RESEARCH ARTICLE

Shh Expression in a Rudimentary Tooth Offers New Insights into Development of the Mouse Incisor

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ABSTRACT

For teeth as for any organ, knowledge of normal development is essential for the proper interpretation of developmental anomalies in mutant mice. It is generally accepted that tooth formation is initiated with a single signaling center that, in the incisor region, is exclusively related to the development of the functional adult incisor.

Here, using a unique combination of computer-aided three-dimensional reconstructions and whole mount in situ hybridization of mandibles from finely staged wild-type mouse embryos, we demonstrate that several Sonic hedgehog (*Shh*) expression domains sequentially appear in the lower incisor region during early development. In contrast to the single *Shh* expression domain that is widely assumed to be present in each lower incisor area at ED12.5–13.5, we identified two spatially distinct regions of *Shh* expression that appear in an anterior–posterior sequence during this period. The initial anterior, more superficially located *Shh* expression region represented the rudimentary (so-called deciduous) incisor, whereas only the later posterior deeper situated region corresponded to the prospective functional incisor. In the more advanced embryos, only this posterior *Shh* expression in the incisor bud was detectable as a precursor of the enamel knot.

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This study offers a new interpretation of published molecular data on the mouse incisor from initiation through ED13.5. We suggest that, as with *Shh* expression, other molecular data that have been ascribed to the progressive development of the mouse functional incisor at early stages, in fact, correspond to a rudimentary incisor whose development is aborted. *J. Exp. Zool. (Mol. Dev. Evol.) 314B, 2011*. © 2011 Wiley-Liss, Inc.

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Early tooth development is controlled by reciprocal interactions between the epithelium and mesenchyme (Tucker and Sharpe, '99; Jernvall and Thesleff, 2000; Chai and Maxson, 2006; Caton and Tucker, 2009). It is generally accepted that at around ED (embryonic day) 11.5 in mice, the epithelial thickenings that are detected in the developing upper and lower jaws represent the first morphological signs of tooth development. During the initiation of tooth development, Sonic hedgehog (*Shh*) is expressed in these epithelial thickenings (Bitgood and McMahon, '95; Hardcastle et al., '98). It has been suggested that this expression domain presages the prospective tooth bud (Hardcastle et al., '98; Cobourne et al., 2001), which is clearly formed by ED13.5 (Kieffer et al., '99).

Mice have only one incisor in each jaw quadrant and this tooth is characterized by continuous growth and asymmetrical abrasion caused by the thick enamel on its labial surface. This growth is fueled by stem cells in the posterior (apical) end of incisor, whose progeny generate the various cell types in the tooth (Smith and Warshawsky, '75; Harada et al., '99, 2002; Suomalainen and Thesleff, 2010). Recently, a number of studies have been performed to elucidate the development of mouse incisors (Kaufman et al., '95; Miard et al., '99; Jarvinen et al., 2006; Klein et al., 2008; Liu et al., 2008; Munne et al., 2010; Nakatomi et al., 2010; Ohazama et al., 2010).

Early development of the lower incisor and adjacent oral vestibule in wild-type mice has been described on histological sections starting at ED11.5 (Hinrichsen, '59; Hay, '61; Pourtois, '61). The morphogenesis of the lower incisor from the bud to early bell stage has previously been studied using three-dimensional (3D) reconstructions and correlated with the distribution of mitoses and apoptosis (Kieffer et al., '99). Already Nakatomi et al. (2010) have shown that the *Shh* expression domain can also be localized in the epithelium separating incisor bud and the vestibular lamina at ED12.25. However, there is still a lack of detailed data at the earliest stages of lower incisor development in wild-type mice, especially regarding the formation of the incisor bud.

Abnormalities at the earliest stages of morphogenesis usually lead to severe morphological changes in an adult tooth and to the formation of different pathologies (Ranta, '88). Therefore, a full appreciation of the sequential steps of early morphogenesis is very important both for the full understanding of normal tooth development and to study the origin of tooth anomalies. Recently, it has become apparent that elucidating the formation of the large incisor placode as well as explaining the origin of the incisor will be essential to future progress in the field (Munne et al., 2010). For these reasons, we focused on the earliest steps of lower mouse incisor development in wild-type mice from the early epithelial thickening to the formation of the dental bud (from ED11.5 through ED13.5). We used a combination of histology, 3D reconstructions, and *Shh* whole mount in situ hybridization (WISH) analyses to search for the developmental origin of the lower incisor in the wild-type mouse mandible. In addition, we investigated the development of the adjacent epithelium of the oral vestibule.

The study reported here has provided new insights into the early lower incisor development in wild-type mouse. According to the generally accepted view, there is only one *Shh* signaling center in each half of the mandible at early stages, which is involved in functional incisor development. In contrast, we clearly documented several *Shh* expression domains concentrated in two (anterior and posterior) regions that differed in time and position. We showed that these regions reflect the sequential development of the rudimentary and functional mouse incisor, respectively. These findings strongly suggest the necessity to re-interpret previous results based on either morphology alone or on the detection of signaling molecules alone, and offer a new insight into the interpretation of existing data from mutant mice.

MATERIAL AND METHODS

Mouse Embryos

CD1 mice (Charles River, Germany) and ICR mice (Velaz, Czech Republic) were used for routine histology and WISH.

The mice were mated overnight and midnight before the morning detection of the vaginal plug was regarded as embryonic day (ED) 0.0. The embryos were harvested at ED11.5, 11.75, 12.0,

12.5, 13.0, 13.5, 14.5, 15.5, and 16.0. The pregnant mice were killed by cervical dislocation, and the embryos were removed from the uterus. Immediately after the removal of embryos, their wet body weight was determined to refine the chronological staging (Peterka et al., 2002).

The embryos at ED11.5–13.5 were used for 3D reconstructions and for WISH. The data obtained by 3D analysis and WISH at early stages (ED11.5–13.5) were correlated with morphogenesis of the lower incisor on histological sections at ED14.5–16.0.

