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Cusp Patterning Defect in *Tabby* Mouse Teeth and Its Partial Rescue by FGF

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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.**

INTRODUCTION

In 1952, Falconer isolated a spontaneous mouse mutation that was named *Tabby* and that displayed a phenotype in three kinds of ectodermal organs: teeth, hair, and some glands. In general, the organs are missing, reduced in size, or abnormal in structure. The tooth phenotype is characterized by missing incisors and third molars, albeit with a variable penetrance, and the cusps of the first and second molars are reduced in size and number (Grüneberg, 1965). The hair follicle composition is abnormal as of the four different hair types normally found in mice—zigzags, guard hairs, auchenes, and awls—only hairs resembling abnormal awls are found in *Tabby*. The timing of the follicle development is also abnormal as the development starts later than in wild-type mice (Kindred, 1967; Claxton, 1967). Over 20 different glands are affected. For example, sweat glands, normally found at the pads of mouse feet, are absent. The lacrimal glands are smaller (exorbital lacrimal gland) or not developed (intraorbital lacrimal gland) and the submandibular gland is reduced in mass (Grüneberg, 1971; Blecher *et al.*, 1983). The human syndrome EDA (anhidrotic ectodermal dysplasia) was suggested to be the human counterpart of *Tabby* because of its similar phenotype and corresponding X-chromosomal location (Blecher, 1986) and subsequent cloning of the genes confirmed this (see below). A link between Tabby and the epidermal growth factor (EGF) pathway has been proposed as injections of EGF into the footpads of newborn mice can rescue the sweat gland phenotype (Blecher *et al.*, 1990) and dermal fibroblasts from *Tabby* mice and EDA patients have a lower amount of EGFR protein than their wild-type controls (Vargas *et al.*, 1996).

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The gene behind EDA was cloned in 1996 and *Tabby* 1 year later (Kere *et al.*, 1996; Ferguson *et al.*, 1997; Srivastava *et al.*, 1997). *Tabby* turned out to code for three splicing forms, *TaA*, *TaB*, and *TaC*. *TaA* is the longest of these and the protein it codes for is called ectodysplasin. It is a nearly 400 amino acid long type II transmembrane protein that is homologous to tumor necrosis factor (TNF)-like proteins (Mikkola *et al.*, 1999) and has a short collagen-like stretch of glycine repeats. The TNF ligand superfamily consists of at least 15 molecules that are trimeric ligands for receptors that regulate, e.g., the immune response and host defense via mechanisms such as apoptosis and cell survival (Smith *et al.*, 1994). Ectodysplasin appears not to be involved in apoptosis; instead it promotes adhesion to the extracellular matrix (Mikkola *et al.*, 1999). The two other main splicing forms of *Tabby*, *TaB* and *TaC*, share with *TaA* a common exon 1 that contains the transmembrane domain but not the glycine repeats or TNF domain (Srivastava *et al.*, 1997). The functional relevance of these forms is uncertain. In humans eight different EDA transcripts have been identified, but only the corresponding *EDA-A* has a TNF domain or glycine repeats (Bayes *et al.*, 1998).

All organs affected in *Tabby* mice are epithelial appendages developing from ectoderm and underlying mesenchymal tissue. The mechanisms involved in the regulation of development are shared to a great extent between all epithelial appendages (Thesleff *et al.*, 1995). In particular, inductive interactions between the epithelium and mesenchyme constitute a mechanism of central importance, and during recent years the molecular basis of the signaling between epithelial and mesenchymal tissues has been elucidated in great detail. It is apparent that the same conserved 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).

