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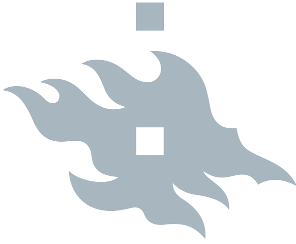

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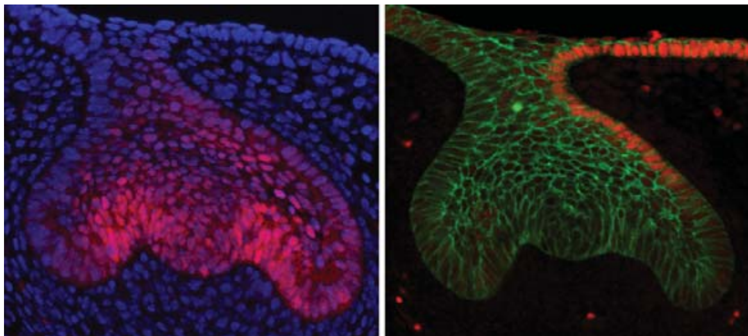
MARIA JUSSILA

Transcription Factors Foxi3 and Sox2 in the Regulation of Tooth Development



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AND
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DEPARTMENT OF BIOSCIENCES
FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES
AND
DOCTORAL PROGRAMME IN INTEGRATIVE LIFE SCIENCE
UNIVERSITY OF HELSINKI

Transcription factors Foxi3 and Sox2 in the regulation of tooth development

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Academic dissertation

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Cover images
Left: Detection of Foxi3 protein (red) by immunofluorescence in an E14.5 molar tooth epithelium. Nuclei of all cells are stained blue with DAPI.
Right: Detection of Sox2 protein (red) by immunofluorescence in an E14.5 molar tooth epithelium. Membranes of all epithelial cells (green) are visualized by E-cadherin immunofluorescence.

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List of original publications

I Shirokova V*, **Jussila M***, Hytönen MK*, Perälä N, Drögemüller C, Leeb T, Lohi H, Sainio K, Thesleff I & Mikkola ML. 2013. *Expression of Foxi3 is regulated by Ectodysplasin in skin appendage placodes.* Developmental Dynamics 242: 593–603. *equal contribution

II **Jussila M**, Aalto A, Sanz Navarro M, Shirokova V, Ohyama T, Groves A, Mikkola ML, Thesleff I. *Transcription factor Foxi3 regulates dental epithelial morphogenesis.* Manuscript.

III Juuri E*, **Jussila M***, Seidel K, Holmes S, Wu P, Richman J, Heikinheimo K, Chuong CM, Arnold K, Hochedlinger K, Klein O, Michon F & Thesleff I. 2013. *Sox2 marks epithelial competence to generate teeth in mammals and reptiles.* Development 140: 1424–1432. *equal contribution

IV **Jussila M**, Crespo Yanez X & Thesleff I. 2014. *Initiation of teeth from the dental lamina in the ferret.* Differentiation 87: 32–43.

The publications are referred in the text by their Roman numerals.
In addition, unpublished data is presented.

Contributions:

I The author performed the experiments with VS, MH and NP, and participated in planning the experiments, analyzing the results and writing the manuscript with other authors.
II The author planned the experiments with IT, MM, and VS, performed majority of the experiments, supervised AA and MSN, and wrote the manuscript.
III The author planned the experiments with EJ and IT, performed the experiments with other authors, analyzed the results with EJ and IT, and wrote the manuscript with EJ and IT.
IV The author planned the experiments and wrote the manuscript together with IT, performed the experiments, and supervised XCY.

Abbreviations

$^{-/-}$ or $^{+/-}$	Homozygote or heterozygote animal for a mutated gene
APC	Adenomatous polyposis coli tumor suppressor protein
Bmp	Bone morphogenetic protein
cKO	Conditional knock-out mouse
dC	Deciduous canine
Dkk	Dickkopf, Wnt antagonist
dP	Deciduous premolar
Dusp	Dual-specificity phosphatase, Fgf antagonist
E	Embryonic days, days from fertilization
Eda	Ectodysplasin
Edar	Ectodysplasin receptor
Fgf	Fibroblast growth factor
Fox	Forkhead box DNA-binding domain containing factor
Gli	Gli family zing finger factor
GSK3-β	Glycogen synthase kinase 3 β
HED	Hypohidrotic ectodermal dysplasia
Id	Inhibitor of DNA binding, Bmp agonist
Idl	Interdental lamina
IEE	Inner enamel epithelium
K10	Keratin-10
KO	Knock-out mouse
Lef1	Lymphoid enhancer binding factor 1
Lgr	Leucine-rich repeat containing G protein coupled recptor
LRC	Label-retaining cell
Lrp	Low density lipoprotein receptor related protein
M1 to M3	First to third molar
Msx	Msh homeobox
OEE	Outer enamel epithelium
Osr	Odd skipped related
PEK	Primary enamel knot
Pitx2	Paired-like homeodomain transcription factor 2
Runx	Runt related transcription factor 2
SEK	Secondary enamel knot
Sfrp	Secreted frizzled related protein, Wnt antagonist
Sostdc1	Sclerostin domain containing 1
Shh	Sonic Hedgehog
Sox	SRY-related high-mobility group box
TGF-beta	Transforming growth factor β
Wnt	Wingless-type MMTV integration site family member
wmish	Whole mount in situ hybridization

Summary

Teeth are ectodermal organs, which form from the embryonic epithelium and mesenchyme. Reciprocal interactions between these two tissues, regulated by the conserved signaling pathways, guide tooth morphogenesis. Activity of each signaling pathway is mediated by transcription factors, which activate or repress target genes of the pathway. During morphogenesis, the shape of the dental epithelium undergoes dramatic changes as it proceeds though placode, bud, and cap stages, finally forming the shape of the mature tooth crown. These events are regulated by signaling centers, which are groups of signaling molecule-secreting cells that appear in sequence during tooth development. These include the placode and bud signaling centers, as well as the primary enamel knot and the secondary enamel knots. The location of the enamel knots determines the future tips of the cusps of the tooth crown.

In mammals, teeth are replaced only once, whereas most reptiles and fish replace their teeth throughout the life of the animal. Regardless of number of tooth replacements, in all animals, replacement tooth development already begins during morphogenesis of the previous tooth generation. Replacement teeth are initiated from an extension of the epithelium called the successional dental lamina. It forms from a dental lamina that is embedded on the tongue-side epithelium of the primary tooth. Wnt signaling is one of the pathways linked to regulation of tooth replacement both in mammals and in reptiles. In reptiles, tooth replacement has been shown to involve stem cells. However, no markers for these cells have been identified.

The aims of this thesis are to study the roles of transcription factors Foxi3 and Sox2 in tooth morphogenesis and replacement, as well as to investigate the molecular regulation of mammalian tooth replacement. A mutation in the gene coding for the transcription factor Foxi3 was identified in hairless dogs, but its function and regulation in ectodermal organ development has not been studied. Here, I showed that *Foxi3* is expressed in the dental epithelium. It lies downstream of several signaling pathways including Ectodysplasin, which regulates development of all ectodermal organs. I studied molar development of conditional epithelial *Foxi3* knock-out (*Foxi3* cKO) mice, and showed that Foxi3 regulates the morphological changes that the dental epithelium undergoes during morphogenesis from bud formation to crown shape patterning by intervening in the activity of several signaling pathways in the signaling centers.

The transcription factor Sox2 has been shown to mark the epithelial stem cells of the continuously growing mouse incisor. By combining modern mouse genetics tools with histological and molecular analysis in the ferret and in several reptilian species, I studied how Sox2 is related to the formation of new teeth from the existing dental epithelium. I showed that *Sox2* is currently the only gene linked specifically to tooth replacement in mammals and reptiles. Using the mouse molar as a model of successional tooth formation, I showed that *Sox2*-expressing cells give rise to the epithelium of newly forming teeth, and that Sox2 regulates epithelial proliferation. *Sox2* expression during tooth replacement was complementary to Wnt pathway activity, which links stem cell maintenance and initiation of tooth formation. Finally, using the ferret as a model, I characterized the morphology and some aspects of molecular regulation of the mammalian interdental lamina, and show that this non-tooth forming epithelium has characteristics of tooth-forming potential such as *Foxi3* and *Sox2* expression and Wnt pathway activity.

This thesis links two epithelial transcription factors, Foxi3 and Sox2, to the regulation of tooth morphogenesis and formation of new teeth. Knowledge on the function of transcription factors that act downstream of signaling pathways in tooth development and replacement is essential for the understanding of the complex regulation of organ morphogenesis, and can ultimately be applied to the treatment of human syndromes and to the field of tissue regeneration.

Introduction

Tooth development begins with the initiation and morphogenesis of primary teeth and continues with the formation of replacement teeth. Teeth are formed from epithelial and mesenchymal tissue compartments. During morphogenesis the dental epithelium undergoes remarkable shape changes and new teeth are always initiated from pre-existing epithelium. Dentition varies among species, and the potential to replace the teeth also varies from one round of replacement in most mammals to continuous life-long replacement in reptiles.

Conserved signaling pathways regulate all aspects of tooth development. Much of this regulation is mediated by signaling centers that form within the dental epithelium during development. The changes in gene expression that are induced by the signaling pathways are mediated by transcription factors inside a signal-receiving cell.

Here, as a background for my own work, I will review the different aspects of tooth development from primary tooth initiation and morphogenesis to tooth replacement. I will start by describing the different stages of tooth development. Then I will give an overview of the current knowledge on the signaling pathways and transcription factors regulating tooth morphogenesis. Most of this information has been gained from analysis of the phenotypes of different mutant and transgenic mouse lines. In addition, some gene modifications affect the dentition as a whole, and lead to either missing teeth or supernumerary tooth formation, or fusion of teeth. These phenotypes tell about the signaling pathways regulating tooth initiation and number.

As mice do not replace their teeth, other model animals have been used to uncover the mechanisms of replacement tooth development. I will give a summary of the current data on tooth replacement from these different models. The ferret has a similar dentition to humans, and it has been used as a model of mammalian tooth replacement. Reptiles replace their teeth continuously, and this continuous tooth replacement is fueled by stem cells, but these cells are still poorly characterized. Additionally, human syndromes with missing or supernumerary teeth give information on the regulation of permanent tooth development in mammals.

In my own work I have analyzed the role of two epithelial transcription factors, Foxi3 and Sox2, and showed that the function of these two factors covers all aspects of tooth development from initiation and morphogenesis to replacement tooth formation. In addition, I have used the ferret as a model animal to study the molecular regulation of mammalian tooth replacement.

1. Review of the literature

1.1. Regulation of organ morphogenesis by conserved signaling pathways

Organ morphogenesis begins when undifferentiated cells within the embryo are induced to an organ-specific cell fate. Most organs are formed by epithelial and mesenchymal cells that communicate with each other via conserved signaling pathways. A discrete signaling center often forms within the developing organ and guides morphogenesis. These centers instruct the surrounding cells to proliferate and determine their positioning within the developing organ by changes in adhesion, migration, and apoptosis, and ultimately induce the cells to differentiate into organ-specific cell types. Some adult organs harbor undifferentiated cells, stem cells that can support tissue renewal throughout the life of the individual. These organs include for example the intestine, skin, hairs, and teeth.

Conserved signaling pathways mediate communication between cells and tissues within a developing embryo. Many of these pathways regulate stem cells and tissue homeostasis later in the adult, and dysfunction in cellular communication often leads to cancer. The most common signaling pathways include Wnt, Fibroblast growth factor, TGF-β superfamily, Sonic hedgehog, Tumor necrosis factor and Notch pathways. Collectively, they regulate the different aspects of morphogenesis. Each pathway consists of ligands, cell surface receptors, and an intracellular signaling cascade. Pathway-specific transcription factors mediate activation or repression of target genes in the nucleus. The pathway ligands either act as morphogens that form gradients within tissues and function from a distance from the ligand-secreting cell, or some require cellular contact and function between neighboring cells. Tissue- and cell-specific responses are achieved by specific expression of ligand, receptor, or intracellular mediator molecules. Secreted or intracellular agonists and antagonists bring an additional layer of complexity to the regulation of pathway activity.

1.1.1. Wnt pathway

Wnt ligands can activate two types of intracellular signaling pathways, the canonical and non-canonical Wnt pathways. Here, I focus only on the canonical pathway. The canonical Wnt pathway regulates β-catenin; in the absence of the ligand, β-catenin is bound to a protein complex consisting of adenomatous polyposis coli tumor supressor (APC), Axin, and protein kinases GSK3-β and CK1 that facilitates β-catenin phosphorylation and subsequent degradation (Nusse 2012). Binding of Wnt to Frizzled-receptors and Lrp co-receptors dissociates the β-catenin from the protein complex and allows it to translocate to the nucleus. In the nucleus β-catenin binds Lef/TCF transcription factors, which act as repressors of transcription, thus permitting activation of target genes. The Wnt pathway has several antagonists (Cruciat and Niehrs 2013). The secreted Dickkopf (Dkk) proteins inhibit signaling by binding to Wnt co-receptors, whereas the sFRPs (secreted Frizzled-like proteins) bind Wnt ligands. Sostdc1 (sclerostin domain-containing 1) can also inhibit Wnt signaling by binding to Lrp co-receptors. R-spondins are secreted growth factors, which seem to act by binding Lgr receptors, another type of Wnt co-receptors.

1.1.2. Fibroblast growth factor pathway

The fibroblast growth factor (Fgf) pathway functions through receptor tyrosine kinases that dimerize upon ligand binding and activate a phosphorylation cascade inside the cell (Pownall and Isaacs 2010). This cascade leads ultimately to phosphorylation of MAPK kinase, which phosphorylates and activates transcription factors. A negative feedback loop involves activation of *Sprouty* genes that antagonize the Fgf signaling cascade intracellularly. Dusp (dual specificity phosphatase) proteins are another group of Fgf antagonists that dephosphorylate the MAP kinases (Urness et al. 2008). Their expression patterns correlate with Fgf signaling activity in tissues.

1.1.3. TGF-β/BMP pathway

The TGFβ superfamily includes TGFβ, Bmp (bone morphogenetic protein) and Activin signaling pathways (Massagué 1998). The ligands bind to a type II serine-threonine kinase receptor, which phosphorylates a type I receptor. There are several type I and type II receptors, and each ligand binds a different combination of receptors. The specificity of the intracellular signals for the different ligands is determined by the receptor combination they bind. Activated type I receptor induces phosphorylation of particular Smad proteins. The phosphorylated Smads interact with mediator Smads and translocate to the nucleus to activate transcription of target genes. Bmp antagonist Noggin inhibits signaling by binding directly to Bmp ligands (Walsh et al. 2010). Id proteins are targets of Bmp signaling, and they promote cell growth (Miyazono and Miyazawa 2002).

1.1.4. Sonic hedgehog pathway

The sonic hedgehog (Shh) pathway has several means of signal transduction, but the canonical Gli-protein mediated signaling functions through the primary cilium (Sasai and Briscoe 2012). In the absence of Shh, its receptor Patched (Ptch) inhibits a membrane protein called Smoothened (Smo) from entering the cilium, and the Shh effector Gli transcription factors are processed to a repressor form inside the cell. Binding of Shh to Ptch releases Smo, and allows its translocation to the primary cilium, and the following signaling cascade leads to activation of the Gli factors, which then further activate target genes of the pathway.

1.1.5. Ectodysplasin pathway

The Ectodysplasin (Eda) pathway is a tumor necrosis factor pathway that regulates the development of ectodermal organs (Mikkola 2008). When the ligand, Eda, binds to its receptor, Edar, an intracellular signaling cascade is activated by adaptor proteins Edaradd and Traf (TNFR associated factor). This activates the IKK (IκB kinase) complex, which phosphorylates IκB. Normally IκB maintains NF-κB in an inactivated state, but upon pathway activity, NF-κB is released from IκB and it can then activate Eda target genes in the nucleus.

1.1.6. Notch pathway

The Notch pathway signals through the transmembrane Notch receptors (Hori et al. 2013). The Notch ligands, Jagged and Delta proteins, are also transmembrane proteins, indicating that the pathway is activated between cells that are in close proximity to each other. Ligand-receptor

binding results in cleavage of Notch intracellular domain (NICD), which translocates to the nucleus and activates pathway targets with other cofactor proteins.

1.1.7. Transcription factors

The changes in gene expression that occur in cells upon signaling pathway activity are regulated ultimately by transcription factors. They both activate and repress target genes, and the final outcome is often determined by a combination of different factors binding to each gene enhancer or promoter. The individual transcription factors can pair with others, as well as with different cofactors, and the repertoire of these factors present in a cell at a given time point controls how the cell will respond to the signals it receives. Some transcription factors act as pioneer factors that bind to repressed chromatin and make way for other transcription factors to bind the gene enhancer and promoter sequences.

Each transcription factor binds to a specific DNA sequence, and therefore transcription factors are grouped into families based on the structure of their DNA binding site. The major families include the homeodomain factors, such as Hox, Pax, and Forkhead factors, helix-loop-helix factors, basic leucine zipper factors, and zinc finger factors, such as Sry-Sox factors (Gilbert 2014).

