

An FGF signaling loop sustains the generation of differentiated progeny from stem cells in mouse incisors

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Rodent incisors grow throughout adult life, but are prevented from becoming excessively long by constant abrasion, which is facilitated by the absence of enamel on one side of the incisor. Here we report that loss-of-function of sprouty genes, which encode antagonists of receptor tyrosine kinase signaling, leads to bilateral enamel deposition, thus impeding incisor abrasion and resulting in unchecked tooth elongation. We demonstrate that sprouty genes function to ensure that enamel-producing ameloblasts are generated on only one side of the tooth by inhibiting the formation of ectopic ameloblasts from self-renewing stem cells, and that they do so by preventing the establishment of an epithelial-mesenchymal FGF signaling loop. Interestingly, although inactivation of *Spry4* alone initiates ectopic ameloblast formation in the embryo, the dosage of another sprouty gene must also be reduced to sustain it after birth. These data reveal that the generation of differentiated progeny from a particular stem cell population can be differently regulated in the embryo and adult.

KEY WORDS: Ameloblast, Enamel, FGF signaling, Sprouty genes, Stem cells

INTRODUCTION

Rodent incisors are unusual among mammalian teeth in that they grow continuously throughout the life of the animal. This growth is fueled by stem cells in both the mesenchymal and epithelial compartments of the incisor, the progeny of which perpetually generate the various cell types in the tooth (Gronthos et al., 2002; Smith and Warshawsky, 1975). Incisor growth is counterbalanced by abrasion, without which the tooth would become excessively long and interfere with feeding. It would be difficult to abrade the incisors if enamel, the hardest component of the tooth, covered the entire surface of the incisor as it does in the molar. However, in rodent incisors enamel is normally present on the labial surface (facing the lip) and is absent on the lingual surface (facing the tongue) (see Fig. 1A). This asymmetry not only facilitates the abrasion that keeps incisor length relatively constant, but also ensures that the tooth will be filed down primarily on one side, thus generating a sharp tip (Addison and Appleton, 1915).

Little is known about the stem cells that generate enamel-producing ameloblasts (ameloblast stem cells, or ASCs), because markers for them have not yet been identified. It has been proposed that ASCs reside in a niche located within a region called the cervical loop (CL) at the posterior end (base) of the incisor (Harada et al., 1999) (see Fig. 1A), but it is not known if ASCs give rise only to ameloblasts or also to the other epithelial cell types that must be continuously generated as the tooth grows. Based on models for the generation of differentiated progeny from stem cells in other tissues, such as the crypt of the intestinal villus (Fuchs et al., 2004), it has further been speculated that ameloblast formation begins when ASC

progeny move out of the putative niche and develop into transit-amplifying (T-A) cells that undergo a limited number of cell divisions (Harada et al., 1999; Wang et al., 2007). It is known that, once formed, ameloblasts move anteriorly along the length of the incisor (toward the tip) as they differentiate. After producing and depositing enamel, ameloblasts either undergo apoptosis or shrink in size (Smith and Warshawsky, 1975). Genetic analysis has shown that members of the fibroblast growth factor (FGF) family of secreted signaling molecules play a role in regulating ameloblast development or function, as *Fgf3*^{-/-} mice have defective enamel and *Fgf3*^{-/-}; *Fgf10*^{+/-} mice have very thin or no enamel (Wang et al., 2007). Furthermore, based on data from studies of incisors developing in vitro it has been suggested that *Fgf10* regulates epithelial stem cell survival (Harada et al., 2002; Yokohama-Tamaki et al., 2006).

In wild-type incisors, the lack of lingual enamel is due to the absence of ameloblasts on that side (Smith and Warshawsky, 1975). However, it is not yet known whether lingual ameloblasts are absent because their formation from ASCs is blocked or because there are no ASCs in the lingual CL. Interestingly, the lingual CL differs in morphology from the labial CL (see Fig. 1A), which might reflect a lack of either T-A cells or ASCs. These morphological differences are correlated with asymmetries in the expression patterns of genes encoding members of the FGF family. For example, *Fgf3* is detected in the mesenchyme surrounding the labial but not the lingual CL, and *Fgf10* is expressed at a higher level on the labial side than on the lingual side (Harada et al., 1999). Furthermore, ectopic *Fgf3* expression in lingual mesenchyme is associated with the formation of lingual ameloblasts in embryos homozygous for a null allele of follistatin (*Fst*), which encodes an extracellular inhibitor of signaling by transforming growth factor β (TGF β) superfamily members (Wang et al., 2007; Wang et al., 2004). Together, the available data suggest that suppression of FGF signaling on the lingual side is necessary to prevent ameloblast formation.

Here we identify sprouty (*Spry*) genes, which encode intracellular antagonists of FGF and other receptor-tyrosine kinase signaling pathways (Mason et al., 2006), as essential for establishing and sustaining the asymmetry of enamel deposition necessary for normal incisor length and shape. We provide genetic evidence that sprouty

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genes prevent the generation of lingual ameloblasts by inhibiting an FGF-mediated epithelial-mesenchymal signaling loop on the lingual side. Furthermore, our data suggest that the earliest ameloblasts that form in the incisor do not arise from ASCs, but instead are derived from a transient embryonic ameloblast progenitor cell population that does not self-renew.

MATERIALS AND METHODS

Mouse lines

Mouse lines carrying mutant alleles of *Fgf9* (Colvin et al., 2001), *Fgf10* (Min et al., 1998), *Fst* (Matzuk et al., 1995), *Spry1* (Basson et al., 2005), *Spry2* (Shim et al., 2005), *Spry4* (Klein et al., 2006) and the *K14-cre* (Dassule et al., 2000) and *Wnt1-cre* (Danielian et al., 1998) transgenes were maintained and genotyped as reported, except that *Fst* wild-type and null alleles were genotyped using PCR rather than Southern blotting (primer sequences available on request). Age-matched CD1 embryos and adults were used as wild-type controls in all cases.

Gene expression and histological analysis

To stage embryos, noon of the day when a vaginal plug was detected was considered embryonic day (E) 0.5. RNA in situ hybridization was performed according to standard protocols on paraffin sections (10 μ m) using digoxigenin-labeled probes. Tissue was prepared for sectioning by fixing embryonic heads in 4% paraformaldehyde (PFA) or postnatal jaws in 4% PFA, and then decalcifying in RNase-free EDTA for 2–4 days before sectioning. For postnatal histology, jaws were fixed in Bouin's solution, decalcified using a solution of 50% formic acid and 0.7 M sodium citrate (mixed 1:1), embedded in paraffin, sectioned at 7 μ m, and stained with Heidenhain's Azocarmine-aniline Blue (AZAN) stain. For photography of intact adult jaws, mouse heads were boiled for 30 minutes in distilled water and soft tissues were carefully removed.

X-ray computed tomography

X-ray computed tomography (XTM) was used to assess the degree of mineralization as previously described (Kinney et al., 2000). Briefly, mouse incisors ($n=5$ per group) were scanned at the Advanced Light Source (Lawrence Berkeley National Laboratory) and two-dimensional radiographs were obtained as the specimens were rotated through 180° in 0.5° increments. The radiographs were reconstructed into 1000 slices by Fourier-filtered back projection with a 10.5 μ m resolution. The attenuation coefficient (mm^{-1}) of each pixel is represented by false colors and relates directly to mineral concentration.