Teeth develop from oral ectoderm and underlying neural crest-derived mesenchyme and the epithelial–mesenchymal interactions regulating tooth morphogenesis have been characterized in great detail. All four signal families mentioned above have been implicated in the mediation of epithelial–mesenchymal interactions. Several transcription factors have also been identified which are targets of the signals and which are necessary for normal tooth development. Tooth development is arrested early in mice deficient for Msx1, Pax9, Lef1, and double knockouts of Gli2/3, which are targets of BMP, FGF, Wnt, and Hh signaling, respectively (Jernvall and Thesleff, 1999; Peters and Balling, 1999). Several members of the FGF family are involved in tooth development, some as epithelial signals and some in the mesenchyme. FGF-8 and -9 are involved in the epithelial signaling regulating tooth initiation, *Fgf-4* and *-9* are expressed in the primary enamel knot in cap stage tooth germs and *Fgf-4* in the secondary enamel knots during the bell stage (Jernvall *et al.*, 1994; Kettunen and Thesleff, 1998). The primary enamel knot is thought to act as a signaling center regulating tooth shape, and the secondary enamel knots mark the initiation of tooth cusps (Thesleff and Jernvall, 1997).

Although ectodysplasin, the protein lacking in *Tabby* mice, apparently functions in early developmental regulation of the affected organs and most probably is associated with epithelial–mesenchymal interactions, little is known about its function. The aim of our studies is to clarify the developmental role of ectodysplasin. To this end we have decided to analyze the pathogenesis of the dental phenotype in *Tabby* mice both in adults and in the embryo and to use experimental approaches developed in our laboratory (Vainio *et al.*, 1993; Jernvall *et al.*, 1998) to analyze the possible associations of ectodysplasin with the known signaling pathways. In this paper we show that the *Tabby* molar develops abnormally already at the bud stage and at cap stage has a smaller enamel knot. Signaling molecules important in normal tooth development are expressed although the expression levels appear reduced as the expression domains are smaller, corresponding to the smaller enamel knot in the *Tabby* molars. The secondary enamel knot pairs determining the sites of buccal and lingual tooth cusps are fused to each other thus dictating the molar crown phenotype in *Tabby* mice. Expression of the *TaA* transcript is found in the outer enamel epithelium. Finally, EGF has no effect on the development of *Tabby* tooth buds *in vitro* but FGF-10 can partially rescue the phenotype.

MATERIALS AND METHODS

Animals

Wild-type mice were either (a) NMRI adults or (b) embryos from a CBAT6T6 × NMRI cross. The *Tabby* allele used, B6CBACa-A^{w-J}/A-Ta, 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%

H_2O_2 at 4°C for 45 min after which the mandibles were rinsed in H_2O 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 anti-digoxygenin 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 pCRTMII (Invitrogen), from D. E. Lee; *Fgf-3* (Wilkinson *et al.*, 1988), from D. G. Wilkinson; *Fgf-4* (Jernvall *et al.*, 1994), from C. Basilico; *Fgf-10* (Bellusci *et al.*, 1997), from S. Bellusci; *L-fng*, 860-bp 5′coding region of mouse Lunatic Fringe cloned into pBSSKII(−) and linearized to obtain an antisense probe of 570 bp, from Y. A. Wang; *Shh* (Vaahtokari *et al.*, 1996), from T. Edlund; *Wnt-10a* (Dassule and McMahon, 1998), from A. MacMahon; *Tabby* exon 1, plasmid pTaNoBg (Srivastava *et al.*, 1997); *Tabby TaA*, plasmid pTE2A, a 0.8-kb *Ta-A3* fragment (nucleotides 2371–3164, EMBL Accession No. AJ 243658, Mikkola *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 [^{35}S]UTP (Amersham) except for the *Tabby* probes where [^{33}P]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^{w-J}/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

TABLE 1
Frequency Distribution of the Cusp Number of *Tabby* First Molars

	Cusp number					
	2	3	4	5	6	Total
Number of cases	2	8	25	12	2	52
Percentages	4	15	48	23	4	100 (%)

Mean: 3.8 cusps.

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.

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).

