Developmental patterning of the circumvallate papilla

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ABSTRACT Organogenesis is regulated by the sequential and reciprocal interactions between epithelial and mesenchymal tissues. Many molecules, including growth factors, transcription factors, extracellular matrices, cell surface receptors, and matrix degrading enzymes, have been found to be involved in this process. To investigate the molecular mechanism responsible for morphogenesis of the circumvallate papilla/von Ebner’s gland complex, we examined the expression patterns of selected cell adhesion molecules, extracellular matrix molecules, innervation and cell division in the circumvallate papilla of mouse embryos from embryonic day 11.5 (E11.5) to E14. At E11.5-E13.5, the lingual epithelium, the site where the circumvallate papilla will develop, is negative for BrdU labeling. At E14-E15, we detected cell division in the papillary area, especially in the epithelial invagination where von Ebner’s minor salivary gland will form. The basement membrane component, laminin, is expressed as a continuous thin line separating the epithelia from the underlying mesenchyme, but it is broadly and strongly expressed in the area where the nerve fibers penetrate into the circumvallate papilla. At the E12-E12.5 stage of development, the epithelial thickening shows intense E-cadherin staining in the superficial and basal layers, but weak E-cadherin staining in the suprabasal layer. E-cadherin is strongly expressed, but appears dispersed among the basal layer of lingual epithelium, the site where nerve fibers will innervate. At E13, nerve fibers reach the circumvallate papilla. These nerve fibers penetrate into and split the epithelial cell mass into two stalks which will later differentiate to form the von Ebner’s gland. These results suggest that 1) the formation of the circumvallate papilla does not initially depend on cell division, 2) cell migration likely plays a major role during circumvallate placode formation, 3) E-cadherin and laminin may play a role in nerve guidance and 4) innervation impacts the final morphogenesis of the circumvallate papilla.

KEY WORDS: circumvallate papilla, E-cadherin, laminin, PGP 9.5, morphogenesis

Introduction

Morphogenesis and cytodifferentiation in developing vertebrate organs is controlled by the sequential and reciprocal interactions between epithelial and mesenchymal tissues. During the early stage of morphogenesis, many organs share common morphological features that begin with the thickening of an epithelial sheet, followed by invagination of the epithelium into the underlying mesenchyme. With advancing morphogenesis and cytodifferentiation, such as folding and/or branching of epithelia or secretion of cell specific products, epithelial and mesenchymal interaction is critical for mediating the signal(s) exchanged during each developmental process. Ultimately, these interaction(s) will give rise to different types of organs, such as hair/feather, scale, tooth, limb, kidney, and glands (Chuong, 1993; Jernvall and Thesleff, 2000; Warburton et al., 2000).

The tongue is an organ that is subject to pattern formation with unique rostral-caudal and dorsal-ventral characteristics, as well as striking morphogenetic identity (Jung et al., 1999). Gustatory papillae are one of the specialized and complex structures found on the dorsal surface of the tongue. Due to the presence of taste buds, the epithelia of the gustatory papillae are distinguished from adjacent tongue epithelia. Taste buds contain sensory cells for the chemical sense of taste and require innervation to convey sensory information to the central nervous system. There are three types of gustatory papillae in mammals distributed in a rostral-caudal axis. The fungiform papillae are distributed on the anterior two-thirds of the tongue, the foliate papillae are found at the junction of the anterior two-thirds and the posterior one-third of the tongue, and the circumvallate papillae are found in the posterior one-third of the tongue. The molecular mechanism that directs the rostral-caudal axis and hence directs papilla formation, growth, and maintenance is not well understood (Jung et al., 1999).

Abbreviations used in this paper: BrdU, bromodeoxyuridine; CVP, circumvallate papilla; E, embryonic; ECM, extracellular matrix; VEG, von Ebner’s gland.

