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Review

Epiprofin/Sp6: A new player in the regulation of tooth development

Lucia Jimenez-Rojo[2], Gaskon Ibarretxe[1], Maitane Aurrekoetxea[1], Susana de Vega[3], Takashi Nakamura[3], Yoshihiko Yamada[3] and Fernando Unda[1]

[1]Department of Cell Biology and Histology, Faculty of Medicine and Dentistry, University of the Basque Country, Vizcaya, Spain, [2]Ecole Polytechnique Fédérale de Lausanne (EPFL), ISREC-Swiss Institute for Experimental Cancer Research, NCCR Molecular Oncology, Lausanne, Switzerland and [3]Laboratory of Cell and Developmental Biology, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA

Summary. Odontogenesis is governed by a complex network of intercellular signaling events between the dental epithelium and mesenchyme. This network leads to the progressive determination of tooth shape, and to the differentiation of these tissues into enamel-producing ameloblasts and dentin-producing odontoblasts respectively. Among the main signaling pathways involved in the regulation of tooth development, Bone Morphogenetic Protein (BMP), Sonic hedgehog (Shh) and Wingless-type MMTV integration site (Wnt) pathways have been reported to play significant roles. Recently, the phenotype of mice deficient in Epiprofin/Sp6 (Epfn) has been found to present striking dental abnormalities, including a complete lack of differentiated ameloblasts and consequently no enamel, highly altered molar cusp patterns and the formation of multiple supernumerary teeth. In this article, we review the interaction of Epfn with the BMP, Shh and Wnt pathways in the regulation of tooth development, based on the data obtained from the study of several genetically modified mice.

Key words: Odontogenesis, Supernumerary teeth, Ameloblast, Epiprofin/Sp6, Shh, Wnt, BMP

Normal tooth development

The tooth is an organ of ectodermal origin and its development is driven by serial and reciprocal inductive interactions between the dental epithelium and the underlying mesenchyme. Histologically, the first noticeable event of mouse odontogenesis occurs around embryonic day 11.5 (E11.5) with a local thickening of the oral epithelium at the sites where teeth will form. This epithelium, also known as dental lamina, is the source of the inductive signals that initiate tooth development and regulate the expression of different mesenchymal transcription factors. These molecules will reciprocally act upon epithelial cells which, in turn, form a signaling center called the dental placode. At E12, due to the signals secreted by the dental placode, the odontogenic potential shifts from epithelium to mesenchyme, which acquires the ability of tooth formation even when recombined with non-dental epithelium (Kollar and Baird, 1970; Mina and Kollar, 1987). Thus, the mesenchyme induces the proliferation of the dental epithelium giving rise to the epithelial bud. At E13, the tooth bud ceases growing vertically and a new signaling center called the primary enamel knot (PEK) starts to form at the tip of the epithelial bud (Vaahtokari et al., 1996). This non-proliferative structure induces the formation of the epithelial cervical loops that invaginate and give rise to a cap-shaped structure containing the dental papilla mesenchyme. At this stage, the epithelium is called the enamel organ, which is composed of the stellate reticulum, stratum intermedium, as well as the inner and outer enamel epithelia. The PEK is a transient structure and when it disappears due to apoptosis, new secondary enamel knots (SEK) are formed during the so-called bell stage around E16.5. The function of the SEKs is directly linked to the regulation of the shape and size of tooth cusps (Jernvall et al., 1994). At E17.5-E18.5, cells from the dental papilla (preodontoblasts) that are facing the inner enamel epithelium polarize and differentiate into dentin-secreting odontoblasts. It has been shown that similarly to what happens in earlier stages of tooth development,

Offprint requests to: Dr. Fernando Unda, Department of Cell Biology and Histology, Faculty of Medicine and Dentistry, University of the Basque Country, Vizcaya, Spain. e-mail: fernando.unda@ehu.es

preodontoblast differentiation requires signals from dental epithelium (Thesleff and Hurmerinta, 1981; Martin et al., 1998; Ruch, 1998). Thus, the presence of predentin or dentin-secreting odontoblasts is essential for the complete differentiation of the inner enamel epithelium into ameloblasts. Ultimately, these cells form the enamel matrix and eventually disappear after the tooth eruption process.

The epithelial-mesenchymal interactions that govern tooth development require a well-controled secretion of signaling molecules, including BMPs, FGFs, Shh and Wnts. However, transcription factors play an essential role in the regulation of the paracrine mechanisms that underlie tooth formation. Epiprofin/Sp6 (Epfn) is a zinc-finger transcription factor expressed not only in dental tissues, such as the inner enamel epithelium and odontoblasts, but also in other ectodermal appendages (Nakamura et al., 2004). Recently, the study of *Epfn*-deficient mice has demonstrated that in fact this transcription factor is essential during tooth morphogenesis and differentiation (Nakamura et al., 2008). Hereby, we review the role of Epfn throughout odontogenesis, as well as its relationship with some of the main signaling pathways, which regulate tooth development such as the BMP, FGF, Shh and Wnt/ß-catenin pathways.

