RESEARCH ARTICLE

Suppression of epithelial differentiation by Foxi3 is essential for molar crown patterning

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ABSTRACT

Epithelial morphogenesis generates the shape of the tooth crown. This is driven by patterned differentiation of cells into enamel knots, root-forming cervical loops and enamel-forming ameloblasts. Enamel knots are signaling centers that define the positions of cusp tips in a tooth by instructing the adjacent epithelium to fold and proliferate. Here, we show that the forkhead-box transcription factor Foxi3 inhibits formation of enamel knots and cervical loops and thus the differentiation of dental epithelium in mice. Conditional deletion of Foxi3 (Foxi3 cKO) led to fusion of molars with abnormally patterned shallow cusps. Foxi3 was expressed in the epithelium, and its expression was reduced in the enamel knots and cervical loops and in ameloblasts. Bmp4, a known inducer of enamel knots and dental epithelial differentiation, downregulated Foxi3 in wild-type teeth. Using genome-wide gene expression profiling, we showed that in Foxi3 cKO there was an early upregulation of differentiation markers, such as p21, Fgf15 and Sfrp5. Different signaling pathway components that are normally restricted to the enamel knots were expanded in the epithelium, and Sostdc1, a marker of the intercuspal epithelium, was missing. These findings indicated that the activator-inhibitor balance regulating cusp patterning was disrupted in Foxi3 cKO. In addition, early molar bud morphogenesis and, in particular, formation of the suprabasal epithelial cell layer were impaired. We identified keratin 10 as a marker of suprabasal epithelial cells in teeth. Our results suggest that Foxi3 maintains dental epithelial cells in an undifferentiated state and thereby regulates multiple stages of tooth morphogenesis.

KEY WORDS: Foxi3, Multituberculata, Epithelium, Morphogenesis, Signaling center, Tooth development

INTRODUCTION

Tooth development begins with the formation of an epithelial thickening or placode. Tooth placodes consist of a basal cell layer facing the mesenchyme and several layers of suprabasal cells covered by periderm. The basal and suprabasal cells differ in their gene expression patterns (Palacios et al., 1995; Mitsiadi et al., 1995, 2010; Fausser et al., 1998; Nieminen et al., 1998; Ohazama and Sharpe, 2007). The mechanism of suprabasal cell formation has not been studied extensively in tooth development. After induction, placodal cells proliferate and form a bud that grows to the underlying mesenchyme. The connection of the bud to the oral epithelium constricts and forms the dental cord, where only a thin layer of suprabasal cells remains between the basal cells (Jussila and Thesleff, 2012). At this time, the suprabasal cells become loosely organized in the center of the bud. These cells, called stellate reticulum cells, resemble mesenchymal cells morphologically, while still maintaining an epithelial gene expression profile.

From the cap stage onwards, the basal epithelium is divided into inner enamel epithelium (IEE), which faces the dental papilla mesenchyme, and the outer enamel epithelium (OEE), which faces the dental follicle mesenchyme. A signaling center called the primary enamel knot (PEK) forms within the IEE and can be identified histologically as a condensed group of epithelial cells. It expresses several signaling molecules and is non-proliferative, as demonstrated by the expression of genes such as the cyclin-dependent kinase inhibitor p21 (Cdkn1a) and absence of 5′-bromo-2′-deoxyuridine (BrdU) incorporation (Jernvall et al., 1998; Keränen et al., 1998). The signals secreted by the PEK stimulate, either directly or via mesenchyme, the flanking basal epithelium to grow and form the cervical loops, which eventually form the roots. During subsequent morphogenesis of multicuspid molars, secondary enamel knots (SEKs) are induced at the sites of the future cusp tips. Like the PEK, the SEKs secrete signaling molecules that instruct the surrounding intercuspal IEE to proliferate. These IEE cells will eventually differentiate into ameloblasts. Mathematical modeling has shown that the pattern of the SEKs results from a balance of activating and inhibiting signals coupled with tissue growth (Salazar-Ciudad and Jernvall, 2002, 2010). Consequently, the local patterns of proliferation, instructed by the signaling centers, generate the shape of the tooth crown. The initiation of the second and third molars differs from the first molar in that they do not arise from a placode, but from a posterior extension of the epithelium associated with the previously formed tooth (Juuri et al., 2013). However, after initiation, their morphogenesis proceeds in a similar manner to the first molar.

A heterozygous mutation in the Foxi3 transcription factor was identified in hairless dogs, which have missing and abnormally shaped teeth (Drögelmüller et al., 2008). Foxi3 belongs to the superfamily of Forkhead box transcription factors that act as transcriptional activators, repressors, pioneer factors or chromatin modifiers (Benayoun et al., 2011; Lam et al., 2013).
expression is restricted to the epithelium during the development of several ectodermal appendages, including teeth (Shirokova et al., 2013). Foxi3 is expressed in the dental epithelium at all stages of development, but is downregulated in the differentiated ameloblasts (Shirokova et al., 2013). Foxi3 knockout (KO) mice die at birth as a result of craniofacial malformations, which arise from the lack of branchial arch-derived structures (Edlund et al., 2014).