1.2. Teeth as ectodermal organs

1.2.1 Ectodermal organ development

Ectodermal organs derive from the embryonic ectoderm and the underlying mesenchyme. In addition to teeth, these organs include scales in reptiles and fish, feathers in birds, hairs and mammary glands in mammals, and exocrine glands such as sweat and salivary glands in all vertebrates, among others. The most comprehensive knowledge on the molecular regulation of development of ectodermal organs comes currently from studies on teeth, hairs, and mammary glands (Biggs and Mikkola 2014). All ectodermal organs begin their development from a local thickening of the ectodermal epithelium, called a placode. The placode grows down into the underlying mesenchyme to form a bud, and the mesenchyme condenses around the bud epithelium. Up to the bud stage, the development of all the ectodermal organs is very similar, and the same signaling pathways regulate it. After the bud stage, each organ undergoes particular morphogenetic events that lead to the differentiation of organ-specific cell types. The adult ectodermal organs show remarkable differences in shape and function. For instance, human teeth do not renew, whereas hairs undergo a continuous cycle of growth, regression, and rest, and mammary glands go through extensive tissue remodeling during pregnancy and lactation (Macias and Hinck 2012, Rompolas and Greco 2014).

1.2.2. Diversity of dentitions

Teeth are one of the most diverse organs in vertebrates. They come in all kinds of shapes and sizes (Fig. 1). In addition, their renewal and replacement capacity shows a great deal of variation between species. Due to the hardness of the mineralized tissues in teeth, they preserve well, and thus there is an extensive fossil record of teeth of ancient animals. From this we can learn about the diet and therefore the ecology of extinct species, as well as about the changes in tooth morphology that have occurred during evolution. The shape of a tooth is generated during embryogenesis, and it does not change in adult animals except due to wear. Experimental work

on the molecular regulation of embryonic mouse teeth and comparative analysis with other species has helped to uncover the possible genetic changes that have taken place during evolution to generate the dental diversity among vertebrates (Jernvall and Thesleff 2012).

Teeth develop from the epithelium, which in most species derives from the embryonic ectoderm, but in some cases from the endoderm (Soukup et al. 2008), and from neural crest cell-derived mesenchyme (Chai et al. 2000). Teeth can have a single or multiple cusps. In general, fish and reptiles have simple-shaped teeth, whereas mammals have more complex tooth shapes, as well as teeth belonging to different tooth families, namely incisors, canine, premolars and molars. The trend throughout evolution has been a decrease in the number of teeth, so that for example some rodents, such as mice, have only one pair of incisors and three pairs of molars, and some animals, such as birds, have lost teeth completely (Davit-Beal et al. 2009). Teeth can be replaced continuously throughout the life of some animals as in many reptiles and fish, just once as in the majority of mammals, or not at all. There are many interesting adaptations to the diet and lifestyle of a species, such as the posterior replacement of molars in the silvery mole rat, and continuously growing teeth in some rodents, among others (Rodrigues et al. 2011, Tummers and Thesleff 2003, Tummers and Thesleff 2008).

Humans have two incisors, one canine, two premolars and three molars in each half of the jaw in the permanent dentition (Fig.1). Humans replace incisors, the canine, and premolars once, but similar to all mammals, molars are never replaced. Mice have only one incisor and three molars, which are separated by a toothless diastema region and their teeth are not replaced (Fig. 1). The ferret (*Mustela putorius furo*) has a similar dentition to humans, but the number of teeth in each tooth family is different (Fig. 1). It has three incisors, one canine, three premolars, and two molars. Reptiles do not have teeth from different tooth families, and their teeth are replaced continuously (Fig. 1).

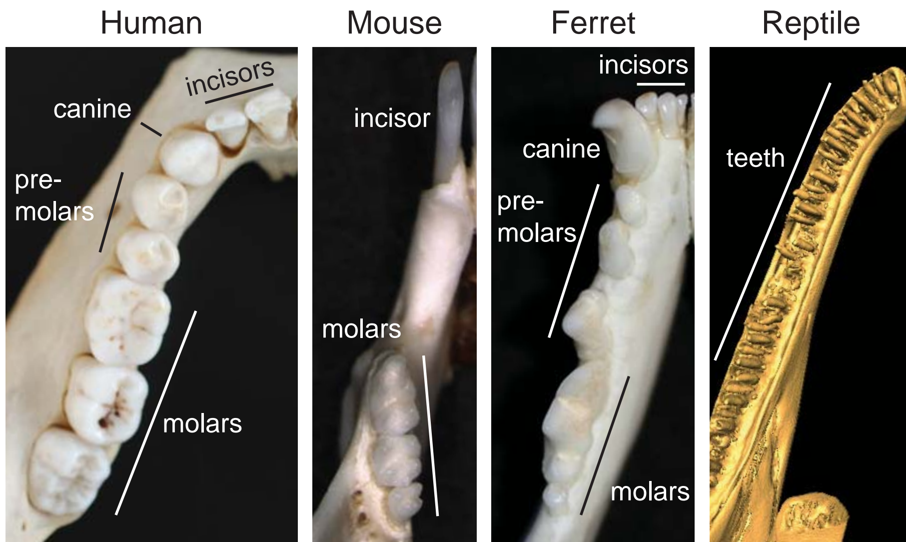


Figure 1. Dentitions of human, mouse, ferret, and a reptile
Lower jaw halves of human, mouse, and ferret, and a CT (computed tomography) scan of an upper jaw half of leopard gecko, a reptile. Humans have teeth belonging to all the mammalian tooth families; incisors, canine, premolars, and molars. Mice have only one incisor and three molars. Ferret dentition is similar to human dentition. Reptile teeth do not belong to different tooth families. Leopard gecko image courtesy of Joy Richman, University of British Columbia.

1.2.3. Tooth morphogenesis

There are many similarities in tooth morphogenesis among different types of teeth and among different species. Here, I will focus on the initiation and morphogenesis of molars in the mouse dentition (Fig. 2 on page 6), but I will point out events that are different for other kinds of teeth.

Tooth development takes place during embryogenesis both in the lower jaw, or the mandible, which arises from the first branchial arch, and in the upper jaw, or the maxilla, which derives from the first branchial arch and the frontonasal process (Mina and Kollar 1987). The craniofacial mesenchyme is formed by neural crest cells that migrate to the branchial arches during early development (Chai et al. 2000). Tooth development begins on embryonic day 11.5 (E11.5) in mouse by formation of the primary dental lamina, also called the odontogenic band, which is a horse-shoe shaped thickening of the epithelium along the embryonic jaws (Fig. 2A). The individual tooth placodes form within the dental lamina (Fig. 2A). In mice, the dental lamina between incisors and molars disappears after this stage, and therefore they do not have an interdental lamina connecting the different teeth. In most reptiles and mammals, however, the primary dental lamina grows down into the mesenchyme together with the forming teeth and forms an interdental lamina that connects the tooth germs to each other within the jaw (Fig.5 on page 17). Once the placode of the first molar has formed it continues its morphogenesis involving cross-talk between the epithelial and mesenchymal tissues. However, the second and third molars do not arise from a placode, but from the posterior epithelium of the previously formed tooth (Fig. 4 on page 16, Kavanagh et al. 2007). After initiation, their morphogenesis proceeds through the same stages of development as in the case of the first molar. Similarly, in species that replace their teeth, replacement teeth are initiated from the dental lamina embedded in the lingual side, or tongue side, of the primary tooth germ epithelium. This dental lamina splits to form a successional dental lamina, where the replacement tooth bud forms and undergoes similar morphogenesis as the primary teeth (Fig. 4 and 5, Järvinen et al. 2009, Richman and Handrigan 2011).

The tooth placode consists of two epithelial cell populations: the basal and the suprabasal cells (Fig. 2B). The cells that are derived from the basal layer of the oral epithelium have an columnar morphology. The suprabasal cells appear squamous and lie between the elongated cells and the single-cell layer thick periderm covering the surface of the embryo. When skin becomes stratified, basal cells differentiate to give rise to the suprabasal cells (Koster and Roop 2007). In tooth, it is not known what the relationship between these two cell populations is. When the placode forms a bud, the underlying mesenchyme condenses and instructs the epithelium to grow down (Fig. 2B). At the same time the neck area of the bud starts to constrict. This region forms the dental cord, a thin epithelial connection between the oral epithelium and the tooth. After the bud stage, morphogenesis of the tooth crown begins and the epithelium is further divided into distinct cell populations. The dental cord has formed by the cap stage, connecting the tooth epithelium, now called an enamel organ, to the oral surface (Fig. 2B). Here, the two opposing layers of basal cells are separated by a few layers of suprabasal cells. The suprabasal cells in the core of the enamel organ are called stellate reticulum cells (Fig. 2B). They have a loose organization and a mesenchymal, star-like shape.

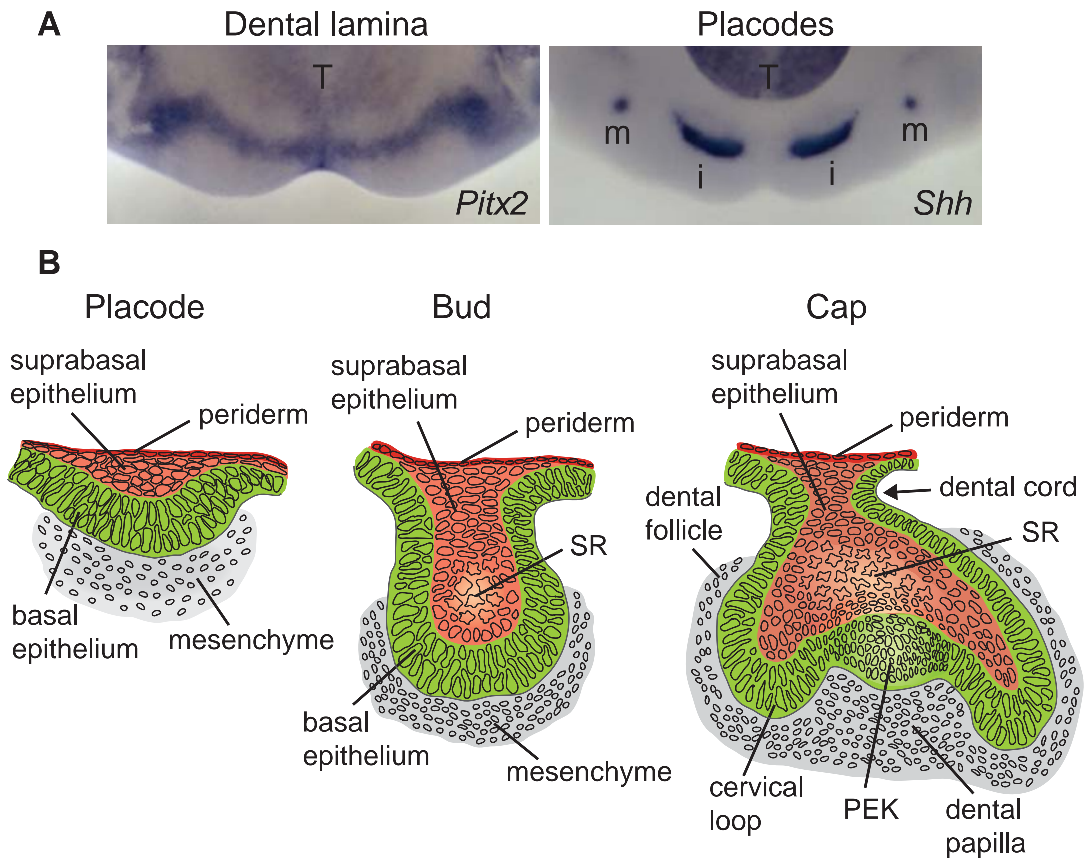


Figure 2. Tooth development

A: Whole mount in situ hybridization of embryonic lower jaws with *Pitx2* and *Shh*. Tooth development is initiated in mouse at E11.5 by formation of the primary dental lamina, which expresses markers such as *Pitx2*. Individual tooth placodes of molars (m) and incisors (i), visualized by *Shh* expression, form within the dental lamina by E12.5. T = tongue. Pictures courtesy of Anne Aalto.

B: At placode stage, a developing tooth consists of suprabasal and basal epithelial cell populations, which lie under the periderm. Mesenchyme has started to condense under the placode. At bud stage, the stellate reticulum (SR) begins to form in the core of the bud epithelium. The dental mesenchyme has further condensed around the bud. At cap stage, the enamel organ is connected to the oral surface by a dental cord. Stellate reticulum (SR) is more prominent, the primary enamel knot (PEK) has formed, and cervical loops begin to grow. The dental mesenchyme is divided into the dental papilla, which will become encompassed by the cervical loops at later stages, and the dental follicle, which surrounds the enamel organ.

The primary enamel knot (PEK), a signaling center, forms in the center of the enamel organ (Butler 1956, Fig. 2B and 3A). It secretes a number of signaling pathway ligands, and thus instructs the flanking epithelium to proliferate and form the cervical loops (Jernvall et al. 1994, Vaahtokari et al. 1996a). The cervical loops grown downwards and participate later in root formation (Fig. 3C). There are signaling centers already in the placode and in the tip of the bud, but unlike the PEK, they are not histologically distinct from the surrounding epithelium. The PEK is a round structure running along the length of the enamel organ, and is composed of cells from both basal and suprabasal cell populations. Within the cervical loops, there is a core of stellate reticulum cells (Fig. 2B). The epithelium between the PEK and the cervical loops is called the inner enamel epithelium, and it will eventually give rise to ameloblasts. The enamel organ and the dental mesenchyme are separated by a basal lamina, which is composed of extracellular matrix (Thesleff et al. 1989). The mesenchyme, which starts to become encompassed by the cervical loops, forms the dental papilla, and it will later give rise to odontoblasts (Fig. 2B). The outer enamel epithelium (OEE) faces the dental follicle mesenchyme, which surrounds the enamel organ (Fig. 2B, Fig. 3B). At the bell stage in molars, the secondary enamel knots (SEKs) form at the sites of the future cusps, which are the occlusal tips of the tooth (Jernvall et al. 2000, Fig. 3B). The SEKs instruct the surrounding inner enamel epithelium (IEE) to grow down and fold to generate the shape of the tooth crown (Fig 3B,C). The SEKs form only in the teeth that have multiple cusps.

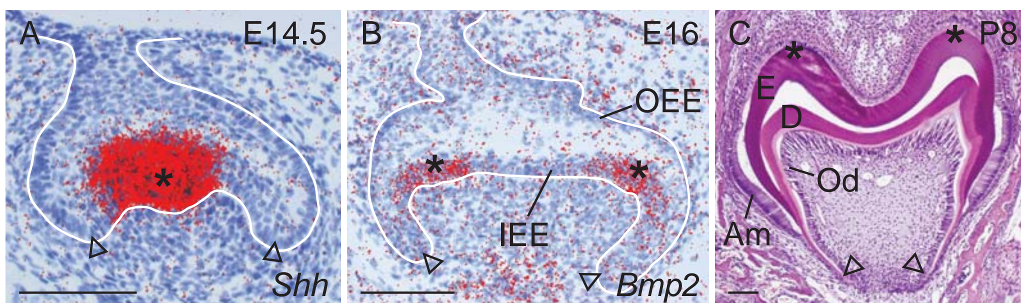


Figure 3. Primary and secondary enamel knots regulate tooth crown shape
A: At the cap stage at E14.5 the primary enamel knot (asterisk) expresses a number of signaling pathway ligands that instruct the cervical loops (arrowheads) to grow. Here, *Shh* expression is detected by radioactive in situ hybridization. The white line indicates the border between epithelium and mesenchyme.
B: At the bell stage at E16 the secondary enamel knots (asterisks) have formed in the inner enamel epithelium (IEE) at the locations of the future cusp tips of the tooth crown. Here, *Bmp2* expression in SEKs is detected by radioactive in situ hybridization. Arrowheads point to growing cervical loops. OEE = outer enamel epithelium. The white line indicates the border between epithelium and mesenchyme.
C: Hematoxylin-eosin staining at P8 shows the unerupted first molar. Ameloblasts (Am) have secreted enamel (E), and the tips of the tooth crown (asterisks) are the sites where secondary enamel knots were located during development. Dentin (D) has been secreted by odontoblasts (Od). The cervical loops have formed roots (arrowheads).
Scale bar 100 μm.

1.2.4. Differentiation of tooth-specific cell types

Differentiation of the tooth-specific cell types begins at the bell stage. Odontoblasts start to differentiate first. They arise from a cell layer of the dental papilla underlying the basement membrane and IEE. Signals from the odontoblasts initiate ameloblast differentiation from the IEE on the opposite side of the basement membrane. Odontoblast and ameloblast differentiation involves important changes in cell shape as the cells proceed through different secretory stages. Differentiating odontoblasts and ameloblasts lay down the dentin and enamel matrices, respectively (Fig. 3C). Mineralization of the tooth crown takes place first at the cusp tips and then moves downwards. The cervical loops begin to make roots once ameloblast differentiation has reached the future crown-root border. Here, the cervical loops become depleted of stellate reticulum cells, and the IEE and OEE form a bilayer called Hertwig's epithelial root sheet (HERS). HERS continues to grow down for a limited length and induces the adjacent dental follicle cells to differentiate into odontoblasts to produce root dentin. Finally the HERS disintegrates into an epithelial network called epithelial rests of Malassez (ERM), allowing the dental follicle cells to come in contact with root dentin and differentiate into cementum-depositing cementoblasts. In addition, the dental follicle cells give rise to the periodontal ligament that connects the tooth to the osteoblasts that form the alveolar bone where the periodontal ligament fibers are embedded.

The ever-growing rodent incisor is asymmetric. More precisely, in these teeth, ameloblasts differentiate only on the labial side to secrete enamel, whereas dentin is present on both labial and lingual surfaces. Consequently, the labial side is called the crown analogue, and the lingual side the root analogue. In the continuously growing molars of animals such as voles and sloths the HERS does not form, and stem cells in the cervical loops support the production of both ameloblasts and root epithelium (Tummers and Thesleff 2003, Tummers and Thesleff 2008).

