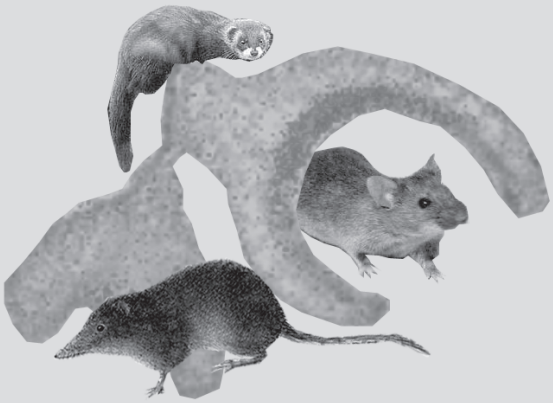

Mechanisms and Molecular Regulation of Mammalian Tooth Replacement

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There is no road to happiness,
happiness is the road.
– Buddha

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

I James MJ, **Järvinen E**, Wang XP, Thesleff I. (2006) Different roles of Runx2 during early neural crest-derived bone and tooth development. *J Bone Miner Res*.21:1034-44.

II **Järvinen E**, Salazar-Ciudad I, Birchmeier W, Taketo MM, Jernvall J, Thesleff I. (2006) Continuous tooth generation in mouse is induced by activated epithelial Wnt/*β*-catenin signaling. *Proc Natl Acad Sci U S A*. 103:18627-32.

III **Järvinen E**, Välimäki K, Pummila M, Thesleff I, Jernvall J. The taming of the shrew milk teeth. *Evol Dev*. In Press.

IV **Järvinen E**, Tummers M, Thesleff I. Formation of tooth placodes and mechanisms of tooth replacement in the ferret *Mustela putorius furo*. Manuscript.

ABBREVIATIONS

BC	Before Christ
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BSA	Bovine serum albumin
Dkk	Dickkopf
Dlx	Vertebrate homologue of *Drosophila* distal-less gene
DNA	Deoxyribonucleic acid
Dspp	Dentin sialoprotein
E	Embryonic day
EDA	Ectodermal dysplasia, anhidrotic
Eda	Ectodysplasin
Edar	Ectodyspasin receptor
EEC	Ectrodactyly, ectodermal dysplasia and cleft/lip palate syndrome
FAP	Adenomatous polyposis
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GIS	Geographic information system
Gli	Vertebrate homologue of *Drosophila* cubitus interruptus gene
HED	Hypohidrotic ectodermal dysplasia
Hh	Hedgehog
Hox	Homebox
K14	Keratin 14
KO	Knockout
Lef1	Lymphoid enhancer factor 1
LRP	Lipoprotein receptor
Msx	Vertebrate homologue of *Drosophila* muscle segment (Msh) gene
M1	1st molar
NF-κB	Nuclear factor kapha B
Pax	Paired-like homeobox
PCR	Polymerase chain reaction
PCP	Planar polarity pathway
Pitx2	Pituitary homeobox 2
PN	Post natal
Ptc	Patched
Smad	Vertebrate homologue of the *Drosophila* mother against decapentaplegic (MAD) gene
Smo	Smoothened
Shh	Sonic hedgehog
TG	Transgenic
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
Tcf	T-cell specific transcription factor
Wnt	Wnt-family member

SUMMARY

In most non-mammalian vertebrates, such as fish and reptiles, teeth are replaced continuously. However, tooth replacement in most mammals, including human, takes place only once and further renewal is apparently inhibited. It is not known how tooth replacement is genetically regulated, and little is known on the physiological mechanism and evolutionary reduction of tooth replacement in mammals. In this study I have attempted to address these questions.

In a rare human condition cleidocranial dysplasia, caused by a mutation in a Runt domain transcription factor *Runx2*, tooth replacement is continued. *Runx2* mutant mice were used to investigate the molecular mechanisms of Runx2 function. Microarray analysis from dissected embryonic day 14 *Runx2* mutant and wild type dental mesenchymes revealed many downstream targets of *Runx2*, which were validated using *in situ* hybridization and tissue culture methods. Wnt signaling inhibitor *Dkk1* was identified as a candidate target, and in tissue culture conditions it was shown that *Dkk1* is induced by FGF4 and this induction is *Runx2* dependent. These experiments demonstrated a connection between *Runx2*, FGF and Wnt signaling in tooth development and possibly also in tooth replacement.

The role of Wnt signaling in tooth replacement was further investigated by using a transgenic mouse model where Wnt signaling mediator β-catenin is continuously stabilized in dental epithelium. This stabilization led to activated Wnt signaling and to the formation of multiple enamel knots. *In vitro* and transplantation experiments were performed to examine the process of extra tooth formation. We showed that new teeth were continuously generated and that new teeth form from pre-existing teeth. A morphodynamic activator-inhibitor model was used to simulate enamel knot formation. By increasing the intrinsic production rate of the activator (*β-catenin*), the multiple enamel knot phenotype was reproduced by computer simulations. It was thus concluded that *β-catenin* acts as an upstream activator of enamel knots, closely linking Wnt signaling to the regulation of tooth renewal.

As mice do not normally replace teeth, we used other model animals to investigate the physiological and genetic mechanisms of tooth replacement. *Sorex araneus*, the common shrew was earlier reported to have non-functional tooth replacement in all antemolar tooth positions. We showed by histological and gene expression studies that there is tooth replacement only in one position, the premolar 4 and that the deciduous tooth is diminished in size and disappears during embryogenesis without becoming functional. The growth rates of deciduous and permanent premolar 4 were measured and it was shown by competence inference that the early initiation of the replacement tooth in relation to the developmental stage of the deciduous tooth led to the inhibition of deciduous tooth morphogenesis. It was concluded that the evolutionary loss of deciduous teeth may involve the early activation of replacement teeth, which in turn suppress their predecessors.

Mustela putorius furo, the ferret, has a dentition that resembles that of the human as ferrets have teeth that belong to all four tooth families, and all the antemolar teeth are replaced once. To investigate the replacement mechanism, histological serial sections from different embryonic stages were analyzed. It was noticed that tooth replacement is a

process which involves the growth and detachment of the dental lamina from the lingual cervical loop of the deciduous tooth. Detachment of the deciduous tooth leads to a free successional dental lamina, which grows deeper into the mesenchyme, and later buds the replacement tooth. A careful 3D analysis of serial histological sections was performed and it was shown that replacement teeth are initiated from the successional dental lamina and not from the epithelium of the deciduous tooth. The molecular regulation of tooth replacement was studied and it was shown by examination of expression patterns of candidate regulatory genes that BMP/Wnt inhibitor *Sostdc1* was strongly expressed in the buccal aspect of the dental lamina, and in the intersection between the detaching deciduous tooth and the successional dental lamina, suggesting a role for *Sostdc1* in the process of detachment. *Shh* was expressed in the enamel knot and in the inner enamel epithelium in both generations of teeth supporting the view that the morphogenesis of both generations of teeth is regulated by similar mechanisms.

In summary, histological and molecular studies on different model animals and transgenic mouse models were used to investigate tooth replacement. This thesis work has significantly contributed to the knowledge on the physiological mechanisms and molecular regulation of tooth replacement and its evolutionary suppression in mammals.

1. REVIEW OF THE LITERATURE

1.1. Developmental biology as a research field

In history there have been two dominating theories of how an organism gets its form, the epigenetic and the preformistic view. The epigenetic hypothesis was first introduced 400 BC by Aristoteles, who had anticipated that the organs of an embryo are developed *de novo* at each generation. However, the epigenetic theory was forgotten in favor of the preformistic hypothesis for almost two thousand years due to the prevailing religious, philosophic and scientific views. As late as in the 1800-century the preformists believed that all humans were inside the germ cells as small fully developed beings called the *homunculus*, and development was merely growth of existing structures, created by god in the beginning of time. During the 19th century comparative embryology, based on observation and description, produced detailed information of the development of embryos in different species, and it was understood that development proceeds gradually. It was also noticed by Ernst Häckel that the early development of different vertebrates resemble each other. Charles Darwin published 1859 in the book *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life* the theory of evolution, where he proposed that different animal species have been developed and changed through natural selection during millions of years. Yet the driving force of development and evolution remained a mystery for a long time. Gregor Mendel, an Austrian monk experimented on peas and formulated in 1866 *the theory of transmissible factors* that are responsible for inheritance of traits. It was the discovery of a predictable mechanism by which inherited characteristics are transmitted from parents to offspring. His work was revolutionary for the times and remained unrecognized for decades. It was rediscovered only in the early 1900s. W.H Sutton anticipated that Mendel's factors recide in chromosomes and T.H. Morgan showed with his experiments on *Drosophila melanogaster*, the fruit fly, that chromosomes are the vehicles of inheritance. W.H. Sutton advanced the notion that only a small part "an inheritance unit" of a chromosome is responsible for one specific trait. During 1920s it was understood that genes are involved in heredity and that genes are composed of deoxyribonucleic acid. In 1953 Watson and Crick published the model of DNA and how it is replicating itself. However, only in the 1970s it was discerned that genes are involved not only in the generation but also the processing of information: the development and the idea of genetic causal sequences was applied to animal developmental biology (Gilbert 2006; Martinez and Stewart 2002; Alcamo 1996; Wilkins 2002).

