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RESEARCH ARTICLE

Lineage Tracing of the Endoderm During Oral Development

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Background: The contribution of the endoderm to the oral tissues of the head has been debated for many years. With the arrival of Cre/LoxP technology endoderm progenitor cells can now be genetically labeled and tissues derived from the endoderm traced. Using Sox17-2A-iCre/Rosa26 reporter mice we have followed the fate of the endoderm in the teeth, glands, and taste papillae of the oral cavity. Results: No contribution of the endoderm was observed at any stage of tooth development, or in development of the major salivary glands, in the reporter mouse during development. In contrast, the minor mucous glands of the tongue were found to be of endodermal origin, along with the circumvallate papilla and foliate papillae. The mucous minor salivary glands of the palate, however, were of mixed ectodermal and endodermal origin. Conclusions: In contrast to urodele studies, the epithelium of murine teeth is derived solely from the ectoderm. The border between the ectoderm- and endoderm-derived epithelium may play a role in determining the position of the lingual glands and taste buds, and may explain differences observed between taste buds in the anterior and posterior part of the tongue. *Developmental Dynamics 241:1183–1191, 2012.* © 2012 Wiley Periodicals, Inc.

Key words: endoderm; oral cavity; lineage labeling; mouse; tooth; gland; taste buds

Key findings:

- The epithelium of murine teeth is derived from the ectoderm.
- The epithelium of the major salivary glands is derived from the ectoderm.
- A V-shaped border at the base of the tongue separates the ectoderm- and endoderm-derived tissue.
- The minor mucous salivary glands of the tongue, circumvallate papillae, and foliate papillae are derived from endoderm.

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INTRODUCTION

In the cranial region, there are several structures that have been proposed to have an endodermal origin, although for many organs the origin of the epithelium is unclear. The oral limit of the endoderm during early stages of development lies behind the buccopharyngeal membrane, however, it has been proposed that once this membrane ruptures, to create a continuous oral cavity, the endoderm cells can migrate forward to take part in more anterior structures. A migration forward of the endoderm across the oral ectoderm for example has been shown in urodeles (Soukup et al., 2008). The position of the buccopharyngeal membrane and original border of the endoderm has also been previously difficult to discern as the embryo develops, with researchers relying on overlapping expression of cytokeratins (Hosoya et al., 2010).

In the teeth, it has been suggested that the molar epithelium might derive from the endoderm, while the incisor epithelium derives from the ectoderm (Tucker and Sharpe, 2004). In support of this, the epithelium of murine molars appears to express several genes generally associated with endoderm, while the incisor

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epithelium does not (Ohazama et al., 2010). Lineage tracing of the murine endoderm in organ culture has lead to variable results, with one study showing invasion of the endoderm up to the tooth region (Imai et al., 1998), while another not identifying such a movement (Ohazama et al., 2010). In axolotls, tissue grafting and DiI (1,1′, di-octadecyl-3,3,3′,3′,-tetramethylindocarbocyanine perchlorate) studies have shown that the posterior teeth are endodermally derived while the anterior teeth are of ectodermal origin, while in the middle of the mouth, teeth of dual epithelial origin are located (Soukup et al., 2008). Teeth derived from ectoderm or endoderm were previously shown in the salamander (Adams, 1924). In many fish, pharyngeal teeth develop deep in the pharynx in an area of endoderm, although it has not been proved that the epithelium of these teeth is derived exclusively from the endoderm (Huysseune et al., 2010).

The origin of the salivary gland epithelium has also been queried (Miletich, 2010). The major salivary glands (parotid, submandibular, and sublingual) develop at the back of the mouth behind the molars (Tucker, 2007). The mesenchyme around the salivary glands has been shown to be of neural crest origin (Jaskoll et al., 2002), but the origin of the epithelium is controversial with papers quoting both an ectodermal (Nordgarden et al., 2003) and endodermal origin (Hisatomi et al., 2004). Text books often suggest that the parotid is ectodermal but submandibular and sublingual are endodermal (Avery, 2002; Halim, 2008; Balaji, 2009). In addition, the epithelial origin of the multiple minor salivary glands, concentrated on the palate and tongue, is unknown.

It is generally assumed that a border between the endoderm and ectoderm in the lower jaw is located near the back of the tongue but its actual position has not been successfully mapped. In fact, it has been suggested that endoderm-derived tissues can be found at the tip of the human tongue, perhaps having migrated rostrally during development to mix with the ectoderm-derived tissue (Zhang and Oakley, 1996).

Mice have three types of gustatory papillae on the tongue (those papillae that contain taste buds). The fungiform papillae are found in rows at the anterior two thirds of the tongue, foliate papillae reside in lateral groves near the posterior of the tongue, and the single circumvallate papillae (CVP) is found at the posterior midline (Petersen et al., 2011). Lineage-labeling studies in the axolotl have shown that the taste buds originate from the endoderm (Barlow and Northcutt, 1994, 1995), agreeing with descriptive studies in elasmobranchs (Cook and Neal, 1921). In teleost fish, at least some taste buds are ectodermal, as in species such as catfish there are external taste buds over the head and trunk (Atema, 1971). However, the origin of the gustatory papillae in mouse has not been clearly shown.