RESULTS

Spry4^{-/-};*Spry2*^{+/-} mice have abnormal, 'tusk-like' incisors due to ectopic enamel deposition on the lingual side of the tooth

We found that *Spry4*^{-/-};*Spry2*^{+/-} mice, obtained by crossing mice carrying combinations of *Spry4* (Klein et al., 2006) and *Spry2* (Shim et al., 2005) null alleles, had excessively long and thick mandibular incisors that resembled tusks (Fig. 1B,F). Animals of this genotype will hereafter be referred to as 'tusk mutants'. Abnormally long incisors can result from a lack of abrasion when the maxilla and mandible are misaligned. However, we detected no obvious craniofacial abnormalities in tusk mutants, and their incisors were thicker than those found in wild-type mice or in animals we sporadically found in our mouse colony in which there was overgrowth due to misalignment (not shown). Thus, we hypothesized that the excessive length and thickness of the tusk-like incisors might be due to deposition of lingual enamel, which would both thicken the mutant incisors and make them resistant to abrasion.

We performed synchrotron XTM, which measures the relative mineral concentration in calcified tissues and distinguishes enamel from dentin and bone. In adult wild-type and *Spry4*^{-/-} (*Spry4* null)

incisors, which were of normal length and thickness, we detected enamel only on the labial surface (Fig. 1C,D). By contrast, tusk mutant incisors had enamel on both labial and lingual surfaces (Fig. 1E, and data not shown). Here we will focus on the phenotype of the mandibular incisors; the maxillary incisors have additional abnormalities, and will be described in a separate study.

Sagittal histological sections of postnatal day (P) 14 tusk mutant incisors confirmed that they had ectopic enamel along much of the anteroposterior length of the lingual surface (Fig. 1G,H). Underlying this enamel we observed an ectopic layer of columnar cells, which resembled the ameloblasts that are normally found exclusively on the labial side (Fig. 1G-L). In addition to the presence of this ectopic layer of lingual ameloblasts, hereafter referred to as the 'lingual ameloblast phenotype', the morphology of the lingual CL was abnormal. In wild-type incisors the lingual CL is flattened and composed of a cuboidal epithelium (Fig. 1M, and data not shown), whereas in tusk mutants it resembled the labial CL in that it was more bulbous and composed of a columnar epithelium (Fig. 1O and data not shown; compare with Fig. 1N,P). These data indicate that sprouty genes function in the incisor to prevent ameloblast production on the lingual side, thus facilitating incisor abrasion and preventing excessive incisor length.

Pre-ameloblasts are present on the lingual side of both *Spry4*^{-/-};*Spry2*^{+/-} and *Spry4*^{-/-} incisors at embryonic stages

As ameloblasts are continuously lost at the anterior end of the rodent incisor as it grows (Smith and Warshawsky, 1977), and we found that lingual enamel was present throughout the life of the tusk mutants, it follows that ameloblasts must be continuously produced on the lingual side in these mice. To determine when lingual pre-ameloblasts (cells that have not yet begun to produce enamel matrix proteins) first appear in the tusk mutants we assayed for expression of sonic hedgehog (*Shh*), which is expressed in ameloblasts from a very early stage in their development and is downregulated as they mature (Bitgood and McMahon, 1995). On the labial side, we detected *Shh*-expressing cells in wild-type and tusk mutant incisors at E15.5 and 16.5, along the length of the epithelium anterior to the CL (Fig. 2A,B,D,E). At E16.5 we also detected expression of amelogenin (*Amelx*), a gene crucial for proper enamel formation (Zeichner-David et al., 1995), on the labial side near the anterior end of the incisor (Fig. 2C,F). Thus as expected, we found that cells in the labial epithelium were differentiating along the ameloblast lineage, with those at the anterior end of the incisor at a more advanced stage.

On the lingual side, *Shh*-expressing cells were not detected in wild-type or tusk mutant incisors near the cervical loops at E15.5 (Fig. 2A,D), but by E16.5 *Shh*-expressing cells were observed in the lingual epithelium of the tusk mutant, in a small domain just anterior to the CL (Fig. 2B,E). These data suggest that lingual pre-ameloblasts begin to form in tusk mutants between E15.5 and 16.5, at least one day after they are present on the labial side. These lingual pre-ameloblasts go on to differentiate, as *Amelx* expression was detected in the lingual epithelium of tusk mutants by E18.5 (not shown).

We assumed that *Spry4* null incisors would not have lingual ameloblasts, because the incisors in *Spry4* null adults were neither excessively long nor thick (not shown) and had no enamel on the lingual side (Fig. 1D). Unexpectedly, we found that lingual *Shh*-expressing pre-ameloblasts were present in *Spry4* null incisors at E16.5 (compare Fig. 2G,H and 2D,E), which matured into *Amelx*-expressing cells by E18.5 (data not shown). However, lingual ameloblasts ceased to be generated in *Spry4* null animals after birth,