Today, in the post-genome era, as the sequence of human genome and the genomes of many other species have been revealed, it has been learned that the number of genes in different mammalian species are very similar. This raises the question: If the number of genes and their actions in development are similar e.g. in mouse and human, what makes us so different?

1.2 Developmental biology and its relation to evolutionary biology

Evolutionary developmental biology is a relatively new scientific field originated in the 1990s. It combines developmental biology and genetics with evolutionary biology and paleontology. The key finding that led to the generation and increasing importance of

this new field was in the 1980s when the Homeobox (Hox) genes were discovered. The Homeobox is a cluster of genes required for setting segmental identities. Hox genes are found in all eukaryotic phyla from yeast to humans. Interestingly the chromosomal order of Hox genes in the genome relates to the spatial order of the expression of the genes in the antero-posterior axis in the developing embryo. This is true for both non-vertebrates and vertebrates (Graham et al. 1989; Duboule and Dolle 1989). These findings showed that all animals share key underlying genes that pattern their embryos. Moreover, it shows that the genes that regulate development have changed only little during evolution. This leads to the questions: What have been the changes in development that create so many different kinds of animals? How is embryonic development changed through the evolutionary changes? Are some evolutionary changes more common than others? Evolutionary developmental biology is trying to find answers to these questions. The aim of evolutionary developmental biology is to trace the mechanisms, processes and events that have generated the diversity of animal forms. Recent findings indicate that the changes in evolution are often driven by small changes in the dynamics of developmental processes (Kavanagh et al. 2007; Kangas et al. 2004).

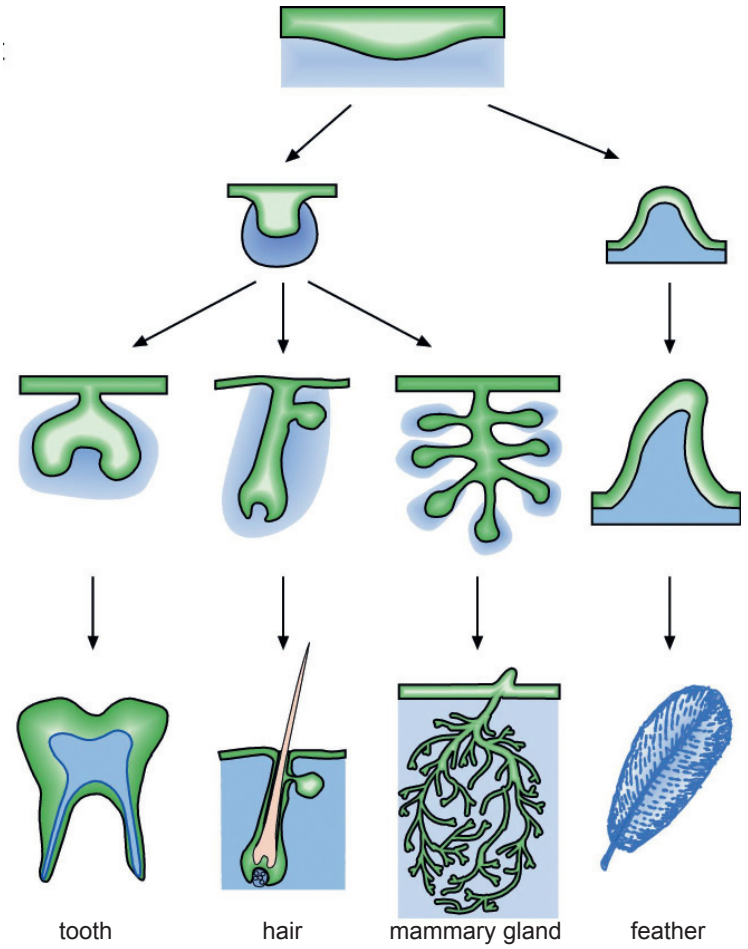

Figure 1. Ectodermal organs and their early development.

1.3 Development of ectodermal organs

Ectodermal organs are a group of organs that are developed from the outer layer of the embryo, the ectoderm, which is formed during vertebrate gastrulation. Ectodermal organs include hairs, teeth, nails, mammary glands, sweat glands, salivary glands, sebaceous glands, feathers, scales, beak and horns. All ectodermal organs share similar early development (Figure 1.). The ectodermally derived epithelium and the underlying mesenchyme interact through reciprocal signaling. The first sign of the developing organ is an epithelial thickening, a placode, which invaginates (or evaginates) into the mesenhcyme. Also the signals regulating the initiation of all ectodermal organs are similar (Pispa and Thesleff 2003).

1.4 Signaling molecules directing development

Development in different organs is directed using a very similar molecular system. There are several signaling molecule families. These signaling pathways are conserved throughout the animal phyla. The same genes that regulate the development of the fruit fly also regulate the development of vertebrates. A signaling pathway consists of soluble ligands, cell membrane receptors, intracellular signaling factors, transcription factors, co-factors and antagonists. These molecule families are named after soluble growth factors that can mediate signals even through multiple cell layers, for short distances or long distances. The growth factors are placed into families, according to their genetic and protein structure similarities. The growth factor signal binds the receptor on the cell surface in the recipient cell and an intracellular transduction cascade is set about that ends up in the cell nucleus where the target genes are activated. It is a common feature that the signaling pathway members, including the antagonists, are regulated by the same family in an autoregulatory fashion.

1.4.1 Wnt signaling

Wnt signaling has been indicated to have a role in embryonic induction, generation of cell polarity, specification of cell fate, tumorigenesis, cell proliferation, migration, cell differentiation and homeostatic self-renewal in adult tissues (Logan and Nusse 2004; Clevers 2006). In ectodermal organs Wnts have been shown to be involved in initiation of tooth, mammary and whisker placodes (van Genderen et al. 1994; Andl et al. 2002), and in feather placode induction (Noramly et al. 1999), in hair placode patterning and initiation of placode formation (Zhou et al. 1995; Gat et al. 1998; Huelsken et al. 2001; Närhi et al. 2008), in hair stem cell differentiation and maintenance (Lowry et al. 2005; Huelsken et al. 2001). Wnt signaling has been connected with ectodermally derived cancers such as skin and hair follicle tumours (Gat et al. 1998; Niemann et al. 2002; Lo et al. 2004). I will discuss the role of Wnt signaling in tooth development later.

Wnts are thought to act as morphogens i.e. importing long range signals whose activities are concentration dependent (Wodarz and Nusse 1998). In mammals all together 21 cysteine rich glycoprotein Wnt ligands and 11 Frizzled receptors with seven transmembrane domains are known today. A single Wnt ligand can bind multiple receptors. The binding of the ligand involves co-receptors Lrp5 and Lrp6. Both the Frizzled and Lrp5/6 receptors are needed for the activation of the pathway. Secreted Dickkopf proteins inhibit Wnt signaling by directly binding to Lrp5/6 (Logan and Nusse 2004; Clevers 2006).

Wnt signaling can be divided into two categories, based on the ability of the Wnt ligands to activate different intracellular pathways, namely the β-catenin dependent signaling and the non-β-catenin dependend signaling. The non-β-catenin dependend signaling can be divided further into the planar cell polarity (PCP) pathway and theWnt/Ca^{2+} pathway. The PCP signaling is a common mechanism for cellular polarization and has a role in the eye and wing development of *Drosophila* (Jenny and Mlodzik 2006). It has also been shown to play a role in carcinogenesis in human (Katoh 2005). TheWnt/Ca^{2+} pathway has been shown to have a role in early embryonic induction and left-right axis determination, neural induction and somite formation (Slusarski and Pelegri 2007).

