Tabby is a mouse mutant characterized by deficient development of the ectodermal organs: teeth, hair, and a subset of glands. Ectodysplasin, the protein encoded by the Tabby gene, was recently identified as a novel TNF-like transmembrane protein but little is known about its function. We have examined the Tabby tooth phenotype in detail by analysis of the adult and embryonic teeth. Tabby first molars had an obvious defect in cusp patterning as the number of cusps was reduced and the buccal and lingual cusps were joined. The disturbance in development was first visible morphologically in the bud stage molar. The primary enamel knot in a cap stage Tabby tooth expressed all enamel knot markers analyzed but was smaller than wild type and the first pair of developing secondary enamel knots was fused. We propose that the Tabby tooth phenotype is due to growth retardation during early stages of development which leads to reduced signaling from the primary enamel knot, followed by deficient growth of the dental epithelium and lack of formation of the last developing secondary enamel knots. The ectodysplasin transcripts were expressed in the outer enamel epithelium and dental lamina. When cultured in vitro Tabby bud/cap stage molars formed fewer cusps than wild-type controls. This phenotype was not rescued by exogenously added EGF despite the previously proposed link between Tabby and EGF. Instead FGF-10 partially restored morphogenesis and stimulated the development of additional tooth cusps in cultured Tabby molars.

Key Words: epithelial-mesenchymal interactions; ectodermal dysplasia; enamel knot; tumor necrosis factor; TNF.
It is apparent that the same signaling molecules regulate the development of all vertebrate organs, including teeth, hair, and glands. The signal families that have been best characterized in this respect are the FGF (fibroblast growth factor), Hh (hedgehog), BMP (bone morphogenetic protein), and Wnt families. All families consist of several members and they bind to specific cell surface receptors that also have been characterized. In addition, numerous transcription factors have been identified which are regulated by the signals (Thesleff and Sharpe, 1997). The downstream targets of the transcription factors are poorly understood, and the cellular responses to signaling have not been thoroughly studied yet. It is however evident that the responses involve regulation of cell proliferation and survival, as well as changes in cell–cell adhesion and cell–matrix interactions (Hogan, 1999).

Materials and Methods

Animals

Wild-type mice were either (a) NMRI adults or (b) embryos from a CBA T6 T6 X NMRI cross. The Tabby allele used, B6CBACa-Aw a TaA, was obtained from Jackson Laboratories (Bar Harbor, ME) (stock No. JR 0314) and was kept by breeding Ta/0 females to Ta/Y males. All embryos from the cross were either Ta/0 or Ta/Ta females or Ta/Y males and displayed the Tabby phenotype.

Analysis of the Adult Tooth Phenotype

Skeletal preparations of mandibles were made by boiling mice heads for 1–2 h, dissecting the mandibles, and clearing them in 5%
H₂O₂ at 4°C for 45 min after which the mandibles were rinsed in H₂O and left to dry.

**Histology**

Tissues were fixed in 4% PFA and embedded in paraffin. Postnatal teeth were decalcified after fixation in 12.5% EDTA containing 2.5% PFA. Ten-micrometer sections were cut and stained with hematoxylin–eosin.

**Three-Dimensional Reconstructions**

Three-dimensional computer reconstructions were done of serial paraffin sections as previously described (Jernvall et al., 1998). The reconstructions of gene expression domains show in situ silver grains that were projected and resampled from aligned dark-field image stacks using the public domain NIH Image 1.61 program (U.S. National Institutes of Health, available from the Internet by anonymous FTP from zippy.nimh.nih.gov). The basement membrane was drawn directly onto the digitized images and rendered in Extreme 3D (Macromedia).

**In Situ Hybridization (Whole Mount)**

The following probes were made and labeled with digoxygenin: Bmp-2 (Vainio et al., 1993), from Genetics Institute; and Fgf-8 (Heikinheimo et al., 1994) from C. A. MacArthur. In situ hybridization was carried out as described in Henrique et al. (1995). Following hybridization, the tissues were incubated with antidigoxygenin Fab conjugated to alkaline phosphatase (Boehringer Mannheim). Positive in situ hybridization signals were detected by incubation with NBT/BCIP substrates (Boehringer Mannheim) in alkaline buffer.