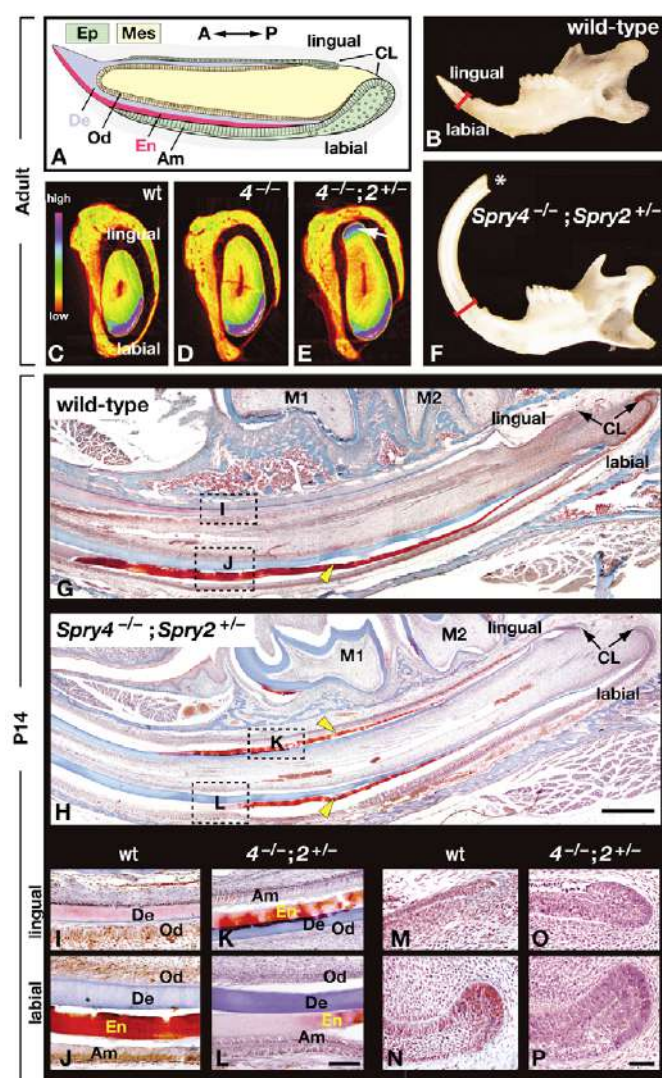


Fig. 1. *Spry4*^{-/-};*Spry2*^{+/-} mice develop a 'tusk'-like incisor due to the presence of enamel on the lingual surface. (A) Schematic diagram of an adult incisor. Enamel and dentin, the calcified tissues of the tooth, are produced by ameloblasts and odontoblasts, respectively. Note that in the normal incisor, ameloblasts and enamel are present only on the labial surface. (B,F) Side views of mandibles from wild-type and *Spry4*^{-/-};*Spry2*^{+/-} adult mice with soft tissue removed. Note the abnormal length and thickness (red bar) of the mutant incisor, as well as the absence of a sharp tip (asterisk). (C-E) Coronal sections from an XTM analysis of wild-type, *Spry4*^{-/-} (4^{-/-}), and *Spry4*^{-/-};*Spry2*^{+/-} (4^{-/-};*Spry2*^{+/-}) incisors. The colors in the bar on the left indicate mineral content. In the XTM sections, enamel is false-colored blue and purple, and dentin and bone are false-colored green and yellow. The white arrow points to ectopic enamel. (G,H) Sagittal sections of the mandibular incisor from postnatal day 14 wild-type and *Spry4*^{-/-};*Spry2*^{+/-} animals. Here, and in all other panels in this and subsequent figures, anterior is to the left and posterior to the right. Yellow arrowheads point to a layer of enamel. Black arrows point to the CLs at the posterior end of the lingual and labial sides of the incisor. (I-L) Higher magnification views of regions boxed in G and H. Note that in the wild-type and mutant incisors, dentin (stained pink and/or blue) and odontoblasts are present on both lingual and labial sides, whereas enamel and ameloblasts are found only on the labial side in wild-type and on both labial and lingual sides in the *Spry4*^{-/-};*Spry2*^{+/-} incisor. (M-P) High magnification views showing lingual and labial CL morphology in wild-type and *Spry4*^{-/-};*Spry2*^{+/-} incisors. Note that the mutant lingual CL is more similar to the labial CL than to the wild-type lingual CL. Scale bars: 500 μ m in H; 100 μ m in L; 50 μ m in P. A, anterior; Am, ameloblasts; CL, cervical loop; De, Dentin; En, enamel; Ep, epithelium; Mes, mesenchyme; M1 and M2, first and second molar; Od, odontoblasts; P, posterior; wt, wild type.

as discussed below. Pre-ameloblasts were not detected on the lingual side in either *Spry4* null heterozygotes, even when they were *Spry2* null, or in *Spry1*^{-/-};*Spry2*^{-/-} embryos at E16.5 (not shown). Together, these data show that of the three sprouty genes known to be expressed in the embryo (Minowada et al., 1999), *Spry4* is uniquely required to suppress the generation of pre-ameloblasts on the lingual side of the embryonic incisor, and that both alleles of *Spry4* must be inactivated to obtain a lingual ameloblast phenotype.

Previous studies have shown that the incisors in *Fst* null embryos have lingual ameloblasts (Wang et al., 2004), although it is not known what the postnatal incisor phenotype would be because *Fst* null embryos die perinatally (Matzuk et al., 1995). Because *Fst* is the only other gene known to be necessary to suppress the formation of lingual ameloblasts in *Fst* and *Spry4* null embryos. When we assayed *Fst* null embryos at E16.5, we detected *Shh*-expressing cells in a domain adjacent to the lingual CL similar to the one we observed in tusk mutant and *Spry4* null incisors. However, there was also a second lingual domain of *Shh*-expressing cells in *Fst* null incisors, which began a short distance anterior to the first domain and extended anteriorly toward the tip of the tooth (Fig. 2I). These data show that there are two discrete domains in the lingual

epithelium, an anterior domain in which the formation of pre-ameloblasts is normally suppressed by FST but not SPRY4, and a posterior domain in which it is suppressed by both genes.

Loss of sprouty function leads to abnormal FGF gene expression on the lingual side of the incisor

As sprouty genes normally function to suppress FGF signaling in different developmental settings (Klein et al., 2006; Shim et al., 2005), we investigated whether loss of sprouty function affects the expression of targets of FGF signaling, which in the molar include FGF genes themselves (Kettunen et al., 2000; Kratochwil et al., 2002). In wild-type incisors at E16.5, we found that *Fgf3* RNA was localized in labial mesenchyme just anterior to the CL (Fig. 3A). *Fgf10* RNA was also detected in labial mesenchyme, but was more broadly distributed around the CL, in a domain that extended further anterior and also posterior to the *Fgf3* expression domain (Fig. 3D). *Fgf9* RNA was localized in a small epithelial domain just anterior to the labial CL (Fig. 3G). By contrast, on the lingual side, only *Fgf10* RNA was detected in lingual mesenchyme anterior to and surrounding the CL, but at a much lower level than on the labial side.

In both tusk mutant and *Spry4* null incisors, the FGF gene expression patterns were indistinguishable from what we observed in wild-type embryos at E15.5 and earlier (not shown). However, by

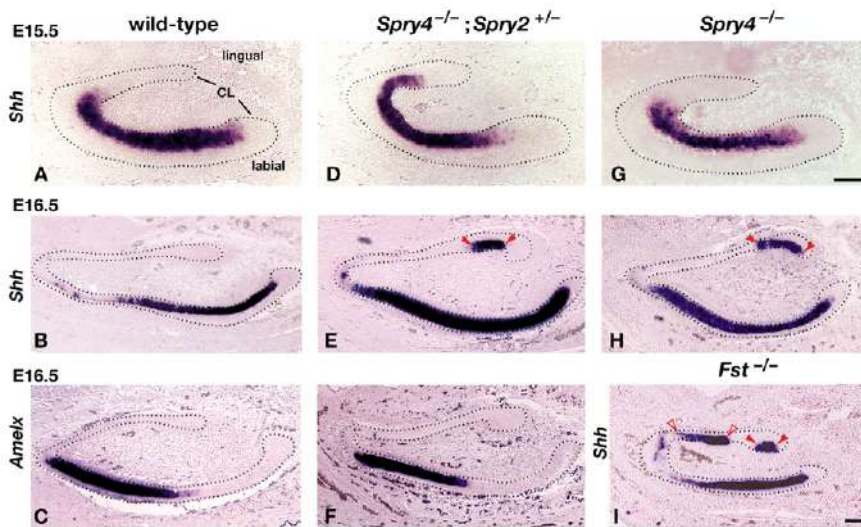


Fig. 2. *Shh* expression reveals the presence of ectopic pre-ameloblasts in *Spry4*^{-/-}; *Spry2*^{+/-}, in *Spry4*^{-/-}, and in *Fst*^{-/-} embryonic mouse incisors. (A-I) Gene expression analyzed by RNA in situ hybridization using the indicated probes on paraffin sections of wild-type and mutant embryonic incisors. The stage at which the embryos were collected is indicated. In this and subsequent figures, a dotted line outlines the incisor epithelium. In D, the *Shh* expression domain appears to extend slightly into the lingual epithelium, but as this was not reproducibly observed it is likely to be an artifact of the plane of section. The red arrowheads in E and H point to a domain in which *Shh* is ectopically expressed on the lingual side of *Spry4*^{-/-}; *Spry2*^{+/-} and *Spry4*^{-/-} incisors. A similar domain is detected in *Fst* null incisors (panel I), together with an additional anterior domain of ectopic *Shh* expression (open arrowheads). Scale bars: 100 μm.