Sp/KLF transcription factors in odontogenesis

Epiprofin (Epfn) is a transcription factor belonging to the Specificity protein (Sp) subfamily of the large Krüppel-like factor (KLF) family that was identified by differential hybridization using mRNA isolated from embryonic mouse molars (Nakamura et al., 2004). *Epfn* sequence showed high homology with the previously described Sp6 gene. Intringuily, *Sp6* was found to be ubiquitously expressed in all adult tissues examined (Scohy et al., 2000), whereas *Epfn* is expressed in a tissue-specific manner during mouse embryogenesis. It has been reported that Epfn and Sp6 are different transcripts perhaps generated by alternative promoters from the same gene, but they ultimately code for the same protein (Hertveldt et al., 2007).

Sp/KLF family members share a DNA-binding domain composed of three tandem C2H2-type zinc fingers at the C-terminus (Kadonaga et al., 1987). Until now, nine members (Sp1-Sp9) have been described within the Sp subgroup in mammals. Their function can be to either induce or repress the expression of their target genes (Philipsen and Suske, 1999).

Regarding tooth development, *Sp3, Sp4, Epiprofin/Sp6 (Epfn) and Osterix/Sp7 (Osx)* are expressed in dental tissues. It has been reported that the Sp3 protein is found in rat odontoblasts and ameloblasts, showing its highest levels at the secretory stage when ameloblasts are actively producing enamel-matrix proteins which include amelogenin and ameloblastin (Lv et al., 2006). In *Sp3*-knockout mice, ameloblasts fail to express these enamel-matrix proteins, and thus they present a disrupted dentin/enamel layer (Bouwman et al., 2000). *Sp4* is expressed by dental mesenchymal cells, but *Sp4* deficient mice show normal tooth development (Supp et al., 1996). Finally, *Osx* is expressed in dental mesenchyme from E15.5 onwards, and it seems that it has no essential role during odontogenesis since, similarly to Sp4-knockout mice, *Osx*-null mice do not present any tooth abnormalities (Nakashima et al., 2002).

During odontogenesis, *Epfn* expression is restricted to the inner enamel epithelium of developing molars and incisors from the bud to bell stages, when it is also expressed in differentiated odontoblasts (Nakamura et al., 2004). The most striking phenotype of *Epfn*-knockout mice is a severe hyperdontia, featuring supernumerary incisor and molar teeth (Nakamura et al., 2008). In addition, tooth roots are not well-developed, the pattern of molar cusps is dramatically altered and, occasionally, fused molars and incisors are found (Fig. 2B,H). A detailed analysis of *Epfn*-knockout mice reveals profound alterations in tooth morphogenesis from the cap stage onwards, with a highly irregular enamel organ that grows more intensively on the lingual

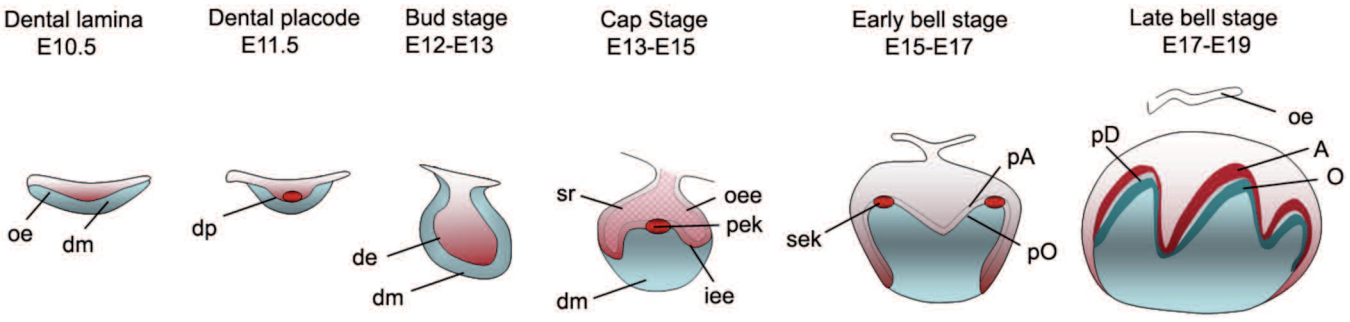

Fig. 1. Stages of molar tooth development. From the initiation stage, when teeth begin to form at specific sites in the dental lamina, continuous interactions between dental epithelium and mesenchyme govern odontogenesis until its last stages when teeth are completely differentiated. A: ameloblasts; de: dental epithelium; dm: dental mesenchyme; dp: dental placode; iee: inner enamel epithelium; O: odontoblasts; oe: oral ectoderm; oee: outer enamel epithelium; pA: preameloblasts; pek: primary enamel knot; pO: preodontoblasts; sek: secondary enamel knot; sr: stellate reticulum.