**In Situ Hybridization (Sections)**

The following probes were used: Bmp-4 (Vainio et al., 1993) from Genetics Institute; EGFR, a mouse cDNA fragment corresponding to the cytoplasmic domain cloned into pCRMII (Invitrogen), from D. E. Lee; Fgf-3 (Wilkinson et al., 1988), from D. G. Wilkinson; Fgf-4 (Jernvall et al., 1994), from C. Basilio; Fgf-10 (Belusci et al., 1997), from S. Bellusci; L-fng, 860-bp 5' coding region of mouse Lunatic Fringe cloned into pBSKII(−) and linearized to obtain an antisense probe of 570 bp, from Y. A. Wang Sh (Vahtokari et al., 1996), from T. Edlund; Wnt-10a (Dassule and McMahon, 1998), from A. MacMahon; Tabby exon 1, plasmid pTaNg (Srivastava et al., 1997); Tabby TaA, plasmid pTE2A, a 0.8-kb Ta-A3 fragment (nucleotides 2371–3164, EMBL Accession No. AJ 243658, Mikkelø et al., 1999) cloned into pGEM3Z (Promega); and Tabby TaB, plasmid pTaB, a 0.8-kb TaB fragment (nucleotides 814–1620, GenBank Accession No. AF 016630, Srivastava et al., 1997) cloned into pGEM3Z (Promega).

Hybridizations were performed according to Wilkinson and Green (1990). Probes were labeled with [35S]UTP (Amersham) except for the Tabby probes where [33P]UTP (Amersham) was used; exposure time was 15 days. The images were digitized with ImagePro and NIH Image 1.60 software and further manipulated with Adobe Photoshop and Micrografx Designer software.

**Immunohistochemistry**

Cryosections were fixed with 4% PFA and acetic acid MeOH (1:3). After washing and blocking with 4% normal donkey serum (Jackson Immunoresearch Laboratories) the sections were incubated first with sheep anti-human EGFR (Upstate Biotechnology Inc.) and then with biotin-conjugated anti-sheep IgG (Jackson Immunoresearch Laboratories). Color reaction was obtained with β-galactosidase-conjugated streptavidin (Boehringer Mannheim) and X-gal.

**Organ Culture Experiments**

E13/E13.5/E14 molars were dissected and grown for 4 days at 37°C in a Trowell-type culture containing Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. FGF-10 (a kind gift from Dr. N. Itoh), FGF-4 (R&D Systems), and EGF (Sigma) were added at the onset of culture either with growth factor-coated beads to local regions of the cultures or in the medium. For coating beads, about 100 heparin acrylic beads (Sigma, 100–200 μm in diameter) were washed with sterile phosphate-buffered saline and soaked in 5 μl of growth factor for 1 h at 37°C. Growth factor concentrations used were 25 ng/μl for FGF-10 and -4 and 5, 25, and 100 ng/μl for EGF. The beads were then carefully placed onto the distal part of the tooth explants using fine forceps. Growth factors were added to the medium at the onset of culture at concentrations 150 ng/ml for FGF-10 and -4 and 10 ng/ml for EGF, chosen from previous dose–response studies. After 4 days the tooth explants were fixed with 4% PFA.

**RESULTS**

**Description of the Tabby Adult Tooth Phenotype**

Mice generally have one incisor and three molars in each half of the maxilla and mandible. In Tabbies the incisors and third molars are often missing and the first molars have a reduced number of cusps. However, the penetrance of the phenotype is affected by the genotype so, e.g., the prevalence of defects in the incisors may vary from 11 to 85% depending on the background strain (Grüneberg, 1965). We analyzed the adult tooth phenotype of our Tabby stock, B6CBAcA-"A"/Ta, with emphasis on the lower first molar as the reduction of the cusp number there appeared to have a nearly 100% penetrance in our strain and as the lower first molar has been mostly used in the analysis of the molecular regulation of tooth development in our laboratory and others, and therefore its development has been well characterized at both morphological and molecular levels (Gene expression in tooth, WWW database, 1999).

Skeletal preparations of mouse mandibles were evaluated. Each half mandible was treated as one entity. While the lower incisors are generally present in our strain, all lower first (M1) and most of the second (M2) molars are smaller than wild type with a reduced number of cusps. The frequency distribution of M1 cusp number is shown in Table 1. Wild-type M1s have six main cusps and one small distal cusp. In Tabby M1 the average cusp number is 3.8
and the cusps usually missing are the two anterior cusps (the anteroconid) and the distal cusp (the hypoconulid). Additionally, the tips of buccal and lingual cusps are close to each other and often joined to a single tip. The size ratio of M1/M2 is also different in the Tabby strain. In wild-type M1 is larger than M2, but in 80% of our Tabby mandible samples (N = 42) M1 is the same size as M2. In 14% (N = 7) M1 is larger than M2 (as in wild type) and in 6% (N = 3) M1 appears to be missing (with M2 and M3 present). Seventeen percent (N = 9) of the lower third molars (M3) are absent. Typical Tabby molars together with wild-type molars as comparison are depicted in Fig. 1.