1.3. Molecular regulation of tooth development

1.3.1. Initiation and regulation of ectodermal organ development

There are some differences in the initiation of ectodermal organ development among the different organs. Development of the teeth and mammary glands is initiated even before placode induction, by the formation of a dental lamina or a milk line. These are locally thickened stripes of epithelium along the jaws or along the flanks of the embryo. There is evidence that the milk line is formed by cell migration rather than cell proliferation (Propper 1978, Propper et al. 2013). Similarly, hair placodes arise from directional migration of epithelial cells towards the center of the placode (Ahtiainen et al. 2014). The cellular mechanism by which the dental lamina and tooth placodes form has not been established. The potential to initiate the organ development has been shown to reside first in the epithelium for teeth, but for hairs and mammary glands the current evidence points at the potential residing in the mesenchyme (Biggs and Mikkola 2014).

The most important regulators of the early ectodermal organ development are Ectodysplasin and Wnt pathways, and transcription factor p63. *P63* is expressed throughout the ectoderm, and when it is deleted in mice, all ectodermal organs fail to form, although the dental lamina develops (Laurikkala et al. 2006). Similarly, overexpression of Wnt pathway antagonist *Dkk1* in the epithelium inhibits hair and mammary gland initiation completely, and tooth development arrests at placode stage (Andl et al. 2002, Chu et al. 2004, Liu et al. 2008). Alternatively, activation

of Wnt pathway in the epithelium leads to formation of ectopic hair and supernumerary teeth (Järvinen et al. 2006, Liu et al. 2008, Närhi et al. 2008, Zhang et al. 2008).

The importance of Ectodysplasin (Eda) signaling pathway in ectodermal organ development is illustrated by hypohidrotic ectodermal dysplasia (HED) syndrome, which results from mutations in the Eda pathway genes (Mikkola 2009). Human patients have sparse hair, missing and abnormally shaped teeth, and absence of sweat glands. HED is also found in mice, cattle, dogs, and even fish (Drögemüller et al. 2003, Iida et al. 2014, Kowalczyk-Quintas and Schneider 2014). Overexpression of *Eda* in the epithelium in mice causes ectopic hair and mammary placodes and a supernumerary tooth to form (Mustonen et al. 2004).

1.3.2. Odontogenic potential and molecular regulation of tooth initiation

Teeth have a remarkable self-organizing capacity, which was demonstrated before any knowledge on the molecular regulation of tooth development was established. Studies by Shirley Glasstone showed that if a mouse first molar tooth germ is dissected from the embryo and grown in vitro, it will develop normally, independent of the surrounding tissues (Glasstone 1936, Glasstone 1967). Placing the first molar germ in the anterior chamber of the eye of a recipient mouse, a common technique used to grown organs in a physiological environment, will result in all three molars developing (Lumsden 1979). The tissue origin of the odontogenic potential has been studied by recombining epithelium and mesenchyme of different stages together and following which of these combinations give rise to teeth. These experiments have shown that the odontogenic potential resides initially in the first brancial arch epithelium, and it shifts to the mesenchyme around the placode stage when the dental mesenchyme condensates. First branchial arch epithelium from E9 to E11 embryos can induce tooth development when recombined with second arch mesenchyme, or even with trunk neural crest cells (Lumsden 1988, Mina and Kollar 1987). After E12, the epithelium can no longer induce tooth formation, but at this time point, the dental mesenchyme can form teeth when recombined with second arch epithelium, or even with limb epithelium (Kollar and Baird 1970, Mina and Kollar 1987). Once mesenchyme has acquired odontogenic competence, it also contains the information for tooth shape (Kollar and Baird 1969).

The early events in the initiation of tooth formation seem to be largely regulated by Fgf, Bmp, and Wnt pathways. Before dental lamina formation, *Bmp4* is expressed in the distal branchial arch epithelium, overlapping with *Fgf8* in the proximal region (Neubüser et al. 1997). Many Wnt ligands are expressed in the epithelium, and Wnt signaling has been shown to induce *Fgf8* in the epithelium (Sarkar and Sharpe 1999, Wang et al. 2009). It is not known how activity of these signaling pathways leads to the formation of the dental lamina and to the restricted gene expression within it. The dental lamina is known to express signaling pathway ligands *Shh*, *Bmp2*, *Wnt10*, *Wnt10b* and the transcription factor *Pitx2* (Dassule and McMahon 1998, Keränen et al. 1999).

Bmp signaling in the early epithelium is important, since overexpression of Bmp inhibitor *Noggin* in the epithelium from the dental lamina stage onwards leads to an arrest of tooth development at placode stage (Wang et al. 2012). When the odontogenic potential shifts to the mesenchyme, *Bmp4* induces its own expression in the dental mesenchyme (Vainio et al. 1993). In the incisors, this shift of *Bmp4* is promoted in addition by Wnt pathway (Fujimori et al. 2010). *Bmp4* regulates also the shift of Wnt pathway transcription factor Lef1 from the epithelium to mesenchyme (Kratochwil et al. 1996). Fgf signaling in the epithelium induces *Activin*, *Fgf3*, and

Fgf10 expression in the mesenchyme, and these signals, together with *Bmp4* and others, regulate reciprocally the subsequent morphogenesis of the epithelium (Ferguson et al. 1998, Kettunen et al. 2000). Fgf signaling in the incisor mesenchyme has been shown to antagonize Wnt signaling in order to support the odontogenic properties of the tissue (Liu et al. 2013).

The induction of odontogenic potential in the mesenchyme is accompanied by induction of expression of several transcription factors by signaling pathways ligands such as Bmp4, Fgf8 and others. These induce *Msx1*, *Pax9*, *Lhx6*, *Lhx7*, *Barx1*, *Dlx1*, *Dlx2, Runx2* as well as Shh pathway mediators *Gli1* and *Gli2* (Bei 2009). Even though none of these factors are expressed exclusively in developing teeth, it is most likely that it is their combination that gives the mesenchyme an odontogenic identity.

1.3.3. Molecular regulation of placode and bud stages

When the placodes form within the dental lamina, a subset of the epithelial cells within the placode forms a signaling center, which continues to express the dental lamina genes *Shh*, *Wnt10a*, *Wnt10b*, and *Bmp2* (Dassule and McMahon 1998). The signaling center also expresses *Lef1*, whereas dental lamina gene *Pitx2* is expressed in the whole placode epithelium (Mucchielli 1997, Sasaki et al. 2005). A number of mouse mutants display arrested tooth development at the transitions from the dental lamina to placode formation or from placode to bud stage, highlighting the importance of several signaling pathways and important transcription factors in this process. The reciprocal signaling between the placode epithelium and the underlying mesenchyme regulates the key transition between the placode and bud stages.

The mutants with a lamina stage arrest include *Msx1*;*Msx2* double knock-outs (KOs), and *p63* KO (Bei and Maas 1998, Laurikkala et al. 2006, Yang et al. 1999). A placode stage arrest is observed in teeth of *K14-Dkk1* transgenic mice, in *Fgfr2IIIb* KO, and in mice that conditionally express the Bmp inhibitor *Noggin* in the epithelium, as well as in all maxillary teeth of *Pitx2* KO, in the maxillary molars of *Dlx1*;*Dlx2* double KO, and in maxillary incisors of *Gli2*$^{-/-}$;*Gli3*$^{+/-}$ embryos (Hardcastle et al. 1998, Hosokawa et al. 2009, Lin 1999, Lu et al. 1999, Thomas 1997, Veistinen et al. 2009, Wang et al. 2012). Unfortunately, in many of these cases, the dental lamina formation has not been analyzed, which makes it difficult to compare the severity of the phenotype of these different mouse models.

There are also mouse mutants where development proceeds past the placode stage and arrests at the bud stage. Here, the next important morphogenetic events, formation of the primary enamel knot (PEK) and the cervical loops, do not take place. In the *Pitx2* KO, mandibular teeth arrest at the bud stage (Lin 1999, Lu et al. 1999). The development of jaws in *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ double KOs seems severely affected, but some tooth development up to bud stage can be observed (Hardcastle et al. 1998). The Wnt transcription factor *Lef1* is important for bud morphogenesis. It is expressed both in the epithelium and mesenchyme, and in *Lef1* KOs both epithelial and mesenchymal genes are affected in the arrested teeth (van Genderen et al. 1994). Deleting the Wnt effector β-catenin only from the mesenchyme leads to downregulation of *Lef1* in the mesenchyme and together with changes in other genes, results in a failure to form the PEK (Chen et al. 2009). Development also arrests at bud stage when the Bmp pathway is affected by deleting *Msx1* or overexpressing *Bmpr1a* in the epithelium (Andl et al. 2004, Satokata and Maas 1994). When *Bmpr1a* is conditionally deleted from the mesenchyme, molars stop at bud stage and incisors even earlier (Li et al. 2011). In addition to a placode stage defect discussed in the previous paragraph, later activation of the Bmp antagonist *Noggin* expression in the

epithelium leads to an arrest of mandibular molar development, where the proper mesenchymal condensation does not form (Plikus et al. 2005). There is also another study on *Fgfr2IIIb*, where instead of a placode stage arrest, only the incisor development stops at bud stage (De Moerlooze et al. 2000). In *Activin bA* KO maxillary molars are arrested, and deleting the mesenchymally expressed *Pax9* leads to a failure to support the dental mesenchyme past bud stage (Ferguson et al. 1998, Peters et al. 1998). In transcription factor *Runx2* KO the arrested tooth begins to form multiple irregular buddings (Åberg 2004a). As all these examples include signaling pathway components and transcription factors that are expressed in both epithelium and in mesenchyme, they clearly illustrate how the reciprocal signaling between the two tissues within the tooth germ is critical for the advance of the proper development.

1.3.4. Cell-level mechanisms regulating placode and bud formation

The signaling pathways that regulate tooth morphogenesis ultimately change cell behavior. There is both proliferation and apoptosis taking place during development. In addition, changes in cell-cell and cell-matrix adhesion must occur when the shape of the epithelium changes and the mesenchyme condenses. It is also possible that cell migration plays a role, but altogether these events have still not been studied in great detail during tooth development.

Proliferation is important for the growth of all developing organs. In some cases, the arrest of tooth development has been linked to decreased proliferation in either the epithelium or in the mesenchyme (Kettunen et al. 2007, Li et al. 2011, Veistinen et al. 2009, Wang et al. 2012). Conversely, tooth development arrests in *Lef1* mutants, but this has been shown to be due to increased epithelial apoptosis, and not decreased proliferation (Sasaki et al. 2005). Changes in proliferation and apoptosis have also been linked to the rescued development of the rudimentary premolar, which forms in the diastema in front of the first molar in some mutant mice (Lagronova-Churava et al. 2013). Even though there are apoptotic cells in the PEK, and already earlier in the tooth bud epithelium, mouse mutants with defects in different apoptosis pathway genes have no tooth phenotype (Jernvall et al. 1998, Matalova et al. 2006, Matalova et al. 2012, Matalova et al. 2012, Setkova et al. 2007, Vaahtokari et al. 1996b). Because of this, it has been difficult to determine what the role of apoptosis in tooth morphogenesis is.

During early development, the tooth placode grows down to the underlying mesenchyme. This process is impaired in the incisors of *Irf6* and *Ikkα* KO embryos, and their incisors evaginate outwards instead of forming a bud (Blackburn et al. 2012, Ohazama et al. 2004). The cell biological mechanism behind this phenotype remains unknown. More is known about the regulation of the mesenchymal condensation. *Fgf8* and *Semaphorin 3F*, secreted by the placode epithelium, induce mesenchymal cells to pack tightly by attracting and repulsing them, respectively (Mammoto et al. 2011). Interestingly, this physical change in the cell shape induces expression of dental mesenchymal markers *Pax9* and *Msx1* in the condensed cells. In addition, the condensed mesenchyme expresses cell surface and extracellular matrix proteins, such as tenascin and syndecan (Vainio and Thesleff 1992).

Distinct cell populations in the developing tooth epithelium express different adhesion molecules, such as the tight junction proteins, claudins (Ohazama and Sharpe 2007). Desmosomal adherence proteins desmoglein and γ-catenin show specific localization to the suprabasal cells at bud stage, suggesting that they may play a role in the bud and dental cord morphogenesis (Fausser et al. 1998). E-cadherin and P-cadherin are expressed in the epithelium with E-cadherin expression being stronger in suprabasal cells and P-cadherin in basal cells (Fausser et al. 1998,

Palacios et al. 1995). Integrins mediate cell-matrix adhesion, and they are also located in different compartments of epithelium and mesenchyme in developing teeth (Jaspers et al. 1995, Salmivirta et al. 1996, Yamada et al. 1994). The exact function of these adhesion molecules in tooth morphogenesis remains unknown, in part because early tooth phenotypes for mutations of these genes have not been reported. In addition, their expression in different mouse mutants has not been analyzed. However, some adhesion molecules have been examined in adult teeth. In mouse incisor, E-cadherin has been shown to be important for incisor renewal (Li et al. 2012), while in molars Nectin-1 is important for late ameloblast differentiation (Yoshida et al. 2010). Deletion of β-1 integrin disrupts cusp formation and ameloblast differentiation in molars, but the early morphogenesis up to bell stage is normal (Chen et al. 2009).

1.3.5. Molecular regulation of the primary enamel knot

The published literature suggests that once the primary enamel knot (PEK) has successfully formed, mutation of important genes will not cause a full arrest of tooth development anymore. The later developmental defects usually relate with problems in the patterning of the secondary enamel knots (SEKs) or with cell differentiation.

The PEK is a histologically visible condensation of cells within the enamel organ that does not proliferate (Butler 1956, Jernvall et al. 1994). It is not known how cells become specified as PEK and what regulates the differential adhesion that forms the PEK shape. It expresses *p21*, a cyclin-dependent kinase inhibitor that suppresses the cell cycle (Jernvall et al. 1994). The PEK expresses a number of Fgf ligands, but it lacks Fgf receptor expression (Kettunen and Thesleff 1998). Therefore only the surrounding epithelium is induced to proliferate by Fgf signaling from the PEK. Other pathway ligands and transcription factors expressed by the PEK include *Shh, Bmp2*, *Bmp4*, *Bmp7*, *Wnt10a, Wnt10b, Follistatin*, *Msx2,* and *Lef1* (Vaahtokari et al. 1996a, Dassule and McMahon 1998, Keränen et al. 1998, Wang et al. 2004). Similarly as there are no tooth-specific genes that determine the odontogenic identity, there are no enamel knot specific genes that determine the identity of the PEK cells. It is not known how these cells acquire their fate, but it is most likely regulated by a combination of the signals that regulate all the steps of tooth development.

Before formation of SEKs, the PEK disappears apoptotically (Jernvall et al. 1998). A novel marker for the PEK is Gadd45g (growth arrest and DNA damage inducible gene), which is expressed already in the placode and bud signaling centers (Ishida et al. 2013). Its functions include regulation of cell cycle inhibition and apoptosis, among others. Adenoviral overexpression of *Gadd45g* in molar cervical loops in tissue culture results in upregulation of *p21* expression (Ishida et al. 2013). However, the *Gadd45g* KO mice have morphologically normal but slightly smaller molars and thus it is not a key gene for PEK formation.

The receptor of Ectodysplasin, Edar, is expressed in the PEK (Laurikkala et al. 2001). The main role of the Eda pathway in tooth is the regulation of tooth shape and size. Overexpression of *Eda* in the epithelium induces larger PEKs and more and bigger cusps, whereas *Eda*$^{-/-}$ mice have smaller PEKs and smaller teeth with fewer cusps (Kangas et al. 2004, Mustonen et al. 2003). *Fgf20* is a direct downstream target of Eda in the PEK, and its deletion results in a similar cusp phenotype as in the *Eda*$^{-/-}$ mice (Häärä et al. 2012). This suggests that Eda signaling functions in the PEK through Fgf20.

The Wnt pathway has also been demonstrated to be critical in PEK development. Forced activation of Wnt pathway in the epithelium leads to formation of ectopic PEK-like signaling

centers, suggesting that Wnt signaling is an enamel knot activator (Järvinen et al. 2006). Wnt pathway is active also in the dental mesenchyme. Nevertheless, the phenotype of a mutant mouse for *Gpr177*, a protein regulating Wnt ligand sorting and secretion, supports the role of Wnts as enamel knot regulators within the epithelium (Zhu et al. 2013). This study showed that conditional deletion of *Gpr177* from the epithelium leads to downregulation of several PEK markers and an arrest in molar development at a stage, where some rudimentary cervical loop growth and epithelial morphogenesis has taken place. This seems to be one of the rare mutations where tooth development indeed arrests at cap stage. The mesenchyme is not affected by a lack of secreted Wnt ligands from the epithelium, as it can form a normal tooth when recombined with a wild type epithelium. Though Wnt pathway is active in both the dental epithelium and mesenchyme, these data support a role for epithelial Wnt to be crucial for PEK development.

YAP is a co-activator of the Hippo pathway, which has been linked to organ growth. Its overexpression in the epithelium results in a very interesting PEK phenotype (Liu et al. 2014). Normally *YAP* is expressed in the epithelium, with a slightly stronger expression in the IEE. In molars overexpressing *YAP* there are cells with PEK morphology in the center of the enamel organ within the stellate reticulum instead of in the normal PEK location. Known PEK signaling molecules, however, are expressed in cells at the normal PEK area in the IEE adjacent to the dental papilla. Only aberrant or no cervical loop growth takes place in these teeth. This phenotype suggests that the PEK cells and the signaling activity of the signaling center may be differentially regulated.

1.3.6. Molecular regulation of tooth shape and number

The shape of the tooth crown is defined by the positions of the secondary enamel knots that secrete largely the same signaling molecules as the PEK. They appear in sequence in the IEE after PEK disappearance, and are also non-proliferative (Coin et al. 1999). The final shape of the enamel organ is in addition regulated by further signals that act during the epithelial morphogenesis. Phenotypes of mouse mutants have revealed genes regulating enamel organ shape, fusion of teeth, and also the number of teeth within the dentition in mice.