E16.5, *Fgf3*, *Fgf10* and *Fgf9* were all abnormally expressed on the lingual side. For *Fgf3* and *Fgf10*, the expression pattern on the lingual side appeared to be a mirror image of that in labial mesenchyme (Fig. 3B,C,E,F). For *Fgf9*, lingual expression was limited to a small domain localized slightly more posteriorly than the labial *Fgf9* domain, extending into lingual CL epithelium (Fig. 3H,I). This domain was similar to that of the lingual *Shh* expression domain (see Fig. 2E,H), suggesting that *Fgf9* is expressed in lingual pre-ameloblasts. The increase in FGF gene expression detected on the lingual side of the mutant incisors was correlated with a marked

alteration in the morphology of the lingual CL, as described for the tusk mutant incisor at P14 (see Fig. 1O); indeed, the lingual CL in the E16.5 tusk and *Spry4* null mutants appeared to be a mirror image of the labial CL (see Fig. 3B,C).

We next assayed for expression of *Etv4* and *Etv5* (previously known as *Pea3* and *Erm*, respectively), which are considered to be direct targets of RTK signaling (O'Hagan and Hassell, 1998; Roehl and Nusslein-Volhard, 2001). In wild-type incisors at E16.5, we detected *Etv4* and *Etv5* RNAs in both labial mesenchyme and epithelium, anterior to the CL, and on the lingual side, only in the

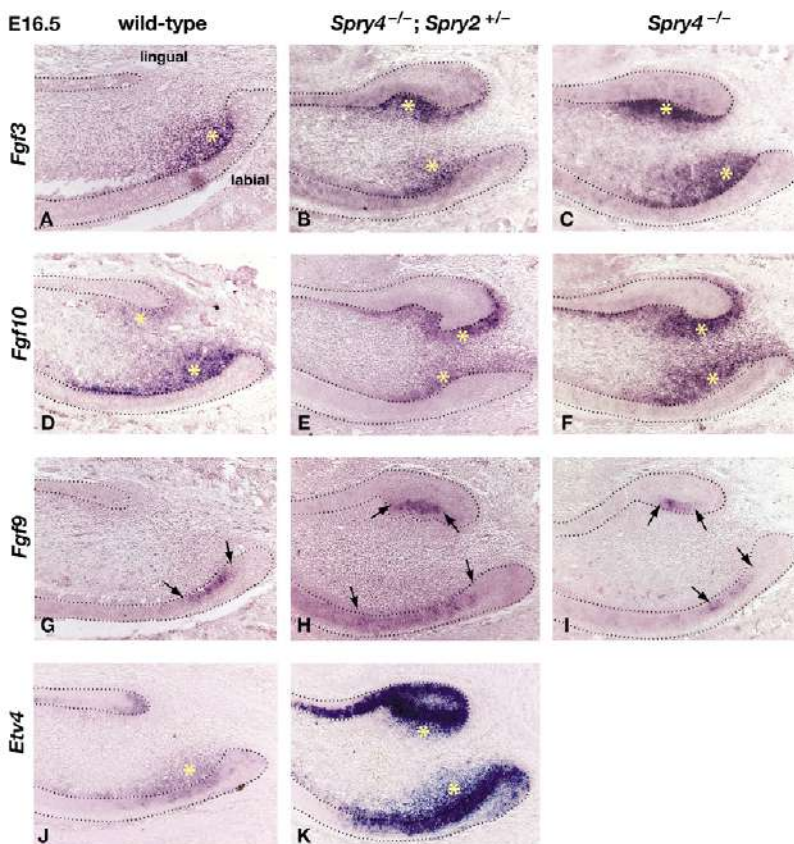


Fig. 3. FGF genes are upregulated or ectopically expressed on the lingual side of *Spry4*^{-/-}; *Spry2*^{+/-} and *Spry4*^{-/-} embryonic incisors. (A-K) Gene expression was analyzed by RNA in situ hybridization using the probes indicated on paraffin sections of E16.5 incisors of the genotypes denoted. Yellow asterisks indicate gene expression domains in the mesenchyme, and arrows point to the anterior and posterior ends of gene expression domains in the epithelium. All samples are shown at the same magnification.

epithelium, in a small domain at the posterior end of the CL (Fig. 3J, and data not shown). By contrast, in tusk mutant incisors, *Etv4* and *Etv5* RNAs were detected at a high level on both the labial and lingual sides, throughout the posterior epithelium and in the mesenchyme adjacent to the CL (Fig. 3K, and data not shown). These data indicate that loss of sprouty function results in an increase in RTK signaling in incisor mesenchyme and epithelium on both the labial and lingual sides.

Together these results suggest that the normal function of sprouty genes is to prevent the establishment of an epithelial-mesenchymal FGF signaling loop on the lingual side of the incisor that can stimulate ameloblast formation from a stem cell population in the CL. We also assayed for expression of a number of other genes that are thought to play a role in tooth development, including *activin*, *Bmp4*, *Dll1*, *Fgf4*, *Fst*, *Jag2*, *Lfng*, *Notch1*, *Notch2*, *Notch3*, *Runx2* and *Twist1*, but found no evidence that they were abnormally expressed in tusk mutant incisors (not shown). These observations further suggest that upregulation of FGF gene expression, and consequently FGF signaling, is the primary cause of the observed phenotype in sprouty loss-of-function mutants.

Continuous formation of lingual ameloblasts in adult mice requires loss of *Spry4* function and reduction in the dosage of another sprouty gene

Although loss of *Spry4* function alone results in the formation of ameloblasts on the lingual side of the incisor at E16.5 (Fig. 2), there is no lingual enamel in *Spry4* null adults (Fig. 1D). To investigate when the generation of lingual ameloblasts ceases, we examined sections of *Spry4* null incisors at P5. In sagittal sections, we observed a long swath of lingual enamel over a layer of ameloblasts (Fig. 4A) extending anteriorly from a point approximately 750 μ m anterior to the lingual CL. The region from this point posterior to the CL appeared devoid of ameloblasts (Fig. 4A,B). This absence of ameloblasts was confirmed by examining serial coronal sections (not shown). By contrast, the labial ameloblast layer extended along the entire length of the tooth up to the CL (Fig. 4A,C). These data confirm that the ectopic *Shh*-expressing pre-ameloblasts detected in *Spry4* null incisors at E16.5 subsequently differentiated into functional, enamel-producing ameloblasts, and show that lingual ameloblasts ceased being produced before P5.