**TABLE 1**

<table>
<thead>
<tr>
<th>Cusp number</th>
<th>Total</th>
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<tbody>
<tr>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
</tr>
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<td>4</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>52</td>
</tr>
</tbody>
</table>

**Number of cases**

<table>
<thead>
<tr>
<th>2</th>
<th>8</th>
<th>25</th>
<th>12</th>
<th>2</th>
<th>52</th>
</tr>
</thead>
</table>

**Percentages**

| 2   | 15  | 48  | 23  | 4   | 100 (100%) |

**Mean:** 3.8 cusps.

Developmental Anatomy of Tabby Tooth Morphogenesis

The timing of the developmental disturbance in the teeth of the Tabby embryos cannot be concluded from the adult tooth phenotype. The defect may occur already at the stage of initiation or at the late bud stage, as shown in some knockout mice (Bei and Maas, 1998; Peters and Balling, 1999), but it may take place even at a quite advanced stage as shown in squirrels in which teeth in the diastema region which have been lost during evolution develop until advanced bell stage and even form some hard tissue before they regress (Luckett, 1985). We examined our Tabby stock at the embryonic level by histology. Sofaer (1969) has reported that Tabby M1 resembled wild type up to E15 (late cap stage) where it appeared smaller and less mature than its wild-type control. Figure 2 shows a panel of our wild-type and Tabby tissues at stages E11, E13, E14, E16, and PN2.

At E11 when the epithelium starts to thicken and to invaginate into the underlying mesenchyme there is no difference between wild-type and Tabby tissues (Figs. 2a and 2b). At the late bud stage (E13) when the mesenchyme has condensed around the budding epithelium the epithelial bud of the Tabby tooth was slightly smaller than wild type (Figs. 2c and 2d). This was more obvious in the mesiodistal axis (see below) rather than the buccolingual cross-section shown here. The cap stage (E14) Tabby tooth is clearly smaller than the wild-type control and has an abnormal shape (Figs. 2e and 2f). The enamel knot is shorter as is illustrated by a 3D analysis of the enamel knot marker Fgf-4 (see below and Fig. 4). The abnormal development is also visible at the bell stage (E16) (Figs. 2g and 2h) as the tooth germ is smaller than wild type and individual cusps are not yet visible. At PN2 (Figs. 2i and 2j) the Tabby M1 is smaller than wild type and has fewer cusps forming. The cusps are closer to each other and the differentiation of dentin and enamel is less progressed than in a wild-type molar. However, the height of the tooth crown is nearly normal. Also the relative size difference of M1 and M2 can be seen as in wild-type M1 is approximately three times as large as M2 whereas in Tabby the size of M1 almost equals M2. At this time point the Tabby M2 is also less differentiated than the wild-type M2.

In conclusion, in our Tabby stock we found the first disturbances in tooth development at E13 (bud stage) where the Tabby first molar is abnormally small. The development continues to be delayed and disturbed resulting in an irreversible reduction in the size of the tooth crown and number of cusps. Differentiation of odontoblasts and ameloblasts appears not to be affected allowing normal formation of the hard tissues (judged by the adult phenotype).
Expression of Signaling Molecules

The development of the Tabby molar was followed with markers for signaling molecules known to be important for tooth morphogenesis. Early tooth development was studied by whole mount in situ hybridization analysis of Fgf-8 and Bmp-2 expression in mandibular arches. Cap stage development was analyzed from sections of mandibular molars by radioactive in situ hybridization with probes for Bmp-4, Shh, Wnt-10a, Fgf-4, Lunatic Fringe (L-fng), Fgf-3, Fgf-10, and EGFR. In addition, EGFR protein expression was analyzed by immunohistochemistry.

E10.5 and E12 Tabby and wild-type mandibles were hybridized with probes Fgf-8 and Bmp-2, which are expressed in the epithelium during the initiation and early budding stage. FGF-8 and BMP-2 proteins can both mimic some effects of the early epithelium on presumptive dental mesenchyme during the early epithelial–mesenchymal interactions (Vainio et al., 1993; Kettunen and Thesleff, 1998; Peters and Balling, 1999). Fgf-8 is expressed in the developing molar regions with a gradient of weaker expression toward the incisor region in E10.5 wild-type (wt) mandibles (Fig. 3a) and an identical expression pattern is seen in a Tabby mandible (Fig. 3b). Bmp-2 is expressed in both the molar and the incisor forming regions in an E12 mandible (Fig. 3c). Again similar expression is seen in a Tabby mandible (Fig. 3d).