Where the ectodermal/endodermal border is established in mammals is important, as it has been suggested that different types of taste buds develop according to their ectodermal/endodermal origin, with the circumvaliate papilla (CVP) forming from endoderm and fungiform papilla from ectoderm (Barlow and Northcutt, 1995; Zhang and Oakley, 1996; Petersen et al., 2011). These two types of papilla are innervated from different sources, the anterior fungiform papillae by the chorda tympani nerve and the circumvallate papilla by the glossopharyngeal nerve (Liu and Lee, 1982). Evidence from the rat shows that the anterior foliate trenches are innervated jointly by the chorda tympani and glossopharyngeal nerve, while the more posterior trenches are innervated by the glossopharyngeal nerve alone (Whiteside, 1927). Therefore, the boundary between the ectoderm and the endoderm might lie at the boundary between the two areas of innervation, and would consequently fall within the foliate trenches.

We have, therefore, studied the boundaries of the endoderm in the developing oral cavity, using Sox17-2A-iCre/R26R mice, paying particular attention to the teeth, tongue, taste papillae, and salivary glands to finally conclude this issue. Sox17 is essential for formation of endoderm and fetal hematopoietic stem cell maintenance (Kanai-Azuma et al., 2002; Kim et al., 2007), and the Sox17-2A-iCre mouse when crossed with a R26R reporter mouse, permanently labels all endoderm-derived tissue (Engert et al., 2009).

RESULTS

Teeth

To investigate the border of the endoderm in the oral cavity, we first investigated the extent of Sox17-2A-iCre-driven Rosa26-lacZ reporter expression in the developing pharyngeal arches at embryonic day (E) 10.5 (Fig. 1A). The Sox17-2A-iCre line labels all endoderm-derived tissue, but in addition also labels the endothelial cells that form the blood vessel network and the fetal hematopoietic stem cell lineage (Engert et al., 2009). At E10.5, the buccopharyngeal membrane has ruptured and the ectoderm and endoderm form a continuous epithelium. The endoderm did not extend far into the oral part of the mandible or maxilla, but lined the inner surface of the pharynx of the lower and upper jaw (Fig. 1B,C). At E11.5, the pharynx continued to stain strongly blue as expected (Fig. 1D), but no blue endodermal cells were observed more anteriorly in the oral region (Fig. 1E). At E11.5, the site of molar tooth development can be observed as a thickening of the oral epithelium. In the epithelial thickening on both the maxilla and mandible no blue Sox17-LacZ cells were observed (Fig. 1F). At E15.5, when the molars had reached the cap stage of development, no blue endodermal cells were observed in the dental or oral epithelium (Fig. 2A). Labeled cells, in contrast, were located in the mesenchyme around the tooth germ. To confirm that these cells were endothelial cells, we used Mesp1-Cre/R26R mice to show the contribution of the mesoderm-derived endothelium to the tooth (Saga et al., 1999; Yoshida et al., 2008). A similar population of labeled cells in the mesenchyme was observed in the Mesp1-Cre/ R26R sections, labeling the forming blood vessel network of the tooth (Fig. 2B; Rothova et al., 2011). These positive cells could be used as a control to show that the lack of labeling in the epithelium was not due to poor penetration of the staining solution. A similar pattern of LacZ staining in the forming blood vessels around the first molar but not in the epithelium-