Breeding of Transgenic Mice

C57BL/6 mice carrying a cassette encoding a Shh-EGFP (Enhanced Green Fluorescent Protein) fusion protein and Cre recombinase inserted into the endogenous *Shh* locus (B6.Cg-*Shhtm1(EGFP/cre)Cjt*/J), as well as a reporter line containing EYFP (Enhanced Yellow Fluorescent Protein) inserted into the *Gt(ROSA)26Sor* locus, where expression of *EYFP* is blocked by an upstream *loxP*-flanked STOP sequence (B6.129X1-Gt(ROSA)26Sor<tm1(EYFP)Cos>/J), were purchased from the Jackson laboratory (Maine). These two strains were genotyped using appropriate Jackson's Lab Protocols and crossed in order to mark cells expressing *Shh* at the beginning of incisors development.

The pregnant mice were killed by cervical dislocation. Embryos were harvested at ED14.5, their wet body weight was determined and the lower jaw arches were dissected. The dissected jaws were photographed under a Leica AF6000 fluorescence microscope (Leica Microsystems GmbH).

Histology

After fixation in Bouin–Hollande fluid, the embryonic and fetal heads at ED11.5–16.0 were embedded in paraffin. Two hundred mouse embryonic heads were cut in a series of 5 µm or 7 µm frontal or sagittal sections. The sections were stained with hematoxylin–eosin and alcian blue.

Whole Mount In Situ Hybridization (WISH)

To visualize the early odontogenic areas in the mouse incisor region, *Shh* expression was detected using WISH in 52 ICR mouse embryos. Mandibles were dissected at ED11.5–13.5, washed in Rnase free PBS (pH 7.4), and fixed in 4% paraformaldehyde (PFA) solution over night at 4°C. Specimens were hybridized according to a standard protocol. The probe for *Shh* was generated by in vitro transcription from cDNA fragment (kind gift from Dr. A. McMahon, Harvard University, Cambridge, MA). The hybridized samples were documented using a Leica MZ6 stereomicroscope connected to a DC480 digital camera Leica (Leica Microsystems GmbH).

Cryosections

Hybridized lower jaws were embedded in a series of graded solutions of sucrose (Sigma, St. Louis, MI) diluted in PBS (pH 7.4). Specimens were embedded in O.C.T. Tissue Tek (Sakura, Tokyo, Japan) diluted 1:1 with 20% sucrose, frozen in isopentane (Sigma, St. Louis, MI) cooled on dry ice to −60°C, and sectioned frontally on a cryostat microtome Mikrom HM 560 (Mikrom Walldorf, Germany) in a series of 10 µm sections. The sections were postfixed in 4% formaldehyde and counterstained by Nuclear fast red (Fluka, Buchs, Switzerland), dehydrated, and mounted in Neomount (Merck, Darmstadt, Germany).

Computer-Aided 3D Reconstructions

For 3D reconstruction of the epithelium of the developing incisor and adjacent oral vestibule, we used 14 series of routinely stained frontal paraffin sections of mouse heads at ED11.5–13.5. The specimens were selected according to the chronological age (ED) refined by a body weight of embryos to create a longitudinal series of successive stages of incisor development. In all these specimens, the epithelium of the right and/or left lower incisor area was reconstructed. In addition, the right or left lower jaw quadrant was randomly selected in seven series of paraffin sections. In these specimens, the entire half of a dental arch was reconstructed to show the spatial relationships of the incisor and molar area in 3D.

Three-dimensional analysis of the developing incisor region was also undertaken in six representative *Shh* hybridized lower jaw quadrants at ED12.5–13.5. For the preparation of drawings, the series of frozen frontal sections of WISH specimens was used.

Contours of the dental and adjacent oral epithelium were drawn from each histological section (magnification of 170 × –310 ×) using a LEICA DMLB microscope (Leica Microsystems GmbH) or a JENAVAL microscope (Carl Zeiss, Jena, Germany) equipped with a drawing chamber.

Superimposition of the drawings was performed by the "best-fit method" (Gaunt and Gaunt, '78), with respect to the middle line and to the horizontal level for correct spatial positioning of the reconstructed structures.

Digitalization of the serial drawings and the correlation of successive images have previously been described (Lesot et al., '96). Three-dimensional images were generated using VG Studio Max 2.0 software (VG Studio Max, Heidelberg, Germany).

The reconstructed mouse incisors were ranked in a longitudinal series of the successive steps of tooth development. The early stages of tooth development (epithelial thickening, dental lamina, tooth bud) were distinguished according to Peterkova et al. ('96).

RESULTS

Morphological Analysis

At ED11.5 and 11.75, a broad swelling of thickened epithelium was present in the anterior part of the right or left mandible on 3D reconstructions (Fig. 1A,B, and D). In the cheek region, a second swelling of the dental epithelium was apparent. This latter

