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The activation level of the TNF family receptor, Edar, determines cusp number and tooth number during tooth development

A.S. Tucker, D.J. Headon, J.-M. Courtney, P. Overbeek, and P.T. Sharpe

Abstract

Mutations in members of the ectodysplasin (TNF-related) signalling pathway, EDA, EDAR, and EDARADD in mice and humans produce an ectodermal dysplasia phenotype that includes missing teeth and smaller teeth with reduced cusps. Using the keratin 14 promoter to target expression of an activated form of Edar in transgenic mice, we show that expression of this transgene is able to rescue the tooth phenotype in Tabby (Eda) and Sleek (Edar) mutant mice. High levels of expression of the transgene in wild-type mice result in molar teeth with extra cusps, and in some cases supernumerary teeth, the opposite of the mutant phenotype. The level of activation of Edar thus determines cusp number and tooth number during tooth development.

Introduction

The arrangements of teeth in the oral cavity (the dentition) determine the feeding habits of every vertebrate species. Changes in tooth shape, size, and number thus constitute one of the major driving forces in evolutionary adaptation. The principal features of morphological change in tooth crown shape are the cusps. The molecular control of cusp formation is thus fundamental to understanding tooth evolution and development. The recent identification of a TNF signalling pathway regulating cuspal morphogenesis in mice and humans has provided first insights into how cusp formation is controlled. Several genes mutated in forms of human ectodermal dysplasias that affect the development of ectodermal structures such as skin, hair, and teeth have been identified (Headon et al., 2002; Kere et al., 1996; Monreal et al., 1998, 1999). Mutations in these same genes have been found in spontaneous mouse mutants, tabby, downless jackson, Downless Sleek, and crinkled (Ferguson et al., 1997; Headon and Overbeek, 1999; Headon et al., 2002; Srivatava et al., 1997). Ectodysplasin (Eda-tabby) is a TNF family ligand that binds to a TNF-like receptor, Edar (downless jackson, Sleek), and transmits an intracellular signal via an adaptor protein, Edaradd (crinkled). Mutations in any of these three genes in mice produce identical molar tooth phenotypes where the number of cusps is reduced and those that form have a rounded appearance (Grüneberg, 1965, Sofaer, 1969a, 1977). Downstream responses to Eda signalling are likely to involve activation of NFκB (Döpfner et al., 2001; Kumar et al., 2001; Schmidt-Ullrich et al., 2001). Edar has been shown to activate NFκB in a dose-dependent manner in transfected cells (Koppinen et al., 2001).

During tooth development, Edar and Edaradd are both expressed in the enamel knot signalling center, a transient epithelial structure found in early tooth germs from E13.5 to E15.5 (Headon et al., 2002; Tucker et al., 2000). Eda, in contrast, is expressed in the outer enamel epithelium but is cleaved from the membrane to generate a secreted form that interacts with Edar (Elomaa et al., 2001; Tucker et al., 2000). Loss of Edar, Edaradd, and Eda leads to defects in the enamel knot. In tabby mice, loss of Eda leads to the formation of a small enamel knot (Pispa et al., 1999), a similar small enamel knot being seen in crinkled mice, after loss of Edaradd (Ohazama et al., 2004). In contrast, in downless jackson mice, loss of Edar leads to the formation
of elongated rope-like enamel knot (Tucker et al., 2000). Different developmental phenotypes have also been shown between tabby and downless jackson mice with respect to their submandibular salivary glands, where the tabby glands are hypoplastic, while the downless glands are dysplastic (Jaskoll et al., 2003). The defects in enamel knot formation seen in tabby, crinkled, and downless mutants have strengthened the evidence for a role of the enamel knot in coordinating cusp formation. In addition to defects in cusp development, tabby, downless, and crinkled mice also show defects in the size of the teeth and in the number of molars. The third molar, for example, is missing in 50% of tabby mice (Grüneberg, 1966; Kristenova-Cermakova et al., 2002).