Here, we show that conditional epithelial deletion of Foxi3 led to fusion of molars with abnormally patterned shallow cusps. Genome-wide expression profiling revealed upregulation of genes related to the differentiation of PEK and SEK and the cervical loop in Foxi3 mutants. During normal development, Foxi3 is expressed at high levels in IEE except for PEK and SEK signaling centers, tips of cervical loops and ameloblasts. Bmp4, a known inducer of PEK and SEK formation and ameloblast differentiation, inhibited Foxi3 expression. In situ analysis showed that markers normally restricted to SEKs were extended, which probably reflected the disrupted balance of activators and inhibitors regulating patterning and cusp formation. In addition, an earlier defect in the suprabasal epithelial cell compartment and budding morphogenesis was evident. Our results suggest that Foxi3 maintains epithelial cells in an undifferentiated state and thereby regulates multiple stages of tooth morphogenesis.

RESULTS

Deletion of Foxi3 led to fusion of molars with an altered cusp pattern

The Foxi3−/− phenotype is embryonic lethal, with severely affected development of branchial arch derivatives (Edlund et al., 2014), precluding analysis of all mandibular teeth and maxillary molars. We detected structures resembling upper incisors in Foxi3−/− embryos in an outbred ICR background, but when the mice were maintained in an inbred C57Bl/6 background, no signs of tooth development were observed (data not shown).

Conditional Foxi3 mutant mice (K14cre43;Foxi3−/floxed, hereafter Foxi3 cKO) in a mixed ICR and NMRI background were viable and fertile, but often smaller than their littermates (Table S2). The adults had erupted molars, but their morphology and cusp pattern were dramatically altered. Furthermore, the first (M1) and second (M2) molars of Foxi3 cKO mice were fused, and the third molar (M3) was sometimes missing or fused with anterior molars (Fig. 1A-C). Micro-computed tomography (micro-CT) scans on 5-week-old control and Foxi3 cKO lower jaws (Fig. 1D-L; Fig. S1) revealed small cusps in two parallel rows. The transverse ridges connecting cusps in the wild-type teeth were missing from mutants. Shallow cusps were easily visible in a lingual view (Fig. 1G-I). From an anterior view, it was obvious that the buccal side of the Foxi3 cKO molars was more affected (Fig. 1J-L). Mesenchymal condensation and ameloblast differentiation was evident. Our results showed that markers normally restricted to SEKs were extended, which probably reflected the disrupted balance of activators and inhibitors regulating patterning and cusp formation. In addition, an earlier defect in the suprabasal epithelial cell compartment and budding morphogenesis was evident. Our results suggest that Foxi3 maintains epithelial cells in an undifferentiated state and thereby regulates multiple stages of tooth morphogenesis.

Epithelial morphogenesis was impaired in Foxi3 cKO mice

To determine the stage of molar morphogenesis when the Foxi3 cKO tooth phenotype arises, we analyzed the histology of control and mutant molars from the M1 initiation onwards, as Foxi3 is already expressed in the epithelium in the molar placode and bud (Shirokova et al., 2013). At embryonic day (E) 12.5, the Foxi3 cKO placode was smaller compared with controls, and the suprabasal epithelium appeared especially poorly developed (Fig. 2A,B). At E13.0 and E13.5, budding morphogenesis was impaired in Foxi3 cKO (Fig. 2C-F). Both the dental cord and the stellate reticulum were missing from Foxi3 cKO molars (Fig. 2E-J). Cervical loops formed in Foxi3 cKO, but they were smaller in size than controls (Fig. 2G,H). At E16, the IEE did not show normal folding, indicating that the shape of the crown was not forming correctly (Fig. 2I-L). At E18, some embryos showed normal M3 bud formation despite the fusion of M1 and M2 (Fig. 2M,N). Mesenchymal condensation and ameloblast differentiation appeared normal in Foxi3 cKO embryos (Fig. 2). In postnatal day (P) 8 molar crown, there was secreted enamel and dentin, and root formation had been initiated normally (Fig. 2O,P). We analyzed the efficiency of the Cre-driver used and noticed mosaic Foxi3 expression in the Foxi3 cKO molars at E12.5 (Fig. 2A-C). At later stages (E13.5 and E14.5), qRT-PCR and in situ hybridization confirmed the downregulation of Foxi3 expression (Fig. 5A; Fig. S2D,E). In conclusion, loss of Foxi3 affects several epithelial cell populations in the developing molar and impairs tooth morphogenesis.