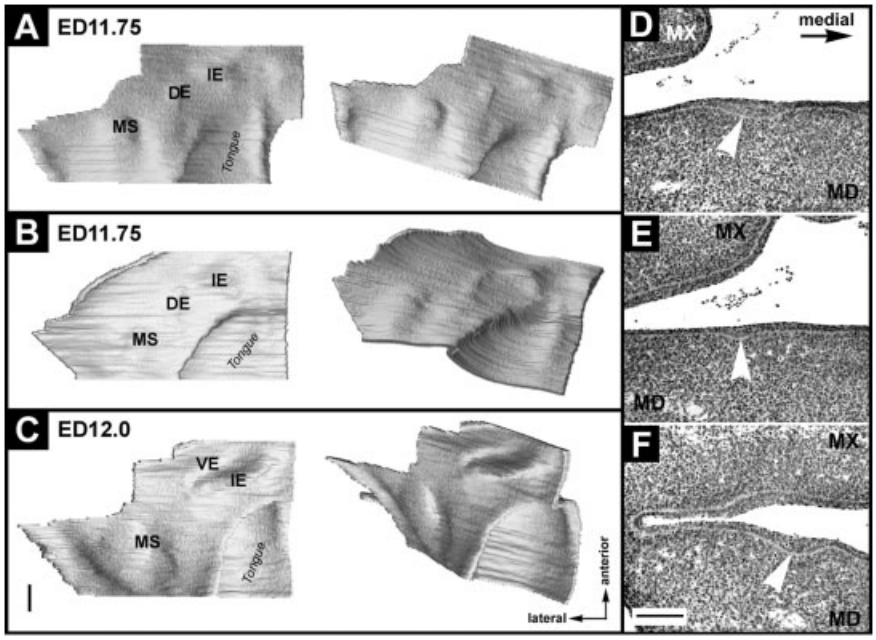

Figure 1. Early epithelial structures on the oral surface of mouse embryonic mandible. (**A–C**) 3D reconstructions, (**D–F**) frontal histological sections. (A) At ED11.75 (62 mg), three epithelial swellings appear in the lower jaw quadrant: in the prospective incisor area (IE), in the cheek region (MS), and as a smaller button-shaped epithelial swelling (DE) located between IE and MS. (B) At ED11.75 (70 mg), the button-shaped swelling elongated in a ridge, whose anterior limit joined IE swelling, and the posterior limit was located anteromedially from a rudimentary (premolar) tooth primordium (MS). The premolar tooth primordia appear chronologically before and positional anteriorly to the first molar (Peterkova et al., 2000; Prochazka et al., 2010). (C) At ED12.0 (86 mg), in the lip region, anteriorly (externally, labially) to the incisor swelling (IE) the vestibular epithelium (VE) appears. In the cheek region, MS is detectable. The prospective toothless diastema between IE and MS is free of epithelial swellings. (D–F) Morphology of the dental epithelium is shown along the anteroposterior course of the mandible at ED11.75. Arrowhead points the dental epithelium. MX, maxilla; MD, mandible. Bar indicates 100 μm.

structure corresponded to the initial anlage (rudimentary tooth primordium MS; see Fig. 1) in the cheek region of the mandible (Fig. 1A–C, F). Interestingly, in the more advanced specimens at ED11.75, a button-shaped epithelial structure (Fig. 1A) was present in the gap between the anterior and MS swellings (Fig. 1A and E). In the most advanced embryos at ED11.75, the button-shaped structure elongated into a low ridge (Fig. 1B), which seemed to join the lateral end of the anterior swelling at ED12.0 (Fig. 1C).

In the developmentally less advanced embryos at ED12.0, the morphology was very similar to ED11.5–11.75. In contrast, in the more advanced embryos at ED12.0, two epithelial swellings were detectable in the very anterior part of each left and right lower jaw quadrant (compare Fig. 2A–C). The inner (lingual) swelling corresponded to the dental epithelium at previous stages and on frontal section, it appeared to be at the lamina stage of tooth development. The outer epithelial swelling appeared externally (labially) to the dental epithelium; it corresponded to the anlage of the oral vestibule. A groove separated the developing dental and vestibular epithelia from each other. Similarly to the earlier stages, the rudimentary tooth primordium MS was clearly distinct in the cheek region of the mandible. The gap between the incisor and MS was free of swellings and its epithelium did not differ from other regions of the epithelium in the embryonic oral cavity.

At ED12.5, the vestibular epithelium formed a distinct ridge, classically called the vestibular lamina (Fig. 2D–F). The dental epithelium increased in size and its posterior end invaded deeper into the mesenchyme. At that place, the incisor bud started to develop (Figs. 2D and 3A). In the anterior part of the dental epithelium, three epithelial bridges formed, extending anteriorly from the anterior base of the incisor bud. They traversed the former groove separating the incisor bud and the vestibular lamina (Fig. 3A) and interconnected the developing tooth with the anlage of the oral vestibule (Fig. 3A–F).

At ED13.5, the incisor bud and the externally (labially) located vestibular lamina were clearly apparent (Fig. 3B). Only two epithelial bridges were distinct, interconnecting the incisor bud and vestibular lamina (Fig. 3B, C, G–I). The third, most laterally located bridge, which was observed at the previous stage, disappeared and was no longer detectable (Fig. 3C, G, H). The

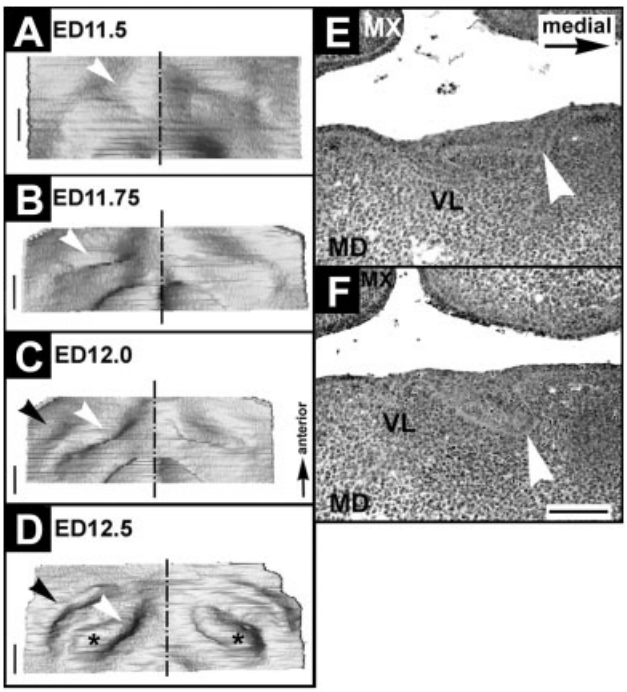

Figure 2. The morphology of the dental epithelium and the epithelium of the oral vestibule in the mouse mandible. (**A–D**) 3D reconstructions, (**E–F**) frontal histological sections. At ED11.5 (A) and ED11.75 (B), a broad epithelial swelling (white arrowhead) became apparent in the anterior part of the lower jaw. At ED12.0 (C), another area of thickened epithelium became apparent externally (labially) to the dental epithelial swelling (white arrowheads) and gave rise to a distinct anlage of the oral vestibule (black arrowheads) at ED12.5 (D). Contemporaneously, the posterolateral part of the dental epithelium (asterisk) started to enlarge giving rise to the incisor bud at later stages (compare to Fig. 4). The dental epithelium and vestibular lamina are documented on frontal sections in the anterior (E) and posterior (F) part of the incisor region at ED12.5. VL, vestibular lamina; MX, maxilla; MD, mandible. A shaded line indicates a midline. Bar indicates 100 μm.