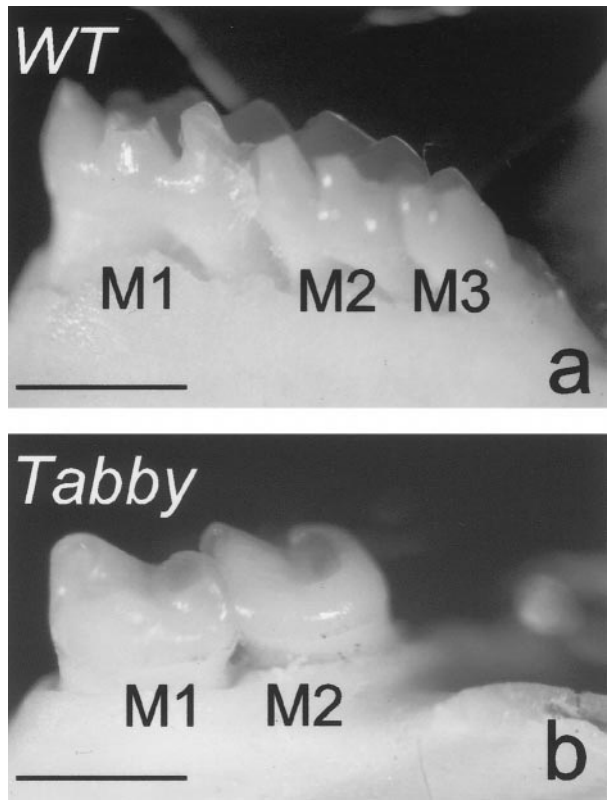


FIG. 1. Wild-type and *Tabby* molar teeth. Lateral views of wild-type (a) and *Tabby* (b) adult mandibles show the reduction in the size of the tooth and the number of cusps in *Tabby* first and second molars. The third molar is missing in *Tabby*. M1, first molar; M2, second molar; M3, third molar. Bar, 1 mm.

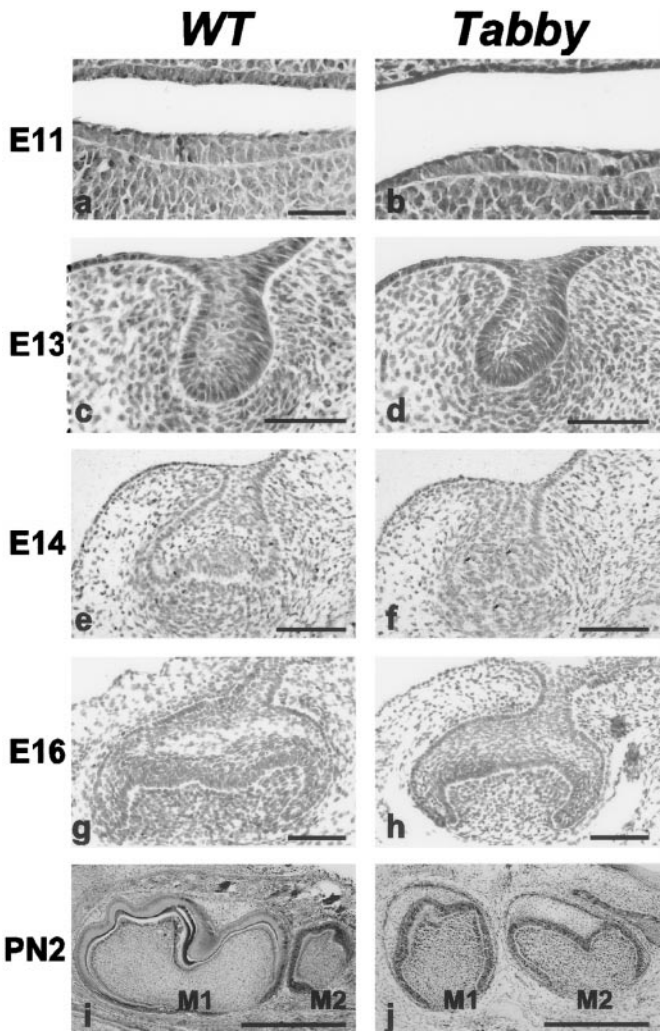


FIG. 2. Comparison of wild-type and *Tabby* embryonic molar development. No difference can be seen between wild-type (a) and *Tabby* (b) tissues during the initiation of tooth development (E11). The bud stage (E13) *Tabby* molar is slightly smaller than wild type (c, d). At cap stage (E14) the *Tabby* molar is clearly smaller and abnormal in shape (e, f) and the disturbance has progressed by the bell stage (E16) (g, h). At PN2 the *Tabby* M1 is delayed in development compared to wild type (i, j). *Tabby* M2 is the same size as M1 compared to the wild-type M2, which is about one-third the size of wild-type M1. M1, first molar; M2, second molar. Bar, 50 μm for a and b; 100 μm for c–h; 500 μm for i and j.