Foxi3 expression was downregulated upon differentiation of dental epithelium and is regulated by several signaling pathways

Next we examined the localization of Foxi3 protein (Fig. 3A-C). In a similar manner to Foxi3 mRNA expression (Shirokova et al., 2013), at E13.5 Foxi3 was strongly expressed on the lingual side of the forming tooth, which grows faster when the epithelium proceeds...
The induction of PEK are known to be regulated by epithelial-ameloblasts was reported previously (Shirokova et al., 2013). From bud to cap, at E14.5, double staining with Lef1 revealed that although Foxi3 was present in most epithelial cells, it appeared largely absent from the Lef1-positive PEK (Fig. 3B). At E16, Foxi3 was present in the IEE and in the stellate reticulum, but only weakly expressed in the Lef1-positive SEKs (Fig. 3C). In addition, the tips of the growing cervical loops were negative for Foxi3 staining (Fig. 3C). These observations indicate that Foxi3 is excluded from the epithelial cells that have started to differentiate and acquire new identities, such as enamel knots and the cervical loops. Downregulation of Foxi3 in IEE before their differentiation to ameloblasts was reported previously (Shirokova et al., 2013).

The morphogenesis of the tooth germ from bud to cap stage and the induction of PEK are known to be regulated by epithelial-mesenchymal interactions. Therefore, we decided to explore which signaling molecules might regulate Foxi3 expression at this time by using a hanging drop culture system, in which E13.5 molars are exposed to a growth factor for 4 h before RNA collection for qRT-PCR. Activin A was previously shown to upregulate Foxi3 in embryonic skin, and it stimulated Foxi3 in tooth epithelium by 2.7-fold (Fig. 3D). Shh induced a 1.7-fold upregulation of Foxi3 (Fig. 3E). Bmp4, a known inducer of PEK (Jernvall et al., 1998), downregulated Foxi3 expression levels to half that of the control samples (Fig. 3F). Fgfl8 had no effect on Foxi3 expression, yet it induced its own target Dusp6 by over fivefold (data not shown). These results suggest that Foxi3 is a target of Activin A, Shh and Bmp4. We have previously shown that ectodysplasin (Eda) also enhances Foxi3 expression at this stage in the tooth (Shirokova et al., 2013). However, after the bud stage Foxi3 expression is significantly broader than Eda receptor expression (Laurikkala et al., 2001), indicating that Foxi3 expression is regulated largely by other signals. All these pathways are known to target the dental epithelium (Jussila and Thesleff, 2012) and might therefore regulate Foxi3 directly.

Suprabasal cell population was reduced in the Foxi3 cKO molar

In order to study more closely the defect in the early morphogenesis of Foxi3 mutant molars, we used E- and P-cadherin (cadherins 1 and 3, respectively) as markers of the suprabasal and basal epithelial cell populations (Palacios et al., 1995; Fausser et al., 1998). We measured the relative areas of the strongly E-cadherin-positive and -negative populations (Palacios et al., 1995; Fausser et al., 1998). We found that suprabasal cell population was reduced in the Foxi3 cKO molar. In control molars, the suprabasal epithelium represented 35% of the total epithelial area, and in the Foxi3 cKO only 28% (Fig. 4C; P = 0.006). Overall, the area of the Foxi3 cKO epithelium was smaller than in control embryos (Fig. S3). These observations raise the possibility that the defect in suprabasal cell layer and budding morphogenesis might be connected.

A putative defect in suprabasal cells was supported by our microarray analysis of E13.5 Foxi3 cKO and wild-type molar epithelia (array results reported in detail below), which showed that Notch2, Krt10 and Krt1 were downregulated in the Foxi3 cKO molar. Notch2, together with Notch1 and 3, is expressed in the
We analyzed expression of K10, and observed few weakly K10-positive cells in control and Foxi3 cKO molars at E13.0 (data not shown). Twelve hours later, there were K10-positive cells in the suprabasal epithelium of control molars, and in addition, some basal cells expressed weaker K10 signal (Fig. 4I, Fig. S4A,B). In Foxi3 cKO molars, only few weakly K10-positive cells were scattered among the suprabasal cells (Fig. 4J, Fig. S4C). At E14.5, both K10-positive and K10-negative cells were present in the control molars, whereas in the Foxi3 cKO the K10-positive cells were largely absent (Fig. 4K,L). At E16, the lack of K10-positive cells in the suprabasal cell compartment (i.e. stellate reticulum) of the Foxi3 cKO tooth was striking compared with the control tooth (Fig. 4M,N). These results indicate that the K10-positive suprabasal cell population in the molars is decreased in the Foxi3 cKOs, consistent with the reduction of the stellate reticulum cell compartment. K10-positive cells associated with the stratification of oral epithelium were present in both control and Foxi3 cKO (Fig. 4N; data not shown).