The cells of the primary enamel knot, which appears at the early cap stage when the tooth crown begins to form, express Fgf-4 (Jernvall et al., 1994). Also E14 Tabby molar enamel knot was histologically discernible and the cells expressed Fgf-4. However, the size of the enamel knot, as judged from histology and Fgf-4 expression domain, was small. The Fgf-4 expression domain was limited particularly along the longitudinal axis, as seen in the 3D reconstruction of serial sections (Figs. 4b and 4f). The difference between wild-type and Tabby molar germs was more pronounced at E16. In an E16 wild-type molar two to three secondary enamel knots expressing Fgf-4 can be detected corresponding to the developmental initiation of individual cusp tips (Figs. 4c and 4g). In an E16 Tabby molar only one large mesiodistally compressed Fgf-4 expression domain is present (Figs. 4d and 4h), corresponding to the first pair of cusps (the protoconid and the metaconid). These two fused secondary enamel knots correlate with the morphology of fully formed Tabby molars in which the tips of buccal and lingual cusps are often joined to a single tip.

Radioactive in situ hybridization was performed on wild-type and Tabby cap stage molar tissue sections also with the primary enamel knot markers Bmp-4, Shh, and Wnt-10a. Like Fgf-4, these signals are expressed in the enamel knot of cap stage Tabby teeth, but the intensity of the expression appears to be reduced as the expression domain is smaller corresponding to the smaller enamel knot (Figs. 5a–5f). Bmp-4 is, in addition, expressed intensely in the dental mesenchyme and this is also the case in Tabby molars, although the dental mesenchyme is smaller in Tabby (Figs. 5a and 5b).

L-fng, a secretory molecule associated with the Notch signaling pathway, is expressed intensely at cap stage in the dental epithelium outside the enamel knot, i.e., the cervical loops (Fig. 5g). Similar expression domains can be found in the Tabby molar epithelium (Fig. 5h); however, the cervical...
FIG. 3. Fgf-8 and Bmp-2 expression in Tabby and wild-type mandibles. Fgf-8 is expressed in the molar region as a gradient (weaker expression toward the incisor) similarly in a wild-type (a) and Tabby (b) E10.5 mandible. Bmp-2 is expressed in the molar and incisor regions in a similar pattern in wild-type (c) and Tabby (d) E12 mandibles. Bar, 700 μm.
loops are smaller and less developed than in wild type. Fgf-3 and Fgf-10 are expressed intensely in the dental papilla mesenchyme (Figs. 5i and 5k) and have been specifically associated with the rapid growth of the tooth germ and epithelial folding morphogenesis during cap and early bell stages (Kettunen et al., 1999). In Tabby their expression pattern remains similar (Figs. 5j and 5l) but appears reduced in intensity.

EGFR is expressed in the dental epithelium and the dental follicle mesenchyme of a wild-type cap stage molar (Figs. 5m and 5o). Unlike Tabby and EDA fibroblasts which have been reported to have reduced levels of EGFR (Vargas et al., 1996), both EGFR mRNA and protein are expressed at normal intensity and pattern in a Tabby cap stage molar (Figs. 5n and 5p).

Hence, all of the molecules studied here are present in the Tabby dental tissues but none show completely altered expression domains. At earlier stages, E10.5 and E12, no difference in the intensity of the expression could be detected. At the later cap stage the clear difference in the morphology of the small Tabby cap stage molar and the wild-type molar of the same developmental stage was accompanied with reduced expression of most signaling molecules analyzed. The enamel knot markers Fgf-4, Shh, and Wnt-10a show that the Tabby molar enamel knot exists but is small compared to the wild-type enamel knot and the expression of L-fng showed that the cervical loops were much less developed than in the wild type. Also, the expression pattern of Fgf-4 indicated that the first pair of secondary enamel knots in the Tabby molars was abnormal and correlated with the disturbed cusp patterns suggesting disturbances in the delineation of individual cusps.

Expression of Tabby mRNA in Developing Teeth

We examined expression of Tabby with in situ hybridization in the developing tooth. The probes used were TaA, which is specific for the TaA transcript, exon 1, which recognizes all splicing forms of Tabby, and TaB, specific for the TaB transcript. As negative controls we used corresponding sections from Tabby mice (not shown). This allele (Jackson Laboratories, JR 0314) has been shown to be a null allele with no detectable mRNA transcription (Srivastava et al., 1997). In general the Tabby expression levels were extremely low requiring the use of 33P isotope and exposure times of 15 days.