Expression of Signaling Molecules

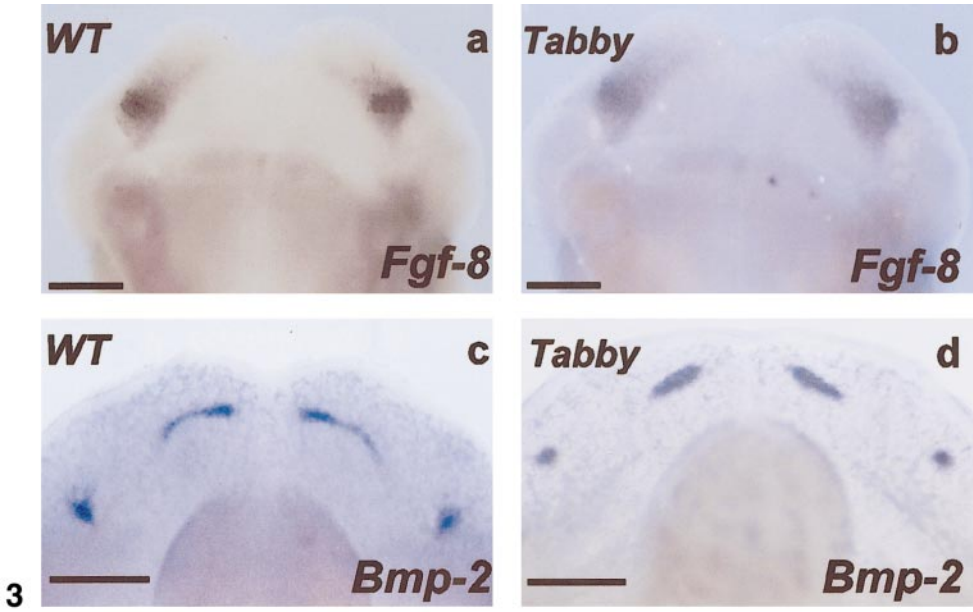
The development of the *Tabby* molar was followed with markers for signaling molecules known to be important for tooth morphogenesis. Early tooth development was studied by whole mount *in situ* hybridization analysis of *Fgf-8* and *Bmp-2* expression in mandibular arches. Cap stage development was analyzed 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