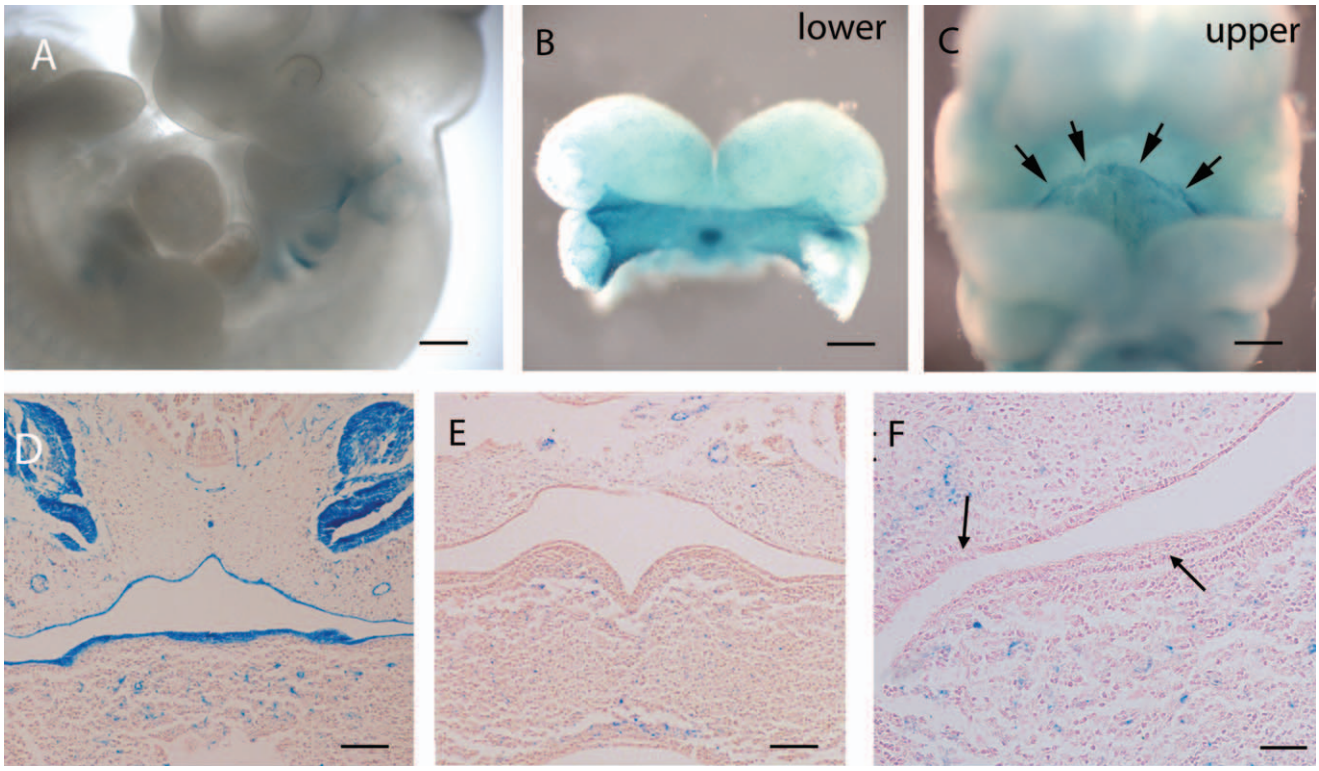

Fig. 1. Boundary of the endoderm during early development of the oral cavity. A–F: Sox17-2A-iCre/R26R mice stained with LacZ. **A**: Embryonic day (E) 10.5 embryo showing blue staining in the endoderm-derived pharyngeal pouches. Embryo weakly stained to highlight endoderm rather than endothelial staining. **B**: E10.5 Dissected mandible and second pharyngeal arch, internal view. **C**: E10.5 upper jaw boundary (outlined with arrows). D–F: Frontal sections through E11.5 heads. **D,E**: Progressive sections through the pharynx moving anteriorly into the oral cavity. **F**: Thickening of the oral epithelium (arrows, upper and lower jaw) at the initiation of molar development. Scale bars = 400 μm in A, 200 μm in B,C, 120 μm in D,E, 60 μm in F.

derived ameloblasts layer was observed postnatally before eruption (Fig. 2C,D). The first molar is, therefore, of ectodermal origin. The second and third molars develop from the first molar, appearing sequentially further back in the jaw. To rule out an endodermal contribution to these later, more proximally developing teeth, we investigated the epithelium of the second and third molar postnatally. From Vibratome and cryostat sections, it was clear that the third molar epithelium was not positive for the Sox17-LacZ, unlike the surrounding endothelial cells (Fig. 2E, data not shown). A similar result was found for the upper molars (Fig. 2F), and also for the upper and lower incisors (data not shown). Therefore, all teeth are of ectodermal origin in the mouse.

Major Salivary Glands

The major salivary glands (parotid, submandibular, and sublingual) develop at the back of the mouth from buds that form behind the molar tooth germs. This more proximal location means they might form from endoderm. At E15.5, when the glands have reached the canalicular stage, the epithelium had no blue Sox17-LacZ endodermal cells (Fig. 3A,B), in contrast to the blue endothelial cells (Fig. 3A–C), which were found throughout the mesenchyme in a branching pattern. As expected, the glands that form more proximally from the pharyngeal pouches, such as the thyroid, were stained blue with the Sox17-2A-iCre/R26R reporter, confirming their endodermal origin (Fig. 3D).

The Endoderm–Ectoderm Border and Minor Salivary Glands

To understand where the ectoderm boundary with the endoderm was located within the tongue, tongues from Sox17-2A-iCre/R26R mice were stained postnatally. In whole-mount, a clear V-shaped blue stain was identified at the back of the tongue (Figs. 4A, 5A). A few blue cells were located scattered more anteriorly but these were found to be blood vessels located within the tongue mesenchyme and a clear boundary within the epithelium between the endoderm and ectoderm was evident on sections (Fig. 4B). To look at the extent of the endoderm in the upper jaw, we examined whole heads in sagittal section. Of interest, the upper jaw boundary was more posteriorly positioned than the tongue boundary (Fig. 4C).

The tongue is host to a collection of minor salivary glands. At E15.5, the minor glands start to bud off from the oral epithelium at the back of the tongue and the posterior palate (Wells et al., 2011). These buds were found to be positive for Sox17-2A-iCre/R26R (Fig. 4E). After birth, the minor glands had formed into little bunches of grapes sitting under the tongue epithelium and unlike the major salivary glands showed strong positive staining (Fig. 4F). To confirm that all mucous minor glands were located in the endodermal part of the tongue, serial