1.4.1.1 β-catenin dependent pathway

β-catenin dependent pathway has been implicated to have a role in most cellular processes during development, tissue self-renewal and cancer (Clevers 2006). The pathway consists of the Frizzed receptor, Lrp5/6 co-receptor complex, cytoplasmic protein Dishevelled, as well as the cytoplasmic destruction complex of GSK-3, APC, Axin and β-catenin. In the absence of a Wnt ligand, the destruction complex binds β-catenin, β-catenin is phosphorylated and targeted to destruction by a proteasome. In the presence of a Wnt ligand activation of the pathway involves the recruitment of Dishevelled to the cell membrane, which in turn acts upstream of GSK-3 and β-catenin. Co-receptor Lrp5/6 interacts with Axin, which functions as a scaffold protein, interacting directly with GSK-3, β-catenin and APC, members of the cytoplasmic destruction complex. The Axin-GSK-3-APC destruction complex is thus recruited to the cell membrane, β-catenin is stabilized and transported to the nucleus where it binds to Lef/Tcf transcription factors

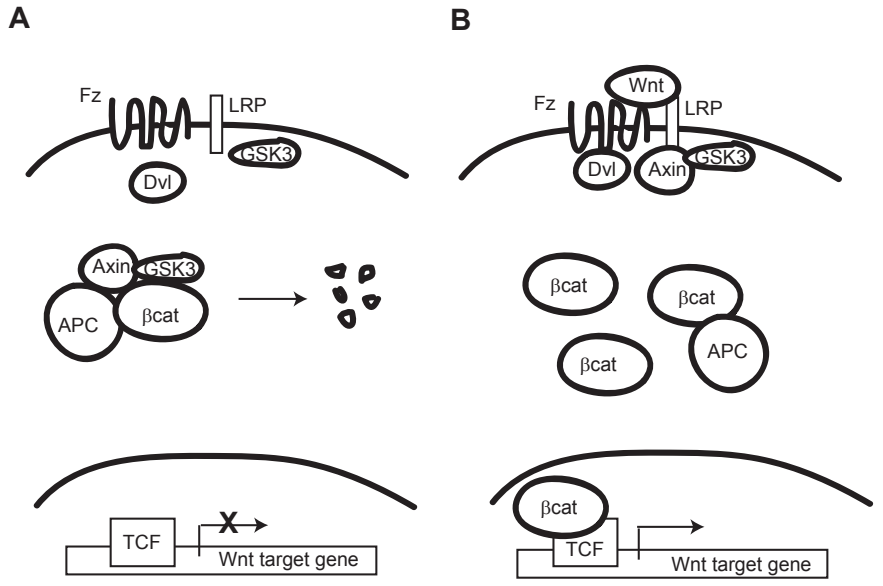

Figure 2. β-catenin dependent pathway. A) When Wnt ligand is not present, β-catenin is degraded. B) When Wnt ligand binds to the Frizzled and Lrp receptor complex, β-catenin goes into nucleus and activates target genes.

and activates the target genes (Figure 2.). There are two members in the Axin family, Axin1 and Axin2. It has been shown that Axin2 is a negative regulator of Wnt signaling and that Wnt/β-catenin/Tcf signaling induces the transcription of *Axin2* (Jho et al. 2002). *Axin2* RNA expression is thus an indicator of active Wnt signaling. β-catenin has a dual role in the cell. In addition to the signaling function, β-catenin also binds E-cadherin, and functions in cell adhesion on the cell plasma membrane (Takeichi 1995; Brembeck et al. 2006).

1.4.2 Other signaling pathways

Other signaling pathways include Hedgehog, TGF-β, Ectodysplasin, FGF and Notch signaling pathways. I will introduce their mode of action briefly and give a few examples of their functions specifically in ectodermal organ development.

1.4.2.1 Hedgehog

Hedgehog proteins have a central role in the development of most organs e.g. the central nervous system, the circulatory system, myogenesis, limb development, and in the formation of face and head (McMahon et al. 2003). There are three ligands in vertebrates, Sonic hedgehog (Shh), Indian hedgehog and Desert Hedgehog. Shh signals through a receptor complex that includes Patched (Ptc) and Smoothened (Smo). The binding of Shh to the receptor Ptc, releases Ptc repression of Smo. Smo activates its intracellular targets including Gli family zinc finger transcription factors. In other words, the receptor Ptc represses the pathway when the ligand is not present. Shh plays a central role in the formation of most ectodermal organs. *Shh* is the only hedgehog ligand expressed during tooth development and it acts as a long range signal, affecting both epithelium and mesenchyme (Hardcastle et al. 1998; Dassule et al. 2000). *Shh* is a late placodal marker and disruption of Shh signaling in the early stages of tooth development does not affect the initiation (Hardcastle et al. 1998). Conditional deletion of *Shh* in the dental epithelium under K14 promoter leads to small and abnormally shaped teeth, where the lingual cervical loop and the dental cord are missing (Dassule et al. 2000). Shh thus regulates the growth and shape of the tooth. When *Shh* is deleted only in dental epithelium by *K14-Smo* approach, teeth have disrupted morphology, and epithelial cells have defects in proliferation, growth, differentiation and polarization (Gritli-Linde et al. 2002). Hair placodes are initiated but hair follicle growth is blocked in *Shh* null allele skin (Chiang et al. 1999). Shh has been shown to be involved in feather formation (Chuong et al. 2000). However, Shh is not needed for mammary placode formation (Gallego et al. 2002; Michno et al. 2003), but repression of hedgehog signaling is required for normal mammary gland development (Hatsell and Cowin 2006).

1.4.2.2 TGF-β superfamily

TGF-β signaling has been implicated to affect embryonic patterning and tissue homeostasis. The superfamily consists of three subfamilies which are TGF-β, BMP and Activin/Inhibins. They all bind to cell surface type I and II serine-threonine kinase receptors. After ligand binding, the type II receptors phosphorylate type I receptors, which then bind and phosphorylate cytoplasmic Smad proteins. Smad proteins mediate the signals into the nucleus and activate the target genes (Massague and Wotton 2000;

Balemans and Van Hul 2002). BMP signals are mediated by Smad 1, 5, and 8. TGF-β and Activin signals are mediated by Smad2 and Smad3.

In ectodermal organs BMP signaling has been associated with lateral inhibition of hair follicles and in the initiation of tooth development. Inhibition of BMP signaling in the dental epithelium leads to changes in number, size and shape of teeth (Plikus et al. 2005). BMP releasing beads inhibit hair and feather follicle formation (Botchkarev et al. 1999; Jung et al. 1998). Ectopic expression of BMP or constitutive active Bmpr1 in chick skin disrupts feather formation (Noramly and Morgan 1998; Ashique et al. 2002). Activin has been shown to be essential for the initiation of incisors and lower molars (Ferguson et al. 1998). Follistatin, an Activin and BMP inhibitor, has been shown to be important in morphogenesis of molars (Wang et al. 2004a). Tooth morphology and cusp patterning are disturbed in molars when the function of the BMP inhibitor Sostdc1 (Ectodin, Wise) is knocked out (Kassai et al. 2005).

1.4.2.3 Ectodysplasin signaling

Ectodysplasin (Eda) belongs to the tumor necrosis factor (TNF) signaling molecule family. Other members of the TNF family are involved in host defence, immunity and inflammation and specifically function in cell survival and apoptosis. Eda is the first TNF family member implicated in ectodermal organ development (Headon and Overbeek 1999; Mikkola et al. 1999). It has a function in initiation, morphogenesis and differentiation of ectodermal organs. The ligand Eda signals through its receptor Edar, and the downstream effects of Edar are mediated through the transcription factor NF-κB (Mikkola 2007). The receptor Edar is one of the earliest markers of ectodermal placode formation and it has been indicated that it is also a potent stimulator of placode formation (Laurikkala et al. 2002). The ligand Eda is an early regulator of placodes thought to act downstream of the inductive signal. Mouse mutants of *Eda* and *Edar* have defects in hair, tooth, mammary glands and sweat glands (Mikkola 2007). Overexpression of *Eda* under the K14 promoter in the ectoderm leads to ectopic teeth and mammary glands, stimulation of hair and nail growth, and increased activity of sweat glands (Mustonen et al. 2003; Mustonen et al. 2004). It has been shown by *in vitro* experiments that Eda regulates hair follicle fate in a dose dependent manner (Pummila et al. 2007). Ectodysplasin signaling is evolutionary conserved in ectodermal organ development. It has been shown that the loss of *Edar* leads to complete loss of scales in teleost fish (Kondo et al. 2001).

1.4.2.4 Fibroblast growth factor signaling

FGF signaling has been implicated in proliferation, cell survival, differentiation, adhesion and migration (Szebenyi and Fallon 1999). Fibroblast growth factor (FGF) family consists of 23 ligands in mammals. Signaling is mediated through a family of tyrosine kinase transmembrane receptors. Four receptors Fgfr1, 2, 3, 4 with multiple isoforms are identified. Ligand binding of FGF receptors depends on the presence of heparan sulfate proteoglycans (HSPG), which act as low affinity FGF co-receptors regulating the diffusion of FGF proteins, and is essential for the formation of active FGF/FGF receptor signaling complex (Ornitz 2000). In ectodermal organs FGF signaling has been implicated in hair, tooth and feather development. Loss of FGF signaling leads to arrest of hair, tooth, mammary gland and feather development. *Fgfr2b* null allele mice

have dysgenic hair formation, and an arrest of tooth development at an early stage (De Moerlooze et al. 2000). FGF2 can induce feathers in chick (Song et al. 1996; Widelitz et al. 1996). FGF10 is required for feather initiation as an early dermal signal (Mandler and Neubuser 2004). Sprouty genes are intracellular inhibitors of FGF signaling (Hacohen et al. 1998). Loss of Sprouty genes leads to the formation of an extra tooth and tooth shape defects in mice (Klein et al. 2006).