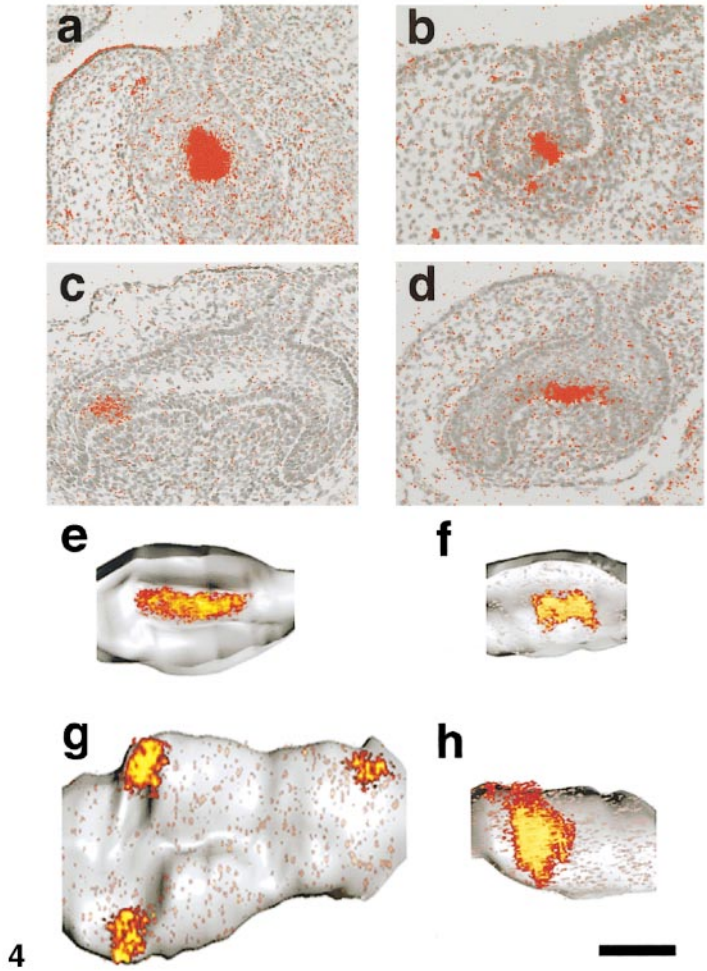


FIG. 3. *Fgf-8* and *Bmp-2* expression in *Tabby* and wild-type mandibles. *Fgf-8* is expressed in the molar region as a gradient (weaker expression toward the incisor) similarly in a wild-type (a) and *Tabby* (b) E10.5 mandible. *Bmp-2* is expressed in the molar and incisor regions in a similar pattern 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 ^{33}P isotope and exposure times of 15 days.

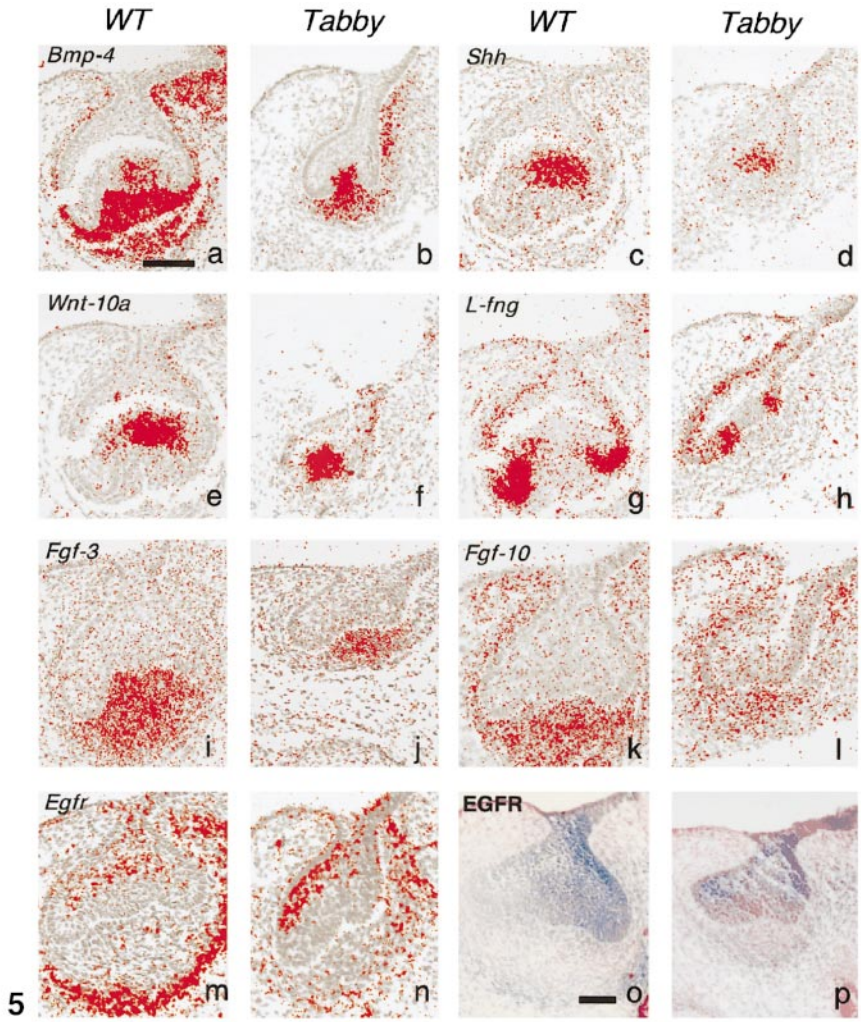
Tooth sections from initiation stage (E11) up to bell stage (E17) were analyzed. No expression can be seen during the initiation and bud stages (Figs. 6a and 6b). The first *Tabby* expression (*TaA* and *exon 1* probes) was detected at the early cap stage (E14) in the outer enamel epithelium and dental 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 buccolingual 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). The