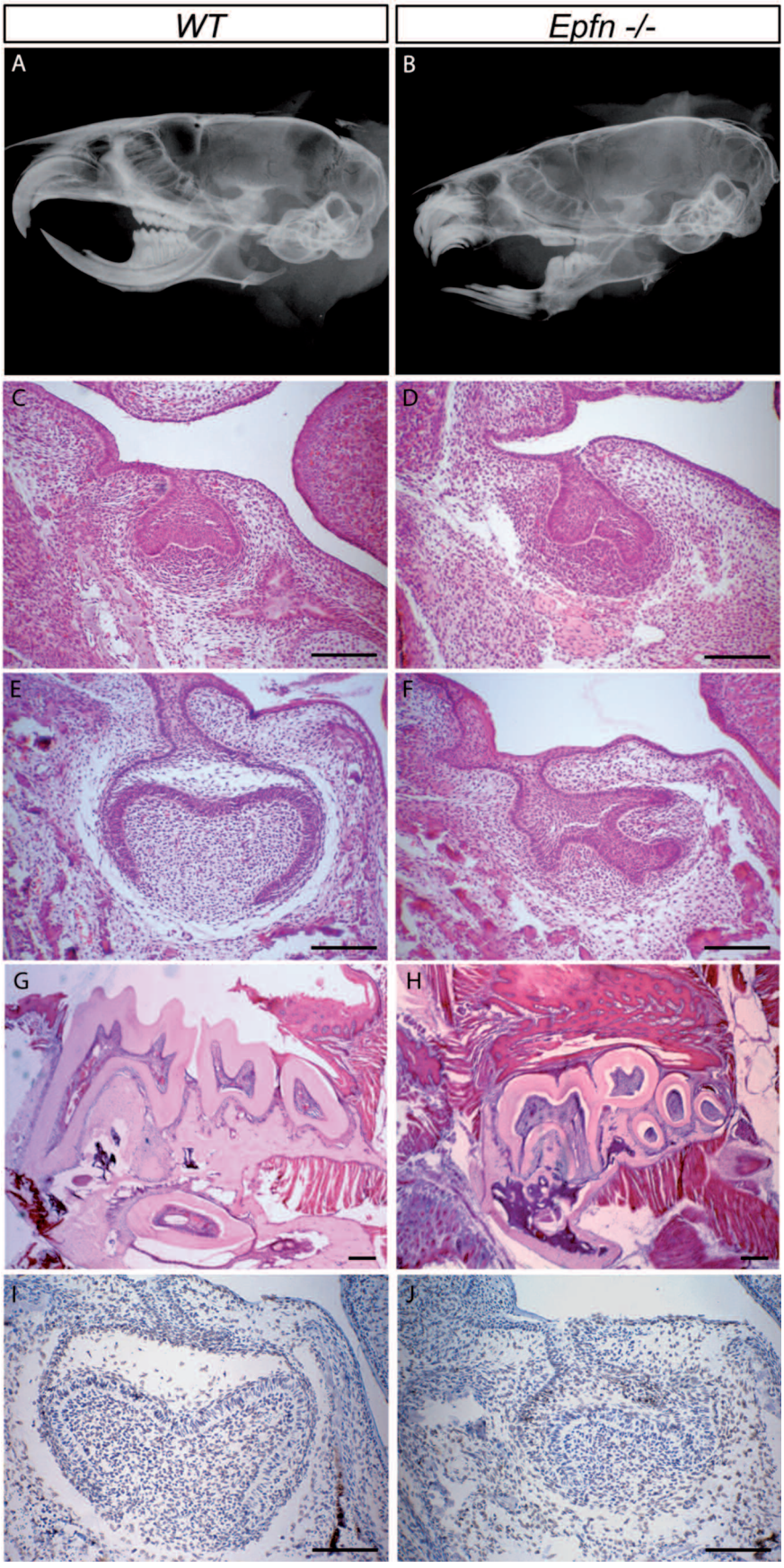

Fig. 2. Alterations in tooth development of *Epfn*-knockout mice. *Epfn*-deficient mice present dental malocclussion, poor dental root development, cusp defects and supernumerary teeth as observed by X-ray **(A, B)**. A detailed histological analysis of *Epfn*-knockout mice showed the first anatomical defects around cap stage **(D)** with extra buds emerging from the primary tooth germ. These extra buds may be a result of the ectopic enamel knots that appear as grouped apoptotic cells detected in the enamel organ by TUNEL assay **(J)**. Ultimately, supernumerary teeth and fused molars erupt with a notable delay in the jaw of mice lacking *Epfn* **(H)**. Scale bars: 100 μm.

than on the labial side (Fig. 2D,F). By the bell stage (E16.5), multiple tooth buds have emerged from one single dental lamina, and several apoptotic zones (Fig. 2I,J), which may correspond to ectopic enamel knots, are evident within this unique enamel organ. As a result of these defects, *Epfn*-knockout mice have extra teeth lacking enamel, owing to a failure in the inner enamel epithelium to differentiate into ameloblasts.

It has been suggested that Sp family members act in a specific manner, but their highly conserved motifs also point to possible overlapping roles, as happens with Sp3 and Sp1 in certain tissues (Ping et al., 2000). *Sp3* and *Epfn* expression overlap in the enamel epithelium during tooth development, and *Sp3* is upregulated in *Epfn*-deficient mice (Nakamura et al., 2008). However, it is obvious that *Epfn* deficiency cannot be compensated by Sp3 for the proper differentiation of the inner enamel epithelium into ameloblasts and tooth number regulation.

Epfn and supernumerary tooth formation

As observed in the study of *Epfn*-knockout mice, *Epfn* deficiency leads to hyperdontia. Apart from *Epfn*-null mice, there are additional mouse models in which the presence of supernumerary teeth can be observed (recently reviewed by Fleming et al., 2010; Cobourne and Sharpe, 2010). This phenomenon can occur by two main different mechanisms, either emerging as rudimentary extra teeth mainly from the diastema region, or arising from the primary tooth germ.