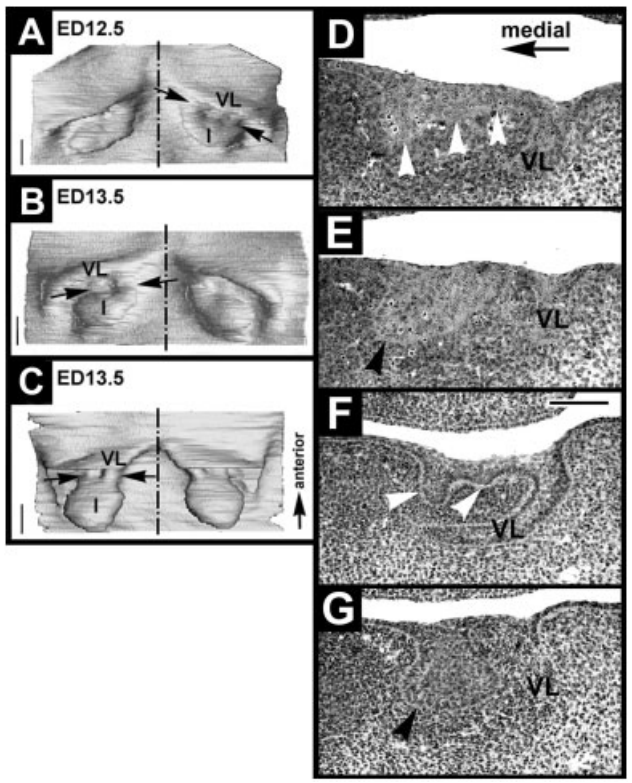

Figure 3. Origin of the epithelial bridges and the incisor bud in mouse embryonic mandible. (**A–C**) 3D reconstructions (**D–G**) frontal sections. (A) At ED12.5 (101–125 mg), the dental epithelium expanded in the posterior direction giving rise to the incisor bud (I) and three epithelial bridges (between black arrows) differentiated in the area adjacent to the vestibular lamina (VL). (B) At ED13.5 (126–150 mg), the most laterally located epithelial bridge disappeared and only two epithelial bridges remained distinct in the developmentally more advanced embryos (156 mg) at ED 13.5 (C). A part of VL in C has been cut artificially to expose the epithelial bridges between I and VL. At this stage of development, the vestibular lamina further grew over the bridges, which are not visible from the mesenchymal aspect. On frontal histological sections of the mandible at ED12.5 (D, E–shown anteroposteriorly), three epithelial bridges (white arrowheads) appear in the area between the incisor bud (black arrowhead) and externally located VL. In the mandible at ED13.5 (F, G–shown anteroposteriorly), the most laterally located epithelial bridge disappeared and only two epithelial bridges were present. A shaded line indicates a midline. Bar indicates 100 μm.

dental bud grew deeper into the underlying mesenchyme and extended more posteriorly (Fig. 3C). In contrast to previous stages, from older ED13.5 embryos (with body weight of 150 mg) onwards, apoptosis was concentrated in the region of epithelial bridges and in the base of the incisor bud (data not shown).

The two epithelial bridges remained clearly detectable on frontal histological sections at the anterior base of the incisor enamel organ until the end of the studied period (ED16.0) and a rudimentary incisor could be traced between the bridges (Fig. 4A and B).

Shh Expression

In the mandibles of the youngest wild-type embryos at ED11.5, only one narrow band of *Shh* expression extended laterodistally. At ED12.5, a band of *Shh* expression in each prospective incisor region remained apparent (Fig. 5A). The sections and 3D reconstruction documented that the *Shh* expression domain was situated in the epithelial thickening present at this stage of development in the lower jaw (Fig. 5B).