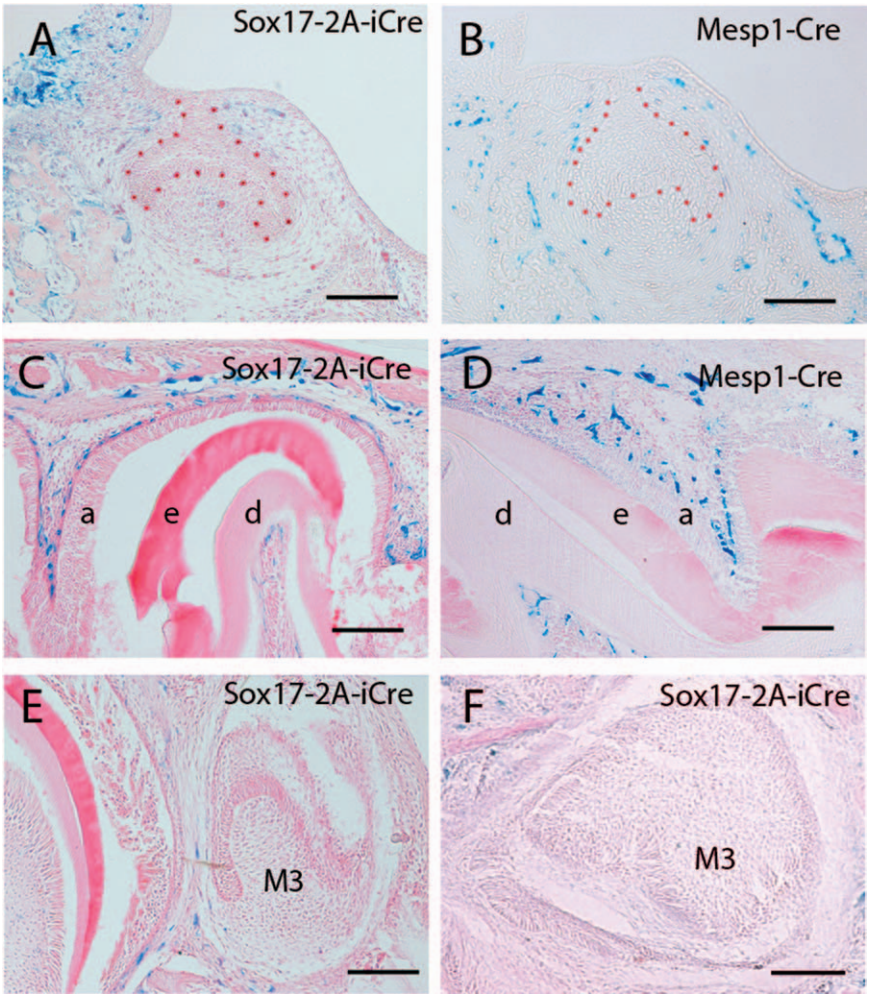

Fig. 2. The molars do not have a contribution from the endoderm. A,C,E,F: Sox17-2A-iCre/R26R stained mice. B,D: Mesp1-Cre/R26R stained mice. **A,B**: Molar tooth germ at the cap stage (frontal section) with the epithelium outlined with red dots. **C,D**: Molar tooth pre-eruption (sagittal section). **E**: Sagittal section through the lower third molar at the cap stage. **F**: Sagittal section through the upper third molar at the cap/bell stage. Scale bars = 100 μm. a, ameloblasts; e, enamel; d, dentine; M3, third molar.

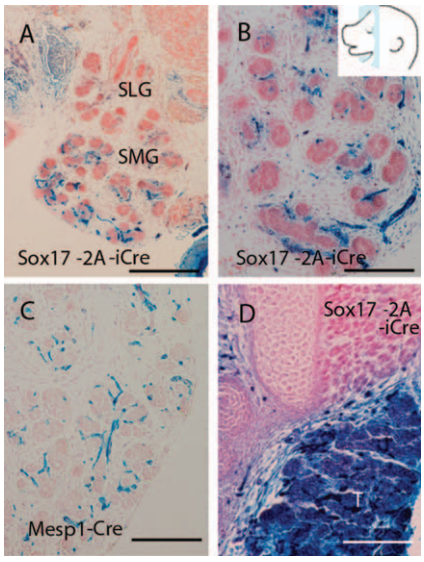

Fig. 3. The major salivary glands are ectodermal organs. A–D: frontal sections at embryonic day (E) 15.5. A,B,D: Sox17-2A-iCre/R26R stained mice. C: Mesp1-Cre/R26R stained mice. **A**: Submandibular glands (SMG) and sublingual gland (SLG). **B**: SMG showing the epithelium is unstained. Inset shows plane of section. **C**: Endothelial cells around the gland stain blue. **D**: The endodermally derived tyroid gland (T) stains strongly blue. Scale bar = 100 μm in A; 50 μm in B–D.

sections were stained with Alcian blue (which stains the mucin rich glands) and for LacZ. Alcian blue staining of the lingual glands completely overlapped with the LacZ staining (Fig. 4C,D), indicating that no mucous minor glands developed anterior to the endodermal border on the tongue. In contrast, the minor mucous glands of the palate were of mixed origin, with the most posterior glands being derived from the endoderm, while the more anterior glands were of ectodermal origin (Fig. 4C,D).