FIG. 4. Three-dimensional reconstructions of *Fgf-4* expression in wild-type and *Tabby* first lower molars. The *Fgf-4* expression in the primary enamel knot at E14 is strong and extends over 200 μm mesiodistally in wild-type molars (a, e). In the E14 *Tabby* molars (b, f), the *Fgf-4* expression is weaker and extends only 100 μm. At E16, the secondary enamel knots corresponding to future cusp tips are present as small *Fgf-4* expressing areas in the wild-type molars (c), and three secondary enamel knots are visible when the molar is viewed from above (g). In contrast, E16 *Tabby* molar is much smaller and has only one large *Fgf-4* expression area (d, h) which corresponds to the first buccal and lingual secondary knots in the wild-type molar (on the left in g). The *Fgf-4* expression is marked with yellow to red corresponding to decreasing density of *in situ* grains. Buccal is toward the right in the histological plates and toward the bottom in the reconstructions (mesial toward the left). Bar, 100 μm.



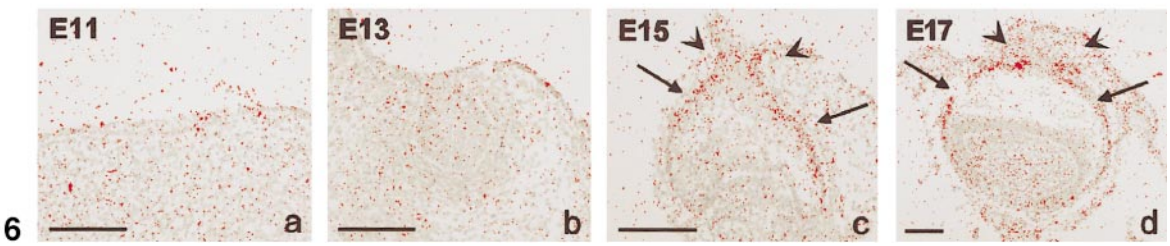
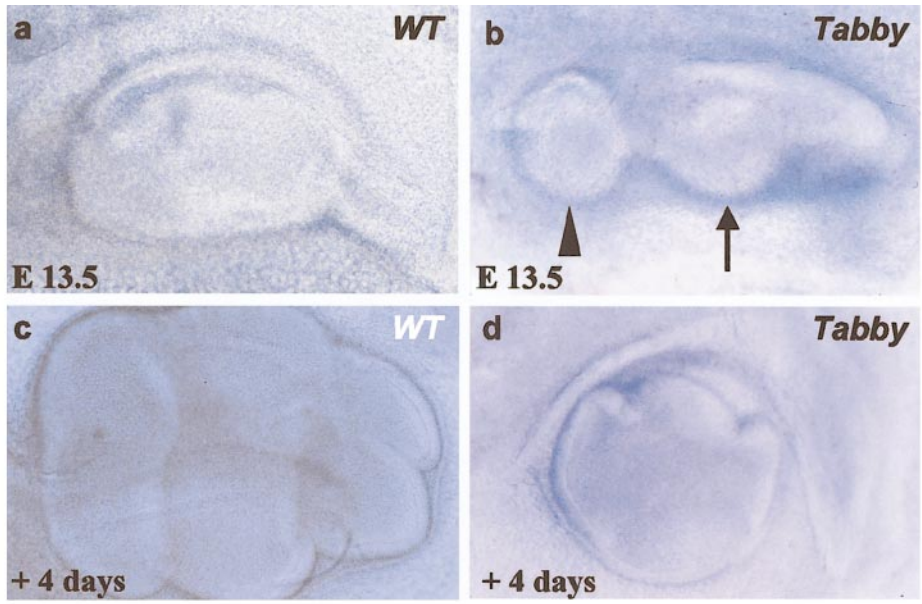