Eda has recently been overexpressed during embryonic development by several groups. Transgenic expression of the mouse Eda-A1 isoform driven by the CMV promoter leads to rescue of the tabby phenotype with respect to hair growth and sweat glands (Srivastava et al., 2001). In addition, the third molar is rescued, so that all three molars are present in the transgenic. However, in this case the cusps remained flattened and the teeth remained smaller when compared to wild types. This transgene thus rescued the hypodontia but not the tooth morphology. A different tooth phenotype was generated after treatment of pregnant tabby mice with a recombinant form of Eda-1 (Gaide and Schneider, 2003). In this case, the third molar remained absent in 50% of cases, as in the tabby mice, but the cusp defect was corrected after addition of protein from E11, so that clear deep cusps were visible.

Because loss of Eda leads to fewer, reduced cusps and fewer, smaller teeth, it is tempting to speculate that overproduction of Eda signalling might lead to extra cusps and extra larger teeth. In wild-type mice, expression of the CMV-driven Eda-A1 transgene leads to no detectable phenotype, possibly because the transgene was not expressed at very high levels (Srivastava et al., 2001). Transgenic mice have also been created where Eda overexpression is driven in wild-type mice by the keratin 14 promoter (Mustonen et al., 2003). Unlike the CMV promoter, which is widely expressed in many tissues, the K14 promoter drives expression specifically in the epithelium, including oral and dental epithelium, at high levels (Dassule et al., 2000; Vassar et al., 1989). These transgenic mice did indeed produce supernumerary molars found distal to the first molar (Mustonen et al., 2003). The molar teeth in general were shorter and wider than controls, but the cusp pattern remained the same. The absence of a change in cusp formation might suggest that the level of the receptor (Edar) acts as a limiting factor in ectodysplasin signalling in this regard. To investigate this and to eliminate any possible complications resulting from an imbalance between ligand and receptor, we have overexpressed a constitutively active form of Edar driven by the keratin 14 promoter to examine the effect on tooth development.
Materials and methods

Generation and analysis of transgenic mice

The transgenic construct consisted of the human keratin 14 promoter driving the expression of a chimeric cDNA encoding the six transmembrane domains of Epstein–Barr virus LMP1 (amino acids 1–187) fused to the intracellular region of mouse Edar (amino acids 211–448). This construct included a generic intron between the promoter and LMP1–Edar1C cDNA and a human growth hormone polyadenylated sequence 3′ to the cDNA. Transgenic FVB/N mice were generated by pronuclear injection. Five transgenic lines were generated in total. Of these two had no obvious phenotype or health problems and were mated to tabby and Sleek mutant mice. Offsprings of these mice were sacrificed at various stages after tooth eruption so that tooth cusp pattern, and tooth size and number could be analyzed. The remaining three transgenic founders had strong phenotypes and were sacrificed after signs of weakness between 10 and 50 days old. None of these founders were strong enough to produce a genetically more stable F1 generation. Mouse heads were fixed in formalin and stored in 70% ethanol. For sectioning, heads were decalcified in a 0.5 M EDTA solution before being mounted in wax. Slides were stained with a tri-chrome stain. The presence of the transgene in the mice was confirmed by PCR from genomic DNA using the oligosequence LMP1 S TCGTTATGAGTGACTGGAC and LMP1TM AS cagggtaatacatccagatta.

35S radioactive in situ

Paraffin wax sections were cut at 8 μm and then prepared for radioactive 35S in situ hybridization as previously described by Tucker et al. (1999). downless (edar) was linearized with BssHI and transcribed with T7. LMP1 was linearized with SpeI and transcribed with T7 (1–564 of the LMP1 open reading frame). Radioactive sections were left to develop after dipping in emulsion for 7 days. Serial sections were stained with hematoxylin/
eosin (Sigma) or hematoxylin alone to visualize tissue morphology.