Microarray analysis revealed alterations in multiple signaling pathway components in the Foxi3 cKO molar
In order to identify putative Foxi3 downstream targets, we performed genome-wide profiling of differentially expressed genes in Foxi3 cKO and control molar epithelia at E13.5, the earliest stage displaying efficient Foxi3 deletion. Of the differentially expressed genes, 292 were downregulated and 431 upregulated (Table S3), but only 30 of the downregulated genes showed more than a 0.6-fold reduction of expression and only 54 upregulated genes showed more than a 1.2-fold difference. To validate the microarray results, we confirmed Foxi3 downregulation and assessed 16 other genes by qRT-PCR. With the exception of one gene, all showed a statistically significant change with qRT-PCR (Fig. 4D; Fig. 5).

The Bmp4 target gene Msx2, as well as three Iroquois-family transcription factors (Irx2, Irx3 and Irx5) that are expressed in the molar epithelium (Houweling et al., 2001), were among the downregulated genes (Fig. 5A). Interestingly, a striking number of the genes upregulated in the Foxi3 cKO in the microarray belonged to different signaling pathways, including Fgf, Shh, Wnt and Bmp. Fgf15 and the Wnt inhibitor Sfrp5 were among the highest upregulated genes in the microarray, and qRT-PCR showed about 15- and 13-fold upregulation of Fgf15 and Sfrp5 transcripts, respectively (Fig. 5B). In addition, we validated upregulation of the Fgft pathway target genes Dusp6 and Etv5 and the Shh pathway genes Shh and Ptc1, whereas the difference in Gli1 expression was not significant (Fig. 5C). Semaphorin 3E (Sema3e) was also upregulated in Foxi3 cKO (Fig. 5C).

Subsequently, we localized some of the differentially expressed genes by RISH in sections of E13.5 control and Foxi3 cKO molars. In the control samples, Fgf and Bmp pathway genes were confined to the tip of the bud epithelium, but in Foxi3 cKO the expression of the Fgft pathway target Dusp6 and Fgf antagonist sprouty 2 (Spry2) spanned the whole depth of the dental epithelium (Fig. 6A-D). Bmp7, as well as the Bmp target Id1, showed a similar broad expression in Foxi3 cKO (Fig. 6E-H). By contrast, Shh was expressed in a patchy pattern compared with the focal signaling center present in control teeth (Fig. 6I,J), and Wnt10a was more restricted to the basal portion of the mutant molar epithelium (Fig. 6K,L). Msx2 showed a similar but weaker expression in the buccal epithelium in the Foxi3 mutants compared with controls (Fig. 6M,N). Sema3e expression has not been reported for developing molars, and unlike the control teeth, Sema3e was expressed ectopically in the Foxi3 cKO epithelium (Fig. 6O,P). In conclusion, our microarray, qRT-PCR
and RISH data suggest that the defect in epithelial morphogenesis of the Foxi3 cKO tooth was associated with incorrect localization and activity of signaling pathways, and in particular, the Fgf, Shh and Bmp pathways were stimulated.

**Foxi3 cKO dental epithelium displayed molecular signs of precocious differentiation**

The PEK and the SEKs are marked by expression of the cyclin-dependent kinase inhibitor p21 (Jernvall et al., 1998). p21 was upregulated in our microarrays, and RISH confirmed a strong expression at E13.5 in the tip of Foxi3 cKO epithelium (Fig. 7A,B). At E14.5, there was p21 expression in the PEK and in the dental cord in control molars, but in the Foxi3 cKO p21 expression spanned the whole epithelium (Fig. 7C,D). This prompted us to investigate other characteristics of the Foxi3 cKO PEK. Analysis of BrdU-negative cells at E14.0 revealed that, in contrast to weight-matched littermates, there was already a clear BrdU-negative area in the tip of the epithelial bud of the Foxi3 cKO molar, indicating precocious PEK formation (Fig. S5A,B). At E14.5, Lef1 and P-cadherin immunostaining revealed that the structure of the PEK was abnormal in Foxi3 cKO (Fig. S5C-F). However, PEK marker genes Shh and Dkk4 were expressed in a similar manner in control and Foxi3 cKO PEK, whereas Wnt10a seemed to be expressed more widely in Foxi3 cKO (Fig. S5G-L).

At E16, p21 was expressed in the SEKs of the control teeth, whereas in the Foxi3 cKO p21 spanned the whole IEE (Fig. 7E,F). The Wnt and Bmp inhibitor Sostdc1 has been linked to PEK formation and it is expressed in a mirror image with p21 (Laurikkala et al., 2003; Kassai et al., 2005). Sostdc1 was downregulated in our microarray, and RISH revealed a reduction of Sostdc1-expressing epithelium in the Foxi3 cKO at E13.5 and E14.5, whereas its mesenchymal expression seemed unaffected (Fig. 7G-J). At E16, Sostdc1 was expressed in the stellate reticulum and in the IEE between the SEKs in the control tooth, but in Foxi3 cKO there was no expression in the IEE (Fig. 7K,L). p21 and Sostdc1 showed mirror-image expression patterns in both the control and Foxi3 cKO teeth.
epithelium at all stages studied. This suggests that in the Foxi3 cKO the intercuspal Sostdc1-positive IEE had undergone a change in cell fate into SEK.