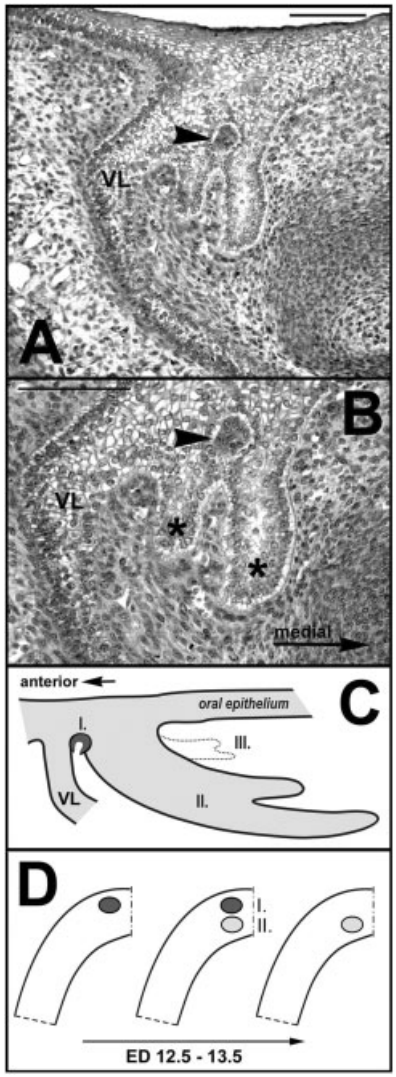

Figure 4. Rudimentary and prospective functional incisor in mouse mandible. (**A**, **B**) Frontal sections at ED 16.0 show the rudimentary prelacteal tooth (arrowhead) between the epithelial bridges (asterisks) at the base of the stalk of the developing lower incisor (compare to position of I. in C). (**C**) A tentative scheme presenting developmental potentiality to form three generations of teeth on a sagittal section of the lower incisor region in the mouse: prelacteal (I.), functional (II.) and a replacement tooth (III.). The criteria for classifying the rudimentary tooth at the labial base of the incisor enamel organ have been described by Fitzgerald ('73). In this rudimentary incisor, no enamel and no differentiated enamel organ have been found, but a shell of irregular dentin surrounded by few differentiated odontoblasts has been described (Fitzgerald, '73). The mouse functional incisor is generally regarded as the permanent incisor; the minute rudimentary tooth is generally assumed to represent a deciduous incisor. However, Woodward (1894) has suggested and Fitzgerald ('73) clearly expressed the opinion that this rudimentary structure represents instead an ancient tooth remnant persisting in modern mammals. Fitzgerald ('73) has related this minute rudimentary mouse incisor to the earliest (also rudimentary) generations of teeth in reptiles. We have supported this opinion by the similarity in morphology and place of origin (in the pits on the inner face of epithelium) between the minute rudimentary mouse incisors and the first formed rudimentary teeth in reptiles (Peterkova et al., 2002). That is why it has been proposed to classify the vestigial incisor in rodents not as a lacteal incisor, but as an element of the prelacteal dentition, with the functional incisor as the tooth corresponding to a lacteal tooth generation (Peterkova et al., 2002, 2006). The prelacteal teeth have been described as minute primitive tooth rudiments located labially/bucally from the regular functional lacteal teeth in many mammalian species including humans (Leche, 1893; Röse, 1895; Adloff, 1909; Moss-Salentijn, '75, '78). (**D**) A scheme summarizing the temporo-spatial pattern of *Shh* expression in the anterior part of the mouse embryonic mandible during ED 12.5–13.5. The dark gray indicates the anterior region of *Shh* expression corresponding to the rudimentary prelacteal incisor (I.) and the light gray indicates the posterior region of *Shh* expression corresponding to the functional incisor (II.). VL, vestibular lamina. Bar indicates 100 μm.

In embryos at ED13.0, the former *Shh* domain (placode) disintegrated into two adjacent spots of *Shh* expression located abreast in the anterior area in each mandible (Fig. 5C). The intensity of the expression was highest in the centers of spots. These two spots were limited to the rising epithelial bridges at the base of the incisor bud (compare Fig. 5D and E, and Fig. 3). In the more advanced specimens at ED13.0, the laterally located *Shh* expression domain intensified and the two spots tended to fuse. The vestibular area was free of *Shh* expression.

In the developmentally less advanced embryos at ED13.5, the former spots fused again. Only one triangularly shaped dark expression domain (Fig. 5F) matched the area of epithelial bridges and reached up to the anterior base of the developing tooth bud (compare Fig. 5G and H and Fig. 3).

In the more advanced embryos at ED13.5, compared with the previous stage, the posterior part of the expression domain at the base of the incisor bud became less intense and smaller (compare Fig. 5F–K). The expression domain was located on the anterior part of the dental epithelium adjacent to the vestibular lamina and matched the epithelial bridges projecting from the incisor bud toward the vestibular lamina (Fig. 5J and K).

Next, a new large spot of *Shh* expression appeared posteriorly. As a result, two *Shh* expression domains were present in each lower jaw quadrant: a smaller (former) one located anteriorly and a larger (newer) one located posteriorly (Fig. 5L). The 3D reconstruction showed that the smaller expression domain was positioned more superficially and matched the epithelial bridges (Fig. 5M and N). This expression domain was a remnant of the

Shh expression domain visible at the previous stage (compare Fig. 5J, K and M, N). The posterior, deeper, and more intense *Shh* expression domain was located at the tip of the incisor bud growing into the mesenchyme (Fig. 5M, N). In the area between the anterior and posterior expression domains, less intense staining was detected.

In all specimens at ED13.5, the vestibular epithelia were free of *Shh* expression. In the developmentally most advanced embryos at ED13.5, the anterior *Shh* expression domain, which matched the epithelial bridges originally, diminished (Fig. 5O–Q). The *Shh* expression remained apparent only at the tip of the well-formed incisor bud (Fig. 5P and Q). This later *Shh* expression domain preceded the enamel knot appearing at ED14.0 morphologically.

The Origin of the Prospective Oral Vestibule

To trace the lineage of cells expressing *Shh* from the beginning of incisor development, we crossed the mice carrying Cre recombinase in the endogenous Shh locus with reporter mice carrying enhanced Yellow Fluorescent Protein (EYFP) inserted into the *ROSA26* locus. In these mice, the expression of *EYFP* is blocked by an upstream *loxP*-flanked STOP sequence except in cells expressing *Shh*, in which case the STOP sequence is excised. Thus, in these mice, cells expressing *Shh* and all their descendants expressed YFP. We found that at ED14.5, YFP-positive cells were present not only in the area of the developing incisor germ but also in the epithelial anlage of the oral vestibule (vestibular lamina) developing externally to the tooth (Fig. 6A).

DISCUSSION

Tooth development is thought to be initiated between ED9 and ED11, as the oral epithelium signals to the underlying neural crest derived mesenchyme through FGFs (Fibroblast Growth Factors), BMPs (Bone Morphogenetic Proteins), WNTs, and SHH (Sonic hedgehog). These events are accompanied by localized thickenings of the oral epithelium at the position of the developing teeth (for review see: Miletich and Sharpe, 2003).

It is generally assumed that *Shh* expression in the incisor region before ED13.5 is exclusively linked to development of the prospective functional incisor. In contrast our combined analysis by morphological methods and the detection of *Shh* expression in a series of finely staged mouse embryos allowed the identification of two successively appearing anterior and posterior regions of *Shh* expression that corresponded to the primordia of the rudimentary and functional mouse incisor, respectively (Fig. 4C and D).