1.4.2.5 Notch signaling

Notch signaling was first found in Drosophila and has been implicated in lateral inhibition mechanisms, asymmetric cell fate assignation, in boundary formation and lineage decisions in stem cells (Bray 2006; Fiuza and Arias 2007). Notch family of signaling molecules consists of cell membrane receptors that mediate short-range signaling between neighboring cells. In mammals three membrane bound ligands Jagged1, Jagged-2 and Delta1, and three receptors Notch1, 2, 3, and intracellular modulator Lunatic fringe, and target genes including transcription factors of the Hes family has been shown to play a role in ectodermal organ development. It has been suggested that Notch signaling plays a role in the determination of odontoblasts and ameloblasts and in the morphogenesis of molars (Mitsiadis et al. 1998; Mitsiadis et al. 2005), and also in maintenance of epithelial stem cell niche in the continuously growing mouse incisors (Harada et al. 1999), and in hair follicle maintenance (Estrach et al. 2006). Notch signaling has been shown to be involved in establishing the A-P asymmetry of feather buds in chick (Chen et al. 1997).

1.5 Tooth development

Teeth develop from the oral ectoderm and the underlying neural crest derived mesenchyme. Neural crest cells originate at the dorsalmost region of the neural tube. They migrate into the first branchial arch where they take part e.g. in the formation of the bones and cartilage of the head, and of teeth. The early tooth development can be divided into four main stages: initiation, morphogenesis, differentiation of the tooth type cells, and secretion of dentine and enamel matrices (Figure 3.). Today there are over 300 genes known to regulate tooth development (http://bite-it.helsinki.fi). Most of them belong to the signaling molecule families. Tooth development and its molecular regulation have been studied mostly in the mouse (Thesleff 2003).

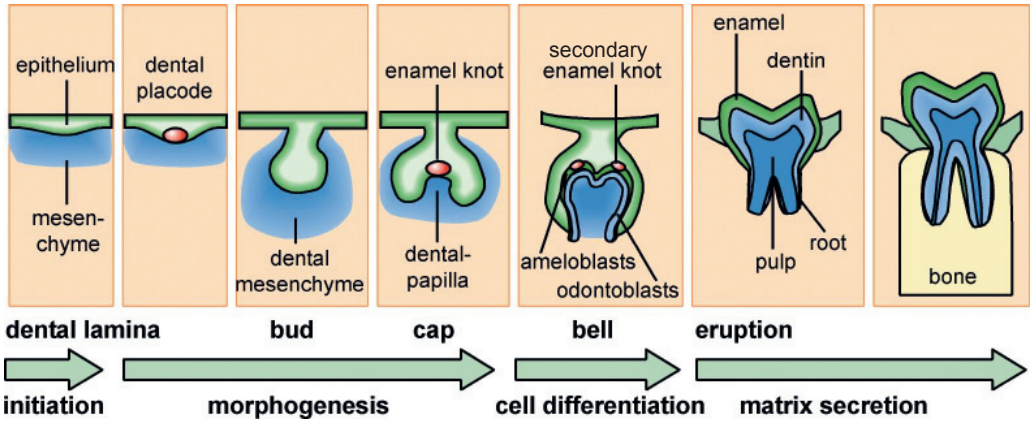

Figure 3. Stages of tooth development.

1.5.1 Initiation

The first morphological sign of tooth development is the formation of the primary epithelial band, a horse-shoe shaped epithelial thickening on the oral ectoderm. In mouse this takes place at embryonic day 11 (E11). It defines the tooth forming region as teeth develop only along this structure. One of the earliest known dental markers is *Pitx2*, which is expressed in the epithelium of the future primary epithelial band, in mouse already at E8.5 (Mucchielli et al. 1997). *Shh* and *Lef1* expression is detected at the primary epithelial band at E11. The expression of *Shh*, *Pitx2* and *Lef1* are downregulated and become restricted to the tooth placodes at E12. Tooth placodes form at incisor and molar areas. The placodes form by reciprocal signaling between the epithelium and mesenchyme. Early signaling centers in the placodes express multiple signals from all signaling molecule families. These early signaling centers have a regulatory role in tooth initiation and morphogenesis (Thesleff 2003). Tissue recombination experiments have shown that the first inductive signal comes from the epithelium and after the early signaling centers form at E12, the induction switches to the mesencyme (Mina and Kollar 1987; Lumsden 1988). It has been shown that the mesenchyme defines the tooth identities (Kollar and Baird 1970), but the molecular signals are not known. It has been suggested that Hox genes may play a role in the tooth identity specification (Thomas and Sharpe 1998), but this has not been proven experimentally. The placode development involves the integration of all signaling pathways (Laurikkala et al. 2006).

1.5.2 The enamel knot and morphogenesis

As tooth development proceeds, the early tooth bud grows down into the mesenchyme. At the late bud stage (at E13 in the mouse) a signaling center called the enamel knot forms at the tip of the bud (Jernvall et al. 1994; Butler 1956). It has been shown by *in situ* hybridization that the non-dividing cells of the enamel express multiple signals that belong to all signaling molecule families (Thesleff 2003). There are over 50 genes known to be transcriptionally active in the enamel knot. There are molecules that belong to the BMP, FGF, Shh and Wnt signaling families (http://bite-it.helsinki.fi). The molecular signals from the enamel knot direct the morhogenesis of the tooth (Figure 3.). Surrounding cells proliferate and the flanking epithelium grows deeper into the mesenchyme forming the cervical loops. Mesenchymal cells that are surrounded by the cervical loops form the dental papilla. There are multiple transgenic mouse models where tooth development is stopped at the bud stage and no enamel knot is formed, indicating the importance of this step in tooth development (Peters et al. 1998; D'Souza et al. 1999; van Genderen et al. 1994; Chen et al. 1996). The enamel knot is a transient structure, which is removed by apoptosis (Vaahtokari et al. 1996). After the removal of the primary enamel knot the morphogenesis proceeds and at the bell stage the secondary enamel knots form, which define the places for the future cusps. Thus this makes the bell stage important for development of the final shape of the tooth crown and enables the formation of teeth with multiple cusps (Jernvall et al. 2000). Disruption of the BMP, Eda or FGF signaling pathways leads to malformed cusp patterns (Kassai et al. 2005; Klein et al. 2006; Kangas et al. 2004).

By mathematical modeling an activator-inhibitor loop has been shown to result in the formation of enamel knots and to account for some aspects in the development and evolution of teeth. The activator-inhibitor concentration gradients reproduce the patterns

of expression of known genes, the nested patterns around the knots, activation of enamel knot formation and the sequence of enamel knot formation. Changes in the activator-inhibitor balance lead to variable cusp patterns and tooth shapes in different species (Salazar-Ciudad and Jernvall 2002).

1.5.3 Formation of dental hard tissues

Differentiation of tooth specific cells, the odontoblasts and ameloblasts, is initiated during the bell stage at the secondary enamel knots and proceeds in cervical direction. In the mesenchyme the cells next to the epithelium differentiate into pre-odontoblasts and odontoblasts. The odontoblasts start to secrete predentin which later mineralizes into dentin. In the inner enamel epithelium, the epithelial cells differentiate to pre-ameloblasts and ameloblasts, which later start to secrete extracellular matrices of enamel (Figure 3.). Roots are formed after the completion of crown development. The mesenchymal dental follicle cells differentiate into cementoblasts, which secrete bone-like cementum, which covers the root. The surrounding dental follicle forms the periodontal ligament that links the tooth with alveolar bone. Teeth erupt into oral cavity after birth (Nanci 2007).

1.5.4 Wnt signaling in tooth development

Seven Wnt ligands have been reported to be expressed in developing teeth, including *Wnt3*, *Wnt4*, *Wnt5a*, *Wnt6*, *Wnt7b*, *Wnt10a* and *Wnt10b*. Wnt ligands are expressed mainly in dental epithelium at all stages from initiation to the late morphogenesis (E12-E17). During the initiation *Wnt10a* and *Wnt10b* are expressed in the early signaling centers and later in the primary and secondary enamel knots. *Wnt3* and *Wnt7b* are expressed in the flanking oral ectoderm of the early signaling centers (Dassule and McMahon 1998; Sarkar and Sharpe 1999). *Wnt4*, *Wnt6* are expressed in oral epithelium during initiation and morphogenesis (Sarkar and Sharpe 1999). These expression patterns suggest that the role of Wnts is solely in the epithelium. *Wnt5a* is the only Wnt ligand known to be expressed in dental mesenchyme. However, Wnt5a has a dualistic role in Wnt signaling. Wnt5a is shown to activate both β-catenin dependent and non-dependent pathways, which are sometimes shown to antagonise each other (Liu et al. 2005). It has been shown in cell culture experiments that Wnt5a protein activates or inhibits β-catenin/Tcf signaling depending on the receptor context (Mikels and Nusse 2006). Thus as there is no experimental evidence, it is only speculation whether the role Wnt5a is to activate or inhibit Wnt signaling in dental mesenchyme.