The second molar (M2) forms by budding from the posterior epithelium of the M1, and this process is affected in several mutant mice. The two molars become fused in *Sostdc1* KO and *Lrp4* KO (Kassai et al. 2005, Ohazama et al. 2008). Sostdc1 is an inhibitor of Bmp and Wnt pathways, and Lrp4 a cell surface receptor that negatively regulates Wnt pathway. Sostdc1 has been shown to bind Lrp4, and the loss of *Sostdc1* has been linked to elevated Wnt signaling (Ahn et al. 2010, Ohazama et al. 2008). Sostdc1 has also been shown to regulate the number and size of hair and mammary placodes (Närhi et al. 2012). Likewise, in mammary glands, *Lrp4* and *Sostdc1* mutations cause a similar phenotype inducing supernumerary and fused nipples, and they are both linked to upregulated Wnt activity (Ahn et al. 2013, Närhi et al. 2012). These results suggest that Sostdc1 has a widespread role in regulating ectodermal organ number.

A similar fusion of M1 and M2 is observed when *Shh* or its receptor *Smoothened* is conditionally deleted from the epithelium, or when Shh blocking antibodies are administrated to pregnant females (Cho et al. 2011, Dassule et al. 2000, Gritli-Linde et al. 2002). Deletion of *Evc*, a primary cilium component and therefore linked to Shh signal transduction, leads to fully penetrant fusion of maxillary molars, but the phenotype is less severe in mandibular molars (Nakatomi et al. 2013). Studies on the interactions of Sostdc1 and Shh pathway have shown that there is increased Wnt activity in the Shh mutants, linking the *Sostdc1* KO and Shh pathway

mutant phenotypes (Ahn et al. 2010, Cho et al. 2011). In addition to the molars not separating from each other properly, in the *Shh* cKO and *Evc* KO, the tooth germ does not separate from the oral epithelium due to a lack of the dental cord (Dassule et al. 2000, Nakatomi et al. 2013). The cervical loop growth is also affected in these mutants, suggesting that the Shh pathway acts upstream of several important morphogenetic changes taking place in the dental epithelium.

During evolution, the incisor number in the mouse dentition has been reduced from three to one pair. Experimental evidence suggests that the big mouse incisor is actually formed by a fusion of the three ancestral placodes. Inhibiting Bmp signaling by Noggin protein or treating incisors with Activin A leads to a disintegration of the big incisor placode into smaller teeth (Munne et al. 2010). Manipulation of the Bmp pathway can sometimes even lead to a fusion of the two normal mouse incisor placodes. Deletion of Bmp pathway inhibitor Noggin results in a fusion and bud stage arrest of maxillary incisors, whereas other teeth are normal (Hu et al. 2012). Also when *BmprI1* is conditionally deleted from the mesenchyme, maxillary incisors fuse and arrest in development (Li et al. 2011). In addition, in Shh pathway mediator *Gli2* KO embryos maxillary incisors often fuse, but develop otherwise fairly normally (Hardcastle et al. 1998). These examples of molar and incisor fusions illustrate the plastic nature of the dental epithelium, and show that under favorable conditions they can fuse and continue the normal developmental process. The examples of the maxillary incisor fusion also point out how teeth in different parts of the jaws are not equally affected by different mutations. Similar phenotypes exist also for molars. Maxillary molars do not develop in *Dlx1;Dlx2* double KO, but in *Activin bA* KO maxillary molars are unaffected and all the other teeth become arrested in development (Ferguson et al. 1998, Thomas 1997). There are clearly differences in the sensitivity of different teeth to the signaling pathway levels. One factor contributing to this is that the maxillary incisors develop from the frontonasal process, whereas all the other teeth develop from the first branchial arch.

In addition to missing teeth, there are also mouse mutants with extra teeth. The most peculiar one is the *Osr2* KO, where an extra tooth develops next to the normal first molar on the lingual side of the tooth row (Zhang et al. 2009). This is accompanied by expansion of *Bmp4* expression from the buccal, or cheek side, dental mesenchyme to the lingual mesenchyme. Normally, mesenchymal Bmp4 as well as transcription factor Pax9 have been linked to the maintenance of dental mesenchyme during normal molar development, since reduction in their doses leads to a decreased number of molars (Jia et al. 2013, Kist et al. 2005). More often a supernumerary tooth develops in front of the first molar in mutant mice. This rudimentary premolar bud present in the mouse jaw has lost its capacity to give rise to a tooth during rodent evolution. Its development is rescued in mice overexpressing *Eda*, in Fgf inhibitor Sprouty mutants, in *Polaris* and *Gas1* mutants with enhanced Shh signaling, in *Lrp4* KO, and in *Sostdc1* KO (Kassai et al. 2005, Klein et al. 2006, Mustonen et al. 2003, Ohazama et al. 2008, Ohazama et al. 2009). This suggests that though this region is unable to produce a tooth, it is still competent to grow when the balance of signaling pathway activity is manipulated under correct conditions.

1.4. Generation of new teeth in vertebrates

In mice, the generation of new teeth no longer takes place after the development of the incisors and the molars is completed. However, rodents have evolved a mechanism that allows the incisors to remain sharp even with a hard diet that continuously abrades the tooth crown. The keys to maintaining a sharp tooth are the difference in hardness between enamel and dentin, and the asymmetric deposition of these two hard tissues in the incisor. The harder enamel is located only on the labial surface and the wear during gnawing hard food results in faster abrasion of dentin compared to enamel, and in a sharp incisor tip. In order to compensate the continuous wear of the tooth, the incisor renews throughout the life of the animal by a continuous production of new enamel- and dentin-secreting cells from the proximal end of the tooth. These new cells are generated by mesenchymal and epithelial stem cell populations. The epithelial stem cell niche in the labial cervical loop is well characterized, and the stem cells have been shown to express different known stem cell markers, such as *Sox2* and *Bmi1* (Biehs et al. 2013, Harada et al. 1999a, Juuri et al. 2012, Seidel et al. 2010).

After the molars have formed during development, they are normally not replaced in any mammal (Fig. 4A on page 16). Nevertheless, very few species, such as the silvery mole rat, continue to produce new molars from the back of its jaws throughout the life of the animal (Rodrigues et al. 2011). Also, this process has to involve stem cells, but they have not been characterized. Molar number is controlled by an inhibitory cascade, which normally inhibits the development of the posterior molars. This cascade is likely to be unlocked in the mole rat. This seems to take place occasionally in humans and mice as well, as fourth molars are present in some individuals (Shahzad and Roth 2012, and unpublished observations from the Thesleff laboratory). In human cleidocranial syndrome, caused by *RUNX2* mutations, one or more supernumerary molars are also observed (OMIM #119600, Jensen and Kreiborg 1990). However, in humans, it is more common to have missing teeth than supernumerary teeth. The affected teeth are usually permanent teeth that are last to develop in a tooth family, such as second premolars or third molars (Nieminen 2009). Eda signaling pathway and genes including *Wnt10a*, *Msx1*, *Pax9*, and *Axin2* have been associated with human tooth agenesis.

The continuous molar formation in the silvery mole rat is considered to represent a modification of the general vertebrate tooth replacement. New teeth in vertebrates are usually generated through replacement that takes place on the lingual side of the previous tooth. In mammals this happens once, when the deciduous or primary teeth are replaced by the permanent teeth (Fig. 4B). In reptiles and fish this can take place several times during the lifetime of the animal (Fig. 4C).

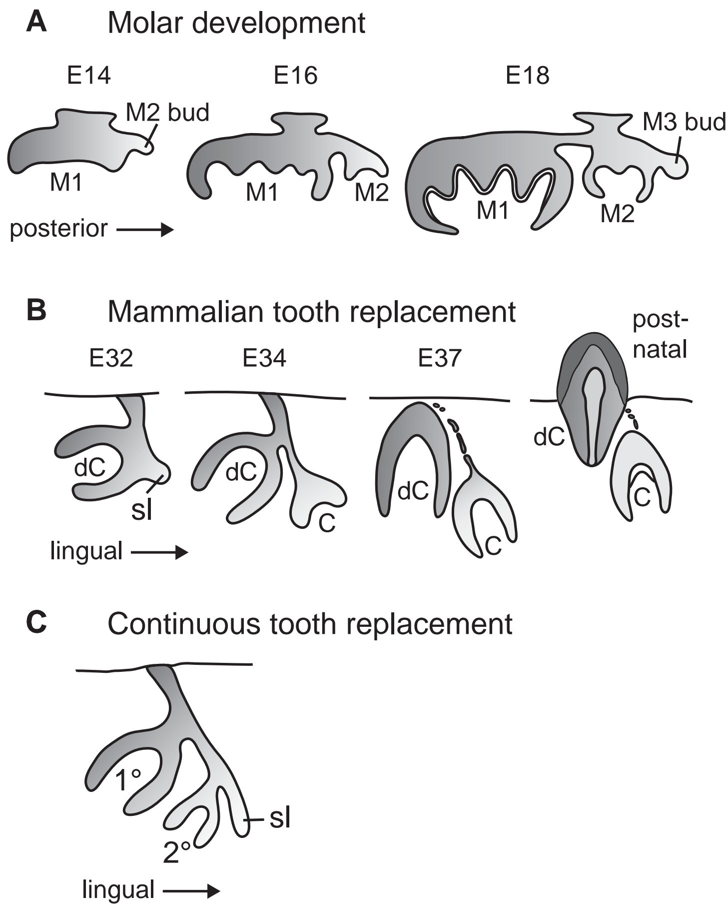


Figure 4. Schematic illustration of molar development and tooth replacement
A: The secon and third (M2 and M3) mouse molar develop from the epithelium of the previous tooth in the anterior-posterior direction.
B: In mammals, permanent (C = permanent canine) teeth are initiated from the successional lamina (sl), which splits from the dental lamina embedded on the lingual side of each deciduous tooth (dC = deciduous canine).
C: During continuous tooth replacement in reptiles, new generations of teeth are formed by repeated initiation of successional lamina (sl) formation (1 and 2 = first and second generation teeth).

1.4.1. Tooth replacement in mammals

Mammals replace their teeth either once or not at all. Some mammals, like rodents, have lost tooth replacement altogether. In others, such as in some shrew species, replacement teeth are the only teeth that become functional, and the primary teeth regress during development (Järvinen et al. 2008, Yamanaka et al. 2010). The most detailed histological description of mammalian tooth replacement is available for the ferret (Järvinen et al. 2009). When the primary dental lamina grows down to the mesenchyme together with the developing tooth germs, it forms the dental lamina, which is embedded in the lingual side of each deciduous tooth (Fig. 5). Between teeth, the individual tooth germs are connected to each other by the interdental lamina (Fig. 5). In ferret embryos, the replacement of different teeth begins at slightly different time points during development. The replacement tooth is initiated from a successional dental lamina that splits

from the dental lamina embedded on the lingual side of the deciduous tooth (Fig. 4B, Fig. 5). The tip of the successional lamina forms a bud, and replacement tooth morphogenesis proceeds. After initiation, morphogenesis of the replacement teeth undergoes the same developmental steps as the primary teeth. Therefore the molecular regulation of replacement tooth morphogenesis is most likely largely the same that I have already described. For that reason, most research has focused on the initiation of tooth replacement and its regulation.

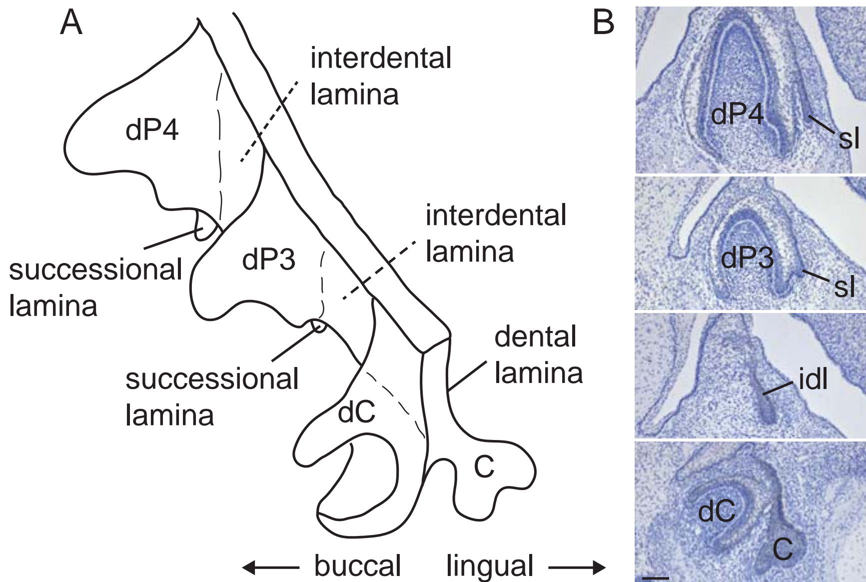


Figure 5. Relationship of deciduous teeth, the successional lamina, and the interdental lamina in the ferret.
A: A schematic illustration of the ferret tooth row between the deciduous canine (dC), deciduous third premolar (dP3), and deciduous fourth premolar (dP4) at E34. The dental lamina is embedded in the lingual side of each deciduous tooth epithelium. Interdental lamina connects the enamel organ of each deciduous tooth. Permanent canine (C) has reached the cap stage. Successional lamina has split off of the lingual side of the dP3 and dP4 to give rise to the permanent premolars.
B: Histological sections of the developing dP4, dP3, interdental lamina, and dC+C at E34.
Sl = successional lamina, idl = interdental lamina. Scale bar 100 μm.

Currently the expression patterns of only a few genes have been studied during ferret tooth replacement. *Sostdc1* is expressed in the epithelium between the splitting successional lamina and the deciduous tooth (Järvinen et al. 2009). The Wnt target gene *Axin2* is expressed in the successional lamina tip and in the condensed mesenchyme surrounding it. *Shh* is neither expressed during replacement tooth initiation in the ferret nor in shrews (Järvinen et al. 2008, Järvinen et al. 2009, Yamanaka et al. 2010). Other pathways have not been studied during ferret tooth replacement, and data on other mammals is not available. It is also not known why tooth replacement stops in mammals. During development, the dental lamina connecting the deciduous and permanent tooth disappears. It has been shown in the minipig that this process involves both apoptosis and an epithelial-to-mesenchymal transition of the dental lamina epithelium (Stembirek et al. 2010). This might not explain the lack of further initiation of replacement tooth

formation from permanent teeth, since in permanent teeth of human embryos a rudimentary successional lamina is visible (Ooë 1981).

1.4.2. Tooth replacement in reptiles

Studies on non-mammalian tooth replacement have focused on lizards and snakes (Richman and Handrigan 2011). Even though the shapes of reptilian teeth are usually simpler than those of mammalian teeth, the mechanism of replacement tooth initiation is thought to be similar. Like in mammals, a successional lamina gives rise to the replacement tooth in reptiles (Fig. 4B, C). The bearded dragon is a reptile species that develops only two generations of teeth. Similarly to human permanent teeth, the second generation teeth of the bearded dragon have a rudimentary successional lamina (Richman and Handrigan 2011). In contrast to other reptilian species, there is apoptosis in the tip of this successional lamina, and no Wnt pathway activity, which may contribute to the loss of further tooth replacement in this species and explain the similar loss of continuous replacement in mammals.

Expression of *Axin2* and *Lef1* indicate that there is Wnt activity in the tip of the successional lamina in reptiles, similar to the ferret (Handrigan and Richman 2010b). The Bmp pathway is also active in the successional lamina tip, and it has been shown to positively regulate Wnt activity (Handrigan and Richman 2010b). As in ferret and in shrews, Shh signaling is not active in the successional lamina in reptiles, but it has nevertheless been shown to play a role in replacement by negatively regulating the Wnt pathway (Handrigan and Richman 2010a, Handrigan and Richman 2010b). Further, a label-retaining experiment, which identifies slow-cycling cells, located putative stem cells in the dental lamina in the leopard gecko (Handrigan et al. 2010). These cells reside in the upper parts of the dental lamina next to the previous generation of teeth as well as in the interdental lamina between them, but not in the successional lamina. This area where these putative stem cells reside expresses some known stem cell markers of the hair follicle stem cell niche, and further, Wnt activity was shown to stimulate proliferation in this area. Together, these data provide solid evidence that reptiles utilize stem cells to produce replacement teeth.

More molecular data on gene expression in the reptilian dental lamina is available for the primary dental lamina when it grows down into the mesenchyme during the early stages of tooth development. The snake and human primary dental lamina share similar morphological features (Buchtova et al. 2008, Hovorakova et al. 2007). Also, the primary dental lamina and the successional dental lamina in reptiles are very similar structures, and therefore it is possible that the same signals regulate their initiation. However, as there is no primary dental lamina downgrowth in mice, the only molecular data available for the mammalian dental lamina are collected from studies on the ferret. In the interdental regions the ferret dental lamina expresses *Sostdc1* on the buccal side and *Axin2* in the tip, similar to the successional lamina (Järvinen et al. 2009). In reptiles, the primary dental lamina has Bmp and Wnt activity in the epithelium similar to the successional lamina, but also some Shh activity, and expression of *Edar* (Richman and Handrigan 2011). The expression of these pathway components in the epithelium is asymmetric, which probably relates to the asymmetric downgrowth of the dental lamina. Unlike in the successional lamina, the Shh pathway has been linked to the regulation of the asymmetric primary dental lamina growth in reptiles (Buchtova et al. 2008).