Tooth sections from initiation stage (E11) up to bell stage (E17) were analyzed. No expression can be seen during the initiation and bud stages (Figs. 6a and 6b). The first Tabby expression (TaA and exon 1 probes) was detected at the early cap stage (E14) in the outer enamel epithelium and dental lamina. This expression pattern persists throughout the late cap and bell stages (E15, E17) (Figs. 6c and 6d) and expression is also seen in postnatal teeth (data not shown). No expression is seen with the TaB probe in any tooth section (data not shown). We did not examine the expression of the TaC isoform, but the corresponding human EDA-C form was shown to be proteolytically unstable when expressed in 293 cells suggesting that this type of cDNA may be a result of aberrant RNA processing rather than a functional transcript (Ezer et al., 1999). We conclude that TaA is the main splicing form expressed in the tooth and possibly also in other tissues as we found no TaB expression in other embryonic tissues examined (Mikkola et al., 1999).

**FGF but Not EGF Can Partially Rescue the Morphogenesis of Tabby Tooth Germs in Vitro**

EGF has been shown to rescue Tabby sweat gland development when injected locally to footpads of postnatal mice (Blecher et al., 1990). We wanted to know whether EGF (or other growth factors) could similarly rescue the Tabby tooth phenotype in vitro. Therefore, we analyzed the effects of exogenous EGF, FGF-10, and FGF-4 on Tabby teeth in an organ culture system.

E13.5 Tabby molars were dissected and their development was examined for 4 days in vitro. The Tabby molars differed from the wild-type controls already at the onset of culture (Figs. 7a and 7b). First, although the buccolinguinal axis appears to be only slightly reduced in Tabby molars (see also Figs. 2c and 2d), the mesiodistal axis is reduced to approximately two-thirds of its length (N = 43). Second, contrary to the one tooth bud visible in a wild-type molar segment, two buds can be seen in Tabby molars, corresponding to the future M1 and M2, and there is a clear boundary between the M1 and M2 forming regions. After 4 days of culture wild-type first molars develop five to six cusps, whereas Tabby molars behave as in vivo forming an average of three to four cusps (Figs. 7c and 7d).
wild-type molar was also larger than the Tabby molar, twice as long on the mesiodistal axis and two-thirds longer on the buccolingual axis.

For the growth factor rescue experiments E13 and E14, Tabby and wild-type molars were cultured for 4 days in the presence of EGF, FGF-10, or FGF-4. The proteins were

**FIG. 5.** Comparison of the expression patterns of Bmp-4, Shh, Wnt-10a, L-fng, Fgf-3, Fgf-10, and EGFR in wild-type and Tabby E14 mouse lower first molars. The primary enamel knot markers Bmp-4 (a, b) (also expressed intensely in the dental mesenchyme), Shh (c, d), and Wnt-10a (e, f), the cervical loop marker L-fng (g, h), and the mesenchymally expressed Fgfs, Fgf-3 (i, j), and Fgf-10 (k, l), are all expressed in appropriate locations in Tabby teeth but in a reduced area compared to wild type. EGFR mRNA (m, n) and protein (o, p) are expressed in the dental epithelium and the dental follicle of both a wild-type and a Tabby molar. Bar, 100 μm.

**FIG. 6.** Tabby expression. No Tabby mRNA is seen at the initiation (E11, a) nor bud (E13, b) stages. At the cap (E15, c) and bell (E17, d) stages expression is in the outer enamel epithelium (arrows) and dental lamina (arrowheads). Probe used TaA. Bar, 100 μm.
applied either locally to the distal part of the explants with beads or they were added to the culture medium. A summary of the results of the bead experiments is shown in Table 2. Wild-type molars form an average of 5.1 (E13) and 5.3 (E14) cusps when cultured in control medium, and the addition of FGF-10 does not affect the number (Figs. 8a and 8b). Tabby molars, without added growth factor, form an average of 2.8 (E13) and 3.4 (E14) cusps per tooth. EGF-

FIG. 7. Development of wild-type and Tabby E13.5 first molars in organ culture. (a) Wild-type E13.5 molar at onset of culture. (b) Corresponding Tabby M1 is shorter along the mesiodistal axis (arrowhead). Note also that M2 has already developed nearly to the size of M1 (arrow). (c) After 4 days of culture six cusps have formed in the wt M1 but the Tabby M1 (d) has only four cusps. M1, first molar; M2, second molar.