**Results**

*Edar* initially has a fairly wide expression pattern in the oral epithelium at E10.5 before becoming restricted to the dental epithelium (Laurikkala et al., 2001; Tucker et al., 2000). At the cap stage, the expression is restricted still further to a small group of cells at the center of the inner enamel epithelium, the enamel knot (Figs. 1A and B). Using the keratin 14 promoter, expression of *Edar* was driven in all the oral epithelium to assess the role of *Edar* on tooth cusp development. Immunohistochemical studies have shown that keratin 14 is expressed intensely in the dental epithelium but not in the mesenchyme of the tooth germ itself or any of the surrounding tissues in the mandible (Tabata et al., 1996). The keratin 14 promoter element has therefore been subsequently used to drive ectodermal expression in the tooth (Dassule et al., 2000; Mustonen et al., 2003). To generate a form of *Edar* that signals in the absence of ligand, we replaced its extracellular domain with the transmembrane domains of the Epstein–Barr virus protein LMP1. LMP1 mimics a constitutively active TNF receptor (Gires et al., 1997) and confers ligand-independent activity when fused to the intracellular domains of TNFRs (Hatzivassiliou et al., 1998). The transduction of the *Eda* signalling pathway could thus occur in all epithelial cells, not only in those that had come into contact with soluble Eda. Overexpression of Edar in tissue culture has previously been shown to have no effect on cell death, as might be predicted from the presence of a death domain, thus supporting the fact that apoptosis is unaffected in tabby and Downless mutants (Koppinen et al., 2001; Tucker et al., 2000).

To visualize the level of expression of the transgene, skin sections were taken from sacrificed mice or from tail cuts and in situ hybridisation using a probe specific for the LMP1 extracellular domain of the transgene carried out. In non-transgenic littermates, no expression was observed in the skin, as would be expected (Figs. 1C and D). In those transgenic founders who had become weak and had to be sacrificed early, expression of LMP1 was shown to be very high in the basal layer (Figs. 1E and F). In those mice who displayed no detrimental effects, but were shown to express the transgene after PCR, only weak expression of LMP1 was observed in the skin (Figs. 1G and H). A correlation could thus be drawn between expression level of the transgene and health of the resulting transgenic mouse.

**Rescue of tabby (Eda) and Sleek (Edar) mutant mice**

The efficiency of the activated Edar protein was first tested by making activated Edar transgenics on a *tabby* or *Sleek* mutant background. A male transgenic founder with no obvious phenotype and low levels of LMP1 expression (as shown in Fig. 1H) was mated to *tabby*/+/*C0* females crossed to K14-activated Edar transgenic founder male. The founder male had no visible phenotype on a wild-type background. (A) Close up showing rescue of cusps. (C) Non-transgenic littermate (*Sleek*/+/*). (D) Close up showing rounded cusps. Cusps are indicated by *.

Fig. 3. Rescue of *Sleek* mutant phenotype by transgene. (A–D) Lower molar tooth development in mice at 25 days after birth. (A) *Sleek* –/– female crossed to K14-activated Edar transgenic founder male. The founder male had no visible phenotype on a wild-type background. (B) Close up showing rescue of cusps. (C) Non-transgenic littermate (*Sleek*/+/−). (D) Close up showing rounded cusps. Cusps are indicated by *.
Downless Sleek mice produce a truncated form of the receptor which is thought to act as a dominant-negative disrupting normal Eda signalling (Headon and Overbeek, 1999), as such Sleek+/− mice have the same tooth phenotype as downless jackson homozygotes, resulting in smaller molars with reduced cusps. Loss of the tertiary molar, as in tabby mice, is frequent (Sofaer, 1977).

