noggin counteracts BMP activity *within* the fungiform papilla (Fig. 7). In this model, BMP diffuses to act as an inhibitor of fungiform papillae in the surrounding inter-papilla space. However, BMP does not inhibit formation of the papilla where it is localized, because intra-papilla BMP is antagonized by noggin. Noggin has much higher affinity binding to BMP receptors than the BMPs have (Botchkarev, 2003). Furthermore, because noggin also has a strong affinity for heparan sulfate proteoglycans at cell surfaces, while remaining active in BMP neutralization (Paine-Saunders et al., 2002), we propose that noggin action is confined primarily within papillae. Therefore, the BMPs can diffuse further than noggin and thereby inhibit fungiform papilla formation in inter-papilla epithelium. Overall, our results imply that for the full complement of fungiform papillae to form on the native embryonic tongue, BMP signaling must be suppressed or limited to some extent and a balance of BMP and noggin must obtain. Ratios of BMP and the antagonist become important also in determining papilla size, seen for example in the larger fungiform papillae that develop in the presence of increased noggin.

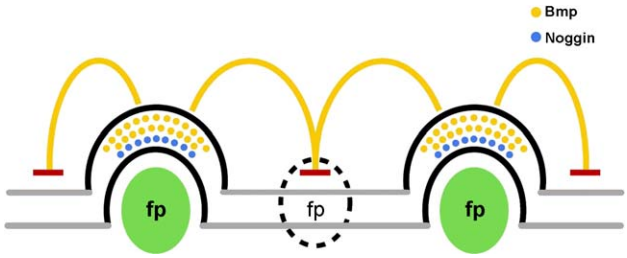


Fig. 7. Model for proposed BMP and Noggin actions in E14 tongue cultures, during the time of papilla formation from the pre-papilla placodes. BMP molecule is in yellow, noggin is in blue. Fungiform papillae (fp) that have formed are represented in green, whereas an inhibited fp is within a hatched surround. The papilla epithelium, black, is distinguished from the inter-papilla epithelium, gray. In the model, BMPs within fungiform papillae (fp) are secreted to inhibit fp development in the inter-papilla epithelium. Within the fungiform papillae per se, BMP inhibitory effects are antagonized by noggin. Noggin is "tethered" within fps through binding with proteoglycans in the basal lamina region, and therefore is not secreted to the inter-papilla epithelium as widely as the BMPs.

For organ patterns to emerge, a system must include inducing molecules to form organs, and inhibiting molecules to develop spacing between organs (Meinhardt and Gierer, 2000; Jiang et al., 2004). Spatial patterns require an activation or induction effect at one location and inhibition in the surround. In such patterning models, based on reaction-diffusion theory, a structure can inhibit the same structure in the surround without inhibiting itself (Meinhardt and Gierer, 2000). A balance of activator and inhibitor molecules is required and both molecules may be expressed in the same structure. In feather bud formation, Jiang et al. (2004) use reaction-diffusion principles in illustrating pattern formation through presence of both activator and inhibitor molecules within the bud. A zone of "lateral inhibition" occurs around each forming bud.

In fungiform papilla development, BMPs and noggin apparently direct lingual epithelial cells toward an inter-papilla or papilla fate, respectively. From our prior work, we know that the between-papilla epithelium is competent to form fungiform papillae. Thus, if noggin predominates over BMPs, superabundant papillae form on the embryonic tongue leaving little inter-papilla space. The opposing actions of BMPs and the antagonist, noggin, can regulate fungiform papilla development and spatial arrangements in rows.

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