In the microarray, Fgf15 and Sfrp5 were significantly upregulated in Foxi3 cKO at E13.5, but we could not detect their expression with RISH at this stage. At E14, however, Fgf15 was weakly expressed in control molars and upregulated in the Foxi3 cKO epithelium (Fig. 7M,N). At E15, Fgf15 expression was present in the SEKs of the control tooth, whereas in the Foxi3 cKO there was ectopic Fgf15 expression in the cervical loops (Fig. 7O,P). Sfrp5 was expressed in both the control and Foxi3 cKO molars at E14.5 around the PEK and in the cervical loops (Fig. 7Q,R). At E15 in the control molar, there was expression only in the cervical loops, but in the Foxi3 cKO there was strong expression spanning from the cervical loops towards the enamel knots (Fig. 7S,T). During normal development, Foxi3 is downregulated in the cervical loops at E16 (Fig. 3C). Taken together, upregulation of differentiation markers, such as p21, Fgf15 and Sfrp5, points to a precocious differentiation of the Foxi3 cKO epithelium.

In order to investigate whether the precocious epithelial differentiation had an effect on ameloblasts, we localized expression of the enamel protein ameloblastin. At E18 in control lower molars, ameloblastin expression had started at the cusp tips and spread cervically in some sections, whereas in Foxi3 cKO lower molars the expression covered a wider area and appeared more intense (Fig. S6A-D). In upper molars, there was no ameloblastin expression in controls, but in Foxi3 cKO the expression was evident (Fig. S6E,F). These observations suggested an earlier onset of ameloblast differentiation in the Foxi3 mutants. However, owing to the restricted number of samples the results should be considered preliminary.

Crown patterning signals were misexpressed in the Foxi3 cKO molar
The cusp phenotype of the adult Foxi3 cKO molars and the expression pattern of the SEK marker p21 at E16 (see above) indicated a dramatic defect in SEK patterning. Also, the expression of other SEK markers, such as Lef1, Dkk4 and Wnt10a, spanned the entire IEE in Foxi3 cKO molars as evidenced by analysis in both sagittal (Fig. 8A-D) and frontal (Fig. 8E-H) sections. However, Fgf4 was expressed focally in the SEKs in both the control and in Foxi3 cKO molars (Fig. 8I,J). Thus, most but not all SEK markers analyzed were ectopically expressed in the Foxi3 cKO IEE.

DISCUSSION
We have shown that Foxi3 affects all major stages of tooth morphogenesis and has several different functions during tooth development (Fig. 9). Early on, it controls the formation of K10-positive suprabasal cells. Later on, Foxi3 acts as an inhibitor of differentiation that prevents the epithelium from precociously acquiring the fates of cervical loops and enamel knots and probably also of ameloblasts. This kind of function has not been suggested previously for any transcription factor expressed in the dental epithelium (Bei, 2009).

Foxi3 regulates formation of suprabasal cells
The earliest manifestation of the Foxi3 cKO molar phenotype was an abnormally shaped placode with a reduced suprabasal compartment. We saw a specific downregulation of Notch2 but not Notch1, which suggests that in addition to being fewer in number, the suprabasal cells are abnormally specified. Little is known about the cellular mechanism driving tooth placode formation.
formation and the origin of the suprabasal cells. In the embryonic skin, asymmetric cell divisions of basal cells give rise to suprabasal cells, which express differentiation markers, such as K1, while remaining proliferative (Lechler and Fuchs, 2005), as do dental suprabasal cells. We observed K10 expression in some basal cells, suggesting that also in teeth, the K10-positive cells might originate from the basal layer. The lack of stellate reticulum and the downregulation of K1 and K10 in Foxi3 cKO would indicate a failure in this process. However, K10 expression was detectable in developing teeth only after E13.0, whereas the reduction in suprabasal cell population was already noticeable at the placode stage, suggesting an alternative or additional explanation. Hair placodes arise from cell migration and intercalation (Ahtiainen et al., 2014), and it is possible that a similar mechanism exists in teeth and is impaired in the Foxi3 cKO. This could lead to a failure in the specification of the initial suprabasal cells. Further studies will be required to uncover the causative mechanism, but unfortunately, the inefficient deletion of Foxi3 prior to E13.5 hampers these efforts.