Of the Frizzled receptors *MFz6* has been detected in the oral epithelium, enamel knot and outer dental epithelium. *MFz3* and *MFz4* are detected in presumptive dental mesenchyme at E11.5. Wnt antagonists *MFrzb1* and *Mfrp2* have been detected in dental mesenchyme (Sarkar and Sharpe 1999). Of soluble Wnt antagonists, *Dkk1* is expressed in dental mesenchyme, *Dkk2* in dental papilla and *Dkk3* in enamel knots (Fjeld et al. 2005).

None of the investigated *Wnt* null allele mice (*Wnt1, 2, 3, 3a, 4, 5a, 7a*) have a reported tooth phenotype, possibly because of redundancy of the ligands. Transgenic mice approach has revealed some information on the role of Wnt signaling in the initiation of tooth development. *Lef1* deficient mice have arrested tooth development at the bud stage (van Genderen et al. 1994). This phenotype was rescued by FGF4 (Kratochwil et al. 2002), indicating that the Wnt and FGF pathways interact. Overexpression of Dkk1 in

oral epithelium leads to the arrest of tooth development at the early bud stage (Andl et al. 2002). Wnt signaling has been implied to the differentiation of dental cell types. Wnt10a has been suggested to link the differentiation of odontoblasts and cusp morphogenesis (Yamashiro et al. 2007).

1.6 Tooth development in non-mammalian vertebrates

Non-mammalian vertebrates, fish, amphibians and reptiles are homodont i.e. all teeth share the same tooth shape of simple conical teeth (unicusped condition). The function of the teeth is to catch and hold food which is swallowed as a whole and digested first in the stomach. Another typical characteristic is that non-mammalian vertebrates have continuous tooth replacement and renewal (polyphyodonty) throughout their lifespan. Teeth are added in two directions, to the back of the jaw as the animal and the jaw are growing, and on the place of lost or damaged teeth between existing teeth. A common feature is that each replacement tooth is bigger than its predecessor tooth. This is in line with the mode of growth in non-mammalian vertebrates. They have no determinate adult size, but grow throughout their lifespan and thus the teeth have to meet these requirements (Osborn 1998).

The molecular mechanisms regulating non-mammalian vertebrate tooth development are very similar as in mammals. Moreover, it has been shown in the trout, *Oncorhynchus mykiss,* that *Shh* and *Bmp4* are expressed in a similar way both in oral and pharyngeal teeth (Fraser et al. 2006). Interestingly, after initiation of tooth development *Pitx2* expression is downregulated in the trout posterior pharyngeal teeth, but not in the oral teeth, suggesting a possible function for *Pitx2* in an early tooth-commissioning role (Fraser et al. 2006). Inhibition of FGF signaling leads to arrest of zebrafish tooth development. *Pitx2* expression in the primary epithelial band is the first indication of tooth development. However, inhibition of FGF signaling does not affect *Pitx2* expression, suggesting that the earliest steps of tooth development in zebrafish are FGF independent. *Fgf8* and *Fgf9* expression is not detected in zebrafish tooth germs. Also there is no *Pax9* expression (Jackman et al. 2004).

1.6.1 Mechanism of tooth replacement in non-mammalian vertebrates

The research of non-mammalian tooth replacement has been concentrating on two main issues, the patterns of tooth replacement and the developmental mechanisms of the replacement itself. Also the role and evolution of the dental lamina in replacement tooth generation has been studied in non-mammalian species. Replacement which occurs in waves that pass through alternate tooth positions in front to back directions is the most commonly suggested mechanism. The advantage of this is that a large region of the jaw is never devoid of teeth. This is shown in a reptile species *Lacerta viridis* (reviewed by Berkovitz 2000). However, in *Lacerta vivipara* no such pattern is detected and replacement is random (Osborn 1971). In *Alligator mississipiensis* there is evidence for alternation, but the overall sequence does not show perfect regularity (Westergaard and Ferguson 1990; Westergaard and Ferguson 1987). Successive replacement waves may show local variations leading to change of an existing pattern. Also the direction of the wave may vary. In many cases there are random patterns of tooth replacement, the randomness increasing with age (Berkovitz 2000). In zebrafish (*Danio rerio*) tooth replacement does not occur randomly, but follows a pattern in most cases (van der

Heyden et al. 2001). Factors controlling the replacement patterns are not known. It has been suggested that there is a field of morphogen gradients along the jaw that create the alternate replacement patterns or that there is a single cell mass, a clone, which initiate a tooth primordium (Osborn 1978). It has been suggested as part of the clone theory that there is a margin of inhibition surrounding the existing teeth, by the presence of new teeth, leading to alternate tooth replacement patterns (Osborn 1971). As it has recently been suggested that there is a link between the eruption of a tooth and the development of a successional lamina, indicating that tooth replacement is under local control (Huysseune 2006; Huysseune and Witten 2006), and as the results concerning tooth replacement patterns are so variable, it may be more informative to examine tooth replacement in individual tooth positions and not in a whole dentition.

In the common lizard *Lacerta vivipara* teeth arise from the free edge of dental lamina, which is connected to pre-existing teeth, and the dental lamina is reformed on the lingual side of the pre-existing teeth (Osborn 1971 and Figure 4). Also in zebrafish *Danio rerio* replacement tooth generation involves the formation of a successional dental lamina (Huysseune 2006). In zebrafish, amphibians and lizards the successional dental lamina is discontinuous and it disappears when the replacement tooth erupts. The successional lamina can remain quiescent for some time before the replacement tooth is initiated (Huysseune 2006). It has been proposed that the region differentiating to the successional lamina might contain a stem cell niche that regulates the replacement tooth formation (Huysseune and Thesleff 2004). However, there is no evidence for this from molecular or lineage tracing experiments. It has been proposed based on observation in salamander *Pleurodeles waltl* that tooth replacement is initiated in relation to a particular developmental step of the previous tooth (Davit-Beal et al. 2007; Davit-Beal et al. 2006) and that the upper region of the dental organ of the predecessor tooth has conserved the ability to differentiate into a successional dental lamina (Davit-Beal et al. 2007). However, there is no genetic information on the regulation of any of these events. There is not much information on the differential regulation of first generation teeth and the replacement teeth. The zebrafish *Eve1* gene has been shown to be expressed in the epithelium during the inititation of the first generation but not during the second generation of teeth (Laurenti et al. 2004). *Eve1* has not been reported to be expressed during mammalian tooth development. There are several studies where the molecular regulation of tooth number has been investigated in the mouse toothless diastema region. It has been shown that by alternating either Eda, BMP or FGF pathway it is possible to induce the generation of an extra tooth anterior to the first molar (Kangas et al. 2004, Mustonen et al. 2003, Mustonen et al. 2004, Klein et al. 2006, Tucker et al. 2004, Kassai et al. 2007). It remains to be tested if these same molecules and pathways are involved in the regulation of tooth replacement. Taken together, it seems that the physiological mechanisms of

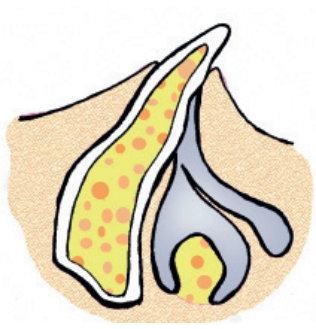

Figure 4. Tooth replacement in *Lacerta vivipara*, the common lizard. Replacement teeth arise from the free edge of the dental lamina lingual to the pre-existing teeth. (Schematic picture by Kalle Karinen, from Osborn 1971).

tooth replacement are fairly well understood, but there is only little information on the molecular regulation of tooth replacement or on the determination of the replacement patterns.

1.7. Tooth development in mammals

Mammalian teeth are morphologically heterodont, with multiple shapes. A typical mammalian dentition consists of incisor, canine, premolar, and molar teeth, with a species specific number of each tooth type. Incisors, the front teeth, are conical, unicusped teeth which are located most mesially. Canines are unicusped, but usually larger and sharper than incisors and they are located laterally to the incisors. Premolars and molars, the cheek teeth, belong to the post-canine tooth family and they have the most complicated morphology of multiple cusps (multicusped). The function of mammalian teeth is different from the non-mammalian vertebrate teeth. Mammals grind their food, which requires a precise occlusion and more complex teeth. Upper and lower teeth have to meet to grind food smaller for easier digestion. Teeth with more cusps better serve this function. In evolution a small change in the amount of a specific gene, such as *Eda*, can alter the tooth shape as well as cusp and tooth number radically (Kangas et al. 2004). It has been shown by geographic information systems (GIS) analysis that the surface complexity of tooth crowns directly reflects the foods the animal consumes (Evans et al. 2007).

