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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