FIG. 8. FGF-10 rescues Tabby tooth germs in vitro but EGF does not. (a) An E13 wild-type molar grown for 4 days with an FGF-10 bead. (b) Cross-section of the same tooth shows three well-developed cusps. (Development of wild-type teeth was similar with control BSA beads and beads releasing EGF.) (c) A Tabby E13 molar grown for 4 days with an EGF bead. (d) Cross-section of the same tooth shows a poorly developed tooth with only two cusps visible. (e) A Tabby E13 molar grown for 4 days with an FGF-10 bead. (f) Cross-section of the same tooth shows three cusps; however, they are not as developed as in the wild-type tooth. Bar, 150 μm.
coated beads (soaked in a concentration range of 5 -100 ng/μl) do not have an effect on the cusp number of Tabby teeth, the average cusp number being 2.8 (E13) and 3.3 (E14) (Figs. 8c and 8d). FGF-10 beads (25 ng/μl), however, increase the cusp number to 3.6 (E13) and 4.3 (E14) (Figs. 8e and 8f) thus partially rescuing the Tabby phenotype. FGF-4 beads (25 ng/μl) have a similar effect but to a lesser degree, the cusp number being 3.1 (E13) and 3.7 (E14). Similar results were obtained when EGF (N = 78), FGF-4 (N = 24), and FGF-10 (N = 32) were added to the culture medium (EGF, 10 ng/ml; FGF-4 and -10, 150 ng/ml) (data not shown).

### DISCUSSION

**The Tabby Tooth Phenotype May Result from Defective Enamel Knot Signaling**

Our comparisons of Tabby and wild-type molar tooth morphology from dissected tooth germs and from histological sections revealed the first differences at bud stage. During the initiation of tooth development the thickening of the epithelium was histologically similar in Tabby and wild-type embryos and the subsequent budding of the epithelium and condensation of the mesenchyme appeared to occur similarly in all embryos. However, the fully developed molar bud was shorter on the mesiodistal axis, and the budding of the second molar appeared to occur earlier in the Tabby teeth than in the wild-type teeth. The epithelial enamel organ, which develops from the bud during the cap stage, was hypoplastic and abnormal in shape and it was progressively disturbed in development during the late cap and early bell stages. During advanced bell stage, however, after the initiation of all cusps and determination of the crown area, the growth of the tooth in height was not so severely affected, and the differentiation of the odontoblasts and ameloblasts proceeded normally even if it was delayed. Dentin and enamel matrices that were deposited also appeared to be normal. Based on these observations we conclude that the lack of Tabby gene function disturbs tooth morphogenesis during the bud and cap stage and results in an irreversible defect in cusp patterning seen as a reduction in the size of the first molar crown and number of cusps, whereas later development appears not to be affected allowing normal formation of the hard tissues and growth of the cervical loop epithelium resulting in relatively normal height of the tooth crown.

The transition from the bud to the cap stage appears to be a key event in tooth morphogenesis. In several knockout mice tooth development is arrested at the bud stage (Thesleff and Sharpe, 1997; Peters and Balling, 1999). Classic tissue recombination studies have shown that the mesenchyme instructs epithelial morphogenesis from bud to cap stage (Kollar and Baird, 1970), and more recently we have shown that this transition involves the induction of the enamel knot in the epithelium (Jernvall et al., 1998). The enamel knot, which is a transient cluster of epithelial cells, expresses at least nine different signals belonging to the FGF, BMP, Hh, and Wnt families, and hence it is believed to act as a signaling center regulating tooth shape. It stimulates the growth of the tooth germ and it is thought to instruct the formation of secondary enamel knots which determine the sites of tooth cusps (Thesleff and Jernvall, 1997).

Interestingly, in Tabby teeth the primary enamel knots were small. Also the rest of the cap stage epithelium was hypoplastic, especially the cervical loops forming at the labial and lingual sides of the enamel knot appeared to grow slowly, although they did express L-fng, the cervical loop marker. We examined by in situ hybridization the expression of several enamel knot signals, including Bmp-4, Fgf-4, Shh, and Wnt-10a and showed that they were all expressed in the Tabby enamel knots but clearly in a reduced area. Hence, the defective development of Tabby tooth germs during the cap stage was associated with the reduced expression of signal molecules in four different families, and the hypoplastic development of Tabby teeth during the cap stage could well be a consequence of defective enamel knot signaling. The enamel knot is a transient structure only present during cap stage, exactly when the Tabby tooth development was progressively affected. Furthermore, as shown by Fgf-4 expression domains, the first pair of secondary knots was fused demonstrating that individual cusp morphogenesis is disturbed already by E16. Thus, cusp patterning, as detected via secondary enamel knots, is affected early in Tabby molars rather than by reduced growth at later stages alone. It is also noteworthy that the cusps and teeth most commonly missing in Tabby mice are the last developing ones (the anteroconid and hypoconulid cusps and the third molar), further suggesting the progressive effect of early developmental disturbances.