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The circumvallate papillae (CVP) differ from other gustatory papilla. They contain progenitor cells that may differentiate into taste buds, and into serous cells in a tubulo-acinar salivary gland, von Ebners' gland (VEG). Because of this unique developmental pathway, the organ provides an insightful model for the study of patterning and cell-differentiation during organogenesis. CVP number varies across species; 9-13 in human, 1 in mouse and rat, and 2-3 in rabbit, pig and horse (Spielman and Brand, 1995). Mouse CVP forms initially from the thickening of dorsal epithelia in the middle of the posterior one-third of the tongue between embryonic day-11 and -12 (E11 and E12). This placode will later be innervated bilaterally by the nerve fibers from the glossopharyngeal nerve and acquire a mesenchymal core forming a "dome-shaped" structure with two adjacent epithelial stalks. These two stalks invaginate into the underlying mesenchyme, and give rise to the von Ebner's minor salivary gland. The sequence of developmental events from the earliest placode formation, through cytodifferentiation, to finally forming the adult structure, is similar to other placode-derived structures such as hair, teeth and whiskers (Iseki et al., 1996; Thesleff et al., 1996). However, to undergo terminal cytodifferentiation to form taste cells responsive to chemical stimuli, the gustatory papillae requires innervation that other placode-dependent organs do not (Thesleff et al., 1990; Farbman and Mbiene, 1991; Oakley, 1993; Oakley et al., 1993; Mbiene et al., 1997; Morris-Wiman et al., 1999).

In this study we correlate the expression pattern of cell adhesion molecule and extracellular matrix molecule with the changes in CVP morphology and innervation during CVP initiation and morphogenesis. These investigations are an initial step towards the understanding of regulatory roles of cell-cell and cell-matrix interactions during CVP development.

Results

Morphogenesis of Circumvallate Papilla (CVP) in the Mouse

At embryonic day 11.5 (E11.5), the two lateral lingual swellings of the anterior portion of the tongue are separated from the posterior portion of the tongue by a deep sulcus. The dorsal lingual epithelial surface bears no morphological evidence of CVP formation. However at the microscopic level, a broad thickening of the dorsal tongue epithelia can be seen in the middle posterior one-third of the tongue where the CVP will form (Fig. 1A). At E12 and E12.5, the tongue is now recognizable. The midline and is distinguished as a 10-20 cells thick placode (Fig. 1B). From E13 to E14, the CVP placode expands into the underlying mesenchyme. Starting from E13 to E14, CVP morphology is dramatically changed from a placode-like structure into a "dome-shaped" structure. At E13, three different morphologies are observed. In the posterior aspect (see line C, Scheme 1) of E13-E14, the CVP placode evaginates to form a “ball” of tissue, (Fig. 1C) while in the middle aspect (see line B, Scheme 1), CVP forms a "ring-like" structure (see Fig. 4 C,D) and/or a “dome-shaped” structure with a loose-tissue core (Fig. 1D). However, at the anterior aspect (see line A, Scheme 1), the CVP remains as a placode-like structure (Fig. 1E). From E15-E16, a “ball” of cells at the posterior portion and the “ring-shaped” structure at the middle portion of the CVP disappear, leaving only the “dome-shaped” structure with its mesenchymal core (Fig. 1F). The epithelium overlying the “dome” of the CVP becomes thinner, about 3-5 cells...
Developmental Patterning of the Circumvallate Papilla

thick, and at the base of the “dome” two stalks are formed. At E17, these two stalks invaginate deeply into the tongue mesenchyme (Fig. 1G). These two invaginations will later contribute to the von Ebner’s minor salivary gland (VEG). By E18 of development, branching morphogenesis of VEG is observed (Fig. 1H).

Cell Proliferation Activity during CVP Development

BrdU incorporation was used to detect cell proliferation within the CVP placode from developmental stages E11.5 to E15. From E11.5-E12.5, BrdU-labeled cells are distributed generally in both the tongue mesenchyme and the epithelium. However the epithelial placode of the CVP is spared from labeling (Fig. 2 A-C). At E13, a few BrdU-positive cells are observed within CVP core (arrowhead in Fig. 2D). At E13.5, a few cells of the epithelial “dome” (arrows in Fig. 2E) and the cells inside the mesenchymal core (arrowhead in Fig. 2E) are BrdU-labeled. At E14 and E15, BrdU-labeling remains largely negative in the epithelia on the roof of the “dome”, but there are BrdU-positive cells found at the invaginating stalks at the base of the “dome” (arrows in Fig. 2 F,G). These two stalks will later invaginate and undergo development to form the paired von Ebner’s minor salivary gland (see Fig. 1 G,H).