Among the first group, mice lacking *Sprouty2 (Spry2), Sprouty4 (Spr4), Tg737, Gas1, Ectodin, Lrp4* and those which overexpress *Ectodysplasin (EDA)* or its receptor *EDAR* are found. Spry2 and Spry4 are negative-feedback regulators of fibroblast growth factors (FGFs) and other receptor tyrosine kinase signaling. FGFs are crucial at different stages during odontogenesis, but specifically during the transition from the bud to cap stage, when mesenchymal FGFs signal to the enamel epithelium, thus inducing the expression of *Shh*. It has been suggested that Spry2 and Spry4 prevent the expression of *Shh* in the diastema, leading to the arrest of tooth development at the bud stage in this region (Klein et al., 2006). Mice expressing a hypomorphic allele of the intraflagellar transport protein Tg737 (also known as *Polaris*) and Gas1 (a *Shh* antagonist) knockout mice, present ectopic Shh signaling in the diastema region, inducing the formation of extra teeth (Zhang et al., 2003; Ohazama et al., 2009). *Ectodin* (a Wnt/BMP signaling antagonist also named as *Wise, USAG-1, Sostdc1*) and *Lrp4* (a receptor that suppresses Wnt signaling) mutant mice present both supernumerary molars and incisors (Jonhson et al., 2005; Kassai et al., 2005; Murashima-Suginami et al., 2007; Ohazama et al., 2008). Similar to the above-mentioned mice, formation of extra molars is, in both cases, a consequence of ectopic *Shh* expression in the diastema region. However, supernumerary incisors are the result of BMP and Wnt signaling upregulation (Munne et al., 2009). Finally, overactivation of EDA signaling leads to the appearance of vestigial extra molars for which *Shh* overexpression is responsible (Mustonen et al., 2003; Kangas et al., 2004; Tucker et al., 2004).

On the other hand, the appearance of supernumerary teeth observed in Epfn-knockout mice is more likely a consequence of the multiple buds observed in the enamel organ during late developmental stages. This phenomenon highly resembles the phenotype of ß-*cat*$^{\Delta ex3K14/+}$ and *K14-Cre;Apc*$^{cko/cko}$ mice. In both mouse models, the Wnt/ß-catenin pathway is overactivated in the dental epithelium as a result of the stabilization of ß-catenin (Wnt effector) or the depletion of Apc (an inhibitor of the Wnt pathway), respectively (Järvinen et al., 2006; Kuraguchi et al., 2006). Similar to what we observed in *Epfn* mutants, multiple supernumerary teeth arise continually from a single dental lamina. It is possible that when due to overactivation of the Wnt/ß-catenin pathway or loss of *Epfn*, new teeth form during later stages of odontogenesis, thus reproducing the inductive signals required for tooth replacement.

Epfn is involved in the crosstalk between BMP and Wnt signaling

It has been recently reported that the expression of *Epfn* is controlled by Wnt/ß-catenin signaling in the embryonic limb ectoderm (Talamillo et al., 2010). Similarities in the tooth phenotype regarding supernumerary teeth formation between *Epfn*-deficient and Wnt/ß-catenin-overactivated mice point out a possible link between *Epfn* and the Wnt/ß-catenin pathway during odontogenesis.

The importance of Wnt signaling during tooth development has been proved by the study of different mouse models. Inhibition of the Wnt/ß-catenin pathway can lead to the arrest of tooth development. This occurs after abrogation of the signaling transcriptional effector *Lef-1* (*Lymphoid Enhancement binding factor-1*); constitutive *Dkk1* (Dickkopf 1) overexpression in dental epithelium; inducible *Dkk1* overexpression and *ß-catenin* depletion in epithelial cells (van Gederen et al., 1994; Andl et al., 2002; Liu et al., 2008). Odontogenesis is blocked in all of these mice at the early bud stage, demonstrating that epithelial Wnt/ß-catenin signaling is required for the progression of tooth development beyond this stage.

The BMP and Wnt signaling pathways are interconnected at different levels during the development of several organs, including teeth. For instance, *Lef-1* and *Ectodin* are common regulators of both pathways, as a target gene and as an inhibitor, respectively. In addition, the BMP-4 recombinant protein induces the expression of *Dkk-1*, a Wnt/ß-catenin signaling inhibitor, in the embryonic limb appendage and osteogenic mesenchyme (Grotewold and Rüther, 2002; James et al., 2006). Our data show that similarly, BMP4 strongly induces Dkk1 expression in E11.5 mandibles (Fig. 3G).

At this stage, *Epfn* presents the same expression pattern as *Wnt10b*, being restricted to the presumptive dental epithelium. The expression analysis of these genes in Epfn mutant mice revealed that while *Wnt10b* expression was not altered, *Bmp4* and *Dkk1* presented an abnormal expression pattern in comparison to wild-type embryonic mandibles (Fig. 3B-F). Downregulation of BMP4 may lead to a decreased expression of *Dkk1*, which could ultimately cause overactivation of the Wnt/ß-catenin pathway. In fact, *Lef-1* expression is dramatically increased in *Epfn*-null mandibles at both E11.5 and E13.5 (Nakamura et al., 2008), when an ectopic *Lef-1* expression in the dental mesenchyme can be observed. Intriguingly, these findings fit with the previously mentioned similarities in tooth number phenotype between *Epfn*-knockout and ß-cat$^{\Delta ex3K14/+}$ mice.