To determine whether this change had occurred by P2, we assayed for the expression of two of the genes that we found were affected by loss of *Spry4* function at E16.5. *Shh* was robustly expressed in labial epithelium in all samples (Fig. 4D,E), but very few *Shh*-expressing cells were detected in the lingual epithelium of *Spry4* null incisors (Fig. 4D). By contrast, there was strong lingual expression of *Shh* in tusk mutant incisors (Fig. 4E). Concomitant with the absence of *Shh* expression in *Spry4* null lingual epithelium, *Fgf3* was no longer abnormally expressed in lingual mesenchyme (Fig. 4F), whereas ectopic *Fgf3* expression was detected in lingual mesenchyme of tusk mutant incisors at this stage (Fig. 4G). The absence of lingual *Fgf3* expression in *Spry4* null postnatal incisors was accompanied by a morphological change in the lingual CL from labial-like to lingual-like (Fig. 4D, compare with Fig. 3C). These data show that formation of lingual pre-ameloblasts ceases around the time of birth in *Spry4* null incisors, but continues in *Spry4* null mice that also lack one copy of *Spry2*. Interestingly, we found that *Spry4*^{-/-} animals that carried one null allele of *Spry1* (Basson et al., 2005) likewise displayed a 'tusk mutant' phenotype (not shown). Thus, the production of lingual ameloblasts that is initiated prenatally as a result of loss of *Spry4* function continues after birth only when the dosage of another sprouty gene, either *Spry1* or *Spry2*, is reduced.

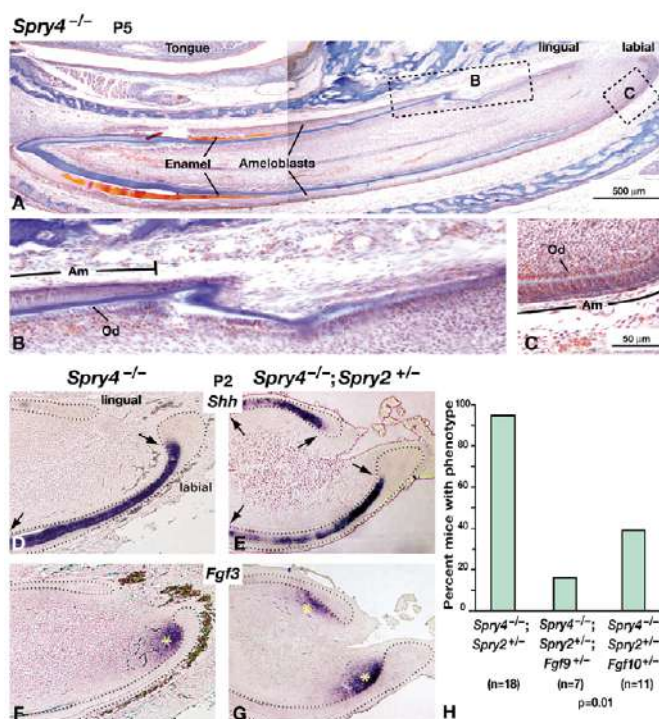


Fig. 4. Loss of *Spry4* function alone is not sufficient to maintain continuous production of ameloblasts on the lingual side of the incisor. (A-C) Sagittal section of postnatal day (P) 5 *Spry4*^{-/-} incisor. The areas boxed in A are shown at higher magnification in B and C. Note that unlike on the labial side, the lingual ameloblast layer does not extend to the posterior end of the incisor. (D-G) RNA in situ hybridization assays for *Shh* and *Fgf3* expression in paraffin sections of P2 incisors of the genotypes indicated. Yellow asterisks indicate mesenchymal expression, and black arrows demarcate the extent of epithelial expression in these panels. Note that no ectopic expression of either gene is detected in the *Spry4*^{-/-} incisors at this stage. (H) Frequency of the tusk phenotype (presence of lingual enamel) in *Spry4*^{-/-}; *Spry2*^{+/-} adult mice, and the effects of reducing the dosage of *Fgf9* or *Fgf10*. The *Spry4*^{-/-}; *Spry2*^{+/-} animals evaluated were pooled from both crosses used to generate animals with reduced FGF gene dosage. P-values were calculated using Fisher's exact test. Am, ameloblasts; Od, odontoblasts.

The lingual ameloblast phenotype can be rescued in the adult by reducing FGF gene dosage

To determine the extent to which lingual ameloblast formation is sensitive to FGF signaling, we produced tusk mutants heterozygous for an *Fgf9* null allele (Colvin et al., 2001) (*Spry4*^{-/-}; *Spry2*^{+/-}; *Fgf9*^{+/-} adults), and examined their incisors by XTM. We found that only 14% had lingual enamel, compared with 100% of their *Spry4*^{-/-}; *Spry2*^{+/-} littermates ($P < 0.01$). Likewise, heterozygosity for a null allele of *Fgf10* (Min et al., 1998) had a similar, although less dramatic, effect, in that 37% of *Spry4*^{-/-}; *Spry2*^{+/-}; *Fgf10*^{+/-} adults had lingual enamel, compared with 93% of their *Spry4*^{-/-}; *Spry2*^{+/-} littermates ($P < 0.01$) (Fig. 4H). Thus, continuous formation of ameloblasts on the lingual side in *Spry4*^{-/-}; *Spry2*^{+/-} adults is dependent on persistent abnormal expression of *Fgf9* and *Fgf10* at high levels.

By contrast, when we assayed for *Shh* expression at E16.5, all sprouty mutant embryos that were heterozygous for an *Fgf9* null allele (*Spry4*^{-/-}; *Fgf9*^{+/-}, $n=4$; *Spry4*^{-/-}; *Spry2*^{+/-}; *Fgf9*^{+/-}, $n=3$) had lingual *Shh*-expressing pre-ameloblasts (not shown), as was