Morphological Considerations

At ED11.5 (Fig. 2A), one broad swelling of thickened dental epithelium was present in the anterior part of each half of the mandible. At ED12.5, the epithelial anlage of the oral vestibule started to differentiate externally to the dental epithelium (Figs. 1C and 2C,D). Dassule and McMahon ('98) have shown that *Shh* expression initiates as a single linear domain in the prospective incisor area at ED11.0. Using WISH, we did not find any *Shh* expression in vestibular epithelia during the period under observation (ED11.5–13.5). However, using reporter mice, we documented that both dental and vestibular epithelia at ED14.5 are descendants of the cells expressing *Shh* (Fig. 6A). Thus, we suggest that the cells of the first *Shh* expression domain contribute to the vestibular epithelium. Previously, a close relationship of the developing murine dentition to the vestibular epithelium has been reported (Hay, '61; Peterkova, '85). A common origin of the teeth and oral vestibule has been found also in the lip region of the human lower jaw (Hovorakova et al., 2007). In contrast, separate development of the dental and vestibular epithelia has been described in the upper jaw (Hovorakova et al., 2005) and in the cheek region of the lower jaw in human (Hovorakova et al., 2007). Further studies should focus on this topic and try to determine the developmental origin of the cell population that gives rise to vestibular lamina.

In the area between the incisor bud and vestibular lamina, epithelial bridges connected the tooth bud with the adjacent anlage of the oral vestibule (see Fig. 3). The location of the epithelial bridges at the labial (anterior) base of the incisor enamel organ corresponds to the area where a rudimentary tooth occurred at later prenatal stages (Fig. 4A, B). This tooth (also called "milk," "lacteal," "deciduous" or "prelacteal" incisor; see Fig. 4C) has been described in the upper and lower jaws in mouse (Woodward, 1894; Fitzgerald, '73; Peterkova et al., 2002) and also in rat (Moss-Salentijn, '75, '78).

In the mouse upper jaw, multiple buds of epithelium are observed during the earliest stages of incisor development, and these can be detected on the mesenchymal surface of the incisor bud as well. These swellings reflect the composite origin of this tooth based on fusion of multiple placodes that reflect ancestral tooth primordia (Peterkova et al., '93, '95, 2002). Interestingly, incisors with grooved enamel surfaces have been described in a mouse carrying a hypomorphic mutation of Lrp4, a cell surface receptor that modulates multiple signaling pathways (Ohazama et al., 2010). Based on the origin of the mouse upper incisor from fusion of primordia of several incisors present in mouse ancestors (Peterkova et al., '93, '95), the grooves on the mutant incisor could manifest an incomplete fusion of incisor subunits (ancestral tooth primordia) during tooth formation. In this study, the remnants of the initial epithelial budding (three epithelial bridges) in the mandible were reflected on the mesenchymal surface of the anterior base of the lower incisor bud at ED13.5 (Fig. 3). The epithelial bridges increased in height and entered the incisor bud proper. Based on the present data, the epithelial ridge described along the whole anteroposterior extent of the labial surface of the incisor enamel organ from ED13.5 by Kieffer et al. ('99) can now be interpreted as a chronological successor and