1.4.3. Supernumerary tooth formation in humans

There are several human syndromes that involve supernumerary tooth formation, and these also support the role of Wnt pathway and Runx2 in the regulation of tooth replacement. In humans, familial adenomatous polyposis (FAP) is caused by mutations in *APC*, and the increased Wnt activity in these patients causes potentially cancerous polyps to form in the intestine, but also results in formation of supernumerary teeth as well as odontomas, benign tumors that are composed of several small teeth (OMIM #175100, Wang and Fan 2011). Surprisingly, mutation in another Wnt pathway inhibitor *AXIN2* leads to missing teeth in humans (OMIM *604025, Lammi et al. 2004). The qualitative differences between these two mutations on Wnt signaling activity in humans is not known, but it is possible that they affect the epithelium and mesenchyme differently. Transcription factor Sox2 has been shown to inhibit Wnt signaling, and *SOX2* mutation in humans has been shown to lead to supernumerary tooth formation (OMIM *184429, Mansukhani et al. 2005, Numakura et al. 2010). *RUNX2* is mutated in cleidocranial dysplasia syndrome (CCD), and the supernumerary teeth in these patients have been shown to form as a third dentition and as posterior molars arising from the previously generated teeth (OMIM #119600, Jensen and Kreiborg 1990). Another gene causing supernumerary tooth formation in humans is *Interleukin receptor 11 α*, but the role of Interleukin signaling in tooth development has not been studied (OMIM *600939, Nieminen et al. 2011). These examples show that similar to mice, humans also have capacity for the generation of new teeth.

1.4.4. Supernumerary tooth formation in mice

Even though mice do not replace their teeth, phenotypes of different mutant mice have given us information on the possible regulation of tooth replacement. The examples of the *Osr2* KO and the mutant mice that form a supernumerary premolar suggest that there is potential in the mouse dentition to initiate formation of new teeth upon correct signals (Kassai et al. 2005, Klein et al. 2006, Mustonen et al. 2003, Ohazama et al. 2008, Ohazama et al. 2009, Zhang et al. 2009). Similar to data from the ferret and reptiles, evidence from mouse points to the important role of the Wnt signaling pathway in the regulation of tooth replacement. When Wnt pathway effector β-catenin is stabilized in the epithelium, Wnt signaling is activated. This leads to formation of supernumerary teeth in mice (Järvinen et al. 2006, Liu et al. 2008). The shape of these teeth resembles fish and reptilian teeth, and they were shown to form in succession from the previously formed teeth (Järvinen et al. 2006). Deletion of Wnt inhibitor APC from the epithelium results in a similar phenotype (Kuraguchi et al. 2006, Wang et al. 2009). Deletion of epithelial transcription factor *Sp6* also leads to supernumerary tooth formation, but it is not known if Sp6 interacts with the Wnt pathway (Nakamura et al. 2008). In addition, tooth development arrests in mice in which mesenchymal *Runx2* is deleted, and the arrested tooth epithelium begins to form epithelial buddings similar to those found in Wnt mutant mice (Åberg 2004a). The upper molars are less affected, but they form lingual epithelial protrusions resembling a successional lamina (Åberg 2004a, Wang et al. 2005). The link between Runx2 and the Wnt pathway is also not known. In mice, Runx2 was shown to mediate Fgf signaling from the epithelium to the mesenchyme (Åberg 2004b). Together, these data suggest that there is capacity for further tooth formation even within mouse dentition.

1.5. Integration of signaling pathways during tooth development

The complexity of the molecular regulation of tooth development is clearly illustrated by the examples of the numerous pathways involved in repressing the development of the rudimentary premolar in mouse jaw, as well as by all the numerous mouse mutants with defects in different aspects of normal tooth development. The current knowledge on the regulation of tooth replacement comes mostly from gene expression pattern data, and functional studies are required to uncover the hierarchy of the different signaling pathways in the regulation of formation of new teeth. Expression patterns and expression levels of the currently known molecular regulators of tooth development are available in two databases; Bite-it and ToothCODE (http://bite-it.helsinki.fi, http://compbio.med.harvard.edu/ToothCODE/, Nieminen et al. 1998, O'Connell et al. 2012)

Some experimental work has been done in order to understand the integration of the different signaling pathways during development. In mutant mice, the cusp patterns are usually less complex than in wild type mice, even though the complexity of tooth shapes has increased during evolution. Multiple cusps can be induced experimentally only after several signaling pathways are manipulated simultaneously (Harjunmaa et al. 2012). Mathematical modeling has been used to understand the regulation of cusp patterning. A model combining activating and inhibiting signals with tissue growth can explain the positioning of SEKs and it can even recapitulate the emergence of ectopic signaling centers that form as a result of activated epithelial Wnt signaling in mutant mice (Järvinen et al. 2006, Salazar-Ciudad and Jernvall 2002, Salazar-Ciudad and Jernvall 2010). Similarly, an inhibitory cascade functioning during molar development defines the size ratio between the successionally forming teeth. There are two signals acting on the successionally forming tooth. The previously formed molar expresses still unknown inhibitors, whereas signals, such as mesenchymal Bmp4 and Activin A, promote the growth of the subsequent molar (Kavanagh et al. 2007).

Recent technological progress has allowed the complex analysis of substantial amounts of data, and revealed simple principles inside the large number of potential signaling pathway interactions during tooth development. A systems biology approach to early tooth morphogenesis identified an epithelial-mesenchymal Wnt-Bmp circuit as the main determinant of odontogenic signaling interactions (O'Connell et al. 2012). It shows that these two pathways are upstream of the Shh and Fgf pathways, and the circuit activates Wnts, Shh, Fgfs, and Bmp4 in the epithelium and Fgfs and Bmp4 in the mesenchyme. In addition, even though there is such variation in tooth shapes and numbers among vertebrates, a network of certain core genes seems to have been in place already when teeth appeared in evolution (Fraser et al. 2009). The differences between species and different teeth most likely arise from small changes in the spatiotemporal regulation of gene expression, which can be achieved by differential promoter and enhancer activity. For instance, an enhancer region has been identified for Bmp4, which targets its expression to the early tooth epithelium, but not to the mesenchyme at later stages of development (Jumlongras et al. 2012). An additional complexity to the molecular regulation of tooth development comes from post-transcriptional and translational modifications such as the micro-RNAs, which regulate targeted degradation of mRNA. They have been shown be important regulators of tooth development both in the epithelium and in the mesenchyme (Cao et al. 2010, Cao et al. 2013, Jheon et al. 2011, Michon et al. 2010, Oommen et al. 2012).

1.5.1. Transcription factors in the regulation of tooth development

The same signaling pathways regulate tooth development from initiation to cell differentiation and to replacement tooth formation. They induce changes in gene expression of their target cells, which are mediated by transcription factors. During tooth development, a multitude of transcription factors is expressed both in the epithelium and mesenchyme. Many of them are known signaling pathway effectors, such as Wnt pathway factor Lef1 or the Gli-factors downstream of the Shh pathway. For others, the upstream regulators are still unknown. Similarly, the target genes of many transcription factors in tooth have not been identified.

Studies on known dental transcription factors have revealed a glimpse of the complex regulatory network controlling gene expression during tooth morphogenesis. *Pitx2* is an important epithelial transcription factor in tooth (Lin 1999, Lu et al. 1999), and it has been shown to bind a specific enhancer of *Bmp4* gene, which drives *Bmp4* expression in the dental lamina epithelium (Jumlongras et al. 2012). Furthermore, Pitx2 has been shown to interact with Wnt pathway effectors Lef1 and β-catenin in tooth (Amen et al. 2007, Vadlamudi et al. 2005). Pitx2 is also involved in its own regulation as it activates the expression of two other transcription factors, *Lhx6* and *Dact2* that both bind Pitx2 and inhibit its function (Li et al. 2013, Zhang et al. 2013). In the mesenchyme, epithelial Bmp4 induces *Msx1* and *Tbx2*, and these two factors have been shown to bind each other (Saadi et al. 2013). They regulate mesenchymal *Bmp4* expression, but, whereas Msx1 induces *Bmp4*, Tbx2 represses it, suggesting that these factors fine-tune the odontogenic identity of the dental mesenchyme. Msx1 has also been shown to interact with several other transcription factors, such as Lhx6 and Lhx8, in the dental mesenchyme, and depending on its interaction partners, control the cell cycle progression either by repressing or activating the cyclin-dependent kinase inhibitor $p10^{ink4d}$ (Zhao et al. 2013). Another mesenchymal factor, Osr2 negatively regulates tooth formation. In its absence, an extra lingual tooth develops (Zhang et al. 2009). It has been shown that Osr2 binds mesenchymal factors Msx1 and Pax9 possibly suppressing the dental identity in the mesenchyme during normal development (Zhou et al. 2011). On the whole, the interactions between the different transcription factors, their upstream regulators, and their target genes in the developing teeth remain to be uncovered.

1.5.1.1. Fox transcription factors

The Fox (forkhead box DNA-binding domain containing) transcription factor superfamily contains 50 genes and 19 subfamilies from Foxa to Foxs. The Fox factors function in development and in adult homeostasis, as well as in disease such as cancer (Benayoun et al. 2011, Lam et al. 2013). They act as traditional transcription factors, as well as pioneering factors and chromatin regulators (Lalmansingh et al. 2012). Pioneering transcription factors bind condensed chromatin opening it up for other factors to bind. Fox-factors have been shown to act as mediators of signaling pathways such as the Shh, TGF-β, and Wnt pathways (Benayoun et al. 2011).

In tooth, Foxo1 and Foxj1 are the only Fox factors whose function has been studied. Their deletion from the epithelium leads to defects in ameloblasts and enamel, and Foxj1 has been shown to interact with Pitx2 and Dlx2 (Poche et al. 2012, Venugopalan et al. 2008, Venugopalan et al. 2011). The Foxi-family contains three members, Foxi1, 2, and 3. *Foxi*-genes have been shown to be expressed during the early craniofacial development (Ohyama and Groves 2004). Mutations in zebrafish *Foxi1* cause defects in ear and jaw development (Nissen et al. 2003). In addition, *Foxi3* has been shown to be expressed in tooth buds and hair placodes in mouse (Drögemüller et al. 2008). In the same study, an identical mutation in *Foxi3* was identified in three

different hairless dog breeds, but the function of *Foxi3* during ectodermal organ development is unknown.

1.5.1.2. Sox transcription factors

Sox transcription factors (SRY-related high-mobility group box) regulate various aspects of embryonic development and adult homeostasis (Kamachi and Kondoh 2013, Sarkar and Hochedlinger 2013). There are 20 Sox-genes in the mouse. Sox genes are known to regulate neural and skeletal development, neural crest cells, hair follicle development, and sex determination in mammals (Kamachi and Kondoh 2013).

One of the Sox-genes, *Sox2*, belongs to the four transcription factors needed to generate induced pluripotent stem cells (iPS cells) from differentiated adult cells (Takahashi and Yamanaka 2006). *Sox2*-expressing stem cells have been found in several adult organs (Arnold et al. 2011). In tooth, *Sox2*-positive epithelial stem cells were shown to give rise to all epithelial cell lineages of the renewing mouse incisor, and in humans, mutation in *SOX2* causes supernumerary tooth formation (Juuri et al. 2012, Numakura et al. 2010). However, the expression of *Sox2* during molar development as well as its function has not been studied.

2. Aims of the study

The transcription factor *Foxi3* is mutated in hairless dogs, and it is expressed in bud stage teeth (Drögemüller et al. 2008). Otherwise, nothing is known about its role in tooth development. The transcription factor *Sox2* has been shown to mark the epithelial stem cells in the mouse incisor, and mutation in human *SOX2* causes supernumerary tooth formation (Juuri et al. 2012, Numakura et al. 2010). Its expression or function during mouse molar development has not been studied, and its potential role in dental stem cells in tooth replacement is not known. Altogether, the expression patterns of the important signaling pathways known to regulate tooth development have not been characterized in detail during tooth replacement in mammals. There are also additional features of the mammalian dentition, such as the formation of the interdental lamina, which cannot be studied in mice.

The aims of this thesis are:

1. To study the expression and regulation of *Foxi3* during tooth development and to analyze the phenotype of mice with a conditional epithelial deletion of *Foxi3* (*K14-cre43*;*Foxi3*$^{-/floxed}$).

2. To study the role of *Sox2* in mouse molar development, and investigate its role in tooth replacement in species with different replacement potential.

3. To study mammalian tooth development using the ferret as a model to describe the interdental lamina morphogenesis as well as to characterize gene expression patterns of putative regulators of tooth replacement in the successional lamina and interdental lamina.

3. Materials and methods

Mouse strains	**Reference**	**Used in publication**
β-*catenin*^{ex3flox}	Harada et al. 1999b	I, unpublished
Dermo1-cre	Yu et al. 2003	unpublished
Eda$^{-/-}$	Pispa et al. 1999	I, II
Foxi3$^{-/-}$ and $^{+/-}$	Edlund et al. 2014	II
Foxi$^{floxed/floxed}$	Andrew Groves, unpublished	II
K14-Eda	Mustonen et al. 2003	I
K14-cre	Huelsken et al. 2001	I
K41-cre43	Andl et al. 2002	II
NMRI	Jackson	I, II, III
Nude	Harlan	unpublished
R26R^{lacZ}	Soriano 1999	III
Shh::GFPCre	Harfe et al. 2004	III
Sostdc1$^{-/-}$	Kassai et al. 2005	I
Sox2$^{fl/fl}$	Smith et al. 2009a	III
Sox2-gfp	D'Amour and Gage 2003	III
Sox2CreERT2	Arnold et al. 2011	III

Other species used in the study		**Used in publication**
Human	(*Homo sapiens*)	III
Ferret	(*Mustela putorius furo*)	III, IV
Leopard gecko	(*Eublepharis macularius*)	III
Corn snake	(*Elaphe guttata*)	III
Ball python	(*Python regius*)	III
American alligator	(*Alligator mississippiensis*)	III
Green iguana	(*Iguana iguana*)	III

Mouse-specific in situ hybridization probes	**Reference**	**Used in publication**
Bmp7	Åberg et al. 1997	II
Dkk4	Fliniaux et al. 2008	II
Dusp6	James et al. 2006	II
Edar	Laurikkala et al. 2001	I
Fgf15	Kettunen et al. 2011	II
Foxi3	Ohyama and Groves 2004	I, II
Id1	Rice et al. 2000	II
Lef1	Travis et al. 1991	II
Msx2	Jowett et al. 1993	II
Notch1	Lardelli et al. 1994	II
Notch2	Lardelli et al. 1994	II
p21	Jernvall et al. 1998	II
Semaphorin3E	Jussila et al., manuscript	II
Sfrp5	Witte et al. 2009	II
Shh	Vaahtokari et al. 1996a	I, II
Sostdc1	Laurikkala et al. 2003	II
Sox2	Ferri et al. 2004	III
Sprouty2	Zhang et al. 2001	II
Wnt10a	Dassule and McMahon 1998	II

Antibodies	**Host species**	**Company**	**Used in publication**
β-catenin	mouse	BD Biosciences	IV
BrdU	mouse	Neomarkers	II
E-cadherin	mouse	BD Biosciences	II
Keratin-10	rabbit	Abcam	II
Lef1	rabbit	Cell Signaling Technology	unpublished
Sox2	rabbit	Millipore	III, unpublished
Sox2	goat	R&D Systems	III
P-cadherin	goat	R&D Systems	II

Method	Reference	Used in publication
Histology	II, III, IV	II, III, IV
3D reconstruction	Juuri et al. 2010, IV	IV
Gene expression induction experiment with beads	I	I
Gene expression induction experiment in hanging drop	Pummila et al. 2007, I, II	I, II
Quantitative RT-PCR	Pummila et al. 2007, I, II	I, II
Affymetrix Mouse Exon 1.0 ST Microarray analysis	II	II
Whole mount in situ hybridization	Wilkinson and Nieto 1993, I, IV	I, III, IV
Non-radioactive in situ hybridization on sections	Wilkinson and Nieto 1993, I	I
Radioactive in situ hybridization on sections	I, II, III, IV	I, II, III, IV
Cloning of ferret-specific in situ hybridization probes	IV	IV
Immunohistochemistry	III	III, unpublished
Immunofluorescence	II, IV	II, IV
Cell proliferation assay (BrdU)	II	II
Genetic lineage tracing with LacZ reporter mice	Seidel et al. 2010, III	III
Tissue culture of fluorescent reporter mice	III	III
Kidney capsule culture of embryonic tooth germs	Järvinen et al. 2006	unpublished

4. Results and discussion

4.1. Expression and upstream regulation of *Foxi3* (I and II)

The transcription factor *Foxi3* was identified as a causative mutation behind the phenotype of three different hairless dog breeds: the Mexican hairless dog, the Peruvian hairless dog, and the Chinese crested dog (Drögemüller et al. 2008). All these breeds have the same 7-bp duplication in the first of the two exons of *Foxi3* gene. This frameshift mutation produces a premature stop codon in the coding sequence. The hairless dogs are heterozygous for the mutation, and their homozygote littermates die in utero. Of the three, the phenotype of the Chinese crested dogs is the best characterized (Wiener et al. 2013). In this breed, the hair phenotype varies from completely hairless to semi-coated fur. All Chinese crested dogs have missing and misshapen teeth, but the correlation between the severity of the hair and tooth phenotypes has not been studied (Wiener et al. 2013). Furthermore, it is not known whether the deciduous or permanent teeth are affected. In the Chinese crested dogs, other ectodermal organs, such as sebaceous glands and sweat glands, are normal.