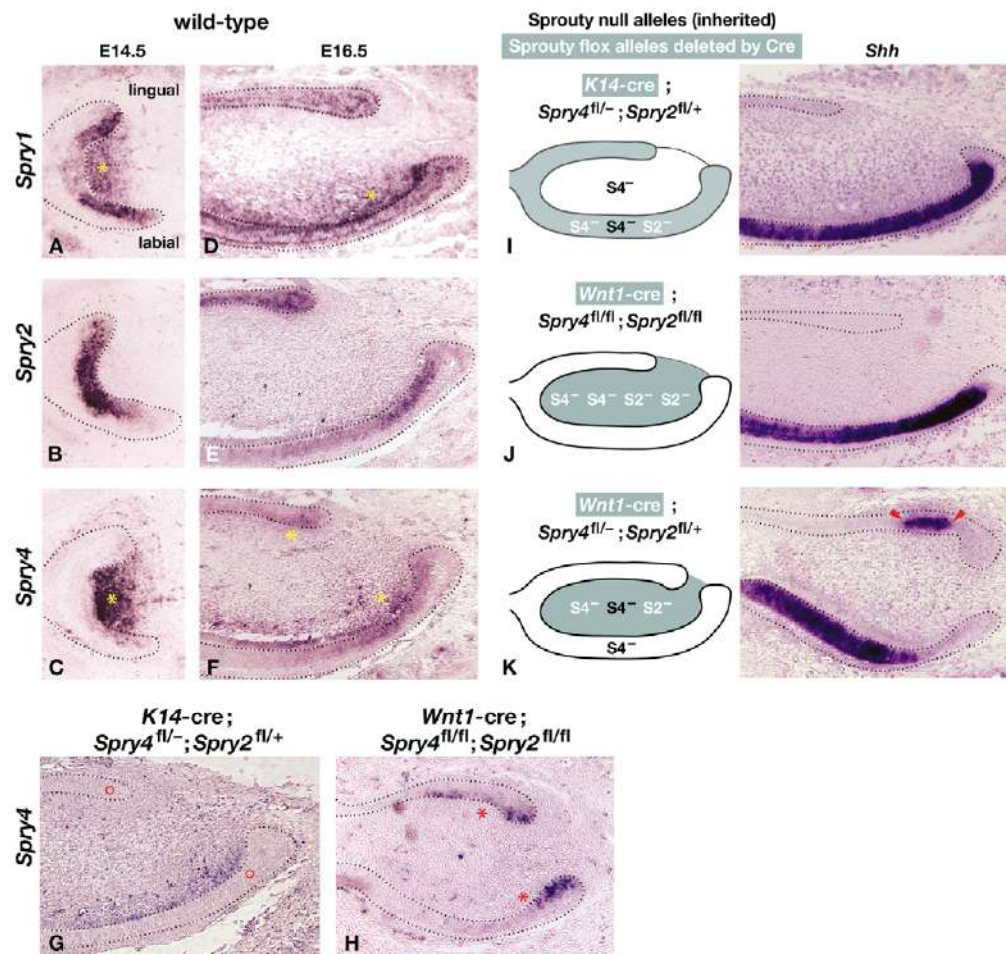


Fig. 5. sprouty gene expression and tissue-specific inactivation in the developing mouse incisor.

Gene expression was analyzed by RNA in situ hybridization using the probes indicated on paraffin sections of embryonic incisors of the genotypes denoted at E16.5 or 17.5. (A-F) A comparison of the expression domains of sprouty gene family members in the incisor at the stages indicated. Yellow asterisks indicate mesenchymal expression. (G-K) Tissue-specific inactivation of *Spry4*. The absence of *Spry4* expression is indicated by red circles in the epithelium of an incisor carrying *K14-cre*, one *Spry4*^{fl} and one *Spry4*⁻ allele (G) and by red asterisks in the mesenchyme of an incisor carrying *Wnt1-cre* and two *Spry4*^{fl} alleles (H). For each genotype shown in I-K, the diagram illustrates the tissue in which Cre-mediated recombination occurred (green fill), the sprouty alleles that were inactivated by Cre (white lettering), or that were inherited as nulls (black lettering). The photograph shows *Shh* expression, which marks cells that are differentiating along the ameloblast lineage. The red arrowheads point to the ectopic lingual *Shh* expression domain.

observed in all *Spry4* null and tusk mutant incisors in which *Fgf9* dosage was normal. Preliminary results on *Spry4* null embryos with reduced dosage of *Fgf10* were similar. Thus reducing FGF gene dosage in sprouty mutant embryos did not rescue the prenatal lingual ameloblast phenotype. These data raise the possibility that loss of sprouty function causes lingual ameloblast formation in embryonic incisors as a consequence of effects on non-FGF-mediated RTK signaling. Alternatively, there may be higher levels of FGF signaling in the embryonic than in the adult incisor, and therefore reducing FGF gene dosage by one copy does not sufficiently decrease FGF signaling in the embryo to prevent lingual ameloblast formation in the absence of sprouty gene function.

***Spry4* is required in both mesenchyme and epithelium to suppress the initiation of lingual ameloblast formation**

To determine where in the embryonic incisor *Spry4* might function to suppress the initiation of lingual ameloblast formation, we assessed the expression pattern of *Spry4* as well as *Spry1* and *Spry2* in wild-type embryos. At E14.5, we found significant differences in the expression domains of the three sprouty genes: *Spry1* RNA was detected in both epithelium and mesenchyme (Fig. 5A); *Spry2* RNA was detected exclusively in the epithelium (Fig. 5B); and *Spry4* RNA was detected exclusively in the mesenchyme (Fig. 5C). However, by E16.5 not only *Spry1* and *Spry2*, but also *Spry4*, expression was detected in the epithelium on both the labial and lingual sides of the incisor. In the mesenchyme, *Spry1* and *Spry4*, but not *Spry2*, expression was detected on the labial side, and only

Spry4 expression was detected, albeit at a low level, on the lingual side (Fig. 5D-F). As *Spry4* expression was detected in both lingual epithelium and mesenchyme, we next sought to determine in which tissue(s) *Spry4* function is required.

To test whether *Spry4* function is required in the epithelium, we performed Cre-mediated conditional loss-of-function experiments using a *K14-cre* transgene that has previously been used to inactivate floxed alleles in dental epithelium from early stages of tooth development (Dassule et al., 2000). First, we produced *K14-cre; Spry4*^{fl/fl}; *Spry2*^{fl/+} embryos, and assayed for *Shh*, *Fgf3*, *Fgf10* and *Fgf9* expression at E17.5, to determine the effect of epithelium-specific inactivation of *Spry4* on lingual gene expression. As expected, *Spry4* RNA was detected in incisor mesenchyme but not epithelium (Fig. 5G, compare with Fig. 5F). However, no abnormal lingual gene expression was observed in such embryos (Fig. 5I and data not shown), indicating that a complete loss of *Spry4* function in the epithelium, even when the mesenchyme is heterozygous for *Spry4*, is not sufficient to allow the formation of lingual pre-ameloblasts.

To test whether *Spry4* function is required in the mesenchyme, we employed a *Wnt1-cre* transgene (Danielian et al., 1998) that has previously been used to inactivate floxed alleles in dental mesenchyme from early stages of tooth development (Chai et al., 2000). We produced *Wnt1-cre; Spry4*^{fl/fl}; *Spry2*^{fl/fl} embryos, and assayed to determine the effect on lingual gene expression at E16.5. As expected, *Spry4* expression was detected in incisor epithelium but not mesenchyme (Fig. 5H, compare with Fig. 5F). However, despite inactivation of both alleles of *Spry4* in the mesenchyme, we

detected no lingual expression of *Shh* or the other genes assayed in these embryos (Fig. 5J and data not shown). Thus, elimination of *Spry4* function in only the mesenchyme is not sufficient to cause the formation of lingual pre-ameloblasts.

We next assayed *Wnt1-cre; Spry4^{fl/-}; Spry2^{fl/+}* embryos, which inherited one *Spry4* null allele and one floxed allele. In these embryos, in which the mesenchyme was null for *Spry4* as a consequence of *Wnt1-cre* activity, and the epithelium was heterozygous for *Spry4*, we observed abnormal lingual gene expression (Fig. 5K and data not shown). These data indicate that inactivation of both *Spry4* alleles in the mesenchyme and one *Spry4* allele in the epithelium is required, when there are two functional *Spry2* alleles (floxed and wild-type) in the epithelium, for the generation of *Shh*-expressing pre-ameloblasts on the lingual side of the embryonic incisor. Because these animals do have two functional *Spry2* alleles in the epithelium, where *Spry2* is exclusively expressed they would presumably cease producing ameloblasts after birth and thus would not display the tusk mutant phenotype.