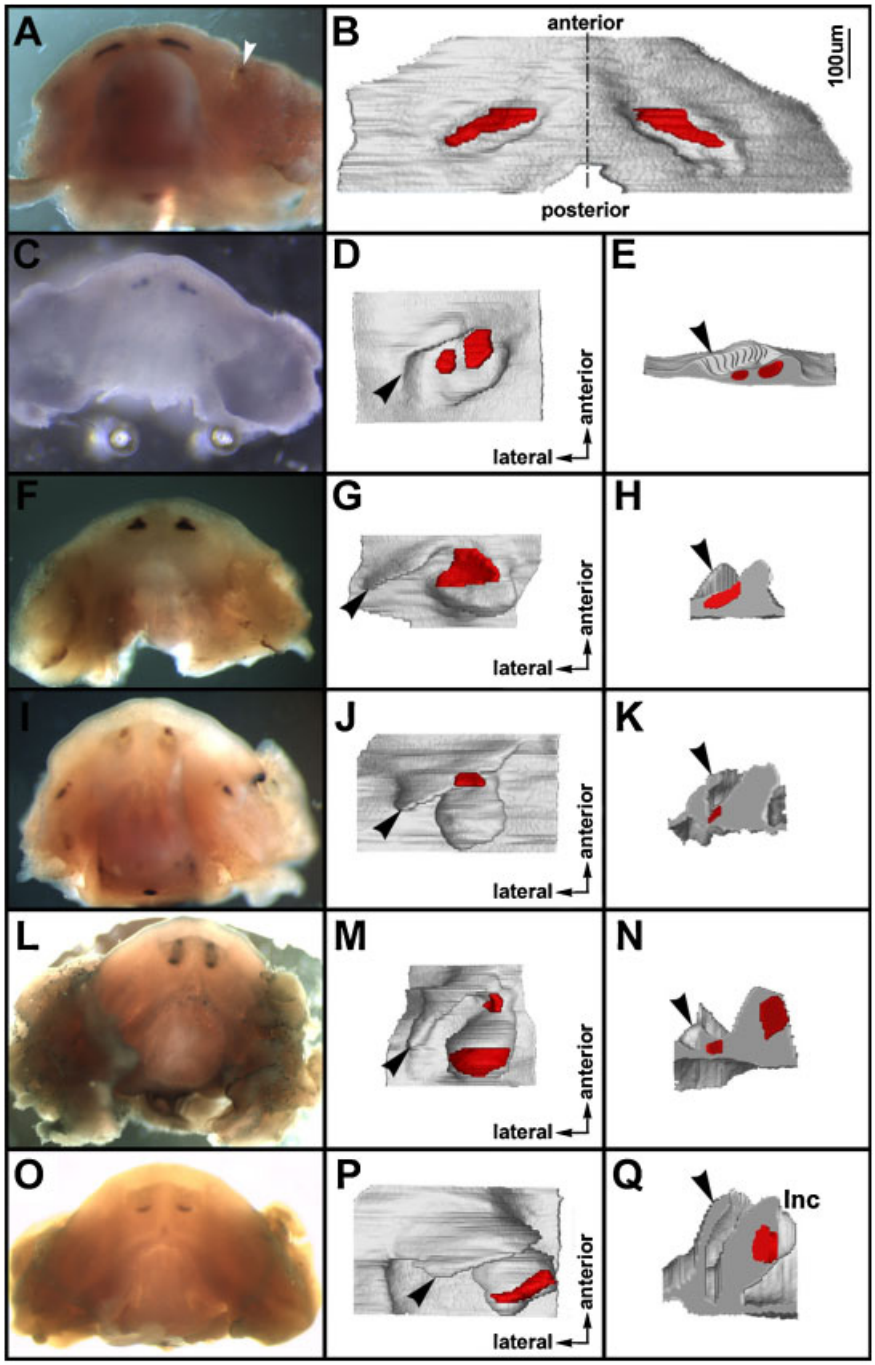

Figure 5. *Shh* expression in the incisor region of the mouse embryonic mandible. **(A, C, F, I, L, O)** WISH hybridized lower jaws in wild-type mice **(B, D, G, J, M, P)**, mesenchymal aspect of the dental and adjacent epithelium in 3D reconstructions with red-labeled *Shh* expression, **(E, H, K, N, Q)** virtual sections through the 3D reconstructions to expose the *Shh* expression. (A) At ED 12.5 (98 mg), WISH showed one domain of *Shh* expression in the anterior part of each lower jaw quadrant. In the cheek region, the small spot of *Shh* expression corresponded to the anlage of MS rudiment (white arrowhead), which appears chronologically before and positional anteriorly to the first molar (Peterkova et al., 2000; Prochazka et al., 2010). The 3D reconstruction documented that the *Shh* expression domain matched an epithelial thickening (B). (C) At ED13.0 (118 mg), WISH showed two *Shh* expression domains located abreast in each lower jaw quadrant. The position of these two *Shh* expression domains was limited to the epithelial budding adjacent to the vestibular lamina indicated by black arrowhead (D, E). (F) In the

continuation of the middle (most pronounced) epithelial bridge (Fig. 3C). We propose that the lower functional mouse incisor also has a composite origin. It develops as a lingually located successor to the multiple epithelial budding at the place of the three epithelial bridges (Fig. 3). The multiple budding and related *Shh* expression domains represent the initial developmental potential to form several smaller teeth of the first (prelacteal) generation (Figs. 3–5). The origin of a single composite tooth from fusion of several ancestral tooth primordia (concrescence theory and dimmer theory) has been suggested long ago (Kükenthal, 1892; Röse, 1892; Adloff, '16; Bolk, '15, '22). We have previously proposed that conjoint development (concrescence) of tooth primordia may be regulated by activator–inhibitor signal gradients (Peterkova et al., 2000, 2002). Indeed, concrescence during development of the mouse first molar has been studied experimentally by visualization of shared development of epithelia that are marked by separate *Shh* expression domains (Prochazka et al., 2010).

Kieffer et al. ('99) found a region of apoptosis in the base (stalk) of the lower incisor enamel organ and adjacent oral epithelium from ED13.5 (150 mg) through the end of the observation period (ED16.0), in wild-type mice and this observation was confirmed in this study. Moreover, we documented that the above-mentioned apoptosis concentrates after the disappearance of *Shh* expression in the epithelial bridges and adjacent base of the enamel organ of prospective functional incisor. The apoptosis in the base (stalk) of the lower incisor enamel organ (Kieffer et al., '99) has also been found by Munne et al. (2009) in E14 wild-type mice, and the disappearance of apoptosis in *Sostdc1* mutant mice has been correlated with ectopic epithelial Wnt activity and appearance of a supernumerary incisor (Munne et al., 2009). This extra tooth has been found lingually (in tandem) with the main incisor and interpreted as a rudimentary replacement tooth of the main incisor, whose lingual epithelium has the capacity of tooth formation (Munne et al., 2009). In a similar fashion, suppression of epithelial apoptosis is involved in the revitalization of a rudimentary premolar tooth primordium and development of a supernumerary tooth in *Spry2* null mice (Peterkova et al., 2009).

Together, these data suggest that the base of the prospective incisor is the origin of the rudimentary tooth labially and of a potential replacement incisor lingually. We propose that there is developmental capacity to the formation of three tooth generations in the lower incisor region in mouse (Fig. 4C). The apoptosis located in front of and in the base of the prospective incisor not only blocks development of a replacing tooth but also impairs full development of the rudimentary incisor. Future studies will help to determine which of the three potential incisor generations (Fig. 4C) can be implicated in the origin of the extra incisors in mice after experimental or genetic manipulations or under pathological conditions.

Shh Expression

In each right and left lower incisor area, we detected two distinct sequentially appearing *Shh* expression regions in tandem (see Figs. 4D and 5). The anterior *Shh* expression region was present in the epithelial bridges and adjacent epithelial base of the growing incisor enamel organ, and the posterior one was at the tip of the incisor bud. At ED11.0, a single strip of *Shh* expression in the anterior part of the mouse mandible has been shown previously (Dassule and McMahon, '98). At ED12–12.5, the single *Shh* expression domain has been called a "large incisor placode" (Munne et al., 2010). This single *Shh* expression domain is generally thought to be related to the formation of a functional large incisor in mice.

This study clearly documented that this "large incisor placode" only corresponds to the anterior *Shh* expression domain at ED12.5 (Fig. 4D and 5A, B) in the epithelial bridges. Thus, this domain is related to the initiation of the rudimentary (prelacteal) tooth, which is an ancient structure persisting in mouse dentition. The *Shh* expression in the epithelial bridges may predetermine the later position and formation of the functional lower incisor. Similarly, the earliest generations of rudimentary teeth in reptiles and sharks have been proposed to play a role in determining the position of sequentially initiated teeth (Smith, 2003), and the rudiments of prelacteal dentition in mammals may have similar function (Peterkova et al., 2006). Thus, rudiments appear to have general importance in the development of structures in extant species (Darwin, 1859; Peterkova et al., 2006; Prochazka et al., 2010).