Localization of Laminin

In this study we examined the changing distribution of laminin during CVP morphogenesis and innervation. At E11.5 laminin is present as a continuous thin line separating the tongue epithelium from the underlying mesenchyme (data not shown). Laminin immunoreactivity is also found in the basement membrane of the nerves and blood vessels in the tongue at all stages examined in this study (see asterisks in Fig. 3 A-C).

The localization of laminin at E12.5 CVP placode is similar to the laminin distribution observed at E12. At the posterior portion (see line C, Scheme 1) and also in the area anterior to E12 and E12.5 CVP placode (anterior to line A, Scheme 1), laminin is distributed in the basement membrane as a continuous thin line separating the tongue epithelia from the underlying mesenchyme (data not shown). In the area closest to the anterior portion (see line A, Scheme 1) of E12 and E12.5 CVP placode, the localization of laminin is broad and extends from the cells along the basement membrane into the cells positioned well above the basement membrane (arrows in Fig. 3A). In the middle portion of E12.5 CVP placode, laminin immunodetection is weaker, but remains traceable, in the area where nerves almost reach the basement membrane (arrows in Fig. 3B).

At the posterior portion (see line C, Scheme 1) of E13 and E13.5 CVP, laminin is distributed as a continuous line clearly separating epithelia from underlying mesenchyme (data not shown). In the area where the “ring-shaped” structure forms, a broadening of laminin immunostaining in the epithelial cells in the middle of the “ring” and in some cells on the top of the “ring” surface are observed (arrows in Fig. 3C). At the area closest to the “dome-shaped” structure, we observed three different laminin immunostaining patterns. First, laminin is surrounded the central core of the “ring” and is broadly distributed at the opening site where the epithelial cell mass will separate to form the “dome-shaped” structure (arrow in Fig. 3D). Second, laminin immunostaining is found surrounded the central core and there are two broad area of laminin immunoreactivity extending from middle of the “ring” to the basement membrane of CVP at both sides of the opening area of the “ring” (arrows in Fig. 3E). Lastly, laminin is observed as two broadly defined areas at the right and left sides of the CVP placode (arrows in Fig. 3F). At the “dome-

Scheme 1. Plane of sections used in this study. The anterior (A), middle (B) and posterior (C) portions of the CVP.

Fig. 2. BrdU labeling of the mouse embryonic circumvallate papilla from E11.5-E15. BrdU-labeled cells are generally found in both the tongue epithelium and the tongue mesenchyme at all developmental stages, except the CVP epithelium. The epithelium of E11.5 (A), E12 (B) and E12.5 (C) of the CVP epithelial placode are spared from labeling. (D) At E13, the entire CVP epithelium remains negative for BrdU-labeling, but a few BrdU-positive cells are observed in the mesenchymal core (arrowhead). (E) At E13.5, a few cells of the CVP epithelial “dome” (arrows) and the cells in the mesenchymal core (arrowhead) are BrdU-labeled. At E14 (F) and E15 (G), BrdU-labeling remains negative in the epithelium on the top of the “dome”, but BrdU-labeled cells are found in the invaginating stalks (arrows). Bar, 20 µm for all images.
shaped” area, laminin is distributed as a continuous line along the basement membrane (arrows in Fig. 4G). At the area closest to the anterior end of the E12 CVP placode, laminin is expressed irregularly and broadly extending from the cells along the basement membrane to the cells positioned well above the basement membrane (arrows). (B) In the middle portion of the E12.5 CVP placode, at the site where nerves approach the basement membrane, laminin immunoreactivity is very weak, but remains traceable (arrows). (C) At the “ring” area of the E13 CVP, laminin is broadly distributed among the epithelial cells at the middle of the “ring” and in some epithelial cells on the top of the “ring” surface (arrows). (D-F) In the middle portion of the E13 CVP, laminin is broadly distributed in the area where the epithelium separated to form a “dome” structure (arrow in D), or laminin immunostaining is extended from the middle of the “ring” to the basement membrane at both sides of the opening area (arrows in E) or laminin is broadly distributed at the left and the right sides of the CVP placode laments in F. (G) In the “dome” shaped area of the E13 CVP, laminin is distributed as a continuous thin line along the basement membrane (arrows). (H) At the area closest to the anterior end of the E13 CVP, laminin is broadly distributed and extends from the basement membrane toward the cells positioned well above the basement membrane (arrows). Bar, 20 µm for all images.