Foxi3 was shown to be expressed in mouse hair and tooth placodes (Drögemüller et al. 2008), but its expression during ectodermal organ morphogenesis and its function in organ development are unknown. We studied the expression of *Foxi3* during development of teeth, hairs, ectodermal glands and other organs (I). In all ectodermal organs, *Foxi3* was expressed in the epithelium, and it was not expressed in the skin or oral ectoderm. I studied *Foxi3* expression in tooth, and observed *Foxi3* expression in the dental lamina. As the placodes formed, *Foxi3* expression covered the whole placode epithelium. During the bud and cap stages, *Foxi3* was expressed more intensely on the lingual side of the tooth epithelium as compared to the buccal side. At bell stage there was no asymmetry in *Foxi3*, and it was expressed in all the epithelial compartments of the enamel organ: inner enamel epithelium (IEE), outer enamel epithelium (OEE), and stellate reticulum. At the second postnatal day, *Foxi3* was not expressed in the differentiating ameloblasts, but expression continued in other epithelial cells. We did not detect expression of the two other Foxi family genes, *Foxi1* or *Foxi2*, in the developing teeth or hair.

There are not that many genes known to be expressed in the dental lamina, but the reason for this may be that most genes have not been studied at this early stage. In addition to *Foxi3* and *Sox2* (I and III), at least *Shh*, *Pitx2, Wnt10*, *Wnt10b*, and *Bmp2* have been shown to be expressed in the dental lamina (Dassule and McMahon 1998, Keränen et al. 1999). It is not known how gene expression in the dental lamina is initiated, and whether all these genes are under the control of the same signaling pathways at this stage. The expression patterns of these genes change at the following placode stage, suggesting that at this point they are differently regulated. Together with *Foxi3*, *Pitx2* is expressed in the placodal epithelium, but its expression also stretches into the surrounding oral epithelium (Keränen et al. 1999), while *Wnt10a*, *Wnt10b*, *Bmp2*, and *Shh* are expressed in the signaling center of the placode (Dassule and McMahon 1998). *Sox2* becomes restricted to the lingual side of the placode (III). Other factors, such as *Fgf8* and *Lef1,* are expressed in a wider area than the dental lamina: at placode stage *Fgf8* is expressed in a similar pattern to *Pitx2*, and *Lef1* is expressed in the signaling center (Kettunen and Thesleff 1998, Kratochwil et al. 1996). *Edar* is not expressed in the dental lamina, but in the signaling center of the placode and later the bud (Laurikkala et al. 2001). As *Foxi3* was expressed both in the dental lamina and in the whole placode epithelium, it could regulate the dental identity of these cells, downstream of pathways expressed in the mesenchyme and in the signaling center

of the placode. At the cap stage the enamel organ is asymmetric, because the lingual cervical loop is bigger. At this stage *Foxi3* had an interesting asymmetric expression pattern. There are other genes expressed asymmetrically at this stage, such as *Msx2* in the buccal epithelium, *Osr2* in the lingual mesenchyme, and *Bmp4* in the buccal mesenchyme (Zhang et al 2009). *Foxi3* can possibly participate in the regulation of the asymmetric shape of the tooth.

The phenotype of the hairless dogs resembles dogs that have hypohidrotic ectodermal dysplasia (HED), caused by mutations in the Eda pathway (Casal et al. 2007). We therefore wanted to know if Eda regulates *Foxi3*. By whole mount in situ hybridization (wmish) we showed that expression of *Foxi3* was downregulated in *Eda* null teeth and mammary glands at E13.5, and upregulated in teeth, hairs, and mammary glands of embryos overexpressing *Eda* in the epithelium under *keratin-14* promoter (I). Treating embryonic E14.5 *Eda* null back skin or bud stage molars with recombinant Eda protein in hanging drop culture for four hours induced *Foxi3* expression in both tissues (I and II). The induction in skin was markedly higher than in tooth. The reason for this was that in *Eda* null skin, there are no hair placodes forming at this stage, and therefore no expression of placode markers such as *Foxi3* (Laurikkala et al. 2002). In *Eda* null molars, in contrast, we showed that there was some *Foxi3* expression present. In addition, *Edar* is expressed in the tip of the bud, whereas *Foxi3* was expressed in a wider area (Laurikkala et al. 2001). As a result, *Foxi3* was not induced in all molar epithelial cells. There are several observations that suggest that Eda is not the only pathway regulating *Foxi3*. The HED dogs are viable, whereas the *Foxi3* homozygote hairless dogs die before birth. If *Foxi3* would be a key target gene of Eda, one would expect that deletion of the Eda pathway or *Foxi3* would result in a similar phenotype. This means that all *Foxi3* functions cannot be downstream of Eda. Also, we observed *Foxi3* expression in Eda null embryos. Most importantly, as *Edar* and *Foxi3* are expressed only partly by the same cells in the tooth, other pathways must regulate *Foxi3*.

To study additional pathways that regulate *Foxi3* in tooth, I used Activin A, Shh, Wnt3a + R-spondin, Fgf8, and Bmp4 in the same hanging drop culture system as Eda (II). We showed by qRT-PCR that Activin A induces *Foxi3* expression in embryonic skin (I), and I confirmed this result by treating wild-type E13.5 molars with Activin A for four hours, and observed a 2.7-fold induction of *Foxi3*. Activin A is expressed in the dental mesenchyme, and it is thought to target the epithelium (Ferguson et al. 1998, Laurikkala et al. 2001). Shh induced 1.7-fold induction of *Foxi3* expression. Recombinant Wnt proteins are poorly available, so I used Wnt3a, which is not expressed in bud stage teeth, to study the effect of Wnt pathway on *Foxi3* expression in combination with a secreted Wnt agonist R-spondin2. Four-hour treatment induced a significant increase in *Foxi3* expression in wild-type E13.5 teeth, but surprisingly the positive control genes *Axin2* and *Lef1*, which are known to be induced by Wnt, were not significantly induced. We also observed that *Foxi3* was induced in the ectopic signaling centers of K14-cre;βcatenin^{ex3flox} embryos where Wnt signaling is activated in the epithelium, but the expression levels were comparable to wild-type teeth (I). *Sostdc1* is a modulator of both Wnt and Bmp signaling (Kassai et al. 2005). We studied *Foxi3* expression in E13.5 *Sostdc1* KO embryos by wmish, but we saw no difference in expression compared with wild-type embryos (I). Interestingly, in the hanging drop culture, Bmp4 downregulated *Foxi3* expression in only four hours, suggesting that it might be a direct target of the pathway (II). The complementary patterns of *Bmp4* in the buccal mesenchyme and *Foxi3* in the lingual epithelium support this finding. *Fgf8* is expressed in the same cells as *Foxi3* at the placode stage, but it becomes downregulated from the bud stage onwards (Kettunen and Thesleff 1998). Fgf8 was not able to induce *Foxi3* expression, and I did not study the effect

of other Fgf proteins. These experiments suggested that multiple pathways that are known to regulate tooth development regulate *Foxi3* both positively and negatively.

4.2. Role of Foxi3 in tooth morphogenesis (II and unpublished)

The *Foxi3*$^{-/-}$ (*Foxi3* KO) phenotype is embryonic lethal with severely affected craniofacial development and lack of tooth development (Edlund et al. 2014). Thus, to study the function of *Foxi3* in tooth development, I analyzed the phenotype of conditional *Foxi3* knock-out mice (hereafter called *Foxi3* cKO). The mice were generated by crossing *Foxi3*$^{+/-}$ males carrying an epithelial *Keratin14*-cre43 transgene (Andl et al. 2002, Edlund et al. 2014) with *Foxi3*$^{floxed/floxed}$ females. In the floxed allele, loxP sites surrounded the second exon of *Foxi3*. Unlike in *Foxi3*$^{+/-}$ mice, which do not have a hair or a tooth phenotype, *Foxi3* is haploinsufficient in dogs and leads to hairless phenotype (Drögemüller et al. 2008). This could be due to a different mutation present in the mice compared to dogs. The variation of the Chinese crested dog phenotype from hairless to semi-coated also suggests that there are additional, but yet unknown modulators of the phenotype in dogs.

I analyzed the phenotype of adult *Foxi3* cKO mice, and found that their lower and upper first and second molars (M1 and M2) were fused. Out of 20 skulls analyzed, only one single individual had fused upper molars but separate lower molars. Third molars (M3) were more frequently present in the upper jaw than the lower jaw (M3 present in 47 % of lower jaw halves and in 83 % upper jaw halves). The fused molars displayed an abnormal crown shape with almost flat surface or multiple mispatterned small cusps.

Both upper incisors were missing in 11 out of 20 animals. I observed that the upper incisors in *Foxi3* cKO embryos became arrested around bud stage, but I did not analyze this phenotype further. To our surprise, upper incisors were always missing in Eda$^{-/-}$;Foxi3$^{+/-}$ animals. This suggests that the upper incisors are more sensitive to loss of *Foxi3,* and that *Foxi3* is an important Eda target in these teeth in particular. Upper incisor development is also arrested in *Gli2*$^{-/-}$*;Gli3*$^{+/-}$ embryos (Hardcastle et al. 1998). Interestingly, Shh is induced by Eda in skin (Pummila et al. 2007), and my results suggested that *Foxi3* is downstream of both Eda and Shh. *Foxi3* expression may therefore be reduced in the Eda$^{-/-}$;Foxi3$^{+/-}$ incisors due to both a lack of Eda activity and decreased Shh activity.

Histological analysis of the *Foxi3* cKO molars during embryonic development revealed several defects. The *Foxi3* cKO molar at placode stage was smaller and shallower in shape compared with control embryos. During subsequent morphogenesis, the *Foxi3* cKO molar did not form a bud, but it continued growing while maintaining a placode-like shape. There were less of the loosely organized stellate reticulum cells in *Foxi3* cKO molars from E13.5 onwards. The abnormal growth of the *Foxi3* cKO epithelium resulted in a lack of the dental cord by E14.5. At this stage, the cervical loops started to form in the *Foxi3* cKO molar, but they were smaller than in the control molar. At E16 and E18, it was evident that the inner enamel epithelium (IEE) of the *Foxi3* cKO tooth was not folding normally to generate the proper cusp pattern of the tooth crown. Despite the abnormalities in the folding of the IEE and cervical loop growth in the *Foxi3* cKO epithelium, cell differentiation appeared to occur normally, as I observed normal ameloblasts and enamel production in these mice. This was not unexpected, as mature ameloblasts lacked *Foxi3* expression indicating it probably is not a key regulator of their differentiation either (I). Interestingly, the *Foxi3* cKO lower incisors did not have ameloblasts or enamel (unpublished

result). In the incisor, *Foxi3* was not expressed in the ameloblasts, but in the cervical loop stem cell niche and in the transient amplifying cells (unpublished result). Therefore, the role of *Foxi3* in the incisor is most likely related to maintenance of the epithelial stem cells or their immediate daughters. Mutations in two other epithelial Fox-factors, *Foxo1* and *Foxj1* lead only to defects in ameloblast differentiation (Poche et al. 2012, Venugopalan et al. 2011). Therefore Foxi3 is currently the only reported Fox superfamily transcription factor that shows a phenotype during early tooth morphogenesis when it is mutated.

The suprabasal cells occupied a smaller area in the *Foxi3* cKO tooth placode compared to control teeth, as studied with an E-cadherin and P-cadherin double staining. Surprisingly, quantification of proliferating cells by BrdU (5-bromo2'-deoxyuridine) incorporation at E12.75 revealed that there was a bigger percentage of proliferating suprabasal cells in the *Foxi3* cKO tooth at this stage. This suggested to us that there could be a cell population missing from the mutant tooth, as the suprabasal layer was smaller despite the difference in proliferation. We compared differences in gene expression in E13.5 *Foxi3* cKO and wild type molar epithelium by Affymetrix Mouse Exon 1.0 ST microarray analysis. One of the downregulated genes in the array was *Keratin-10* (*K10*), and I identified a population of suprabasal cells expressing K10 protein in the control teeth, whereas in the *Foxi3* cKO molar there were less K10 cells with a weaker expression level. The appearance of the strongly K10 positive cells in the control teeth at E13.5 corresponded to the appearance of the loosely organized stellate reticulum cells, which was reduced in the *Foxi3* cKO. When skin becomes stratified, basal cells give rise to suprabasal cells that express both K10 and K1 (Koster and Roop 2007, Wallace et al. 2012), and in our study, K1 was also downregulated in the microarray. Taken together, this data led us to hypothesize that this process of generation of new K10 positive suprabasal cells is impaired in the *Foxi3* cKO, leading to a smaller suprabasal cell layer.

It is tempting to speculate that this process of suprabasal cell formation could also participate in the generation of the bud shape, where a physical force between the new cells occupying the core of the forming bud and the cells of the basal layer would push the epithelium downwards into the mesenchyme. However, comparison of other mutant phenotypes with *Foxi3* cKO challenges this hypothesis. The dental cord does not form in the *Shh* cKO, in *Evc* KO, and in *YAP* cKO, but judged from the histology of the mutant molars, the stellate reticulum seems not to be affected in a similar manner as in *Foxi3* cKO (Dassule et al. 2000, Liu et al. 2014, Nakatomi et al. 2013). Therefore these two events may occur independently of each other. I observed that the epithelium of the *Foxi3* cKO molar was sometimes bulging outwards to the oral cavity at E13.5 and E14.5 (Fig. 6B on page 25). A similar phenomenon is visible in E16 *Shh* cKO molars (Dassule et al. 2000). In *Irf6* and *Ikkα* KO embryos, the whole incisor epithelium evaginates outwards, but the mechanism behind this phenotype is not known (Blackburn et al. 2012, Ohazama et al. 2004). It is possible that the *Foxi3* cKO and *Shh* cKO phenotypes have a similar but milder defect in epithelial morphogenesis. Dental cord morphogenesis clearly requires epithelial cell reorganization during bud formation, and for a still unknown reason, this process failed in the *Foxi3* cKO. In the microarray, I observed differential expression of genes related to cell adhesion, such as claudins and cadherins, and to cell sorting, such as Ephrins, which may further contribute to the molar phenotype of the *Foxi3* cKO (unpublished result).

I did not analyze the mechanism of M1 and M2 fusion in the *Foxi3* cKO. Again, comparison with other mutant phenotypes suggests that it is not connected to the failure of the *Foxi3* cKO epithelium to form a proper bud of M2, which would be similarly affected as the M1 bud in the mutant. Like in *Foxi3* cKO and *Shh* cKO, molars also fuse in the *Sostdc1* KO, but the first molar

morphogenesis is normal in these embryos (Dassule et al. 2000, Ahn et al. 2010, Kassai et al. 2005). The relationship of Foxi3, Sostdc1, and Shh signaling seems complex. In the *Sosdc1* KO, the molar fusion has been linked to increased Wnt signaling (Ahn et al. 2010). Inhibition of Shh by antibodies in pregnant females leads to embryonic molar fusion, and in the molars of these embryos *Sosdc1* becomes downregulated, and Fgf and Wnt pathways upregulated (Cho et al. 2011). I showed that *Foxi3* might lie downstream of Shh. In addition, the microarray and validation by in situ hybridization showed that *Sostdc1* was downregulated and Fgf and Wnt pathways upregulated in *Foxi3* cKO epithelium. However, Shh pathway genes were upregulated in the microarray, but in situ hybridization showed patchy *Shh* expression in *Foxi3* cKO. Therefore it was difficult to determine the exact status of Shh signaling in the *Foxi3* cKO molar. Taken together, I propose that Foxi3 lies downstream of Shh and upstream of Sostdc1 in the regulation of molar fusion, but without further experiments, it is not possible to determine these relationships conclusively.

There was an overall upregulation of Fgf, Bmp, Wnt, and Shh pathway genes in the microarray. In situ hybridization showed that expression of Fgf and Bmp genes were expanded to the whole mutant epithelium at E13.5, whereas *Wnt10a* was restricted to the basal area of the tooth. It is possible that the upregulated signaling pathway activity in *Foxi3* cKO at E13.5 is a result of earlier events that lead to the failure of the bud morphogenesis. In the *Foxi3*$^{-/-}$ mice, development of branchial arches is severely affected (Edlund et al. 2014). This was found to be a result of apoptosis of the neural crest cells within the arches, which is caused by lack of Fgf8 signal secreted from the epithelium. This is in contrast with my results on upregulation of Fgf signaling in the *Foxi3* cKO, and may reflect the different organ- and stage-specific target genes of Foxi3 during development.

P-cadherin staining revealed a cell population with a primary enamel knot -like morphology in the *Foxi3* cKO epithelium at E13.5, and PEK marker gene *p21* was upregulated in the mutant epithelium at this stage. This suggested that the PEK might be differentiating precociously in the *Foxi3* cKO molar. However, at E14.5, PEK markers *Shh* and *Dkk4* were expressed in the *Foxi3* cKO, but they were slightly weaker than in control molars, whereas *Wnt10a* expression was slightly wider. Histologically, the PEK in the *Foxi3* cKO epithelium was not correctly organized, as visualized by P-cadherin and Lef1 staining at E14.5 (Fig. 6). At this stage, *p21* expression had expanded to the whole mutant epithelium, whereas in control teeth it was expressed in the PEK and in the dental cord. The Phenotype of the Hippo pathway co-activator YAP mutant suggests that the PEK signaling activity and the cells with the PEK morphology are not necessarily co-regulated (Liu et al. 2014). In the *Foxi3* cKO, the PEK morphology is abnormal, but it still expresses the correct signals. More cell-level studies are needed to uncover the mechanisms of the suprabasal cell formation, bud and dental cord morphogenesis, and PEK formation in order to understand how these events are linked to each other and to Foxi3.