Recently, it has been proposed that Wnt/ß-catenin signaling induces and maintains *Fgf8* expression in the early oral epithelium (Wang et al., 2009). Our results revealed that FGF8 strongly induces Dkk1 expression in E10.5 mandibles, probably activating a negative

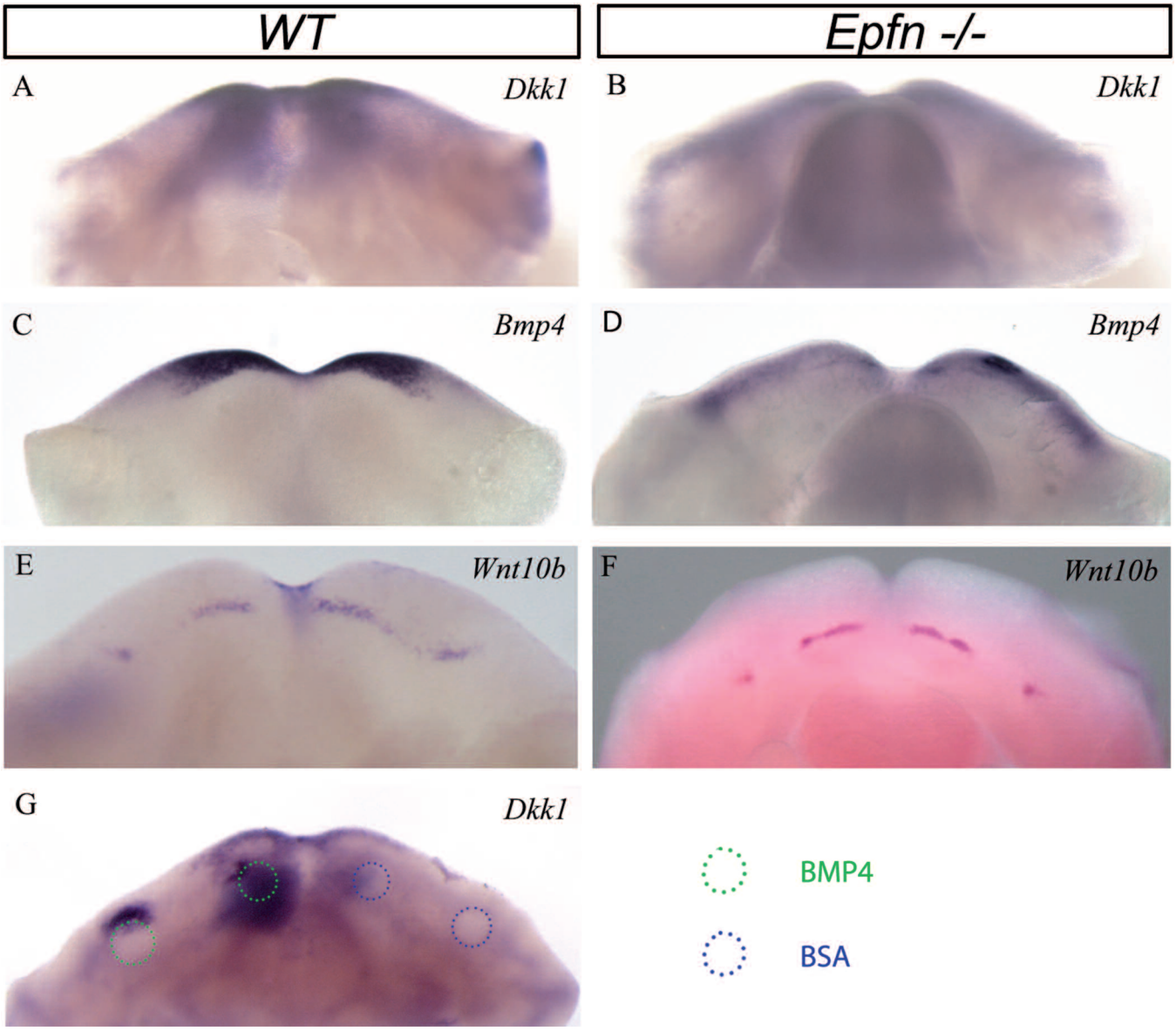

Fig. 3. Epfn regulates BMP and Wnt/ß-catenin signaling. The expression of *Dkk1* is induced by recombinant BMP4 **(G)**. In situ hybridization showed a decrease in the expression of *Bmp4* and *Dkk1* in the distal region of *Epfn*-knockout mandibles at early stages **(B, D)**, whereas *Wnt10b* expression was not altered **(F)**.

feedback mechanism (Fig. 4C). In this context, we found an ectopic *Fgf8* expression in the most distal region of *Epfn*-knockout mandibles at E11.5, which may reflect the above-mentioned Wnt/ß-catenin overactivation (Fig. 4A,B).

Lack of Epfn severely affects ameloblast differentiation

The terminal differentiation of the inner enamel epithelium gives rise to ameloblasts that secrete the enamel matrix. In mouse molars, these ameloblasts ultimately disappear after tooth eruption. In contrast, incisors grow continually throughout the mouse life, due to the presence of an epithelial stem cell niche at the apical end of the labial side that represents the source of odontoblast and ameloblast precursors (Harada et al., 1999). It is important to note that whereas dentin is formed in the labial and lingual sides of the incisors, differentiated ameloblasts and enamel are only present in the labial side.