Examination of the molar teeth of the resulting transgenics showed that the tabby tooth phenotype had been rescued. The number and height of the cusps in the transgenic mutant lines were normal when compared to the reduced number of flat cusps observed in the tabby mutant (Figs. 2A, B, and E). In the tabby mutant, loss of at least one of the third molars (M3) was observed in each

Fig. 4. Development of additional cusps. (A and B) Lower molar tooth development in mice (posteruption). (A) Transgenic mouse sacrificed at 25 days after birth. (B) Close up showing additional cusp formation. (C–F) Upper molar tooth development in mice (preeruption). Photos rotated by 180°. (C) Transgenic mouse sacrificed at 10 days after birth. (D) Close up showing additional cusp formation. (E) Non-transgenic littermate. (F) Close up showing normal cuspal pattern of first molar. Cusps are indicated by *. (G and H) Upper incisor tooth development at 10 days after birth (preeruption). (G) Monocuspid incisor development in transgenic mice. (H) Normal cusp development in wild-type littermate (N = 3, all showing molar cusp phenotype).
head ($N = 3$); however, three molars were found in each quadrant in each transgenic head examined ($N = 5$) (Figs. 2C and D). The size of the molars, in particular that of the first molar (M1), was also rescued (Figs. 2C, D, and F). Rescue of the mutant phenotype, which was caused by the absence of Eda ligand, by the presence of a constitutively active receptor thus indicates that the construct had the desired ligand-independent signalling capacity.

The Sleek cuspal phenotype was also rescued by the transgene with normal deep cusps developing instead of the usual dome (Figs. 3A and B). Non-transgenic littermates retained the Sleek phenotype (Figs. 3C and D). In the head examined, all four third molars were present.

**Cuspal changes**

Having established the efficacy of the transgene, the effect of the activated Edar on the development of normal (wild-type) molars was assayed. Mutations in tabby, downless, and crinkled show the importance of this family in controlling normal cusp development. We wished to investigate whether signalling through this pathway was sufficient to induce additional cusp development. In those transgenic founders with weak expression of the transgene, overexpression of active Edar in the epithelium appeared to have no obvious effect on tooth development. This is a similar result to that seen after Eda-A1 overexpression using the CMV promoter, which gives widespread low expression (Srivastava et al., 2001). However, in other founders where active Edar was expressed at much higher levels (see Figs. 1E and F), several defects were observed ($N = 3$). These founders did not survive well after birth and were sacrificed after signs of weakness together with littermate controls. When the teeth were analyzed, these mice showed abnormal development of molars that were characterized by their unusual cuspal morphology (Figs. 4A–D). The first upper molar normally has eight cusps, while the first lower molar has seven cusps of which a maximum of three can be seen in one plane of section through the tooth when sectioned frontally (Figs. 4E and F). In transgenic mice, the first molars had numerous small, spiky cusps, with up to eight such cusps being visible in a single frontal section through the tooth (compare Figs. 4A and B to Figs. 3A and B). These additional cusps were obvious both before and after tooth eruption. Thus, additional Edar signalling leads to increased numbers of cusps in molars. The incisors, however, remained monocuspid (Figs. 4G and H).

**Supernumerary teeth and loss of tertiary molars**

The presence of supernumerary teeth in front of the first molar has been reported after overexpression of Eda-A1 in wild-type mice (Mustonen et al., 2003). We therefore examined tooth number in our K14 Edar transgenics. Wild-type mice have three molars in each quadrant separated from an incisor by the diastema. Three heads were examined where the expression level of LMP1 was shown to be high. Of these, one mouse had four molars in one quadrant (Fig. 5). In a similar manner to that seen after Eda overexpression, the supernumerary tooth was in front of
(distal to) the first molar and was slightly smaller than the second molar. The ectopic tooth had a rounded appearance with indistinct cusps and therefore looked similar to a premolar (a tooth type not found in the mouse) (Fig. 5A). However, in two out of the three heads examined, instead of the formation of a supernumerary tooth, the tooth number was reduced in at least one quadrant with the third molar (M3) missing, as is often seen in the mutant. A premolar-like ectopic tooth was not only observed in the example with four teeth in the molar quadrant. In two out of the three