Tabby was not expressed in the enamel knot or in the nearby inner enamel epithelium. The only cells where we
detected Tabby transcripts in the teeth were the cells of the outer enamel epithelium. How the loss of function of the Tabby gene is associated with the reduction of enamel knot signaling is therefore not so obvious. Although these cells are not in contact with the enamel knot, the Tabby protein could affect enamel knot cells if it is a secretory molecule. The structural features of ectodysplasin, the protein encoded by the Tabby and EDA genes, indicate that it is a TNF homologue which is anchored in the plasma membrane. We have recently observed that when transfected into cells ectodysplasin increases the adhesion of the cells to extracellular matrix, but we did not find any indications of shedding from the plasma membrane (Mikkola et al., 1999). It is possible that the enzymes needed for proteolytic cleavage of ectodysplasin were not expressed in these cells and that ectodysplasin may be soluble and affect other epithelial cells including those in the enamel knot. The answers to these questions must await the localization of the ectodysplasin receptor. Interestingly, recent data suggest that the gene behind Tabby, a mouse mutation with identical phenotype to Tabby, encodes a member of the TNF receptor superfamily which is very likely the ectodysplasin receptor (Headon and Overbeek, 1999).

The timing of Tabby expression is also somewhat paradoxical. We detected the earliest Tabby expression in E14 cap stage teeth in the outer enamel epithelium, and this clearly is too late to account for the defect already at bud stage. Because the mesenchyme instructs epithelial morphogenesis during the bud stage, it is possible that the induction from mesenchyme was defective. This has been shown to be the case in mouse mutants in which tooth induction from mesenchyme was defective. This has been identified as mesenchymal signals regulating early epithelial tooth morphogenesis (Bei and Maas, 1998; Ferguson et al., 1998; Jernvall et al., 1998; Kettunen et al., 1999).

However, as Tabby expression seems to be confined to epithelial tissue it may not be directly associated with this induction. It could, however, be involved with epithelial development at earlier stages of tooth formation and affect the early induction of dental mesenchyme by epithelium. Although the human embryonic tooth bud is negative for EDA, expression has been detected in the mandibular arch epithelium in embryos (Montoniet al., 1998). It is possible that the level of sensitivity of our in situ hybridization method was not high enough to detect Tabby expression at the early stages. On the other hand, Tabby expression in developing hair follicles seems to follow a similar pattern where the first signal of Tabby is seen at later stages of follicle development (Mikkola et al., 1999). Although our analysis of two epithelial signals, FGF-8 and BMP-2, which are involved in inducing the mesenchyme during the initiation stages of tooth development did not reveal differences between the Tabby and wild-type embryos, small differences in their expression domains would not have been possible to detect with the whole mount in situ hybridizations.

Associations of Ectodysplasin and FGF Signaling

The organs affected in the Tabby mice and human EDA patients are epithelial appendages sharing common developmental mechanisms and therefore it was suggested already decades ago that the defective gene may be associated with epithelial–mesenchymal interactions (Grüneberg, 1971). Today the molecular mechanisms of these interactions have been characterized in great detail at the molecular level. Molecules involved in cell–cell adhesion, cell–matrix interactions, and soluble signal molecules and their downstream targets are all important players in epithelial organ morphogenesis including the tooth and hair (Thesleff et al., 1995; Chuong, 1998; Peters and Balling, 1999). The recent identification of ectodysplasin as a novel member of the TNF superfamily indicates that Tabby codes for a signal molecule (Mikkola et al., 1999). TNFs have been previously associated mainly with the immune system and mostly in the regulation of cell proliferation and apoptosis. On the other hand, they also have direct effects on cell differentiation as recently shown for osteoprotegerin ligand, a novel TNF-like molecule regulating osteoclast differentiation and function (Lacey et al., 1998; Yasuda et al., 1998). Neither the Tabby phenotype nor our expression analysis support associations with apoptosis. Cell proliferation, on the other hand, is obviously associated with hypoplastic organ development in the Tabby mouse, but whether the effects of ectodysplasin on cell proliferation are direct or not is still unknown.