Taste Buds

The single murine circumvallate papilla, which houses the serous von Ebners glands, was found located within the endoderm derived blue section of the tongue (Fig. 5A,B). Our mice were found to have approximately five folliate trenches on each side of the tongue, as stained by alkaline phosphatase (AP) (Liu and Lee, 1982) at postnatal day (P) 13 (Fig. 5C). At this stage, the trenches have all formed and taste bud development has initiated (Toprak and Yilmaz, 2007). The exact pattern of the glands, however, showed some variation, with some mice having long defined trenches, while others had a mix of long trenches and smaller pits, which appeared in some cases to merge into a single trench. This made it difficult to accurately count the number of trenches. The variation appeared not to reflect a difference in stage as variation was observed within the same animal, on either side of the tongue (Fig. 5C). The foliate papillae appeared positioned within the blue region of the posterior tongue (Fig. 5A), whereas, the fungiform papillae were positioned in the un-stained ectodermal anterior region of the tongue (data not shown). To confirm that all the foliate trenches were located within the endoderm, we carried out double labeling for Sox17-LacZ and AP. Tongues were stained until the trenches showed clear LacZ staining (blue; Fig. 5D) and were then stained for AP (purple; Fig. 5E). No Sox17-LacZ-negative/AP-positive trenches were observed, indicating that all the foliate trenches did indeed form within the endoderm.

The nongustatory filiform papillae were found in stained and unstained areas of the tongue, indicating both an endoderm and ectoderm origin (data not shown).