E12-E12.5 CVP placode, E-cadherin is intense in the epithelial cells from the basement membrane toward the epithelial cells in the superficial layer (data not shown). At the anterior end (see line A, Scheme 1) of the CVP placode, E-cadherin is intense in all cell layers (Fig. 4A). However, the staining in the epithelial cells at the tip of placode along the basement membrane is diffuse and the demarcation between the epithelia and the mesenchyme is poorly defined (arrows in Fig. 4A). In the middle portion (see line B, Scheme 1) of the E12 (data not shown) and E12.5 CVP placode, E-cadherin is most intense in the superficial epithelial cell layer and in the epithelial cells along the basement membrane (arrows in Fig. 4B). However, the E-cadherin immunostaining is decreased in the epithelial cells located in the middle layer of the placode (arrowhead in Fig. 4B).

Expression patterns of E-cadherin at E13 and E13.5 are similar. At the posterior end (see line C, Scheme 1) of E13 and E13.5 CVP, E-cadherin immunoreactivity is decreased gradually from the superficial cell layer to the basal cell layer: being most intense in the epithelial cells along the superficial layer and less intense in the epithelial cells along the basement membrane (data not shown). At the “ring” area, E-cadherin immunostaining is intense in the cells that define the middle of the “ring” and the superficial layer (arrows in Fig. 4C), but the staining is weaker among the epithelial cells positioned between these two layers as well as the cells near the basement membrane (arrowheads in Fig. 4C). Staining of E-cadherin in the “ring” area where the nerve will penetrate into the CVP is intense and of approximately equal intensity among all cell layers from the middle cell layer of the “ring” toward the basement membrane (arrow in Fig. 4D). At the “dome” area, E-cadherin staining is very intense in the

**Localization of E Cadherin**

To determine the expression profiles of E-cadherin during CVP morphogenesis and innervation, we performed immunohistochemistry on mouse CVP at developmental stages E12-E13.5. The localization of E-cadherin at E12.5 CVP placode is similar to the distribution observed at E12. At the posterior end (see line C, Scheme 1) of the
Innervation of Developing Circumvallate Papilla

The innervation of developing circumvallate papilla was examined using the pan-neuronal antibody directed against ubiquitin carboxyl terminal hydrolase (protein gene product 9.5; PGP 9.5) (Thompson et al., 1983). We observed nerve fibers in the mandibular process starting from E11.5, but the nerve fibers remain distant from the tongue epithelium (data not shown). At E12 and E12.5 nerve fibers penetrate closer to the tongue epithelium but have not yet reached the tongue epithelium (arrows in Fig. 5 A, B). At E13, the neuronal markers were detected in the middle of the “ball-like” cell mass (arrow in Fig. 5C), in the middle of the “ring-shaped” structure (arrow in Fig. 5D), in the central core of the “dome” (arrow in Fig. 5E), as well as the mesenchymal cells beneath the anterior end of CVP placode (arrow in Fig. 5F). We did not detect this neuronal marker in nerve fibers in the CVP epithelium until E14 (arrows in Fig. 5G). At E15, the central core of the CVP is filled densely with nerves and additional nerves in the CVP epithelium are detected (arrow in Fig. 5H).