DISCUSSION

In a few mammalian species, teeth have evolved the ability to grow throughout adult life (Tummers and Thesleff, 2003). One such tooth is the rodent incisor, in which continuous growth is coupled with a lack of enamel deposition on the lingual side, thereby providing a means of sharpening the tip and limiting incisor length. Here we demonstrate that in mice, sprouty genes are essential for ensuring that this vital asymmetry in enamel deposition occurs. Loss of *Spry4* function results in the generation of enamel-producing ameloblasts on both the lingual and labial sides of the embryonic incisor. We present evidence that this phenotype is due to the establishment of an ectopic epithelial-mesenchymal FGF signaling loop on the lingual side. Interestingly, we found that this lingual ameloblast phenotype, which is robust in the embryo, is not sustained after birth unless the dosage of an additional sprouty gene, either *Spry1* or *Spry2*, is reduced. The additive loss of sprouty function results in 'tusk-like' adult incisors that are resistant to abrasion because enamel is deposited on both the lingual and labial sides.

FGF signaling regulates the generation of lingual ameloblasts from stem cells

It is now well established that loss of sprouty function causes hypersensitivity to FGF and other receptor tyrosine kinase signaling (Mason et al., 2006). Here we provide evidence that in the incisor, sprouty genes antagonize FGF signaling, and that loss of sprouty function results in upregulation of FGF gene expression on the lingual side. Three lines of evidence indicate that this increase in FGF gene expression is responsible for the generation of lingual ameloblasts. First, an increase in the level of lingual FGF gene expression is closely correlated with the initiation of *Shh* expression, a marker for pre-ameloblasts, in the lingual epithelium of late gestation embryos. Second, the reversal of the increase in lingual *Fgf3* expression that we observed in *Spry4* null incisors shortly after birth is closely correlated with cessation of lingual *Shh* expression. Third, reducing the dosage of either *Fgf9* or *Fgf10* substantially rescues the postnatal lingual ameloblast phenotype in tusk mutants, thus providing genetic evidence that high levels of FGF signaling are required to sustain the generation of lingual ameloblasts in adult sprouty mutants.

Based on the results of our tissue-specific knockout experiments and gene expression analyses, we propose the following model to explain how sprouty genes normally function to prevent an increase in FGF gene expression and the consequent establishment of an FGF

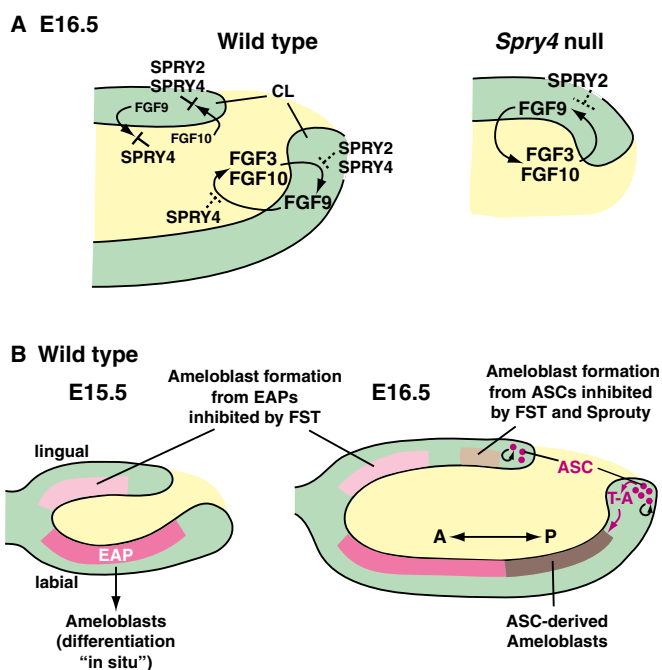


Fig. 6. Models for the role of sprouty genes in controlling FGF signaling in the mouse incisor and for the generation of embryonic ameloblasts. (A) Functions of sprouty genes in inhibiting the establishment of a lingual FGF epithelial-mesenchymal signaling loop. Arrows indicate a stimulatory effect and the symbol \perp indicates an inhibitory effect of one signaling molecule on the expression of another. In wild type, sprouty genes are expressed on the labial side, but they do not prevent (dashed \perp symbol) reciprocal signaling between FGF9 in epithelium and FGF3/FGF10 in mesenchyme. On the lingual side, sprouty genes inhibit signaling to adjacent tissues by the low levels of FGF9 in the epithelium and of FGF10 in the mesenchyme, and consequently there is no upregulation of FGF gene expression in either tissue. However, in *Spry4* null incisors a reciprocal signaling loop between epithelium and mesenchyme is established because, in the absence of SPRY4, these tissues are hypersensitive to the low level of FGF signaling. In turn, the increase in FGF signaling on the lingual side results in the generation of ameloblasts from self-renewing stem cells in the CL. (B) A proposal for how ameloblasts develop in the embryonic incisor. At E15.5, wild-type incisor epithelium contains embryonic ameloblast progenitor (EAP) cells capable of limited proliferation. On the labial side, their descendants (in the domain colored pink) differentiate 'in situ' into enamel-producing cells. Similar cells are present on the lingual side (in the domain colored lighter pink), but their differentiation is inhibited by Follistatin. Between E15.5 and 16.5, an ameloblast stem cell (ASC) population is established in the labial CL. Unlike EAP cells, ASCs have the capacity to self-renew (circular arrow), as well as give rise to ameloblasts. ASC descendants that will develop into enamel-producing cells may first form transit-amplifying (T-A) cells in the anterior CL. After several divisions, their descendants move out of the CL, and begin differentiating. In the E16.5 incisor, these ASC-derived pre-ameloblasts are found in a domain in the labial epithelium (colored dark brown), between the EAP domain and the CL. The diagram illustrates the possibility that ASCs are normally also present on the lingual side. However, no pre-ameloblasts derived from these ASCs are present in the lingual epithelium anterior to the CL (in the domain colored light brown), because the generation of ameloblasts from lingual ASCs is blocked due to the inhibitory effects of sprouty as well as FST function on lingual FGF gene expression.

signaling loop on the lingual side of the incisor between E15.5 and 16.5 (see Fig. 6A). In the epithelium, SPRY4 normally prevents an FGF signal produced in the mesenchyme from inducing/

upregulating FGF gene expression. FGF10 is a good candidate for the mesenchymal signal that is antagonized by *SPRY4* in the epithelium, as *Fgf10* expression is normally detected at a low level in the mesenchyme surrounding the wild-type lingual CL at E16.5. Moreover, FGF10 is known to signal via the b isoform of FGFR2, which is expressed in lingual (and labial) epithelium (Peters et al., 1992). We propose that inactivation of a single allele of *Spry4* in the epithelium renders it sufficiently hypersensitive to the small amount of FGF10 normally produced in the mesenchyme, such that the expression of *Fgf9*, a direct or indirect target of FGF10 signaling, is slightly upregulated in the lingual epithelium. However, if the mesenchyme is not sufficiently hypersensitive, then this increase in epithelial *Fgf9* expression is presumably transitory, because the mesenchymal FGFs are not upregulated in response to the increase in epithelial FGF signal, and a positive-feedback signaling loop is therefore not established on the lingual side.