As previously mentioned, *Epfn*-knockout mice present enamel hypoplasia and a lack of ameloblasts. In Epfn deficient incisors, the inner enamel epithelium starts its normal development from the apical cervical loop, but at the point where cells normally begin to polarize, this epithelium undergoes a profound regression (Fig. 5B,D). Consequently, instead of

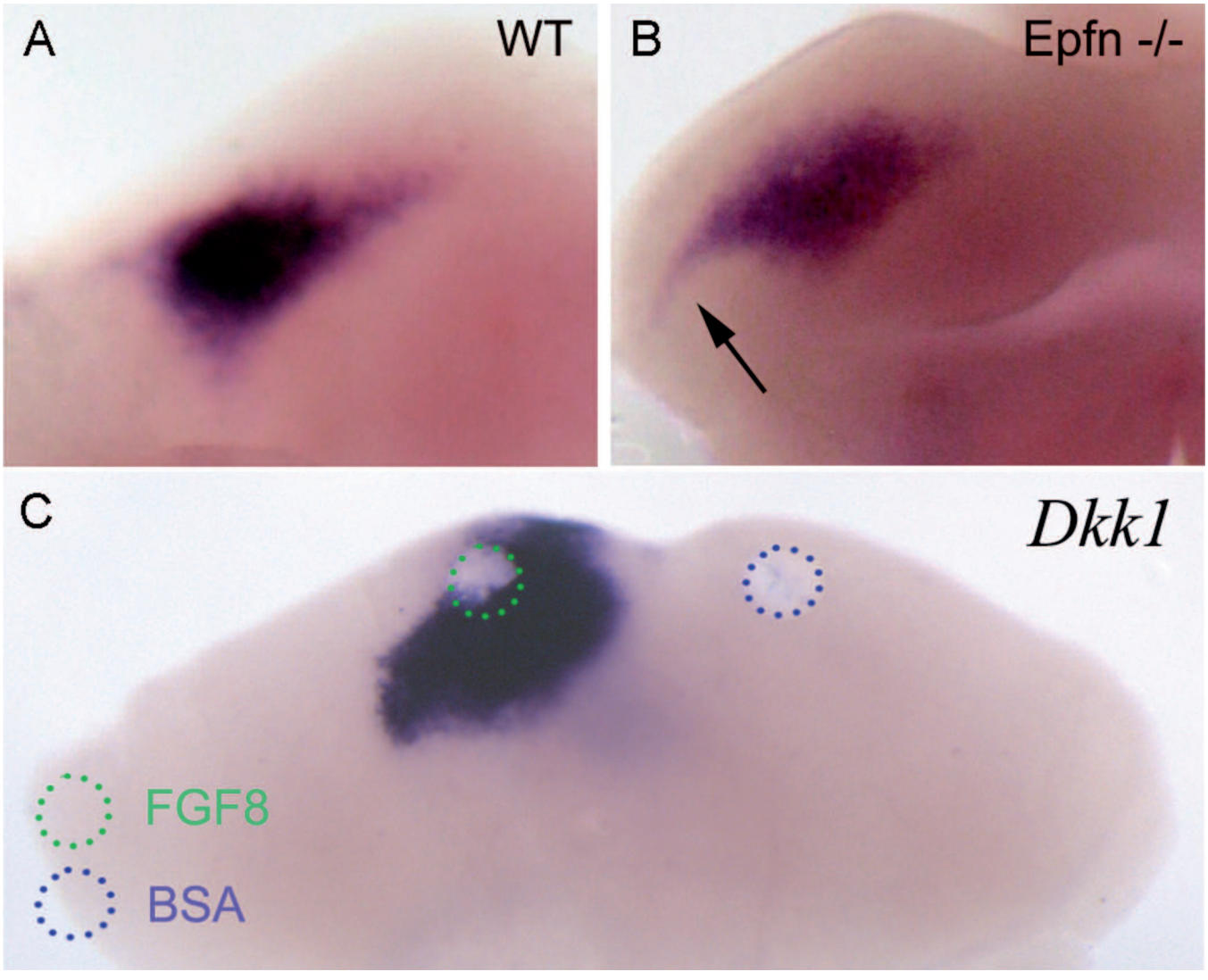

Fig. 4. Interconnection of FGF8 and Wnt/ß-catenin signaling. At E11.5, *Fgf8* expression is ectopically expanded in *Epfn*-knockout (**B**, arrow) in comparison to wild type mandibles **(A)**, and this is in correlation with the Wnt/ß-catenin overactivation that has been demonstrated to be present at these early stages in *Epfn* mutants. In addition, recombinant FGF8 strongly induces *Dkk1* expression **(C)**, acting as a negative regulator of Wnt/ß-catenin pathway.