Contribution of Cranial Neural Crest Cells during CVP Development

To clarify whether the neural crest-derived cells contribute to the CVP placode formation, the Wnt 1-Cre/R26R reporter mice are used to monitor neural crest-derived cell lineages during CVP development. All of the blue-stained cells showed in Fig. 6 are neural crest-derived cells, whereas the cells lacking X-gal staining are non-neural crest-derived. We found that the mesenchymal cells underlying the CVP, within the core of the CVP and also the nerve supply to the CVP is neural crest-derived (Fig. 6 A-F).

Discussion

The development of the circumvallate papilla resembles other systems of organogenesis, such as feather, hair, lung, mammary gland and tooth (Chuong, 1993; Jernvall and Thesleff, 2000; Warburton et al., 2000). These regulated developmental processes involve complex sequential and reciprocal interactions between epithelia and mesenchymal tissues. However, the developmental program for each distinct organ requires tissue-specific regulatory factors and signaling pathways. So far, little is known about the molecular regulatory mechanism during pattern formation of the tongue or circumvallate papilla (Hall et al., 1999; Jung et al., 1999). In order to better understand the development of the CVP, we examined markers of cell division, cell adhesion, a basement membrane component, neural tissue, and neural crest cell-derivatives.

The embryonic stage demarcating the first morphological sign of CVP formation in our study differs from that stage previously reported by Paulson et al. (1985) and AhPin et al. (1989). Paulson et al. (1985) observed the first histological indicator for CVP formation in CD-1 mouse fetuses at E14. However, AhPin et al. (1989) first observed CVP formation in Balb/c mouse fetuses at E13. In our study, we used Swiss Webster (SW) mouse, an outbreed strain, as an animal model. The first histological indicator of CVP formation in SW mice was seen as early as E11.5, starting...
discrepancies in temporal events are likely due largely to differences in murine strains.

The earliest stage of CVP formation starts as a broad thickening of epithelial cells and at latter developmental stages, these cells accumulate and condense in the middle of the tongue as a distinct placode. At E13, innervation and CVP morphogenesis begins. There are three possibilities to explain how epithelial thickening forms a placode: 1) cell division, 2) cell migration, or 3) mesenchymal-epithelial transformation (de longh, et al., 2001). In our study, we used BrdU as a marker to label dividing cells during early CVP placode formation. However, we did not detect BrdU-labeled cells in the epithelial placode from E11.5 to E13. At E13.5 to E14, BrdU incorporation remained negative on the top of the CVP “dome”. However, we detected a few BrdU-positive cells in the epithelial invagination area that will later form von Ebners’ gland as well as BrdU incorporation into cells within the mesenchymal core of the CVP (see Fig. 2). We interpret the negative BrdU-labeling cells in the CVP placode as an indication that cell division does not directly participate in epithelial thickening during the earliest stage of CVP placode formation.

The other possibility underlying epithelial thickening is that the cells in the placode might derive from mesenchymal-epithelial transformation. A mesenchymal to epithelial transformation occurs normally during development such as during somite formation, vasculogenesis or nephrogenesis (Ekblom, 1989; Barasch, 2001). We used a Wnt1-cre/R26R reporter mouse model to identify and thereby to follow the lineages of neural crest cell population within the CVP. The proto-oncogene Wnt1 is expressed in the developing central nervous system and also in the neural crest cell population emigrating from the neural tube (Wilkinson et al., 1987; Echelard et al., 1994). The R26R reporter mouse exhibits constitutive β-gal expression in all cells during embryonic development when crossed with ubiquitously expressed cre (Friedrich and Soriano, 1991; Zambrowicz et al., 1997). Cross mating between the Wnt1-cre transgenic mouse with the R26R reporter mouse created double transgenic line in which lac Z expression is restricted to progenitors of the neural crest cells. In our study, we found no lac Z positive cell in the epithelial placode area. We interpret the absence of a neural crest-derived contribution to the placode as an evidence that mesenchymal-epithelial transformation does not play a significant role in the formation of the CVP placode.