DISCUSSION

The advent of Cre/LoxP technology has allowed the contribution of

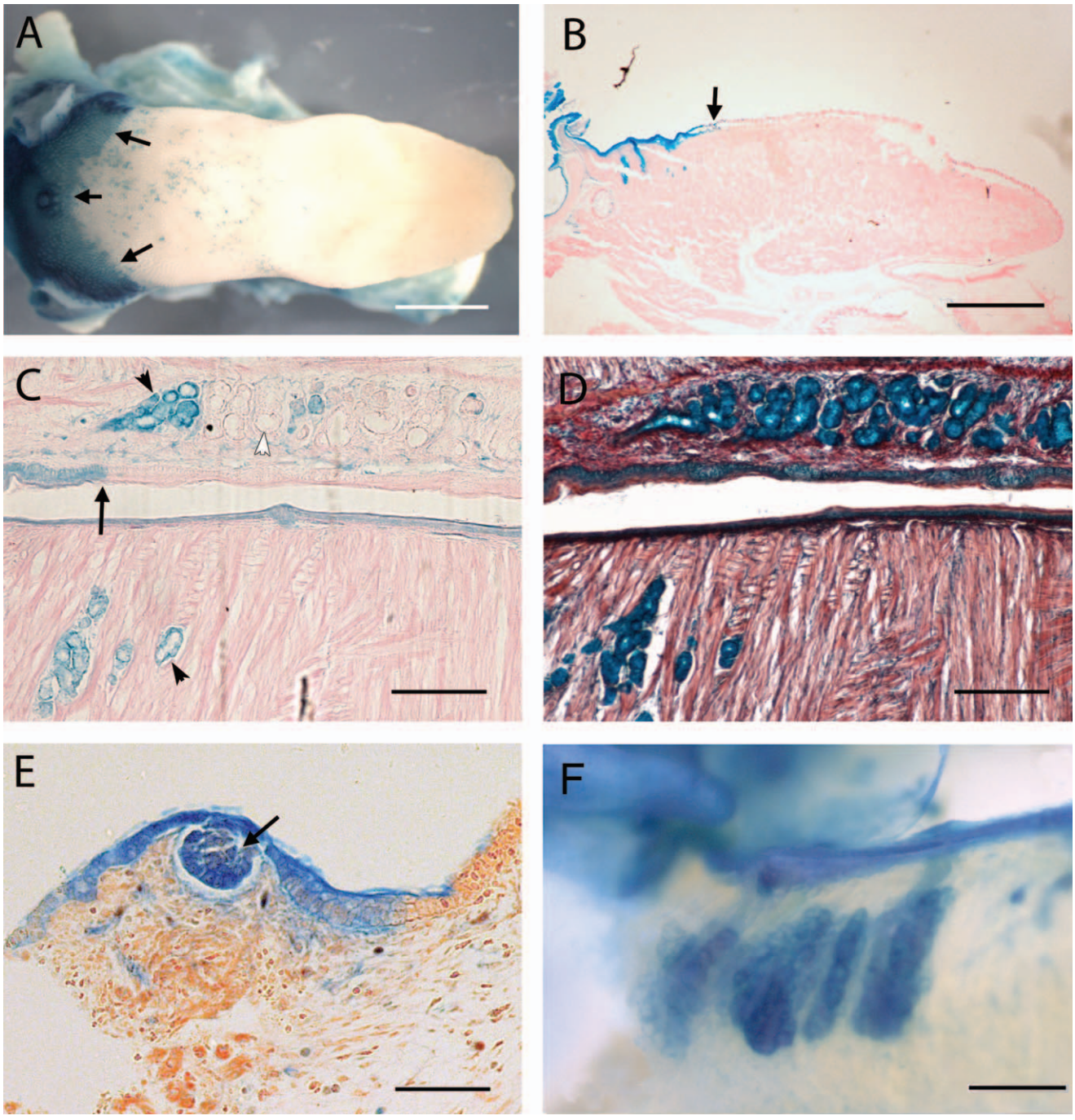

Fig. 4. Boundary of the endoderm after birth and origin of minor glands. A–C,E,F: Sox17-2A-iCre/ R26R stained mice. D: Trichrome stained section. **A**: Whole tongue showing V-shaped border (arrows) of the endoderm in the posterior at postnatal day (P) 12. **B**: Sagittal section through a new-born tongue. The surface epithelium at the back of the tongue stains blue, showing the endoderm. Arrow indicates the border between the endoderm and ectoderm. **C**: P7 sagittal section: The border of the endoderm in the tongue extends more anteriorly than the border in the upper jaw (arrow). The minor mucous glands in the tongue are all positive for LacZ, while only the most posterior palatal mucous glands are endoderm derived (black arrow heads). White arrowheads indicate ectodermal glands. Compare to the trichrome staining in D. **D**: Serial section showing the mucous glands in blue (Alcian blue). **E**: Developing tongue minor salivary gland at E15.5 in frontal section. The gland develops within the endoderm. **F**: Postnatal tongue dissected to reveal the branching minor salivary glands, which are stained in blue. A–D,F: Anterior is right. Scale bar = 2500 μm in A, 500 μm in B, 200 μm in C,D, 100 μm in E, 250 μm in F.

several germ layers to be mapped during craniofacial development. In the head, the Wnt1-Cre mice have shown the contribution of the neural crest to the tooth and jaw (Chai et al., 2000), while the Mesp1-Cre mice have shown the contribution of the mesoderm to the cranial base and skull vault (Yoshida et al., 2008). The K14-Cre mice, have been useful for targeting oral epithelial cells, (Huang et al., 2009), but labels both ectoderm and endoderm-derived tissues. The limit and contribution of the endoderm has not previously been traced due to the

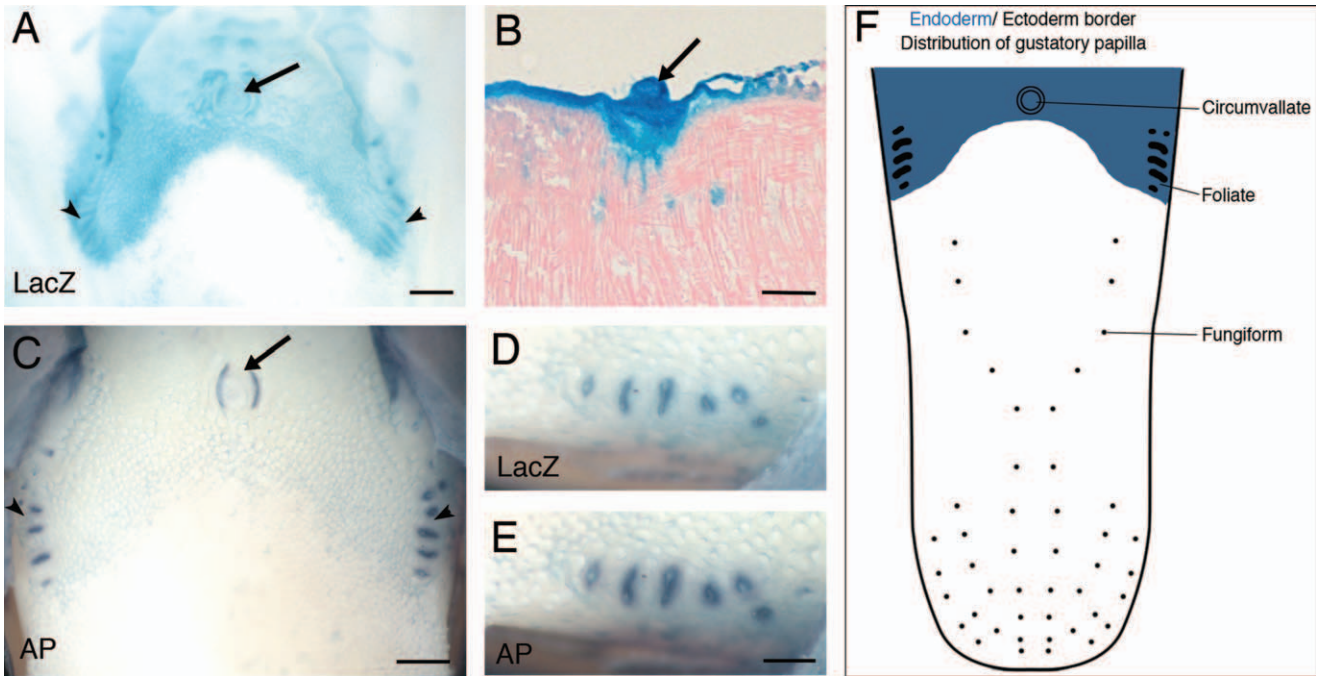

Fig. 5. Gustatory papillae can be divided into ectoderm and endoderm- derived. **A**: Whole-mount stained tongue at birth. The circumvallate papillae (CVP) develops within the blue stained endoderm (arrow). The foliate trenches (arrow heads) are located within the blue, close to the endoderm/ectoderm border. **B**: Frontal section showing Sox17-2A-iCre/ R26R-positive CVP (arrow). **C**: Alkaline phosphatase staining of a tongue (postnatal day [P]13) highlighting the CVP (arrow) and foliate trenches on either side of the tongue (arrowheads). **D**: Detail of foliate trenches stained with LacZ (blue). **E**: Same sample co-stained with alkaline phosphatase (purple). All alkaline phosphatase-positive trenches are also positive for LacZ. **F**: Schematic showing distribution of taste papillae and the border of the endoderm. A,C: Posterior is top. D,E: Posterior is right. Scale bar = 250 μm in A, 300 μm in B, 400 μm in C, 200 μm in D,E.