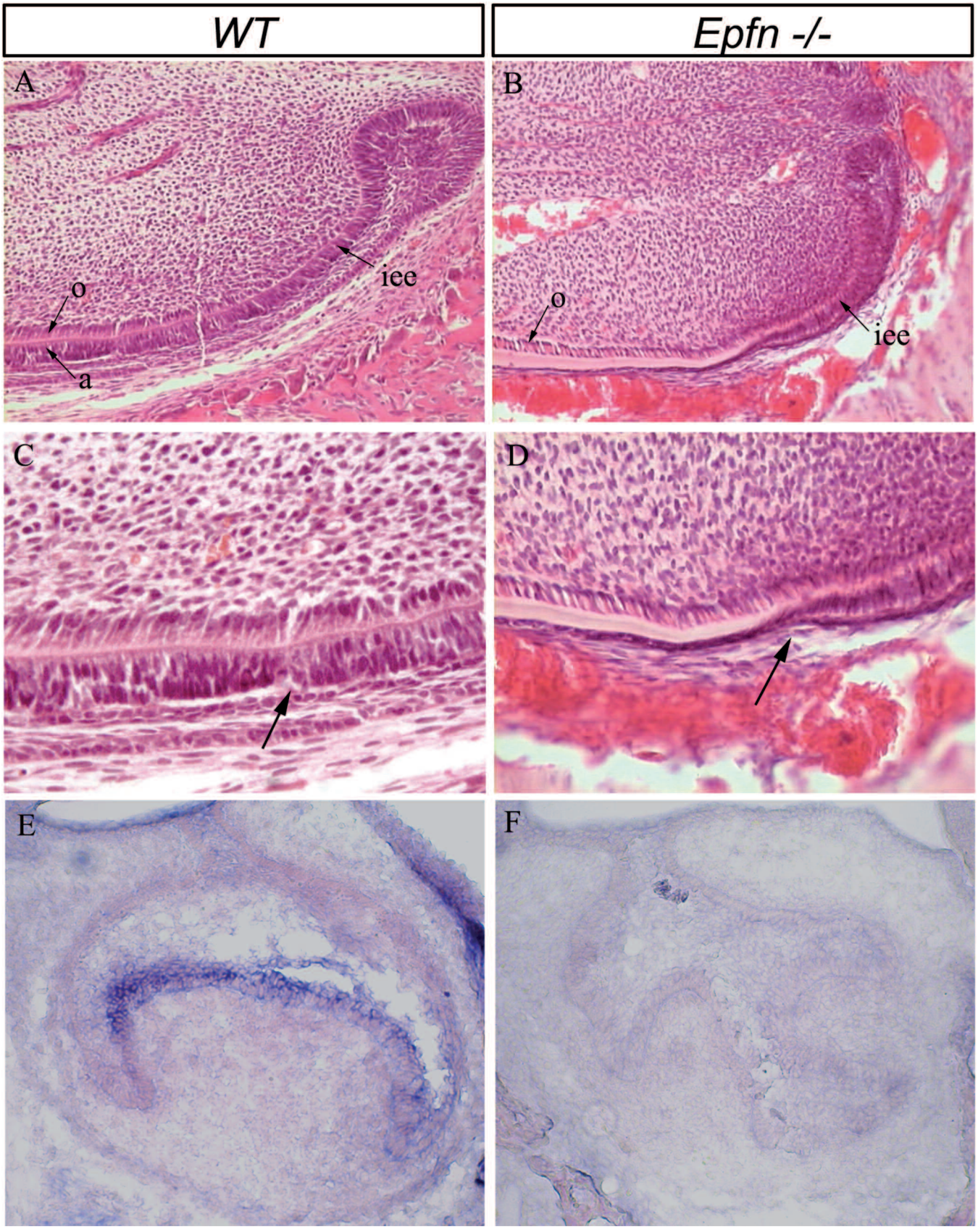

Fig. 5. Epfn is essential for *Shh* expression and ameloblast differentiation. In *Epfn* mutant incisors, inner enamel epithelial cells do not polarize (**B-D**, arrow) and subsequently ameloblast differentiation is severely impaired. In situ hybridization on sections from E16.5 molars demonstrated that *Shh* expression was dramatically decreased in the inner enamel epithelium of *Epfn*-knockout mice **(E, F)**. a, ameloblasts; iee, inner enamel epithelium; o, odontoblasts.

undergoing differentiation, epithelial cells form a stratified layer likely composed of cells from the inner enamel epithelium, stellate reticulum and stratum intermedium (Fig. 5B,D).

There is an absence of enamel in several mouse models, where molecules involved in different signaling pathways such as Shh, BMP or Wnt/ß-catenin are affected. *Shh* is expressed in the tooth epithelium at early stages; it is expressed in the enamel knot at the cap stage and in the inner enamel epithelium from bell stage onwards. A conditional deletion of *Shh* in dental epithelium results in profound anomalies in tooth morphogenesis. Thus, the epithelial enamel organs of the first and second molars appear fused in conditional Shh-knockout mice, and dental ridges show marked irregularity (Dassule et al., 2000). Moreover, the ameloblasts do not fully differentiate in these conditions, and produce only a thin enamel layer. These aforementioned defects are more severe in the case of conditional *Smo* (*Smoothened*)-knockout mice. Smo is a signal transducer of the Shh pathway, and *Smo*-knockout mice show a complete blockade of ameloblast polarization and differentiation and, consequently, no secretion of enamel (Gritli-Linde et al., 2002). In addition, enamel organ fusion is reproduced in *K14-Cre/Smo*$^{flox/flox}$ mice, which eventually develop fused first and second molars (Ohazama et al., 2008).