1.7.1 Tooth replacement in mammals

Mammals have two dentitions. They replace their teeth only once (diphyodont condition)**.** The first set of teeth is called primary, deciduous or milk teeth. The second set of teeth is called secondary, successional, permanent, replacement or adult teeth. Incisors, canines and premolars are replaced, but molars are not replaced. Therefore the classification of molars has been difficult and controversial. Molars are sometimes considered to belong to the first generation of teeth as unreplaced members, as the primary premolars share morphological similarities with the molars. Or they are considered to belong to the second generation of teeth as they are never replaced in modern mammals. It has also been suggested that they should be considered as an entity of their own since they have no predecessors or successors (van Nievelt 2002).

The physiological mechanism of tooth replacement in various mammalian species has been described by many authors already during the 19th century (Leche 1895). However, these reports include only occasional hand drawn pictures of histological sections and as different authors have variable descriptions mostly in German, the replacement mechanism has stayed unclear. In the following I will present few selected central points from the earlier studies concerning the definition and origin of dental lamina and the mechanism of tooth replacement.

Some authors argued that the replacement tooth buds from the primary tooth. Some authors saw already in the primary dental lamina two buds, which later became the primary and the replacement tooth, and some stated that the replacement tooth develops from “superficial epithelial rests” of the dental lamina. According to Leche (1895), the most likely description is that the replacement tooth develops not from the previous tooth, but rather all teeth arise from the same primary dental lamina. There is a deep

end of the dental lamina, which is disconnected from the predecessor tooth, and which grows further down into the mesenchyme. The replacement tooth develops next to the predecessor tooth, to the lingual side. The whole disconnected dental lamina becomes part of the replacement tooth. The replacement tooth is connected to the dental lamina and partly to the enamel organ of the predecessor tooth (reviewed by Leche 1895). The replacement tooth generation has been cited in the modern textbooks as follows: "Also the permanent dentition arises from the dental lamina. The permanent tooth germs form as a result of further proliferative activity within the dental lamina at its deepest extremity. This increased proliferative activity leads to the formation of another tooth bud on the lingual aspect of the deciduous tooth germ" (Nanci 2007). This description leaves room for the imagination of the reader and shows that a detailed modern study of the mechanism of tooth replacement is needed.

1.7.2 Diphyodonty in mammals

The reduction of tooth replacement from the polyphyodont condition of non-mammalian vertebrates to diphyodonty has been linked to the origin of other mammalian characteristics such as small jaw size, rapid growth to adult size, endothermy, formation of hair and glands, lactation, shift to dentary squamosal articulation/middle ear ossicles, changes in the pharynx and oral cavity, mastication, precise occlusion, precise jaw movements and complex cheek teeth (van Nievelt 2002).

A link between dental development and lactation of mammals has been proposed by many authors (Luckett 1985; van Nievelt 2002). It is possible that the mammary line and dental lamina has been initiated at the same evolutionary time period. It is intriguing to think that the same signals might regulate the formation of both structures, and the initiation of both the mammary and dental placodes. Indeed, it has been shown that similar molecular mechanisms are involved. It has been demonstrated that Wnt, BMP, Ectodysplasin and FGF signaling is needed for both processes (Mikkola 2007). Formation of mammary line is dependent on Wnt and FGF signaling (Chu et al. 2004; Hens and Wysolmerski 2005). There are several reports on the requirement of Wnt signaling in the initiation of mammary placodes. The formation of mammary placodes within the mammary line requires Wnt signals (Eblaghie et al. 2004; Veltmaat et al. 2004). Mice overexpressing Dkk1 lack mammary placodes (Chu et al. 2004). Later it was shown that Wnt signaling is required only for the formation of placodes 2 and 3, but not for the initiation of placodes 1, 4 and 5, therefore suggesting differential regulation and identity for different placodes. However, also the three latter placodes need Wnt signaling for the progression of placode development (Boras-Granic et al. 2006). The placodes are thought to form from cell movements within the mammary line (Chu et al. 2004). Ectodysplasin signaling is involved in the formation of mammary placodes. When there is ectopically induced Eda in the epithelium, enlarged and supernumerary mammary placodes form (Mustonen et al. 2004). Eda signaling might thus direct the positioning of the placode or/and promote placode formation. Interestingly, the supernumerary placodes form only along the milk line and near the existing placodes, suggesting that the action of Eda signaling is downstream of the specification of the mammary line. Also the supernumerary teeth arise only along the dental lamina (Kangas et al. 2004; Mustonen et al. 2004; Klein et al. 2006)

1.7.3 Variable tooth replacement patterns

Tooth replacement is reduced in evolution leading to considerable variation in tooth replacement patterns in mammals. Functional replacement takes place when both generations of teeth erupt. Non-functional replacement takes place when the first generation of teeth does not erupt, but its development has been initiated. Usually these rudiments disappear before mineralization. An exception is the seals (*Phocidae*), where the rudimentary teeth mineralize, do erupt, but are shed during embryogenesis (Stewart and Stewart 1987). I will give examples of various replacement patterns in selected mammalian species. Most primates, including humans, have functionally diphyodont teeth. Humans and many domestic animals, cows (*Bos taurus*), sheep (*Ovis aries)*, and goats (*Capra hircus)*, display a primitive pattern of functional tooth replacement, the full replacement of incisors, canines and premolars (Getty 1975). Some *Soricidae*, such as shrews, lost all functional replacement and have non-functional primary teeth, but functional replacement teeth (Kindahl 1959). It was shown that there is non-functional replacement in all tooth positions excluding the molars (Kindahl 1959). Sometimes even inside the same family there is plenty of variation. In the *Mustelidae* there are three different possibilites: primitive eutherian full replacement in the tayra (*Eira barbara*) and in the ferret (*Mustela putorius*), the loss of most functional replacement at the incisor loci in some other weasels (*Mustela*), even the loss of all functional replacement in the striped skunk (*Mephitis mephitis*). The general trend in weasels is to lose all functional lower deciduous incisors and one or two pairs of functional central upper deciduous incisors and to have functionally diphyodont canines and premolars (Habermehl and Röttcher 1967; Moshonkin 1979). Other examples where functional replacement have been reduced only either in canine or premolar positions, but retained in other positions and the horse (*Equus caballus)* has lost functional replacement in the canines, and *Myotis lucifugus,* the little brown bat and *Urothrichus talpoides,* japanese shrew mole have lost some of the functional replacement in the premolars but retain incisor and canine replacement (Hanamura et al. 1988; Getty 1975; Fenton 1970). Muroid rodents, such as the mouse (*Mus musculus*) have lost all tooth replacement. Also tooth number is reduced during evolution and mouse contain only incisor and three molars in each jaw quadrant. A sciurid rodent *Spermophilus parryi*, the squirrel, has a second incisor that develops only until the early cap stage and not further. This is an example of an animal that has a primary tooth developing, but the secondary tooth is rudimentary/non-functional (Luckett 1985). This suggests that mouse incisors may belong to primary dentition. *Sorex araneus* represents an opposite case, where the primary tooth is suppressed and non-functional, but the secondary tooth develops and is functional (Kindahl 1959). It is not easy to define which tooth generation is left in the jaw. In the dog the debate has been going on for over a century. There is only one generation of teeth in dog premolar 1 position and there is evidence both for it being a deciduous tooth or the permanent tooth (Williams and Evans 1978). Also in the pig (*Sus scrofa domesticus*) there is only one generation of P1. However, this information is based only on eruption data (Getty 1975). Taken together, there is great variation in tooth replacement patterns in mammals and a tendency to lose replacement during evolution. Evolutionary reduction of tooth replacement is demonstrated by the number of rudimentary teeth and non-functional replacement in various species. The different replacement patterns are indicative of the precise adaptation of different species.

1.7.4 Continuously growing mouse incisor

Rodents have a large sharp lower incisor in each half of the mandible. A labial-lingual asymmetry in the distribution of enamel keeps the cutting edges of the incisors sharp and the wear is compensated by continuous growth. There is an epithelial stem cell compartment in the cervical loop (Harada et al. 1999). These stem cells differentiate into ameloblasts, the enamel forming cells. It has been shown that follistatin regulates the enamel patterning by asymmetrically inhibiting BMP signaling and ameloblast differentiation in the lingual cervical loop (Wang et al. 2004b). Stem cell proliferation in the cervical loop is controlled by spatial regulation of BMP, FGF, Activin and Follistatin in a complex regulatory network (Wang et al. 2007). The continuously growing mouse and rabbit incisors do not undergo functional replacement (Moss-Salentijn 1978).