The remaining possibility is that placode thickening occurs as a result of cell migration. To test this hypothesis, we examined one of the cell adhesion molecules to see if it was altered during placode thickening. In this study we demonstrated the heterogeneous distribution of E-cadherin during CVP morphogenesis. E-cadherin was intensely expressed in the superficial and basal epithelial cell layers during CVP placode formation. However E-cadherin expression decreased in the epithelial cells within the middle cell layer of the placode (as shown in Fig. 3B). At E13 when CVP morphogenesis and innervation occur, E-cadherin was strongly expressed in the superficial cell layer, in the middle of the CVP “ring” and at the site where nerve processes penetrated into the CVP placode. However, E-cadherin decreased in the cells closest to the basement membrane (as shown in Fig. 3 C-F).
E-cadherin is a member of the cadherin family of transmembrane Ca\(^{2+}\)-dependent, homotypic cell adhesion molecules, which mediates cell adhesion, as well as being regulated during cell movement. In adult tissues, E-cadherin remains expressed at high levels and participates in maintaining tissue architecture (Gumbiner, 1996). The loss of E-cadherin expression can lead to increased cell motility, such as during invasive or metastasis of epithelial tumors (Fri xen et al., 1991), cell migration/rearrangement and during epithelial-mesenchymal transformation during normal developmental processes (Shuler et al., 1992; Sjodin et al., 1995). In this study, the observed decrease in E-cadherin expression in a particular subset of epithelial cells in the CVP placode and in the cells closest to the basement membrane when innervation initiates, is consistent with epithelial cells undergoing migration and rearrangement (Gumbiner, 1996). The strong expression of E-cadherin in the superficial cell layer, the cells surrounding the “ring” and the cells in the area where nerves penetrate might also play a role in maintaining tissue integrity. Our observations are consistent with the hypothesis that cell migration likely plays a role during CVP placode formation.

There is still no molecular explanation for CVP placode morphogenesis into the “dome-like” structure observed (Fig. 1). In this study we attempted to clarify the molecular players that might participate during this process. It is known that nerve fibers enter the tongue mesenchyme starting from E11.5, but what is not known is how nerves find their way to particular sites of lingual epithelia, such as CVP or other gustatory papilla. One candidate that might be involved in this process is the extracellular matrices (ECM). The interactions between cells and the ECM are known to regulate many cellular functions, such as: cell proliferation, migration, morphogenesis and differentiation (Adams and Watt, 1993). Cell migration is among the major events that shape CVP morphogenesis, so components of the ECM might be the major molecular players that participate in this process.

The basement membrane is one example of the highly specialized ECM that lines the interface between epithelia and mesenchyme. Among all of the ECM glycoproteins, laminin is found predominantly in the basement membrane and is the first ECM expressed in the mammalian embryo (Martin and Timpl, 1987). Laminin has been shown to be involved in a variety of biological processes such as cell proliferation, cell migration, morphogenesis and cytokidifferentiation (Kleinman et al., 1985; Aumailler and Smyth, 1998). Moreover, laminin had been shown to stimulate migration of neurons, promote axon extension and promote nerve regeneration (Calof and Lander, 1991; Clark et al., 1993).

In our study, we observed a distinctive expression pattern of laminin during CVP morphogenesis. At the earliest developmental stage, the placode, laminin is distributed as a continuous thin line along the basement membrane. However, at latter developmental stages, as the nerves approach the basement membrane, laminin becomes more broadly distributed at the anterior portion of the placode and is decreased in apparent abundance in the area where the nerve will reach the basement membrane. At E13 when CVP morphology changes dramatically, laminin is intensely and broadly expressed in particular subsets of CVP epithelial cells (as shown in Fig. 3 C-H). However, in the “dome-shaped” structure, where nerves already occupy the central core, laminin is distributed as a continuous thin line along the basement membrane separating the epithelial from the underlying mesenchyme. The expression pattern for laminin appears to correlate with innervation and morphogenesis of the CVP, in that laminin is upregulated at sites where nerve fibers will penetrate into the CVP. It thus appears that innervation participates in the final morphogenetic steps of the CVP.