Epfn-knockout mice display some of the most prominent dental defects that can be found in mice whose Shh pathway is inactivated. Lack of ameloblast differentiation and molar tooth fusion occur in conditional *Smo*-deficient and *Shh*-knockout mice, just as it does in mice defective in *Epfn* (Dassule et al., 2000; Gritli-Linde et al., 2002; Nakamura et al., 2008). Moreover, one striking consequence of *Epfn* depletion is the reduction of *Shh* expression in the dental epithelium, as assessed by *in situ* hybridization on tooth germ sections at the cap stage (Nakamura et al., 2008). *Shh* is prominently expressed in the enamel knots at the cap stage, where it acts to regulate dental morphogenesis. During the bell stage, the expression of *Epfn* and *Shh* coincide at the inner enamel epithelium which later gives rise to ameloblasts. Consequently, the lack of *Shh* expression in the inner enamel epithelium of *Epfn*-knockout mice (Fig. 5F) may be correlated with the failure of ameloblast differentiation. Odontoblasts in turn, are not completely differentiated, producing brittle dentin that shows ultrastructural defects. Therefore, decreased *Shh* expression in the tooth germ of *Epfn* mutant mice may account for fusion of molar teeth as well as for impaired ameloblast differentiation.

Similar defects in ameloblast differentiation are observed when *Follistatin* is conditionally overexpressed in the epithelium under the control of the K14 promoter. In *K14-Follistatin* mice, BMP signaling inhibition in the dental epithelium leads to inappropriate differentiation of the inner enamel epithelium into ameloblasts, and thus gives rise to a complete lack of enamel (Wang et al., 2004). It has been shown that Epfn downregulates *Follistatin* gene expression in a cell line established from rat ameloblasts (Ruspita et al., 2008). It would be interesting to assess whether *Epfn*-knockout mice present an increased expression of Follistatin, mimicking the effect observed in *K14-Follistatin* mice.

It has also been reported that *K14-Wnt3a* mice show a progressive loss of ameloblasts from postnatal lower incisor teeth due to defective differentiation of ameloblast precursors, progressive apoptosis of ameloblasts, or loss of ameloblast stem cells (Millar et al., 2003). Although there is no evidence regarding Wnt signaling overactivation during these late stages in *Epfn*-null mice, *Lef-1* upregulation in earlier stages of *Epfn*-knockout mice suggests that Epfn could also be acting upstream of the Wnt pathway in the regulation of ameloblast differentiation.

Epfn relationship with human ectodermal dysplasias

Although notable differences exist between human and mouse dentition, the use of genetically modified mice is especially useful for the study of the molecular mechanisms that regulate tooth organogenesis. Interestingly, mutations in specific genes causing abnormalities in the tooth development of humans have also been reported to induce a comparable deleterious effect in mice models, as signaling pathways governing odontogenesis are highly conserved in both species (D'Souza and Klein, 2007).

The most common defect in the tooth development of humans is the agenesis of one or more teeth (including anodontia, hypodontia and oligodontia). Although less common than instances of missing teeth, hyperdontia or extra supernumerary teeth generation can also occur. Alterations in tooth number formation can appear in humans either as an isolated feature or as a syndromic condition, wherein normally more ectodermal organs are also affected (D'Souza and Klein, 2007). This is the case of ectodermal dysplasia, a heterogeneous group of rare heritable diseases in which abnormalities in two or more ectodermal organs are affected including the skin, teeth, hair, nails, sweat glands, digits and extremities (Pinheiro and Freire Maia, 1994). Similarly to odontogenesis, the development of these ectodermal organs is driven by inductive interactions between epithelium and mesenchyme (Mikkola and Thesleff, 2003). Mutations in genes involved in this ectodermal organogenesis may lead to ectodermal dysplasia. This is the case of the transcription factor p63, the adhesion molecules P-cadherin and Nectin-1, as well as EDA and EDAR (Itin and Fistarol, 2004).

Epfn-knockout mice show abnormalities in epidermis, hair, digits and teeth (Hertveldt et al., 2008; Nakamura et al., 2008; Talamillo et al., 2010). These findings strongly suggest that a mutation of the *Epfn* human homolog could result in an ectodermal dysplasia. However, further studies are needed in order to link *Epfn* to a particular subtype, since these diseases are part of a large group of rare disorders composed of about 150

different syndromes (Pinheiro and Freire Maia, 1994).

Concluding remarks

In this article, we review the role of the Sp/KLF family member Epfn during odontogenesis, providing some evidence of the interplay between this transcription factor and the BMP, Shh and Wnt/ß-catenin signaling pathways. More specifically, *Epfn* deletion results in a dramatic decrease in *Shh* expression that may lead to molar fusion as well as incomplete epithelium differentiation and absence of enamel. On the other hand, alterations in BMP4 and overactivation of Wnt/ß-catenin signaling detected in *Epfn*-knockout mice could explain the formation of multiple supernumerary teeth. The mechanisms whereby supernumerary teeth arise in *Epfn*-knockout mice highly resemble the tooth renewal process, which has disappeared in mice through evolution. This fact points out a possible role for *Epfn* in tooth replacement, which makes this transcription factor particularly interesting in the tooth engineering field, where strong efforts are made to obtain a completely differentiated tooth from adult tissues. In addition, the results obtained from the study of *Epfn*-knockout mice suggest that mutations in the human homolog of *Epfn*/*Sp6* could result in a type of ectodermal dysplasia. Overall, Epfn is a transcription factor, which has an essential role during tooth development, and an ongoing study of its mechanisms of action would help in the understanding of both normal tooth development and related diseases.

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