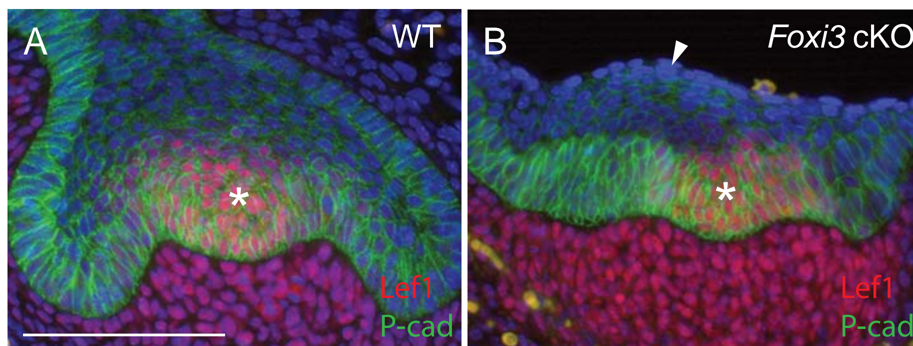


Figure 6. Expression of Lef1 and P-cadherin proteins by immunofluorescence in *Foxi3* cKO cap stage molar

A: Lef1 is expressed in the PEK (asterisk) in the wild-type molar. The PEK has a round shape, visualized by P-cadherin staining.

B: In *Foxi3* cKO, PEK expresses Lef1 (asterisk), but its shape is different compared to wild-type molar in A. In addition, outward bulging of the *Foxi3* cKO epithelium is visible (arrowhead). Scale bar 100 μm.

Normally the PEK induces formation of the secondary enamel knots (SEKs) at the sites of future cusp tips within the IEE. In the *Foxi3* cKO, the SEK markers were not focally expressed, but expanded to the whole IEE. This suggests that the balance of inhibitors and activators in the *Foxi3* cKO molars is perturbed. There is possibly too little inhibitor(s), which leads to the SEK activating signals to spread in the epithelium. This mispatterning leads to the abnormal crown shape of the *Foxi3* cKO molars. In addition, *Fgf15* and *Sfrp5*, which were upregulated in the microarray, were ectopically expressed in the *Foxi3* cKO molar epithelium after E14.0. Normally *Fgf15* is expressed in the PEK and SEKs, and *Sfrp5* in the cervical loops (Kettunen et al. 2011, Porntaveetus et al. 2011, and unpublished). Upregulation of *p21* in the *Foxi3* cKO epithelium as well as ectopic expression of these SEK and cervical loop markers suggest that the normal function of *Foxi3* might be to maintain the epithelium in an undifferentiated state. *Fgf15* was an interesting hit in the microarray, because Foxi3 has been shown to bind to a specific oxidized form of methylated cytosines on *Fgf15* promoter, and this binding is linked to repression of transcription (Iurlaro et al. 2013). Therefore during tooth development, Foxi3 might function as a traditional transcription factor and as an epigenetic regulator of genes such as *Fgf15*.

4.3. Sox2 in the putative stem cells for generation of new teeth (III)

Sox2 was identified as a stem cell marker in the epithelial stem cell niche of the mouse incisor (Juuri et al. 2012). We further characterized *Sox2* expression, and found that it was expressed also in the epithelial cells during mouse molar development. *Sox2* expression was detected in the primary dental lamina upon initiation of tooth development. During subsequent morphogenesis, its expression became confined to the lingual side of the forming molar placodes, and was later restricted to the basal epithelial cells on the lingual side of the enamel organ at cap and bud stages. This was an important finding, as the replacement teeth always form from the successional dental lamina epithelium on lingual side of the previous tooth, and as *Sox2* has been associated with

stem cells (Järvinen et al. 2009, Juuri et al. 2012). Further, we analyzed the expression of Sox2 protein in the ferret, which is a tooth-replacing mammal, in human and in five different reptilian species. Our results showed essentially the same expression pattern as in mouse molars. In the ferret, the strongest Sox2 signal was localized to basal cells in the dental lamina, in the splitting successional dental lamina, and in the lingual side of the interdental lamina. There was weaker Sox2 signal in adjacent stellate reticulum cells in the same areas. The tip of the successional dental lamina and interdental lamina was negative for Sox2. In the reptiles, Sox2+ (Sox2-positive) cells were also present in the dental lamina, but there was no asymmetry in the Sox2+ cell distribution, and there were both basal and stellate reticulum cells with a strong Sox2 signal. Others have reported a similar expression pattern of *Sox2* in mouse molars (Zhang et al. 2012). More recently, Sox2 expression was analyzed during corn snake tooth replacement in tissue culture showing that, similar to our results, the tip of the successional dental lamina is negative for Sox2 (Gaete and Tucker 2013). Together, these data strongly suggest that *Sox2*-expressing stem cells reside in the teeth and they likely contribute to production of replacement teeth.

Are the Sox2+ cells in the dental lamina stem cells? Most of them are likely not. Evidence for this comes from two studies where putative stem cells have been localized during continuous tooth replacement in two reptilian species, the American alligator (*Alligator mississippiensis*) and the leopard gecko (*Eublepharis macularius* Handrigan et al. 2010, Wu et al. 2013). A study analyzing the distribution of label-retaining cells (LRCs) after a 4-week chase period in the American alligator concluded that the stem cells are located in the tip of the successional dental lamina (Wu et al. 2013). Another study on the leopard gecko showed that the biggest percentage of LRCs after a 4-week chase was also located in the tip of the successional lamina, but a longer chase period of 20 weeks revealed a slower-cycling population of cells in the dental lamina and interdental lamina (Handrigan et al. 2010). Expression of known hair follicle stem cell markers *Lgr5*, *Dkk3* and *Igfbp5* was confined to the area of LRCs of the longer chase (Handrigan et al. 2010). There is a functional difference in the dental lamina of the alligator compared with the ferret and the gecko (Järvinen et al. 2009, Handrigan et al. 2010, Wu et al. 2013). The alligator dental lamina does not connect the functional and replacement tooth to the oral surface, but it connects the replacement teeth from different tooth units together within the mesenchyme, like the interdental lamina in the ferret and gecko. This may lead to differences in stem cell localization, but it is at the same time possible that a longer chase period in the American alligator would have revealed a similar cell population as in the leopard gecko.

Regardless, the number of LRCs in these two studies is much smaller than the number of Sox2+ cells we observed in the dental lamina and successional dental lamina of the alligator and the gecko. This suggests that only a small proportion of the Sox2+ cells represent stem cells. It is also possible that the Sox2+ cells are more differentiated progeny of the stem cells, or that they are part of the stem cell niche for the stem cells. In addition, in our study we only used embryonic and juvenile samples, and therefore it is possible that the Sox2+ cell pool decreases in older animals. Genetic lineage tracing in mice has shown that embryonic Sox2+ cells give rise to Sox2+ stem cells in different adult organs (Arnold et al. 2011). In the mouse incisors, there seems to be a number of different epithelial stem cell populations expressing different markers, and their hierarchy is not yet completely understood (Biehs et al. 2013, Harada et al. 1999a, Juuri et al. 2012, Seidel et al. 2010). Future investigations on the model animals that replace their teeth continuously will most likely identify additional stem cell markers expressed in the dental lamina epithelium and characterize the nature and exact location of these cells as well as the role of Sox2 in more detail.

We also analyzed expression of *Sox2* during the posterior addition of molars. Because this process resembles replacement tooth formation, we used it to study the Sox2+ cells in an animal model where tools for genetic manipulation are available. Using a *Sox2*-GFP reporter mouse line in tissue culture, we observed that there was Sox2+ epithelium associated first with the forming M2, and later with the forming M3. With genetic fate mapping, we could show that the Sox2+ cells of the first molar gave rise to the epithelial cells of M2 and M3. These results suggest that besides tooth replacement, Sox2 is associated also with serial addition of new teeth. We therefore propose that tooth replacement and molar addition are variations of the same developmental process, where new teeth emerge from Sox2+ epithelial cells. A recent study showed that as the dental lamina connecting the mouse M2 and M3 becomes fragmented, these epithelial fragments continue expressing *Sox2* (Juuri et al. 2013). In humans, these epithelial fragments have been suggested to be a source for odontogenic epithelial tumors called ameloblastomas. Further, ameloblastomas were shown to contain Sox2+ epithelial cells, supporting this notion (Juuri et al. 2013). This study adds further evidence to the stem cell or progenitor-like nature of the Sox2+ cells in the dental lamina epithelium.

4.4. Sox2 and the Wnt signaling pathway (III, IV, and unpublished results)

We generated animals with a conditional deletion of *Sox2* from the dental epithelium using a *Shh*-Cre line (*Sox2* cKO). We noted that these conditional *Sox2* knock-out animals expressed *Sox2* in random cells within the epithelium so the deletion was incomplete. These *Sox2* cKO animals exhibited a hyperplastic epithelium associated with M2 and M3 (III). This, however, was not accompanied by a marked increase in proliferation in the mutant teeth. However, it is probable that an increase in proliferation took place during an earlier stage of development that we did not analyze. In humans, mutation in *SOX2* causes supernumerary tooth formation (Numakura et al. 2010). This and our results together suggest that if the Sox2+ cells are stem or progenitor cells, Sox2 would act as a maintenance factor of these cells, controlling their cell division or differentiation. Increased Wnt pathway activity has been linked to supernumerary tooth formation in mouse and in human (Wang and Fan 2011), and Sox2 can inhibit Wnt signaling (Mansukhani et al. 2005). A more recent study on submucosal gland development in airways shows that Sox2 can directly bind to the promoter of *Lef1,* a key transcription factor mediating Wnt signaling, and inhibit its transcription (Xie et al. 2014).

In ferret, the Wnt feedback inhibitor *Axin2* is expressed in the tip of the successional dental lamina epithelium and in the mesenchyme surrounding it (Järvinen et al. 2009, and unpublished data). A study on the role of Wnt signaling in regulating corn snake tooth replacement showed that the Sox2 negative tip of the successional dental lamina expresses *Lef1* (Gaete and Tucker 2013). To verify the activity of Wnt signaling in these tissues, I examined the localization of nuclear β-catenin during ferret tooth replacement (IV). I also compared localization of Sox2 and Lef1 in the ferret (unpublished, Fig. 7).

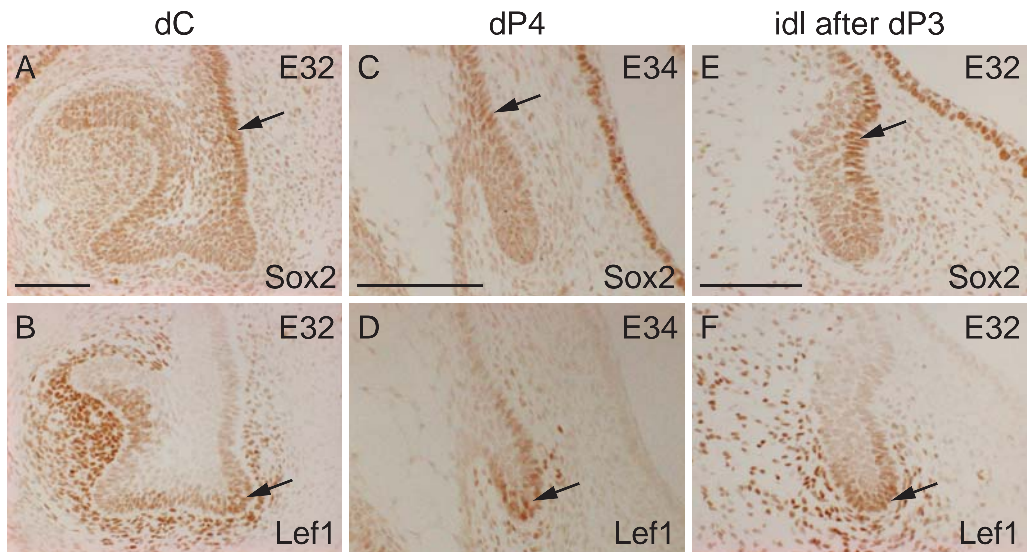


Figure 7. Localization of Sox2 and Lef-1 proteins by immunohistochemistry in the ferret successional lamina and interdental lamina.
A–B: At E32 Sox2 localizes to the dental lamina embedded on the lingual side of the deciduous canine (A, arrow), whereas Lef-1 is expressed in the tip of the successional lamina (B, arrow).
C–D: At E34 Sox2 localizes to the dental lamina embedded on the lingual side of the deciduous fourth premolar (C, arrow), whereas Lef-1 is expressed in the tip of the successional lamina (D, arrow).
E–F: At E32 Sox2 localizes to the lingual side of the interdental lamina (E, arrow), whereas Lef-1 is expressed in the tip of the interdental lamina (F, arrow).
Scale bar 100 μm.

Lef-1 was localized to the tip of the successional dental lamina of both the deciduous canine at E32 and the fourth deciduous premolar at E34, and to the tip of the interdental lamina at E32, and its expression was complementary to Sox2 (unpublished, Fig. 7A-F). Nuclear β-catenin was observed in a small number of cells localized to these same areas (IV). Both Lef-1 and nuclear β-catenin were also present in mesenchymal cells. These results correlate with the *Axin2* expression pattern in ferret, as well as *Axin2* and *Lef1* expression in the ball python successional dental lamina (Handrigan and Richman 2010b, Järvinen et al. 2009). However, in the American alligator, nuclear β-catenin is expressed only in the successional dental lamina epithelium, and not in the mesenchyme (Wu et al. 2013). When Wnt signaling is stimulated by GSK3-β inhibition in corn snake jaw explant culture, *Lef1* expression is expanded, and *Sox2* expression is restricted to the oral areas of the dental lamina (Gaete and Tucker 2013). This treatment induces cell proliferation in the dental lamina close to the oral surface, which results in a wider dental lamina, a phenotype similar to our *Sox2* cKO mice. A more dramatic phenotype is also observed as ectopic tooth germs form upon Wnt activation (Gaete and Tucker 2013). Similarly, GSK3-β inhibition in leopard gecko tissue resulted in increased proliferation and expansion of the dental lamina, where the LRCs have been located, but no ectopic teeth were observed under these conditions (Handrigan et al. 2010).

Altogether, these studies from us and others on Sox2 and Wnt signaling in tooth replacement in different organisms, ranging from human to mouse and ferret and reptiles, link the *Sox2*-expressing progenitor cells in the dental lamina and Wnt pathway activity in the tip

of the secondary dental lamina that gives rise to the newly forming tooth. Inhibiting *Sox2* or activating Wnt disturbs the balance in epithelial proliferation, and when this happens in the correct environment at a favorable time, it can lead to formation of supernumerary teeth.

4.5. Genetic regulation of tooth replacement (III, IV and unpublished results)

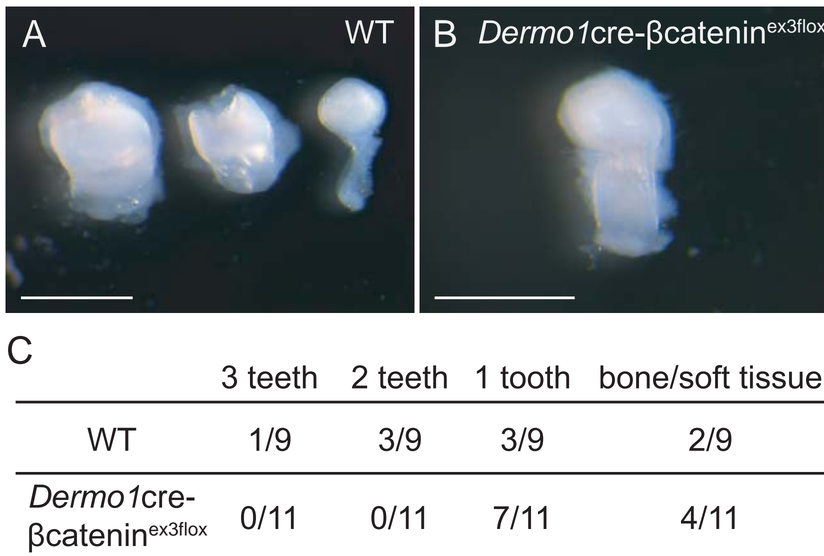
There are several examples that there is capacity for further initiation of new teeth during both primary tooth development and replacement. In mice and humans, fourth molars are occasionally observed (Shahzad and Roth 2012, and our own observations). Some species, such as the silvery mole rat (*Heliophobius argenteocinereus*), form new molars continuously from the back of the jaw (Rodrigues et al. 2011). This continuous serial addition of molars resembles replacement tooth development, but it takes place in a different orientation. In human embryos, a rudimentary successional dental lamina is present in the permanent tooth germs (Ooë 1981), similar to the rudimentary Sox2+ bud we observed in mouse M1 (III). We also saw that in the ferret permanent canine, there is a Sox2+ dental lamina embedded on its lingual epithelium (III).

If there is capacity for further rounds of replacement or tooth initiation, what is normally preventing it from taking place? It has been shown that during the serial addition of mouse molars, there is an inhibitory cascade where the previously formed molar inhibits the growth of the following tooth (Kavanagh et al. 2007). Separating the M2 from M1 in tissue culture results in faster growth of M2 and M3, and can even result in initiation of M4 (Kavanagh et al. 2007). A similar process most likely acts during tooth replacement to regulate the timing of replacement tooth initiation and possibly replacement tooth number, but this has not yet been studied experimentally. In mice, mutations that affect the balance of Eda, Fgf, Shh, Bmp or Wnt pathways can rescue the rudimentary premolar tooth germ that disappears during normal development (Kassai et al. 2005, Klein et al. 2006, Mustonen et al. 2003, Ohazama et al. 2008, Ohazama et al. 2009). This suggests that tinkering with several genetic pathways can overrun the inhibitory mechanism suppressing the development of this rudiment. Similarly, there may be several pathways involved in suppressing continuous tooth formation.