Table 1. Tooth replacement patterns in selected mammalian species. I =incisor, C=canine, P=premolar

Species	Common name	Replacement	Reference
Homo sapiens	human	I, C, P full replacement	(Nanci 2007)
Bos taurus	cow	I, C, P full replacement	(Getty 1975)
Ovis aries	sheep	I, C, P full replacement	(Getty 1975)
Capra hircus	goat	I, C, P full replacement	(Getty 1975)
Canis familiaris	domestic dog	I, C, P, full replacement, but in P1 no replacement	(Evans 1993)
Sus scrofa domesticus	domestic pig	P1 no replacement	(Getty 1975)
Mustela lutreola	European mink	I partly, C and P full replacement	(Moshonkin 1979)
Mustela putorius	polecat, ferret	I, C and P full replacement	(Habermehl and Röttcher 1957), (Berkovitz 1973)
Mephitis mephitis	striped skunk	non- functional replacement in I, C, P	(Verts 1967)
Sorex araneus	common shrew	no functional replacement, non-functional replacement in I, C, P	(Kindahl 1959)
Equus caballus	horse	I, P full replacement, C no replacement	(Getty 1975)
Urotrichus talpoides	Japanese shrew mole	I, C, P, but dp2 no replacement	(Hanamura 1988)
Myotis lucifugus	little brown bat	I, C full replacement	(Fenton 1970)
Spermophilus parryi	squirrel	P, full replacement	(Mitchell and Carsen 1967)
Mus musculus	mouse	no replacement, continously growing lower I	(Moss-Salentijn 1978)

1.8 Mutations affecting tooth number and tooth renewal in humans

Congenitally missing permanent teeth are found in 8% of the population world wide. The most commonly missing teeth are those that develop as last teeth in the different tooth families. Second premolars and upper lateral incisors are thus most often affected. The genes behind this common incisor-premolar hypodontia are not known. Oligodontia, tooth agenesis with more than six missing teeth, is rarer and there are few single gene mutations known to cause this aberration. Supernumerary teeth are less common than developmentally missing teeth. An upper incisor is the most common extra tooth. Multiple supernumerary teeth appear as symptoms in some syndromes.

1.8.1. Mutations causing supernumerary teeth

There are only two known mutations causing supernumerary teeth in human. Mutations in the Runt domain transcription factor *Runx2* (Aml3, Cbfa1, Osf2, Pebp2αA) gene in humans and mice, leads to bone and tooth defects. In humans this autosomal dominant syndrome is called cleidocranial dysplasia. These patients show bone defects and supernumerary teeth (Otto et al. 1997; Mundlos et al. 1997; Jensen and Kreiborg 1990). Interestingly in humans, the primary tooth development is normal, but tooth renewal is not inhibited after the formation of the permanent dentition. These patients also show defects in the eruption of the secondary dentition. In homozygous null-mutant mice the disruption of *Runx2* gene leads to complete loss of bone, and tooth development is arrested at the bud stage (Åberg et al. 2004). Heterozygous mice are similar to wild type mice and show no bone defects. However, sometimes there is an extra lingual tooth bud in the upper molar, and it shows *Shh* expression (Wang et al. 2005). These results indicate that Runx2 is needed both in the formation of the primary dentition and in tooth renewal and that the amount of Runx2 is important.

Autosomal dominant germline mutations in *APC* cause familial adenomatous polyposis (FAP) and its variant the Gardner syndrome (Groden et al. 1991). Gardner syndrome is characterized by dental abnormalities including impacted or supernumerary teeth and compound odontomas (Gardner 1962; Fader et al. 1962; Wolf et al. 1986). *APC* is a tumor suppressor gene associated with the stabilization of β-catenin. Loss of APC leads to increased Wnt signaling causing supernumerary teeth, aberrant hair placode initiation and hair follicle growth in mice (Kuraguchi et al. 2006).

1.8.2. Mutations causing missing teeth

Heterozygous loss of function of the transcription factor *Pax9* causes oligodontia (agenesis of several teeth) in human (Stockton et al. 2000). The primary dentition is normal. Molars are the most affected teeth. Sometimes also some premolars and incisors are missing in the permanent dentition. In *Pax9* null allele mice tooth development stops at the bud stage before the transition to the cap (Peters et al. 1998). It has been shown that FGFs induce *Pax9*. Reduction of *Pax9* gene dosage causes different levels of oligodontia in mice (Kist et al. 2005).

Heterozygous loss of function of transcription factor *Msx1* causes tooth agenesis in human. Affected individuals are reported to have normal primary dentition and the most affected teeth are from the permanent dentition. Premolars and molars are most affected (Vastardis et al. 1996). In *Msx* null allele mice tooth development arrests at bud stage, before the mesenchymal condensation. BMP and FGF induce *Msx1* (Chen et al. 1996;

Bei and Maas 1998). In *Msx1:Msx2* double mutant tooth development stops even earlier, at the lamina stage (Bei and Maas 1998). Mutations in the intracellular Wnt inhibitor *Axin2* lead to severe tooth agenesis in human. Permanent dentition is severely affected, and only sometimes a tooth is missing in the primary dentition. These mutations also predispose to colorectal cancer (Lammi et al. 2004).

1.8.3 Ectodermal dysplasias with tooth phenotypes

Ectodermal dysplasias are congenital defects where the development of two or more ectodermal organs is abnormal. Most common organs affected are hairs and teeth. Mutations in the *Ectodysplasin* (*Eda*) gene cause X-linked hypohidrotic (anhidrotic) ectodermal dysplasia (HED, EDA) leading to defects including the absence of several primary and permanent teeth, delayed primary and permanent dentition, conical tooth crown, sparse and fine hair, premature male balding and the lack of sweat glands (Kere et al. 1996; Headon and Overbeek 1999). When either the ligand Eda, the receptor Edar or the death domain intracellular effector Edaradd are knocked out in mice, the mice display symptoms of the dysplasia (Mikkola and Thesleff 2003). In 20% of *Eda* null allele mice third molars are missing. Sometimes also the incisors are lacking. The crowns of the first molars show a malformed shape. *Eda* null allele mice also lack first wave of hair follicles and have defects in many glands (Mikkola 2007).

Mutations in the transcription factor *p63* of the p53 family, lead to Ectrodactyly-Ectodermal Dysplasia-Clefting (EEC) syndrome characterized by multiple missing and misshapen teeth, and by defects in the skin (Celli et al. 1999). *p63* null allele mice lack all ectodermal organs (Mills et al. 1999). In the mutant mice tooth development is arrested prior the placode stage. However, the primary epithelial band is present, but placodes fail to form (Laurikkala et al. 2006).

A homozygous nonsense mutation in exon3 of *Wnt10a* gene leads to premature truncated protein causing an autosomal recessive ectodermal dysplasia syndrome affecting teeth and hair. These patients have severe hypodontia in the permanent dentition and the primary teeth are peg-shaped. They show also hair and skin defects and reduction of taste papillae (Adaimy et al. 2007).

Table 2. Defects and genes affecting tooth number and renewal in human and mouse. o.e.= oral epithelium.

Gene	Type of protein	Tooth defect	Type of mutation	Organism	References
ActivinβA	TGFβ family ligand	Arrest at the bud stage (not upper molars)	KO	Mouse	(Matzuk et al. 1995)
APC	Wnt family member, intracellular effector	Gardner's syndrome Supernumerary teeth	Autosomal dominant TG, K14 o.e.	Human Mouse	(Fader et al. 1962; Gardner 1962; Wolf et al. 1986) (Kuraguchi et al. 2006)
Axin2	Intracellular Wnt antagonist	Tooth agenesis No phenotype	Autosomal dominant KO	Human Mouse	(Lammi et al. 2004) (Järvinen et al.; unpublished)
Bmpr1a	TGFβ family receptor	Arrest at bud stage	TG, K14 o.e.	Mouse	(Andl et al. 2004)
Dlx1;Dlx2	Transcription factor	Arrest of upper tooth development at the placode stage	KO	Mouse	(Thomas et al. 1997)
Dkk	Soluble extracellular Wnt antagonist	Arrest at the bud stage	TG, K14 o.e.	Mouse	(Andl et al. 2002)
Eda	TNFR superfamily member ligand	Tooth number reduced	X-linked translocation Natural mutant	Human Mouse	(Kere 1996, Headon and Overbeek, 1999) (Srivastava et al. 1997)
Eda	TNFR superfamily member ligand	Extra molar	TG, K14 o.e.	Mouse	(Mustonen et al. 2003)
Edar	TNFR superfamily member receptor	Tooth number reduced	Natural mutant	Mouse	(Tucker et al. 2000)
Edar	TNFR superfamily member receptor	Changes in tooth number	TG, K14 o.e.	Mouse	(Pispa et al. 2004)
Fgf8	FGF ligand	Loss of molars	KO	Mouse	(Trumpp et al. 1999)
Follistatin	TGFβ family ligand	Loss of M3	K14 o.e	Mouse	(Wang et al. 2004)
Fgfr2b	FGF receptor	Arrest at the bud stage	KO	Mouse	(De Moerlooze et al. 2000)
Gli2;Gli3	Transcription factor	Arrest at the bud stage	KO	Mouse	(Hardcastle et al. 1998)
Lef1	Transcription factor	Arrest at the bud stage	KO	Mouse	(van Genderen et al. 1994)
Msx1	Transcription factor	Tooth agenesis Arrest at the lamina stage	Autosomal dominant KO	Human Mouse	(Vastardis et al. 1996) (Chen et al. 1996)
Msx1;Msx2	Transcription factor	Arrest at the lamina stage	KO	Mouse	(Bei and Maas 1998)