Fig. 6. Enamel defects. (A–D) Lower incisor tooth development in mice 10 days after birth (preeruption). (A) Non-transgenic littermate showing normal formation of enamel (purple layer). (B) Close up showing the organized layer of ameloblasts sitting immediately under the enamel (arrow). (C) Transgenic mouse showing normal development of the dentine (red layer) but complete lack of any enamel. (D) Close up showing no evidence of ameloblast development (arrowhead). Alveolar bone encasing the incisor stains red. (E–H) Lower molar tooth development in mice 10 days after birth (preeruption). (E) Non-transgenic littermate showing normal formation of the enamel (purple layer) and polarized ameloblast layer on top (arrow). (F–H) Transgenic mice showing various severities of loss of enamel and disruption of the ameloblast layer. In some regions, the ameloblasts are disorganized and the resulting enamel layer is thinner than normal (arrows) (F and G), while in other cases, no ameloblasts are found resulting in complete loss of the enamel (arrowheads) (G and H) (N = 3, all showing enamel phenotype).
heads examined, one of the teeth developing in the molar field turned out to be a premolar-like tooth, followed by a first and second molar, with the third molar missing. Tooth number was therefore maintained in these examples but tooth type was altered. In one mouse example, four teeth were found in one upper quadrant, two on the opposite side, and three teeth in both lower quadrants, of which one set of three consisted of first, second, and third molars, while the other side consisted of premolar-like tooth, first and second molars. A variety of tooth number and type was therefore present within the same transgenic animal.

Enamel defects

An additional defect seen after overexpression of Eda in wild-type mice was loss of enamel. Both molars and incisors were worn rapidly and incisors completely lacked the enamel-producing ameloblasts (Mustonen et al., 2003). We therefore looked for enamel defects in our active Edar transgenic mice. Immunohistochemistry has shown expression of K14 in developing ameloblasts that are derived from the dental epithelium (Tabata et al., 1996). Sections were cut through the teeth before eruption so that any changes in enamel thickness could be investigated before any wearing could have occurred. In a similar manner to that seen after overexpression of Eda, expression of active Edar leads to complete loss of incisor enamel; in addition, a serious reduction in molar enamel was observed (Fig. 6). In the incisors, the ameloblast layer was absent (Figs. 6A–D), while in the molars, this usually highly polarized cell group was present in unorganized patches resulting in a thin covering of enamel over the dentine (Figs. 6E–H). The dentine in the incisors was also disrupted with reductions around the base of the upper teeth (see Figs. 4G and H). As the transgene is not expressed in the dentine-producing odontoblasts this may reflect a secondary defect caused by loss of the overlying enamel.

Discussion

Overexpression of the Eda ligand and active Edar gives very different tooth phenotypes with respect to cusp formation, with overexpression of active Edar resulting in ectopic cusp formation. The expression pattern and level of activation of the receptor (Edar) rather than the quantity of the ligand therefore determine cusp number during tooth development. Less Edar signalling leads to reduced numbers of cusps while more signalling leads to the formation of additional cusps. The overexpression phenotype is thus the opposite of the mutant phenotype. This is represented schematically in Fig. 7.

Cusp number and size were rescued in the tabby and Sleek mutants after addition of active Edar. Active Edar expression was additionally able to rescue the third molar and the size of the first molar in tabby mutants. Administration of the Eda protein failed to rescue the third molar of tabby mutant mice (Gaide and Schneider, 2003), while overexpression of Eda using the transgenic approach did rescue the third molar (Srivastava et al., 2001). The difference is likely to represent differences in timing of rescue of Eda signalling. Using the transgenic approach, Eda signalling would have been rescued very early in tooth development before the formation of the epithelial thickening, while Eda protein was administered from E11, once the thickening had formed. This implies that Eda signalling is required very early in tooth development in order for the size of the molar field to be correctly established. Given such an early role for Eda signalling in setting up the molar tooth field, it is therefore not surprising that overexpression of Eda driven by the keratin 14 promoter leads to the formation of supernumerary teeth (Mustonen et al., 2003). The formation of supernumerary teeth was also seen after expression of active Edar, however, loss of the third molar was also very common. High levels of Edar signalling thus lead both to tooth loss and ectopic tooth formation. In rare cases of