To gain knowledge on the genetic regulation of tooth replacement, I turned to known human syndromes as well as mouse and dog mutations that display tooth phenotypes and analyzed the expression of these genes during ferret tooth replacement. Supernumerary teeth develop in human patients with mutations in *RUNX2* or *Interleukin 11 receptor α* (*IL11Ra*, Jensen and Kreiborg 1990, Lammi et al. 2004, Nieminen et al. 2011). The *Runx2* KO mouse phenotype is interesting because molar development is ultimately arrested, but the molars form epithelial buddings that resemble the tooth phenotype of the mice with activated Wnt signaling in the epithelium (Åberg 2004a, Järvinen et al. 2006). In addition, in *Runx2* heterozygote and *Runx2* KO mice, the upper molars have a lingual protrusion resembling a successional dental lamina (Wang et al. 2005). In the ferret, I observed both *Runx2* and *IL11ra* expression in the mesenchyme around the tip of the successional lamina (IV). *Il11ra* was additionally expressed in the mesenchyme next to the dental lamina in the ferret (IV), where the Sox2+ cells are present in the ferret and reptiles (III), and the LRCs are present in the gecko (Handrigan et al 2010). These expression patterns suggest that whereas both *Runx2* and *Il11ra* likely play a role in regulating the initiation of the replacement tooth formation, *IL11ra* could also regulate mesenchymal signals that act on the stem cells in the dental lamina. Surprisingly, I observed additional epithelial

expression of *Runx2* in the buccal side of the dental lamina in the ferret (IV). *Runx2* has not been reported to be expressed in the epithelium during early tooth development in mice, and in reptiles its expression has not been studied. Based on the mesenchymal expression in mouse and on the mouse knock-out phenotypes, it has been thought to function in the mesenchyme, with *Fgf3* as one of its targets (Åberg 2004b). The expression pattern of *Runx2* needs to be studied in additional species with tooth replacement to identify its target tissues in more detail.

Mutation in *AXIN2* in humans leads to missing teeth (Lammi et al. 2004). This is unexpected because Axin2 is a Wnt feedback inhibitor. Thus mutations in this gene should lead to activated Wnt signaling, which has been shown to result in supernumerary tooth formation in several different cases where Wnt signaling is activated in the epithelium (Wang and Fan 2011). The fact that *Axin2* is strongly expressed in the dental mesenchyme in both mouse and in ferret may be the reason for this contradictory phenotype. Wnt signaling in the two tissues may have different functions, and the mesenchymal Wnt activity may suppress tooth formation. Support for this hypothesis comes from an unpublished experiment in which I have cultured molar tooth germs in the kidney capsule from E12.5 wild type and *Dermo1*-cre;βcatenin^{ex3flox} embryos (Fig. 8A-C). These embryos have constitutively activated Wnt signaling in the mesenchyme. In short, E12.5 tooth germs were dissected and cultured for 24 hours before transplantation under the kidney capsule of anesthetized nude mice. After three weeks the mice were sacrificed, and the kidneys removed. The cultured teeth were carefully dissected out from the kidney and cleaned. Whereas I obtained between one and three molars from the wild type explants, the mutant tooth germs never gave rise to more than one molar. This indicates that mesenchymal Wnt activity inhibits development of M2 and M3. As we propose that molar addition and tooth replacement are comparable processes, the mesenchymal Wnt activity could have a similar inhibitory role in both cases.



	3 teeth	2 teeth	1 tooth	bone/soft tissue
WT	1/9	3/9	3/9	2/9
*Dermo1*cre-βcatenin^{ex3flox}	0/11	0/11	7/11	4/11

Figure 8. Stabilization of β-catenin in the mesenchyme inhibits successional molar formation.
A–B: Kidney capsule culture of E12.5 wild-type and *Dermo1*-cre;βcatenin^{ex3flox} first molar tooth germs dissected from embryonic jaws and cultured under the kidney capsule of nude mice for three weeks.
C: Quantification of experiments. Wild-type samples formed between one and three molars, whereas the mutant teeth never gave rise to more than one molar in the kidney capsule culture.
Scale bar 1 mm.

I also studied Eda signaling and its putative target gene, *Foxi3*. Mutations in Eda pathway genes cause hypohidrotic ectodermal dysplasia (HED) in humans, as well as in several other species such as dogs, which is characterized by missing and abnormally shaped teeth (Mikkola 2009). In the dog model of HED, the tooth phenotype can be rescued by a postnatal administration of recombinant Eda protein (Casal et al. 2007). Hairless dogs have a mutation in the *Foxi3* gene, and their phenotype resembles the HED dogs (Drögemüller et al. 2008). I did not detect expression of *Edar*, Eda receptor, in the dental lamina or successional lamina in the ferret, indicating that Eda signaling is not active in these tissues (IV). *Edar* was expressed in the enamel knots of the deciduous and permanent tooth, where Eda likely regulates their morphogenesis as in mouse molars. Therefore, as the replacement teeth are initiated during embryogenesis, it is plausible that the postnatal Eda administration in dogs acts on the morphogenesis of teeth. However, in ball python and in American alligator, there is *Edar* expression in the dental lamina and the successional lamina, suggesting that Eda signaling does have a role there (Richman and Handrigan 2011, Weeks et al. 2013). Unlike *Edar*, *Foxi3* was expressed in the ferret dental lamina and successional lamina (IV). Therefore in these tissues, *Foxi3* is regulated by pathways other than Eda. In addition, as *Foxi3* is expressed in the successional lamina, the tooth phenotype of the hairless dogs could be caused by defects in replacement tooth initiation.

During tooth initiation, the odontogenic potential shifts from the epithelium to mesenchyme, and this correlates with a shift in the *Bmp4* expression between these two tissues (Vainio et al. 1993). Mutation in mesenchymal transcription factor *Osr2* induces an ectopic tooth to develop on the lingual side of mouse M1 (Zhang et al. 2009). This is accompanied by downregulation of Bmp4 from the lingual mesenchyme, and Bmp4 has been shown to induce *Osr2* expression (Jia

et al. 2013). In the ferret, both *Osr2* and *Bmp4* were expressed in the mesenchyme around the tip of the successional lamina. In addition, *Osr2* was expressed in the mesenchyme lingual to the dental lamina, and *Bmp4* next to it in the lingual side epithelium of the dental lamina. If *Bmp4* plays a similar role in determining the odontogenic potential during tooth replacement than during tooth initiation, the expression pattern of Bmp4 in the ferret suggests that odontogenic competence would lie in the *Sox2+* lingual dental lamina and in the mesenchyme around the successional lamina. In the early dental lamina of a ball python, *Bmp4* is expressed in the mesenchyme, and in the American alligator it is expressed in the dental lamina epithelium, but this difference could reflect different stages of odontogenic induction of these tissues (Richman and Handrigan 2011, Weeks et al. 2013). Finally, *Bmp4* expression in the American alligator dental lamina is not asymmetric as it is in the ferret, which correlates with our observations on the symmetric expression of *Sox2* in reptiles. Thus, the symmetric and therefore broader *Sox2* and *Bmp4* expression in the reptiles could be an indicator of greater potential for initiation of new teeth in these species compared with mammals.

Fgf signaling has been shown to promote proliferation during tooth development. I observed only weak expression of *Etv4*, an Fgf target gene, during replacement tooth initiation, but strong expression of *Etv4*, *Dusp6* and *Spry2* once the replacement tooth morphogenesis had started (IV). This suggests that the Fgf pathway regulates replacement tooth growth once its development has been initiated. Altogether, there is at the moment a good number of studies on expression of known tooth genes during replacement in the ferret and in other tooth-replacing species. The downside of the analysis of gene expression patterns is that they do not directly characterize the function of the genes. Some functional studies have already been conducted in reptiles such as the leopard gecko, American alligator and the corn snake. Slow reproduction makes ferret a poor model species for developmental biology, and therefore the reptiles most likely continue to be the most informative models to explore the molecular regulation of tooth replacement.

4.6. Structure and gene expression of the mammalian interdental lamina (IV)

During evolution, the mouse dentition has adapted to the characteristic ecology of the species, and therefore the mouse lacks many common features of other mammals, such as tooth replacement that I have discussed above. Another aspect of mammalian tooth development missing in mice is the formation of the interdental lamina (idl). Because of this, the morphogenesis and gene expression patterns of mammalian idl have not been studied before. Furthermore, the finding that LRCs in the leopard gecko reside in the idl suggests that this structure is a functional part of dentition. Therefore I wanted to study idl morphogenesis and expression of known tooth genes in a mammalian species that forms this structure and thus chose the ferret as a model.

To investigate the initiation and growth of the idl, I made 3-dimensional reconstructions of serial histological sections of the developing dental epithelium posterior to the canine at five successive stages of development of ferret embryos. This area included the forming premolars and the idl. I observed that the idl and the premolars grow simultaneously. The deciduous premolar 3 (dP3) was initiated first within the epithelium, and as the deciduous premolar 4 (dP4) started to develop, the idl between the two premolars started to form. During morphogenesis, the idl became longer and narrower, and became tilted towards the lingual side. I analyzed expression

of *Shh* at these same stages, and observed *Shh* expression in the dP3 placode at E25, but in the dP4 enamel knot only at E28. Interestingly, there was a continuous stripe of *Shh* expression along the lingual oral epithelium right next to the idl, indicating it might possibly be involved in the regulation of idl downgrowth and lingual orientation. In reptiles, there are no *Shh*-expressing tooth placodes, but they have a similar *Shh* expression in the oral epithelium on the lingual side of the dental lamina (Handrigan and Richman 2010a). Inhibition of Shh signaling in tissue culture in snake teeth leads to straightening and shortening of the dental lamina (Buchtova et al. 2008). These data are fitting with my findings of *Shh* expression next to the ferret dental lamina and suggest that it might be one of the pathways regulating its asymmetric growth.

Analysis of histological sections at older stages of ferret embryos revealed that the idl between different teeth varied in length and thickness, but that the structure of the idl was constant along the jaw. It resembled a bud stage tooth germ and the cervical loops of the cap and bell stage molars, with two layers of basal epithelium surrounding a stellate reticulum-like core. It also had a small condensed mesenchyme surrounding the tip. The structure of the idl therefore supports its possible dental identity. Moreover, *Pitx2* was expressed in all deciduous and replacement teeth, as well as in the idl in all locations along the jaw.

I characterized the expression of other genes and found the following. *Osr2* and *Runx2* were expressed in the idl mesenchyme and *Runx2* also in the buccal epithelium. Nuclear β-catenin was present in the idl tip and in the mesenchyme, similar to the ferret successional dental lamina. *Bmp4* was expressed in the mesenchyme and in the lingual epithelium. *Foxi3* was expressed in the whole idl, but I did not detect expression of *Edar* or any of the Fgf pathway genes studied. The asymmetric gene expression in the idl is interesting as these genes may regulate the asymmetric structure or some functional asymmetry such as stem cell maintenance in the ferret idl. *Runx2* was coexpressed on the buccal side with the Bmp and Wnt inhibitor *Sostdc1* (Järvinen et al. 2009), and *Bmp4* was expressed on the lingual side together with *Sox2* (III). All the genes that I studied were expressed in a similar manner among teeth from different tooth families and in the idl in different locations along the jaw. As individual teeth and the idl are not morphologically identical, there must be other genes and signaling pathways that regulate their growth and shape, as well as the spacing of the tooth germs within the jaws. Alternatively this differential regulation may be achieved by concentration gradients of signaling pathway ligands within the tissues, which cannot be detected from expression pattern analysis.

5. Conclusions and future perspectives

In this thesis work I have focused on uncovering the role of two transcription factors, Foxi3 and Sox2, in the regulation of tooth development. In addition, I studied gene expression patterns during ferret tooth development and replacement in order to gain understanding on the molecular regulation of the successional dental lamina and interdental lamina. Together with the mouse and the ferret, different reptilian species were used as model animals.

I found that *Foxi3* and *Sox2* were expressed in the epithelium in overlapping patterns. They were both expressed in the primary dental lamina that forms when tooth development is initiated. *Sox2* expression was later limited to a specific cell population both in mouse molars and in teeth of different tooth-replacing animals. In the leopard gecko, this area has been shown to harbor stem cells (Handrigan et al. 2010). It has been proposed that the stem cells for tooth replacement are already present in the primary dental lamina (Smith et al. 2009b). Previously, *Sox2* has been shown to mark the stem cells in the mouse incisor (Juuri et al. 2012), and now our results link Sox2 to the stem or progenitor cells responsible for tooth replacement. Therefore, I speculate that Sox2 is a marker for all dental stem and progenitor cells for tooth renewal and replacement, and these cells are present already at the initiation of tooth development. *Foxi3* was broadly expressed in the epithelium of developing mouse molars during morphogenesis, while in the ferret it was also expressed in the successional dental lamina and interdental lamina. I found that in the *Foxi3* cKO the shape of the molars was abnormal, suggesting that Foxi3 plays an important role in dental epithelial morphogenesis. Foxi3 could therefore regulate the development of all epithelial cells, including the *Sox2*-expressing cells, which are a specific progenitor population within the epithelium.

Sox factors require a binding partner for their function (Kamachi and Kondoh 2013). Currently, no other transcription factor has been found to be expressed specifically in the Sox2+ cells in tooth. Thus, the putative Sox2 binding partners in tooth development and replacement are currently unknown. In future studies, they could be identified using methods such as co-immunoprecipitation. Fox-factors have been shown to have multiple functions: they can act as pioneering factors, traditional transcription factors as well as participate in epigenetic regulation (Lam et al. 2013). Foxi3 has been shown to repress *Fgf15* by binding to a specific type of methylated DNA on its promoter (Iurlaro et al. 2013). Upregulation of *Fgf15* in the *Foxi3* cKO suggests that one of its modes of action in tooth could indeed be transcriptional repression by binding epigenetic methylation marks on gene promoters. It is possible that Foxi3 regulates gene expression in several different ways during tooth morphogenesis, including epigenetic regulation, which adds a new level of complexity to the genetic network regulating tooth development. In order to identify more precisely the genes that Sox2 and Foxi3 are regulating, their binding to specific locations on the genome could be studied using chromatin immunoprecipitation.

We showed that *Foxi3* is expressed in all developing ectodermal organs and that it is a putative target of the Ectodysplasin signaling pathway. Therefore *Foxi3* is a candidate gene in those ectodermal dysplasias in which mutations in the Eda pathway have not been identified. However, I have shown that in tooth, additional pathways, including Activin, Shh and Bmp4, regulate *Foxi3* expression. There were defects in several aspects of epithelial morphogenesis in the *Foxi3* cKO molars such as in the generation of suprabasal cells and in the formation of the dental cord during bud morphogenesis. In addition, Foxi3 was necessary for the patterning of the secondary enamel knots that generate the shape of the tooth crown. *Foxi3* had a seemingly

uniform expression pattern in the epithelium throughout tooth development. Therefore it is unclear why its deletion affects different steps of dental epithelial morphogenesis simultaneously. Either Foxi3 has an important role in converging the activity of several different signaling pathways, or, like other Fox factors, it regulates the different steps of epithelial morphogenesis by several different means of transcriptional regulation.

A common characteristic for the formation of new molars in the primary dentition and for the generation of new teeth in tooth replacement was the presence of *Sox2*-expressing cells. In addition, *Sox2* was expressed in the interdental lamina, which in the ferret developed concomitantly with the primary teeth. Therefore it seems that Sox2+ epithelium is present in all the different parts of dentition throughout development. Expression of *Sox2* in mammalian permanent teeth and the interdental lamina, together with the phenotypes of mice with the Wnt pathway activated in the epithelium and the different human syndromes with supernumerary teeth indicate that there is potential for formation of new teeth even in systems where this normally does not occur. It is possible that initiation and inhibition of new teeth is connected to regulation of the *Sox2* expressing competent epithelium.

Combining modern mouse genetics tools, such as lineage tracing, with gene expression analysis in tooth replacing species proved to be a powerful way to analyze regulation of tooth replacement. Further functional studies using these different models will give information on the maintenance of the competent dental epithelium that can possibly be used for bioengineering new teeth. Methods targeting the genome for making transgenic reptiles are not yet available, but recently developed tissue culture methods (Gaete and Tucker 2013) can be combined with manipulation of gene and protein expression in order to learn about the key signaling pathways and transcription factors that regulate tooth replacement. For the moment the mouse remains the main model to address the function of Sox2. A more efficient cre-line should be used to continue the study of the conditional *Sox2* knock-out molar phenotype. In addition, generation of a mouse line where *Sox2* is overexpressed in the whole dental epithelium would be an interesting tool to analyze what kind of identity *Sox2* expression confers to the cells. The existing *Sox2-gfp* mice could be used to sort the *Sox2*-expressing cells from the lingual side of the molar epithelium in order to identify additional marker genes for these cells by means of microarray or RNA-sequencing approaches.

The expression of *Sox2*, *Bmp4* and *Runx2* in specific cell populations in the ferret dental lamina epithelium, as well as the Keratin-10 expressing cells in the mouse molar add to the growing data of genes expressed not only in the epithelium or mesenchyme, but in specific cell populations within these tissues. The defective suprabasal epithelium and the lack of the dental cord in the *Foxi3* cKO show that there are still unknown morphogenetic events within the tooth bud that require reorganization of the epithelial cells. How these different cell populations are specified, and what kind of cell behavior underlies tooth morphogenesis remains to be investigated. Live imaging of cell level interactions such as migration can be studied in tissue culture. This has already been done in developing skin in which cells labeled with fluorescent markers for proliferating and non-proliferating cells were traced over time (Ahtiainen et al. 2014). A mouse line expressing a transgene composed of the *K10* promoter driving the expression of a fluorescent reporter protein would be useful live-imaging tool to study the behavior of this suprabasal cell population during epithelial morphogenesis. In the future, it will be necessary to apply this kind of new technology to delineate cell-level changes in response to signaling pathway activity regulating tooth development.

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