problems of finding an appropriate Cre recombinase-expressing mouse line. Some endodermal Cre lines only weakly label the pharyngeal endoderm, such as the Amylase 2-Cre (Kockel et al., 2006), while others do not appear to label all endoderm, such as the Alpha-fetoprotein-Cre (Kwon et al., 2006), or are also expressed in ectoderm-derived epithelia of the head in postnatal tissues, such as the tamoxifen-inducible Claudin-6-Cre (Anderson et al., 2008). The Sox-17-Cre line has removed many of these problems but is not completely endoderm specific, labeling also endothelial cells and fetal hematopoietic stem cells (Engert et al., 2009). We have, therefore, compared Sox17-2A-iCre and Mesp1-Cre lacZ stains to identify the endothelial cells.

Teeth: Ectodermal

The boundary shown at E10.5 is similar to that created after DiO labeling of the endoderm before rupture of the buccopharyngeal membrane in the chick, and DiI labeling in the mouse (Ohazama et al., 2010). During later development, this sharp boundary remains with no evidence of endodermal cells moving forward over the ectoderm, as demonstrated in urodeles (Soukup et al., 2008). This movement of the endoderm, therefore, appears not to occur during mouse oral development. Even the third molar, the most proximal of the murine teeth, is formed from epithelium derived from the ectoderm in both the upper and lower jaw. It was previously proposed that the endoderm, in addition to taking part in formation of a tooth, had an important instructive role inducing tooth development. In urodeles, for example, tooth development could only be re-created in culture if endodermal tissue was included in the explant (Graveson et al., 1997), agreeing with earlier work proposing a role for endoderm in urodele tooth development (Sellman, 1946). A role for endoderm was also suggested in the mouse, due to the location of putative endodermal cells near to developing tooth germs (Imai et al., 1998). However, endoderm could not induce tooth development in recombinations of mouse tissue (Ohazama et al., 2010) and did not appear necessary in neural crest–ectoderm grafts (Lumsden, 1987). The lack of an endodermal component in murine tooth development agrees with the mouse recombination experiments that the presence of endoderm is not required for murine tooth development. In teleosts, it has recently been proposed that even the pharyngeal teeth have an ectodermal component, and form at the position of the pharyngeal clefts (Huysseune et al., 2009, 2010). Thus, although the pharynx is lined with endoderm, it is the invading ectoderm from the clefts that may actually take part in tooth development. Thus, ectodermal teeth might be the norm, in mammals and fish.

Whether the molars have a contribution from the endoderm is important for our understanding of tooth evolution, as it has been suggested that an endodermal contribution would indicate that the proximal teeth evolved from pharyngeal teeth (inside out hypothesis; Orvig, 1967; Reif, 1982), while an ectodermal origin would suggest evolution of teeth from skin denticles (outside-in

hypothesis; discussed in Smith and Johanson, 2003; Tucker and Sharpe, 2004). The fact that the molars and incisors in the mouse are of ectodermal origin, therefore, suggests that mammalian teeth may have closer links to ectodermal odontodes than endodermal pharyngeal denticles.

Major Salivary Glands: Ectodermal

The major salivary glands were shown to be derived from the ectoderm. LacZ-positive Sox17-2A-iCre staining could be seen in the numerous blood vessels (endothelial cells) that encase the glands but the epithelium itself was unlabeled. The major salivary glands do not therefore have a contribution from the endoderm during their development. Studies that have used salivary glands to extract endodermal stem cells, therefore, may be flawed (Hisatomi et al., 2004), and many textbooks claiming an endodermal origin for the submandibular and sublingual should be rewritten.

Minor Salivary Glands: Endodermal and Ectodermal

The minor lingual salivary glands, including those associated with the circumvallate papilla (CVP), form at the posterior of the tongue, and here we show that they are confined to the endodermal component. The ectodermal region of the tongue may not be competent to form minor glands. For example, when Eda is added to wild-type tongues in culture, additional ectopic minor glands are induced but these are all found at the posterior of the tongue in the area of endodermal origin (Wells et al., 2011). In contrast, the minor mucous glands along the palate were derived from both ectoderm and endoderm. From histology these glands appear identical, whether the epithelium is ectoderm- or endoderm-derived, therefore, appears to have no consequence for the development of these glands. The mouse only has one CVP at the midline of the posterior tongue. In many other species, including humans, multiple CVP are found positioned in a V-shape at the back of the tongue. This V-shape is very similar to the V seen demarcating the endoderm in the Sox17-2A-iCre/R26R mice, indicating that all the CVPs in such species are endodermal and are initiated in a pattern following the border of the ectodermal and endodermal tissue.