Likewise, in the mesenchyme, *SPRY4* prevents an FGF signal produced in the lingual epithelium from upregulating the expression of mesenchymal FGF gene(s). FGF9, which is normally produced in the lingual epithelium at such a low level that its expression is detected only with a radiolabeled probe [compare Fig. 3G with Wang et al. (Wang et al., 2007)], presumably signals via FGFR1, which is expressed throughout the incisor mesenchyme (Peters et al., 1992). Inactivating two alleles of *Spry4* in the mesenchyme renders it hypersensitive to the small amount of FGF9 produced in the epithelium, such that the expression of *Fgf3* and *Fgf10*, two direct or indirect targets of FGF9 signaling, is induced/upregulated in the lingual mesenchyme. Thus a positive-feedback FGF loop between lingual epithelium and mesenchyme is initiated and maintained for a few days in late-gestation *Spry4* mutant incisors. However, if the epithelium is not also sufficiently hypersensitive, then it will not respond to increases in the level of mesenchymal FGF signals, and a positive-feedback signaling loop will not be established.

Loss of sprouty function did not appear to cause any gross abnormalities on the labial side, where sprouty genes are highly expressed and presumably antagonize the FGF signaling that is required for normal ameloblast development and function. Analogous observations have been made in other developmental settings, where loss of sprouty function apparently affects only cells that do not normally respond to RTK signaling, but has little or no effect on cells that normally respond to high levels of RTK signaling (Basson et al., 2005; Klein et al., 2006; Shim et al., 2005). Precisely how the dynamics of the negative feedback between RTK signaling and sprouty-mediated inhibition of that signaling impacts such developmental systems remains to be elucidated.

Comparison of sprouty and follistatin mutant incisors suggests that ameloblasts form from two different progenitor populations

The data reported here must also be considered in the context of a larger genetic network that includes TGF β family members and their antagonists, such as follistatin. Previous studies of the incisor phenotype in *Fst* null embryos have provided evidence that FST inhibits lingual ameloblast differentiation in the anterior incisor via a direct negative effect on BMP4 signaling (Wang et al., 2004), whereas in the lingual CL region it may do so by negatively regulating *Fgf3* expression indirectly via effects on signaling by other TGF β family members (Wang et al., 2007). Our analysis of *Shh* expression in *Fst* null mutants revealed that there are two physically separate domains in which pre-ameloblasts are found in the lingual epithelium at E16.5 (see Fig. 2I). We suggest that these

anterior and posterior domains correspond to those in which ameloblast formation is thought to be inhibited by direct and indirect effects on TGF β signaling, respectively. Significantly, we found that in embryonic *Spry4* null incisors, lingual pre-ameloblasts are detected only in the posterior domain (see Fig. 2H), thus providing strong evidence that when ameloblasts form in mutant embryonic lingual epithelium, they do so in two domains that respond differently to increased signaling.

To explain these observations, we propose the model illustrated in Fig. 6B. In the anterior domain, ameloblasts are generated from an embryonic ameloblast progenitor (EAP) population capable of giving rise to a limited number of progeny that subsequently differentiate 'in situ' into enamel-producing cells. This process is analogous to that by which all ameloblasts in molars are thought to form (Zeichner-David et al., 1995). Development of ameloblasts from EAP cells in the anterior lingual domain is normally inhibited by FST acting to suppress BMP4 signaling, and is not suppressed by sprouty-mediated inhibition of FGF signaling. In contrast, in the posterior lingual domain, ameloblasts are derived from a self-renewing ameloblast stem cell population located within the nearby CL. The formation of these ameloblasts depends on a high level of FGF signaling, which is normally suppressed by *Spry4* function in the epithelium and mesenchyme. However, ameloblast formation in this domain is also suppressed by FST, most likely by an indirect negative effect on *Fgf3* expression.

One possible explanation for the observation that loss of function of either *Fst* or *Spry4* leads to the induction of *Fgf3* expression in lingual mesenchyme is that *Fst* and *Spry4* act in the same genetic pathway. However, we found that loss of *Fst* function does not result in a decrease in *Spry4* expression or vice versa (not shown), indicating that these genes may instead act in parallel pathways that converge on downstream targets and that can each affect *Fgf3* expression. The increase in lingual *Fgf3* expression that has been detected in *Fst* null mutants could sufficiently increase the level of FGF signaling from the mesenchyme to the epithelium, such that it overcomes the antagonism by sprouty genes that normally prevents the establishment of an FGF-signaling loop.

We further suggest that ameloblast formation on the labial side likewise occurs by two different mechanisms: the first ameloblasts to form in the embryonic incisor develop from EAP cells; their formation requires BMP4 signaling but may occur independent of FGF signaling. Subsequently, ameloblasts are generated from ASCs in the labial CL, and this process depends on signaling via other TGF β family members as well as high levels of FGF signaling. Based on these hypotheses, we speculate that a key event in incisor development is the establishment of the ASC population on the labial side between E15.5 and 16.5. Formation of such stem cells would involve the acquisition, perhaps by a subpopulation of EAP cells, of the ability to self-renew as well as to produce progeny that give rise to ameloblasts (and perhaps other epithelial cell types) throughout the life of the animal.

Possible mechanisms by which FGF signaling controls ameloblast formation

Perhaps the most important question raised by our data is: how does an increase in FGF signaling on the lingual side of the incisor result in the formation of ameloblasts? One possibility is that ASCs are not normally present in the lingual CL, and that ectopic FGF signaling functions to induce their formation. Another possibility is that ASCs are present in the lingual CL, but normally they do not give rise to progeny that develop into ameloblasts. If so, then increased FGF signaling might stimulate them to form such

progeny, perhaps by promoting the formation and/or expansion of T-A cells that subsequently develop into ameloblasts. With respect to the latter suggestion, it is tempting to speculate that the transformation to a more labial CL-like morphology that occurs in the mutant lingual CL when it begins producing ameloblasts might reflect the presence of an expanded T-A cell population. At present, we are unable to explore these and other possibilities because markers for ASCs and T-A cells have not been identified. However, we are inclined to favor the hypothesis that FGF signaling affects T-A cells, if only because the domains in which ectopic *Fgf3* and *Fgf9* expression are detected in *Spry4* null incisors are localized just anterior to the region in the CL where T-A cells are speculated to reside.

One of our most intriguing findings is that a phenotype that appears to be very robust in the embryo – the generation of an ectopic ameloblast population from stem cells on the lingual side of the incisor due to loss of *Spry4* function – is reversed just after birth unless an additional sprouty gene is inactivated. Based on our genetic rescue data, a likely explanation for this phenomenon is that the normal level of FGF signaling on the lingual side, albeit low, is higher in the embryo than in the adult. Thus the removal of an additional sprouty gene is required to render the adult epithelium and mesenchyme sufficiently sensitive to the adult level of FGF signaling to sustain the generation of lingual ameloblasts. These data reveal that the generation of differentiated progeny from a particular stem cell population can be differently regulated in the embryo and adult, and illustrate how manipulating levels of signals or their antagonists can provide a mechanism for enhancing the production of differentiated progeny from adult stem cells.

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