**Foxi3 regulates dental cord formation and prevents molar fusion**

The cellular mechanisms that drive the narrowing of the neck of the tooth bud, the dental cord, are not known. The defect in Foxi3 cKO bud formation could be a result of altered adhesive properties of the epithelium. In support of this hypothesis, several adhesion molecules, such as cadherins and claudins, were differentially expressed in the microarray. The branchial arch phenotype of the Foxi3 KO has been speculated to result from a defect in cell adhesion (Edlund et al., 2014). In addition to Foxi3 cKO, the dental cord does not form in the molars of the conditional epithelial Shh KO (Dassule et al., 2000). In the Shh cKO, also the M1 and M2 fuse and the molars have shallow cusps with an abnormal pattern (Dassule et al., 2000). Deletion of other Shh pathway components, Smo or Evc, leads to milder molar fusion phenotypes (Grillì-Linde et al., 2002; Nakatomi et al., 2013). Our hanging drop induction experiment suggests that Foxi3 might be a downstream target of Shh, and therefore Foxi3 deletion could lead to a similar phenotype to the mutations in the Shh pathway components. Sostdc1 was downregulated in the Foxi3 cKO, and M1 and M2 are fused in the Sostdc1 knockouts (Kassai et al., 2005). However, their cusp phenotype is different from Foxi3 cKO and their early molar morphogenesis appears normal. The molar fusion in Sostdc1 KO was suggested to be caused by ectopic p21 expression, which we observed also in Foxi3 cKO. In general, the mechanism of molar fusion is poorly understood. The buds of M2 and M3 form sequentially from the posterior end of the previous molar, and the separation of molars requires epithelial downgrowth in a similar manner to the growth of cusps in the tooth crown. We suggest that the molar fusion in Foxi3 cKO is related to the failure in the M1 bud formation and to the impaired epithelial downgrowth, which is necessary for proper separation of M2 from M1. Interestingly, Li et al. (2015) showed recently that the downgrowth of cervical loop epithelium in the molar depends on Shh signaling to regulate the maintenance of Sox2-expressing transient stem cells in the loops. It is possible that Foxi3 regulates cervical loop downgrowth as part of this signaling network.

**Foxi3 might function as an epigenetic regulator**

Our microarray revealed a large number of up- and downregulated genes. They are likely to represent both direct and indirect targets of Foxi3, the latter reflecting a deficiency in the suprabasal cell population and precocious enamel knot differentiation. We validated by qRT-PCR downregulation of Irx2, Irx3 and Irx5 in Foxi3 cKO, and in addition, Irx1 was downregulated in the microarray. All four transcription factors have an overlapping expression pattern with Foxi3 (Houweling et al., 2001), making them good candidates for Foxi3 targets, but their function in tooth formation is not known. The highest upregulated gene in the microarray was the enamel knot marker Fgf15 (Kettunen et al., 2002).
2011; Porntaveetus et al., 2011). Fgf15+/− mice have no phenotype (Porntaveetus et al., 2011), but it might act redundantly with other Fgfs. Interestingly, it has been shown that Foxi3 binds to a specific oxidized form of methylated cytosines on the Fgf15 promoter, and this binding is linked to repression of transcription (Iurlaro et al., 2013). This suggests that Foxi3 could normally repress genes such as Fgf15, and perhaps also other microarray hits, such as Sema3e and the Wnt inhibitor Sfrp5, which were also among the most highly upregulated genes. The winged helix domains of Fox factors have structural similarity to linker histones, and some of them have been shown to bind and modulate chromatin in order to induce changes in gene expression (Benayoun et al., 2011). If epigenetic regulation is the main function of Foxi3 in dental epithelium, it is possible that a large amount of microarray targets exhibit this type of regulation. This might also explain why we did not see many genes being strongly up- or downregulated.

**Foxi3 is an inhibitor of epithelial differentiation**

The microarray revealed a general upregulation of components of different signaling pathways, and in particular, PEK markers p21 and Fgf13 in the Foxi3 cKO tooth bud epithelium (Fig. 9). In addition, Sfrp5, which marks the cervical loops initiating root formation at late bell stage (E. Juuri and I.T., unpublished results), was induced precociously at the bud stage in Foxi3 (E. Juuri and I.T., unpublished results), function as an inhibitor of dental epithelial differentiation. Foxi3 in the PEK and ameloblasts, suggests that Foxi3 might be a prerequisite for their induction. The BrdU incorporation assay indicated that PEK induction was accelerated in Foxi3 mutants. Our preliminary data suggested that ameloblasts differentiated slightly downregulated in the cervical loop epithelium. Together, these observations suggest that Foxi3 inhibits the dental epithelium from precocious differentiation towards PEK and cervical loop fates. Additionally, downregulation of Foxi3 in PEK and SEK might be a prerequisite for their induction. The BrdU incorporation assay indicated that PEK induction was accelerated in Foxi3 mutants. Our preliminary data suggested that ameloblasts differentiated slightly earlier in Foxi3 cKO compared with their littermates, supporting a role for Foxi3 as an inhibitor of precocious differentiation. We showed that exposure of bud stage teeth to Bmp4 downregulated Foxi3 expression. Interestingly, Bmp4 has been shown to induce PEK formation (Jernvall et al., 1998) and ameloblast differentiation (Wang et al., 2004) and this, together with the downregulation of Foxi3 in the PEK and ameloblasts, suggests that Foxi3 might function as an inhibitor of dental epithelial differentiation.