Mistretta and Haus (1996) previously reported laminin distribution during sheep gustatory papilla development. They showed that laminin is distributed continuously within the basement membrane, being interrupted only where taste buds form. In our study, we used SW mice, in which the taste buds will not differentiate until late E17 (State and Bowden, 1974). Discrepancies between our study and those of Mistretta and Haus (1996) may be due to animal species and developmental stage that we used. From our observations, laminin expression is highly regulated during CVP morphogenesis and innervation, suggesting that laminin may play a role in specifying an epithelial location where a nerve will penetrate thus providing innervation to taste anexa.

The results of this study suggest that 1) the CVP placode forms from the epithelial cells that migrate toward the midline; 2) E-cadherin may be down-regulated during cell migration to form a CVP placode; 3) laminin distribution may be involved in specifying

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Fig. 6. Detection of the neural crest contribution to CVP development. LacZ staining of Wnt1-Cre/R26R reporter mouse embryos at E11.5(A), E12.5(B) and E13(C-F). The dashed lines define the epithelial outlines. The cells stained blue are neural crest derivatives. Bar, 20 µm for all images.
ethyl uridine (BrdU, 5'-Bromo-2'-deoxy-uridine, Sigma) was nerve dependent, although initiation of gustatory papilla development for 15 min, and washed with PBST. The reaction product was detected by PBST (PBS, pH 7.4, 0.1% Tween 20) and then exposed to an appropriate following day, sections were washed three times, 10 min each, with 1x rinsing solution (2 mM MgCl₂ + 0.1% deoxycholic acid + 0.005% Nonidet P40 in 1x PBS) at room temperature, 2 times, 15 min each. After discarded the rinsing solution, tissues were incubated in X-gal staining solution (1 mg/ml of X-gal in 1x PBS containing 2 mM MgCl₂ + 0.1% deoxycholic acid + 0.005% Nonidet P40 + 35 mM potassium ferricyanide + 35 mM potassium ferrocyanide) at 37°C overnight. Tissues were then refixed in 4% paraformaldehyde at room temperature for 1 hour, paraffin embedded and sectioned to observed lac-Z expression.

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References


the location and provide the guidance for the innervation of epithelial taste buds and 4) morphogenesis of the CVP is dependent upon innervation. Our study supports the observations of other investigators (Farbman and Mbiene, 1991; Oakley, 1993; Oakley et al., 1993; Mbiene et al., 1997; Morris-Wilman et al., 1999) who suggest that maintenance of gustatory papilla morphology is nerve dependent, although initiation of gustatory papilla development is independent of nerve innervation.

Materials and Methods

Embryonic Collection and Staging
Use of vertebrate animals complied with all institutional and federal guidelines. Timed pregnant Swiss-Webster mice were purchased from Charles River (Wilmington, MA). The mouse embryos were collected and staged according to somite pair (Theiler, 1989). Tissues from the posterior tongues of E12 to E15 embryos were dissected and fixed in Carnoy’s solution (Ethanol:Chloroform:Glacial acetic acid = 6:3:1 v/v). Coronal plane of paraffin sections at 5 µm thickness were prepared and stored at 4°C until use. Scheme one demonstrates plane of section and terms used in the result and discussion.