Fig. 7. Schematic diagram showing consequences of altered Edar signalling. (A) Edar mutant. Mutation in receptor leads to loss of signal transduction. This may lead to a reduction in the molar field at the epithelial thickening stage (E11), resulting in fewer molars. No Edar signalling leads to a reduction in number and depth of cusps. (B) Wild-type. Normal size of molar field produces three molar teeth with normal pattern of cusps. (C) Overexpression of the Eda ligand using the K14 promoter. Additional ligand leads to increase signalling through the Edar receptor. This may lead to an expansion of the molar field, resulting in supernumerary teeth. Increased Eda ligand within the enamel knot does not affect cusp formation. (D) Overexpression of a constitutively active Edar receptor using the K14 promoter. Three outcomes with respect to tooth development. Four, three, or two teeth were observed in the molar field. Ectopic expression outside of the normal enamel knot expression domain leads to an increased number of fine cusps. (Top panel) Cell signalling. Eda ligand (red dots). Edar receptor (purple forks). Blue arrows indicate signal transduction. (Middle panel) Epithelial thickening at E11. The green area represents the possible effect on the developing molar field. (Bottom panel) Resulting morphology and number of molar teeth.
enamel defects than molars in the ameloblast cells. The incisors are also more susceptible to comparison to molars which show patchy and disorganized this and show complete loss of the ameloblast layer, in disrupted this balance resulting in loss of these cells. It is expressed in the inner enamel epithelium at E18 as the pre-amaeloblast layer is forming (Tucker et al., 2000) and so may have a role in the differentiation of these cells. It is possible that in a similar way to molar tooth number, a balance of Edar signalling is required for correct development of this layer and that by expressing Edar at high levels we have disrupted this balance resulting in loss of these cells. It is interesting to note that the incisors seem more susceptible to this and show complete loss of the ameloblast layer, in comparison to molars which show patchy and disorganized ameloblast cells. The incisors are also more susceptible to enamel defects than molars in the tabby mutant (Sofaer, 1969a). The molar enamel phenotype in particular appears to be stronger in our Edar transgenics compared to that reported after transgenic overexpression of Eda. A more severe consequence of active Edar expression compared to Eda overexpression is also shown by the fact that the Eda transgenics survive well while many of the Edar transgenics died prematurely or had to be sacrificed after signs of weakness, the cause of which is currently unknown.

In conclusion, overexpression of activated Edar in epithelial cells gives the reciprocal phenotype to loss of Edar signalling, with respect to tooth cusp formation, strengthening the role that this molecule plays in determining tooth shape and suggesting that this pathway may play a fundamental role in evolutionary changes in cusp pattern. At the same time, overexpression of activated Edar gives both the formation of additional premolar-like teeth and loss of the third molar, suggesting that the balance of Edar signalling is crucial and may regulate evolutionary changes involving tooth number and differentiation. It is interesting that excess Eda ligand affects tooth number, while overactive Edar can affect tooth number and cusp number. This suggests that ligand concentration is rate limiting for tooth number but not cusp number. Tooth number appears to be determined before the formation of epithelial thickening by the size and position of the tooth field, while tooth cusp number and shape are determined later from the late bud/cap stage of tooth development. These two processes are both under the control of Eda/Edar signalling, but the dynamics of the signalling relationship at different times appears to be controlled by different modulating mechanisms. Less Edar signalling thus gives few teeth with reduced, low cusps, while high Edar signalling appears to shift the molar field distally giving ectopic distal teeth and loss of the most proximal teeth, along with many, fine cusps in the first and second molar. In this way, tooth number and tooth shape are intrinsically linked to the level of Edar signalling.

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