**Balance of activating and inhibiting signals that regulate crown patterning is perturbed in Foxi3 cKO**

At a later stage, during SEK formation, most IEE took the SEK fate in Foxi3 cKO mutants. This was manifested by the ectopic expression of a number of SEK markers and the absence of Sostdc1 from the intercuspal epithelium, supporting the idea of Foxi3 as an inhibitor of SEKs in addition to PEK. During crown patterning, the cells respond to two types of signals, to activators inducing SEK formation and to inhibitors. The inhibitors repress differentiation and induce proliferation and they prevent new signaling centers from forming in close proximity to the existing centers (Salazar-Ciudad and Jernvall, 2010). The spreading of SEK markers in Foxi3 cKO suggests that there is a decreased level of inhibition in the mutant tooth. Thus, Foxi3 might either suppress the responsiveness of the intercuspal epithelium to activators (such as Bmp4) or promote their sensitivity to inhibitors (such as Shh). As Bmp4 was able to inhibit Foxi3 expression in bud stage teeth, it could likewise also inhibit Foxi3 in the SEKs, allowing differentiation of the signaling centers. At the same time, in intercuspal regions Foxi3 could prevent Bmp4 responses, such as induction of p21 expression (Jernvall et al., 1998) and ameloblast differentiation (Wang et al., 2004). In addition to Bmp4, we identified several other signals that affected Foxi3 expression, namely Shh, Activin and Eda. They have all been linked to modulation of crown shape complexity (Harjunmaa et al., 2012); therefore, changes in Foxi3 levels during evolution could be one way to modify the cusp pattern in different species. Interestingly, the cusp pattern of Foxi3 cKO resembles the multituberculates, an extinct clade of mammals, which had molars with longitudinal cusps (Wilson et al., 2012).

In conclusion, based on the expression pattern of Foxi3 and the phenotype of the Foxi3 cKO, we propose that during tooth morphogenesis Foxi3 has a unique role as a transcription factor that keeps the dental epithelium in an undifferentiated state. Foxi3 might execute its function partly through epigenetic regulation, and it regulates formation of the suprabasal cells and inhibits the differentiation of cervical loops and enamel knots.

**MATERIALS AND METHODS**

**Animals**

Wild-type NMRI mice (The Jackson Laboratory) were used in hanging drop cultures. K14-cre+/−;Foxi3−/− males (Andl et al., 2004; Edlund et al., 2014) were crossed with Foxi3−/− females (Edlund et al., 2014; Foxi3tm1.1Akg/J; The Jackson Laboratory stock 024843) to obtain K14-cre+/−;Foxi3−/− mice (Foxi3 cKO). For analyzing the adult tooth phenotype, cleaned skulls were used in micro-CT scan. For collecting embryonic samples, the day of the vaginal plug was counted as E0.5, and the embryos were staged further according to limb and molar morphology. Foxi3−/− and Foxi3−/− animals were used as controls except in microarray experiments where all controls were Foxi3−/−. All mouse work has been carried out in accordance with the guidelines and approval from Finnish National Board of Animal Experimentation.

**Histology, in situ hybridization, immunohistochemistry and immunofluorescence**

For histology, radioactive in situ hybridization (RISH) and immunohistochemistry, tissues were fixed overnight in 4% paraformaldehyde, dehydrated and embedded in paraffin. Sections 5–7 μm thick were cut in frontal and sagittal planes. Antigen retrieval for immunohistochemistry and immunofluorescence was performed by heating the slides in 10 mM sodium citrate buffer (pH 6.0). Each gene or protein was analyzed in two or three individuals.

RISH was carried out according to a standard protocol. The following [35S]-UTP (Perkin Elmer)-labeled RNA probes were used to detect gene expression: Bmp7 (Åberg et al., 1997), Dkk1 (Flinniaux et al., 2008), Dusp6 (James et al., 2006), Fgf4 (Jernvall et al., 1994), Fgf15 (Kettunen et al., 2011), Foxi3 (Ohyama and Groves, 2004), Id1 (Rice et al., 2000), Lef1 (Travis et al., 1991), Mxi2 (Jowell et al., 1993), p21 (Jernvall et al., 1998), Sfrp5 (Witte et al., 2009), Shh (Vahtokari et al., 1996a), Sostdc1 (Laurikkala et al., 2003), Sprouty2 (Zhang et al., 2001) and Wnt10a (Dassule and McMahon, 1998). Rat ameloblastin probe was a kind gift from Jan C. Hu (University of Texas School of Dentistry, TX, USA). A 645 bp fragment of the mouse Sema3e gene was amplified using the following primers: forward: ACATTGATGCACCTGCTG; and reverse: AGGCAATCAGCTGCAAGAAT. The PCR fragment was cloned into pCRII-pTOPO vector (Invitrogen by LifeTechnologies) and sequenced to confirm fragment identity.