Taste Buds: Endodermal and Ectodermal

In the mouse, we have confirmed both an endoderm and ectoderm origin for the gustatory papillae. It has been previously shown that the gustatory papillae can be divided in to two groups, the anterior fungiform papillae and the posterior circumvallate and foliate papillae (Fig. 5F). The anterior but not posterior papillae express keratin 20 and are effected by loss of follistatin (Zhang and Oakley, 1996; Beites et al., 2009). The posterior and anterior taste buds respond differently to changes in Fgf signaling, with loss of Fgf10 leading to loss of the posterior CVP but expansion of the anterior fungiform papillae (Petersen et al., 2011). Additionally, there are many taste buds on the posterior papillae and these express bone morphogenetic protein 4 (BMP4), but the single taste bud on the anterior papillae are BMP4 negative (Nguyen and Barlow, 2010). These differences, therefore, might be based on origin of the epithelium–endodermal more posteriorly vs. ectodermal more anteriorly, as shown in this study. Despite possible differences in innervation between the anterior and posterior foliate trenches (Whiteside, 1927), all the foliate trenches in our analysis were derived from the endoderm. Therefore, if a difference in innervation is found in the mouse, it does not correspond to the border between endoderm and ectoderm and the origin of the epithelium does not influence the origin of innervation. The number of foliate trenches appears to vary in mice as previous papers have reported between four and eight trenches (Paulson et al., 1985; Royer and Kinnamon, 1988; Toprak and Yilmaz, 2007). In our mice, only five trenches were evident, although some of the trenches appeared divided into smaller pits, making counting difficult. The previously observed variation may be due to strain-specific differences. The number of trenches varies widely between mammals with porcupines having 20 rows of trenches, while flying squirrels have 34 rows (Kubota et al., 1966; Emura et al., 1999). In these species with large numbers of foliate papillae, it would be interesting to investigate whether all the foliate trenches are derived from endoderm.

EXPERIMENTAL PROCEDURES

Mice

Sox17 is expressed in the anterior definitive endoderm and hindgut, during early embryonic development. After E9.5, expression of Sox17 disappears from the endodermal tissue but persists in blood vessels (Matsui et al., 2006). Two Sox17 Cre lines have previously been generated. The original Sox17-iCre line showed expression in arteries but not endoderm (Liao et al., 2009). We have, therefore, used the Sox17-2A-iCre, which contains a viral 2A sequence to allow production of equimolar amounts of Sox17 and iCre. This line shows activity in both the vasculature and endoderm, with expression starting in the extraembryonic visceral endoderm at E6.0, followed by the embryonic visceral and definitive endoderm at E7.5 (Engert et al., 2009).

Heterozygous Sox17-2A-iCre (Engert et al., 2009) and Mesp1-Cre males (Saga et al., 1999; Engert et al., 2009) were mated overnight to homozygous R26R females and the embryos and pups were taken at a range of stages. Noon after the detection of the vaginal plug was considered as embryonic day (E) 0.5. All animals were culled using a schedule 1 method as approved by the Home Office and King's College London. Positive embryos (approximately 50%) were selected using a quick X-gal staining of the bodies/lungs (20 min in X-Gal solution at 37°C). Approximately two litters were taken for each embryonic stage examined and six specimens were used for each postnatal stage.

Whole-Mount LacZ Staining for Wax Embedded Tissue

Sox17-2A-iCre/R26R and Mesp1-Cre/R26R-positive embryo heads were

dissected to show the oral cavity at a range of stages (E10.5, E11.5, E15.5, E16.5). Excess tissue was removed before staining. Tissue was fixed in 4% paraformaldehyde (PFA) for 45 min, washed in phosphate buffered saline (PBS) and stained in X-Gal staining solution for 42 hr at 37°C. After staining, the tissue was post-fixed in 4% PFA overnight and photographed. For sectioning of stained tissue, samples were processed through a methanol series, isopropanol, and tetra-hydro-naphthalene to wax. Oral tissue was then sectioned at 8–10 μm thickness for frontal and sagittal sections. Slides were counter stained with eosin.

LacZ Staining for Cryostat Embedded Tissue

New born and postnatal mouse tongues and heads were fixed in 4% PFA for 30 min, washed in PBS, then cryoprotected with 25% sucrose, embedded in OCT (VWR) and frozen using dry ice. Sections were cut sagittally at 15 μm on a cryostat and mounted onto Superfrost Plus slides. The sections were incubated in X-Gal staining solution for approximately 6 hours at 37°C. After staining, the tissue was counterstained with eosin and mounted in 90% glycerol PBS. Serial sections were stained with trichrome (Alcian blue, Sirrus red, hematoxylin) for histology.

Alkaline Phosphatase Staining

Postnatal mouse heads (n = 4) were fixed in 4% PFA for 30 min, washed in PBS, and the tongues dissected. The tongues were then washed twice in NTMT (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 1% Tween-20), and the alkaline phosphatase activity was detected using NBT (337.5 μg/ml, Roche) and BCIP (175 μg/ml, Roche) in NTMT for ~2 hr at 37°C. After staining, the tongues were washed twice in PBS and imaged.

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