FIG. 5. Comparison of the expression patterns of *Bmp-4*, *Shh*, *Wnt-10a*, *L-fng*, *Fgf-3*, *Fgf-10*, and *EGFR* in wild-type and *Tabby* E14 mouse lower first molars. The primary enamel knot markers *Bmp-4* (a, b) (also expressed intensely in the dental mesenchyme), *Shh* (c, d), and *Wnt-10a* (e, f), the cervical loop marker *L-fng* (g, h), and the mesenchymally expressed *Fgf*s, *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.

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



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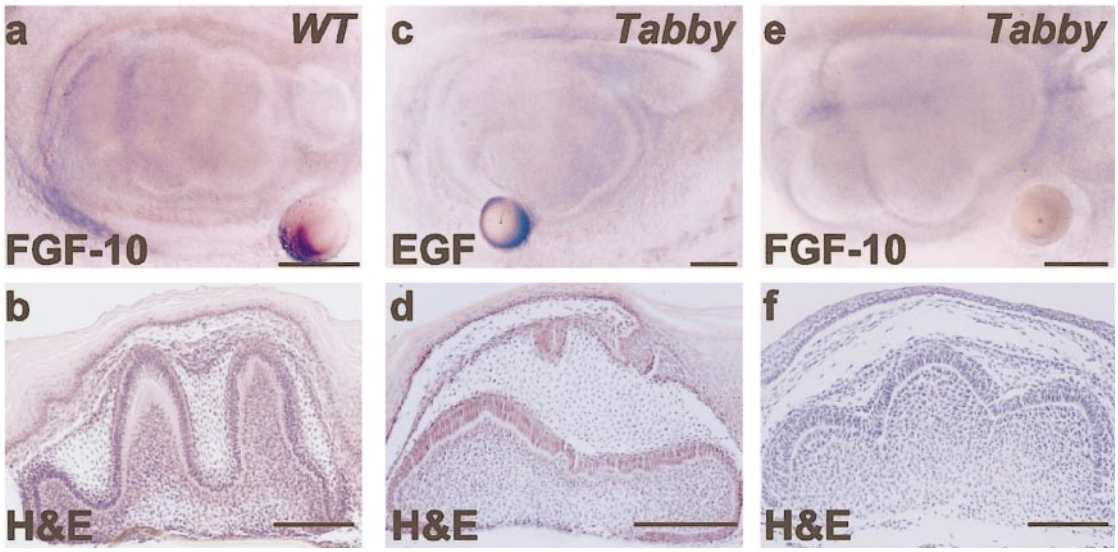

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FIG. 7. Development of wild-type and *Tabby* E13.5 first molars in organ culture. (a) Wild-type E13.5 molar at onset of culture. (b) Corresponding *Tabby* M1 is shorter along the mesiodistal axis (arrowhead). Note also that M2 has already developed 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.

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-

TABLE 2
The Effects of FGF-4, FGF-10, and EGF on the Cusp Number in First Molars of *Tabby* and Wild-Type Embryos Cultured for 4 Days

	E13 (*N*)	E14 (*N*)
WT	5.1 (19)	5.3 (22)
WT + FGF-10	5.0 (12)	5.4 (13)
*Tabby**	2.8 (35)	3.4 (42)
Tabby + FGF-10*	3.6 (27)	4.3 (26)
Tabby + FGF-4	3.1 (24)	3.7 (18)
Tabby + EGF	2.8 (76)	3.3 (68)

Note. The cultures were started at bud (E13) and cap stages (E14) and the growth factors were applied by beads.
* $P < 0.05$.

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 *downless*, 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 development is completely arrested at the bud stage and no enamel knot forms. BMP-4, FGF-3, and activin βA have 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 (Montonen *et 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* molars, 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 (Widelitz *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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