To detect proliferating cells, BrdU (Amersham) was injected to pregnant females at 10 μg/kg, and embryos were collected after 2 h. BrdU was detected with anti-BrdU antibody at 1:1000 (mouse; Thermo Fisher Scientific), the M.O.M. immunodetection kit (Vector Laboratories) and DAB staining (DAB Peroxidase Substrate Kit; Vector Laboratories).

For immunofluorescence, primary antibodies were used at the following dilutions: Foxi3 1:150 (goat; Santa Cruz Biotechnology), E-cadherin 1:1000 (mouse; BD Biosciences), P-cadherin 1:100 (goat; R&D Systems),
Litters. Briefly, molar tooth germs were dissected under a microscope in (Roche Applied Science) with the Lightcycler 480 (Roche Applied Science) and frozen in dry ice before storage at 80°C.

All samples were imaged with a Zeiss Axio Imager M2 microscope equipped with an Axio Cam HR camera (Zeiss) and processed in Photoshop. Frontal images are oriented so that the lingual side is on the left.

Quantification of the epithelial areas was done on E-cadherin- and P-cadherin-stained sections, and ten sections at the widest part of the molar were chosen from three control and three Foxi3 cKO individuals. The whole area of the epithelium, in addition to the basal (strong P-cadherin) and suprabasal areas (strong E-cadherin), was outlined manually using the Zen2011 program (Zeiss). Student’s t-test was used for testing statistical significance, and a P-value of 0.05 was used as the significance threshold.

Hanging drop experiments
Foxi3 induction was studied by treating E13.5 wild-type NMRI molars for 4 h in hanging drop culture with control media (Dulbecco’s modified Eps 80°C or media containing the signaling molecule of interest at the following concentrations: Activin A 0.5 µg/ml (Harrington et al., 2006), Shh 5 µg/ml (R&D Systems), Bmp4 0.1 µg/ml (R&D Systems) and Fgf8 1 µg/ml (R&D Systems). A known signaling pathway target gene was used as a positive control. Left molars or right molars of two to three embryos were pooled together for control and growth factor-treated samples, respectively.

RNA extraction and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)
RNA from the hanging drop experiment samples or from the molar epithelia collected for the microarray validation was extracted using the RNeasy mini kit (Qiagen) and reverse transcribed using 500 ng of random hexamers (Promega) and Superscript II (Invitrogen by Life Technologies) following the manufacturer’s instructions.

qRT-PCR was performed using Lightcycler DNA Master SYBR Green I (Roche Applied Science) with the Lightcycler 480 (Roche Applied Science). Gene expression was quantified by comparing the sample data against a dilution series of PCR products of each gene of interest. Data were analyzed with the manufacturer’s software and normalized against Ranbp. Data are shown as the mean±s.d. Wilcoxon signed-rank test was used for the paired hanging drop experiment samples and the Mann–Whitney U-test for the non-paired microarray validation samples for testing statistical significance, and a P-value of 0.05 was used as the significance threshold. Primers are listed in Table S1.

Microarray experiments and data analysis
For identification of Foxi3 target genes, E13.5 molar epithelium was dissected from Foxi3 cKO and Foxi3+/- littermate embryos from six litters. Briefly, molar tooth germs were dissected under a microscope in Dulbecco’s PBS and transferred to 1:25 Dispase II in 10 mM NaCl, 7.5 mM (stock 5 U/ml, Sigma-Aldrich) and incubated at 4°C for 3–4 h. The separated epithelia of the two molars of each embryo were collected together and frozen in dry ice before storage at −80°C.

Molar epithelia from five embryos were pooled together, and the microarray consisted of four biological replicates of control and Foxi3 cKO samples. RNA extraction is described above. RNA quality was monitored using a 2100 Bioanalyzer (Agilent Technologies). RNA processing and hybridization on Affymetrix Mouse Exon 1.0 ST arrays (Affymetrix) and data analysis were carried out at the Biomedicum Functional Genomics Unit (University of Helsinki, Finland). The data were processed using R/Bioconductor and normalized with the RMA algorithm and CustomCDF-database probe annotations. Differentially expressed genes were searched using the Cyber-T algorithm. P-values were corrected using the Q-value method. Microarray data are available in Gene Expression Omnibus (accession number GSE65725).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
M.J., M.L.M. and I.T. designed the study. M.J., M.S.N., A.S., V.S. and A.K. performed the experiments. M.J. and I.T. analyzed the data. A.G. and T.O. generated the Foxi3 mutant mice. M.J., M.L.M. and I.T. prepared the manuscript. All authors commented on the manuscript.

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References