Shh expression in the epithelial bridges became restricted, then less intense, and finally disappeared in developmentally more advanced embryos at ED13.5. Concomitantly, a new region of *Shh* expression appeared at the tip of the growing incisor bud.

Figure 5. (*Continued*) developmentally less advanced embryo at ED13.5 (128 mg), only one *Shh* expression domain was detectable on WISH mandible. 3D reconstruction showed that it matched the epithelial bridges and adjacent base of the incisor bud (G, H). (I) In the more advanced embryo at ED13.5 (154 mg), the *Shh* expression domain was reduced in size, but remained located at the epithelial bridges (J, K). (L) In the developmentally still more advanced specimen at ED13.5 (164 mg), two *Shh* expression domains in tandem were distinct. A smaller one was still located in the area of the epithelial bridges and was a remnant of the *Shh* expression at previous stages. A new more intense *Shh* expression domain was located at the tip of the incisor bud (M, N). (O) In the most advanced embryo at ED13.5 (174 mg), the anterior *Shh* signaling area disappeared, and only the posterior *Shh* expression domain at the incisor bud tip remained visible (P, Q). A shaded line indicates a midline. Bar indicates 100 μm.

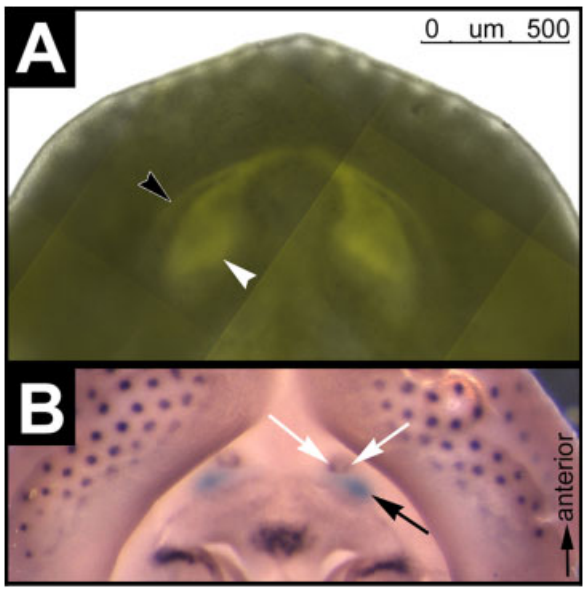

Figure 6. Lineage tracing in the mandible at ED14.5 and *Shh* expression in the upper jaw at ED13.5. (**A**) Mice carrying Cre recombinase in the endogenous *Shh* locus were crossed with reporter mice carrying enhanced Yellow Fluorescent Protein (EYFP) to trace the lineage of cells expressing *Shh* from the beginning of incisors development. All the descendants of the cells expressing *Shh* are YFP positive (yellow). At ED14.5, YFP positive cells were present not only in the area of the developing incisor germ (white arrowhead) but also in the epithelial anlage of the oral vestibule (black arrowhead). (**B**) WISH in the upper jaw in CD1 mouse showed quite similar situation to the lower jaw (compare to Fig. 5). Two *Shh* expression domains (white arrows) appeared in the anterior region and one *Shh* expression domain (black arrow) in the posterior region of the upper incisor area. These anterior and posterior *Shh* expression regions also appear to reflect two generations of teeth (rudimentary and functional) similarly to the mandible (see Fig. 4).

This posteriorly appearing *Shh* expression domain corresponds to the lower functional incisor germ and it precedes the enamel knot, which transiently occurs at ED14.0 (Kieffer et al., '99). Our preliminary data indicate that two generations of expression regions related to two generations of teeth exist also in the upper jaw (Fig. 6B). The anterior and posterior *Shh* expression domains in the incisor region of the upper jaw have been seen also in the figure by Pantalacci et al. (2008).

In the mouse embryonic mandible, Munne et al. (2010) have found a disintegration (splitting) of the incisor placode at ED12.5 into three small placodes after the inhibition of BMP signaling in vitro. In their experiment, the large incisor was replaced by two or three small teeth (Munne et al., 2010). Interestingly, two adjacent BMP2 expression domains in each lower incisor region have been reported in wild-type mice at ED12.25 by Nakatomi et al. (2010). We observed under physiological conditions the trend to disintegration of the initial *Shh* expression domain (placode) in the anterior part of the mandible at ED13.0 in wild-type mice (Fig. 5A–E). This disintegration correlated with the formation of the distinct epithelial bridges (compare Fig. 5D, E and Fig. 3). Thus, the large placode disintegration is not exclusively found during supernumerary teeth formation in mutant mice; it occurs also in wild-type mice at ED13.0. We propose that the early large *Shh* expression domain in wild-type mice arises from fusion of the small signaling centers of separate ancestral teeth. The predisposition of the placode to disintegrate is thus a physiological phenomenon suggesting that the original components keep a certain degree of autonomy. The ancestral subunits can fully develop under experimental conditions and lead to the formation of supernumerary teeth.

CONCLUSION

We propose that the anterior and more superficial *Shh* expression as well as the initial budding in the area of epithelial bridges reflect a more primitive rudimentary (prelacteal) tooth formation, which precedes the formation of the functional incisor. Only the more posteriorly and deeper located epithelial budding and *Shh* expression domain that appear at ED 13.5 (Fig. 5O and P) are the site of the lower functional incisor in wild-type mouse (Fig. 4D). These findings enable re-interpretation of previous results based either only on morphology or only on gene expression patterns and offer a new insight in the interpretation of data obtained in mutant mice. The developmental studies based on *Shh* expression before ED13.5, believed to be performed on early stages of incisor development, may in fact have been performed on the rudimentary incisor, which precedes the formation of the functional incisor.

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