We showed that when bud stage tooth germs from Tabby mouse embryos were cultured in the presence of FGF recombinant protein their growth was stimulated and they developed more cusps. FGFs are potent mitogens to numerous cell types, including dental cells, and it is conceivable that the rescue by FGFs in our experiments resulted from mitogenic effects. Interestingly, FGF-10 had a more potent effect than FGF-4. FGF-10 expression is restricted to dental mesenchyme and we have recently shown that it effectively stimulates cell division in dental epithelium but has no mitogenic effect on dental mesenchyme (Kettunen et al., 1999). Fgf-4 on the other hand is only expressed in the enamel knot and stimulates both epithelial and mesenchymal cell proliferation in dental tissues (Jernvall et al., 1994). This suggests that the effect of FGF specifically on epithelial cells causes the partial rescue of Tabby teeth. The FGFR2b isoform of FGF receptors mediates FGF-10 signaling and is expressed in the dental epithelium (Kettunen et al., 1998). Another FGF receptor, which is expressed in the dental epithelium particularly in the cervical loops, is FGFR1c, which mediates FGF-4 effects. However, no FGF receptor expression is seen in the enamel knot (Kettunen et al., 1998). Hence the rescue effect of exogenously added FGF-10 and FGF-4 proteins conceivably was due to a
stimulation of the growth of dental epithelium in the cervical loops, and we hypothesize that the following extension in the epithelial sheet subsequently allowed the formation of secondary enamel knots and initiation of new cusps. This conclusion is supported by the observation that the cusps missing in Tabby molar, the anteroconids and hypoconulids, are the last cusps to develop. We showed that Fgf-10 and Fgf-3 were expressed in the dental mesenchyme and Fgf-4 in the enamel knot of Tabby teeth. However, the levels appeared reduced and they may have been compensated by exogenous FGF-10 and FGF-4 proteins in vitro. It is also possible that the added FGFs may have mimicked the effects of some other mitogens.

Epidermal growth factor (EGF) has earlier been associated with the Tabby/EDA phenotype. It was shown that EGF injections to newborn Tabby mice induce the formation of sweat glands which normally are missing in these mice (Blecher et al., 1990). Furthermore, the expression of EGF receptors was reported to be reduced in fibroblasts from Tabby mice and EDA patients (Vargas et al., 1996). However, our present observations do not support the proposal that Tabby is directly associated with the EGF signaling pathway. First of all, EGF receptors were expressed at normal intensity and pattern in a Tabby molar and, second, addition of EGF to the cultured Tabby teeth did not rescue development, although EGF receptors show coexpression with Tabby in the outer enamel epithelium during cap stage, and EGF stimulates cell proliferation in cultured dental mesenchymal cells (Partanen et al., 1985). Furthermore, direct interactions between ectodysplasin and EGF were not observed in our recent studies on cultured cells (Mikkola et al., 1999).

In conclusion, we suggest that the patterning defect of tooth cusps in Tabby teeth results from deficient growth of the dental epithelium. Already the budding dental epithelium is hypoplastic and there is a defect in enamel knot formation and subsequent reduction in the expression of signaling molecules regulating tooth morphogenesis during the cap and early bell stages. The rapid and extensive growth of the epithelium during these stages is necessary to allow the formation of secondary enamel knots initiating cusp development and therefore the final shape of the tooth is determined during this phase. The partial rescue of cusp pattern with FGF may not reflect direct associations between the FGF and ectodysplasin signaling pathways and could be explained by stimulation of epithelial cell proliferation compensating for the hypoplastic development of the mutant epithelium. The rescue of sweat gland development by EGF may similarly have been due to general stimulation of cell proliferation (Blecher et al., 1990). On the other hand, FGFs are important mediators of epithelial–mesenchymal interactions during the development of all organs affected by Tabby including hair and glands (Wedelitz et al., 1996). In addition, FGFs are essential for the formation of Rathke’s pouch, which expresses Tabby intensely (Takuma et al., 1998; Mikkola et al., 1999). Hence, there is the interesting possibility that FGF and ectodysplasin signaling are integrated. The heterodimeric transcription factor NF-κB which is activated as a response to numerous TNF-like ligands (Gruss and Dower, 1995) was recently observed to mediate FGF signaling in the limb bud (Bushdid et al., 1998) thus offering one intriguing possibility for convergence of the ectodysplasin and FGF signaling pathways.

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