Immunohistochemistry
Tissue sections were deparaffinized in xylene and rehydrated in a graded series of ethanol solution (100%, 95%, 70% and 50% v/v). All processes were carried out at room temperature unless noted. After rehydration, the sections were submerged in 0.1 M phosphate buffered saline (PBS, pH 7.4), 2 times, 5 min each. Following treatment in 3% hydrogen peroxide in methanol to quench endogenous peroxidase, sections were treated with blocking solution provided by the manufacturer (Zymed). The sections were then incubated with primary-antibody: laminin (1:1000, Sigma), E-cadherin (1:1000, BD transduction laboratories), BrdU (1:200, Sigma), and PGP 9.5 (1:1000, Biogenesis), which was diluted in PBS containing 1% bovine serum albumin (BSA) overnight at 4°C. The following day, sections were washed three times, 10 min each, with 1x PBST (PBS, pH 7.4, 0.1% Tween 20) and then exposed to an appropriate biotinylated secondary antibody (Zymed) for 30 min. Sections were washed 3 times with PBST, incubated with the streptavidin-peroxidase conjugate for 15 min, and washed with PBST. The reaction product was detected by 3-amin-9-ethyl carbazole (AEC) substrate (Zymed). The red color indicates positive staining. Tissue sections were then mounted with glycerol vinyl alcohol (GVA) media.

Control tissue sections were incubated in the absence of primary antibody or in the absence of both primary- and secondary-antibody. In both cases no dye-complex was detected.

BrdU-Labeling and Staining
Bromo-deoxy-uridine (BrdU, 5’-Bromo-Z’-deoxy-uridine, Sigma) was dissolved in PBS and administered into the intraperitoneal cavity of timed pregnant mice at a dose of 100 µg/g body weight. The BrdU-labeled embryos were collected 4 hours after injection, washed twice in PBS and fixed in Carnoy’s solution. The procedure for deparaffinization and to block endogenous peroxidase followed the same protocol as described above. Before exposing the tissue to the anti-BrdU antibody (Sigma), the DNA was depurinated by incubating the sections in 2 N HCl for 60 min at 37°C, then neutralized with 0.1 M Sodium borate pH 8.5 for 30 min and rinsed 4 times, 2 min each, with 1x PBST at room temperature. The non-specific binding of the antibody was blocked according to the manufacturer’s protocol (Zymed). The sections were then incubated with 1:200 anti-BrdU antibody in 1% BSA in PBS at 4°C overnight. The reaction product was detected by AEC substrate-chromogen mixture (Zymed) and the sections were mounted with GVA mounting solution.

Control tissue sections were incubated in the absence of primary antibody or in the absence of both primary and secondary antibody. In both cases no dye-complex was detected.

Wholemount β-Galactosidase (X-gal) Staining
R26R conditional reporter mice are generous gifts from Dr. P. Soriano (Fred Hutchinson Cancer Research Center, Seattle, Washington). Wnt-1 Cre transgenic mice are generous gifts from Dr. A.P. McMahon (Dept. of Molecular and Cellular Biology, Harvard University). Cross mating between the Wnt-1 Cre transgenic mouse with the R26R conditional reporter transgenic mouse created double transgenic wnt-1-Cre/R26R mice which have been used to study the fate of neural crest cells (Chai et al., 2000). The mice were euthanized and embryos from developmental stages E11.5 to E13 were collected. Genomic DNA was purified from each embryonic tail and subjected to genotypes by PCR. The 5’ and 3’ primer pairs used for detecting Wnt1-Cre gene in this study are primer 1 (5’-ATTCTCCACCTGCTAGTG-3’) and primer 2 (5’-CGTTTTCCTGAGCATCCTTGGA-3’) (Chai et al., 2000). The R26R transgenic mice were genotyped according to Soriano (1999) (Soriano, 1999).

Tissues from the posterior tongues of each embryos were dissected and fixed in 4% paraformaldehyde at 4°C, 30 min. Tissues were washed with rinsing solution (2 mM MgCl₂ + 0.1% deoxycholic acid + 0.005% Nonidet P40 in 1x PBS) at room temperature, 2 times, 15 min each. After discarded the rinsing solution, tissues were incubated in X-gal staining solution (1 mg/ml of X-gal in 1x PBS containing 2 mM MgCl₂ + 0.1% deoxycholic acid + 0.005% Nonidet P40 + 35 mM potassium ferricyanide + 35 mM potassium ferrocyanide) at 37°C overnight. Tissues were then refixed in 4% paraformaldehyde at room temperature for 1 hour, paraffin embedded and sectioned to observed lac-Z expression.

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References


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