Table 2 continuing

Noggin	TGFβ family ligand	Loss of M3	TG, K14 o.e.	Mouse	(Plikus et al. 2005)
Pax9	Transcription factor	Tooth agenesis Arrest at bud stage	Autosomal dominant KO	Human Mouse	(Stockton et al. 2000; Peters et al. 1998)
p63	p53 family transcription factor	EEC syndrome Tooth number	Autosomal dominant KO	Human Mouse	(Celli et al. 1999; Laurikkala et al. 2006)
Runx2	Runt-domain transcription factor	Supernumerary teeth Arrest at bud stage	Autosomal dominant KO	Human Mouse	(Jensen and Kreiborg 1990; Otto et al.1997; Mundlos et al. 1997; D'Souza et al. 1999)
Pitx2	Transcription factor	Rieger syndrome, missing teeth Arrest at the bud stage upper, at the placode stage lower	Autosomal dominant KO	Human Mouse	(Semina et al. 1996; Lin et al. 1999; Lu et al. 1999)
Prx1;Prx2	Transcription factor	Lower incisors arrested at bud stage	KO	Mouse	(ten Berge et al. 1998)
Sostdc1	TGFβ family soluble inhibitor	Extra incisor, extra molar	KO	Mouse	(Kassai et al. 2005)
Sprouty 2	FGF intracellular Inhibitor	Extra molar	KO	Mouse	(Klein et al. 2006)
Sprouty 4	FGF intracellular inhibitor	Extra molar	KO	Mouse	(Klein et al. 2006)
Tbx3	T-box family transcription factor	Agenesis of canines	Autosomal dominant	Human	(Bamshad et al. 1997)
Wnt10a	Wnt ligand	Tooth agenesis	Autosomal recessive	Human	(Adaimy et al. 2007)

2. AIMS OF THE STUDY

The aim of this thesis work was to find a model animal for tooth replacement studies and to study the mechanisms and molecular regulation of tooth replacement. The specific aims were:

1. To search for downstream targets of *Runx2* and investigate its role in signaling during tooth development

2. To study the role of Wnt signaling in tooth renewal using transgenic mice with continuously activated β-catenin in the dental epithelium and mesenchyme

3. To study tooth replacement and its evolutionary loss in *Sorex araneus*, the common shrew

4. To study tooth replacement and its molecular regulation in *Mustela putorius furo*, the ferret

3. MATERIALS AND METHODS

3.1 Mouse strains

Strain	Used in article	Purpose
Runx2	I	Search of downstream targets
β-*catΔ*$^{ex3K14/+}$	II	Activation of *β-catenin* in the dental epithelium under the keratin 14 promoter
BATgal	II	Wnt reporter analysis
Rosa26	II	*LacZ* reporter analysis
Nude	II	Kidney capsule transplantation experiments

3.2 Probes

The following probes were used for *in situ* hybridization.

Probe	Reference	Used in article
Ameloblastin	(Wang et al. 2004b)	II
Dkk1	(Andl et al. 2002)	I, II
Dspp	(James et al. 2006)	II
Dusp6	(James et al. 2006)	I
Edar	(Laurikkala et al. 2001)	II
Enpp1	(James et al. 2006)	I
Fgf3	(Kettunen et al. 2000)	II
Fgf4	(Jernvall et al. 1994)	II
Igfbp3	(James et al. 2006)	I
p21	(Jernvall et al. 1998)	II
Lef1	(Kratochwil et al. 2002)	II
Runx2	(D'Souza et al. 1999)	I, II
Shh	(Vaahtokari et al. 1996)	II, IV
Sostdc1	(Laurikkala et al. 2003b)	II, IV
Suncus-Shh	(Ogi et al. 2002)	III
Wnt10b	(Sarkar and Sharpe 1999)	II

3.3. Methods used in articles I-IV

Method	Article
Cloning of genes	I
Tissue culture and bead experiments	I, II
Radioactive *in situ* hybridization on sections	I, II, III, IV
Digoxigenin *in situ* hybridization on sections	IV
Whole mount *in situ* hybridization	I, II, IV
Immunohistochemistry	II
LacZ staining	II
Collection of the *Sorex araneus* samples	III
Stageing of the *Sorex araneus* samples	III
Histology of *Sorex araneus* samples	III
Collection of the *Mustela putorius* samples	IV
Histology of *Mustela putorius* samples	IV
Cloning of *Mustela putorius* specific *Axin2*	IV

4. RESULTS AND DISCUSSION

4.1 Downstream targets of *Runx2* in tooth development (I)

We searched for downstream targets of *Runx2* and analyzed signaling pathway networks. Runx2 is a Runt domain transcription factor known to regulate tooth development at the bud to cap stage transition. In *Runx2* null allele mice tooth development stops at the late bud stage, no enamel knot is formed and the tooth does not continue morphogenesis (D'Souza 1999). To investigate the downstream targets of *Runx2*, we used Affymetrix microarray chips to compare expression patterns in E14 mutant and wild type mandibles. The tissue dissected for the analysis contained the epithelium and the mesenchyme of the first molars and the surrounding osteogenic mesenchyme. In the screen we found 50 downregulated and 13 upregulated genes in *Runx2* mutant tissue compared to the wild type. Of the downregulated genes, genes of interest were selected and radioactive *in situ* hybridization analysis was conducted to verify the microarray results. The expression patterns of these genes were investigated first in the wild type sections. Some of the selected genes were found to be expressed in developing bone and some in developing tooth. *Dkk1*, *Enpp1*, *Igfbp3* and *Dusp6* were all expressed in the dental mesenchyme at E14. The expression patterns of all these genes were overlapping with *Runx2*, and *Dusp6* was expressed also in dental epithelium. The expression pattern of *Dkk1* was interesting as it showed a half-moon like expression at the outer side of the dental papilla mesenchyme but was absent from the vicinity of the epithelium and enamel knot (I Fig. 1). In *Runx2* null allele E14 sections all four genes were completely absent from dental mesenchyme. Also *Dusp6* expression in the epithelium was missing at E14 in the mutant.

To study the regulation of these genes, tissue culture experiments with agarose beads were conducted. Wild type and mutant tooth germs at E14 stage were dissected and the epithelium and mesenchyme were separated. Separated mesenchyme was cultured with FGF4 or control BSA beads (see methods in article I). The expression of target genes was checked by whole mount *in situ* hybridization. FGF stimulated the expression of all four genes in the wild type mesenchyme (I Fig. 1). I asked if *Runx2* is needed for this stimulation and therefore I cultured similarly mutant mesenchyme with FGF4 or control beads and checked the expression of the target genes. Interestingly, *Dkk1* stimulation by FGF4 was significantly reduced in *Runx2* null allele mesenchyme. All the other genes including *Enpp1*, *Dusp6* and *Igfbp3* continued to be stimulated by FGF even in the absence of *Runx2* (I Fig. 1). This indicates that the induction of *Dkk1* by FGF4 is *Runx2* dependent, and *Dkk1* may be a direct target of *Runx2*, and that *Runx2* is not necessary for the stimulation of *Enpp1*, *Dusp6* and *Igfbp3* by FGF4. Shh and BMP4 releasing beads had no effect on the target genes in wild type or mutant mesenchyme.

Similar experiments were conducted for the *Runx2* target genes in developing bone of the mandible. We showed that some of them, including *Dkk1*, were induced by BMP4, but not FGF4. It was shown that *Dkk1* induction by BMP4 in developing bone is *Runx2* dependent at E12, but independent at E11 (I Fig. 2). These results show that the expression of *Dkk1* by *Runx2* is regulated by different signaling pathways in different tissues, the FGF pathway in the tooth and the BMP pathway in the developing bone, and the requirement of *Runx2* is stage dependent.

Our results point a role for *Runx2* in various signaling pathways. As *Dkk1* is a Wnt antagonist, these results indicate that *Runx2* and the Wnt signaling pathways are connected. The *Runx2* downregulation leads to a missing Wnt antagonist in the mesenchyme, suggesting an upregulated Wnt signaling in the mesenchyme. The end result of *Runx2* downregulation is arrested tooth development. Therefore the possible upregulation of Wnt signaling in the mesenchyme may play a role in the progress of tooth development. Moreover, *Dkk1* was induced by FGF4, and the induction was *Runx2* dependent. This is in line with the earlier results where *Runx2* was shown to be induced by FGF4 (Åberg et al. 2004; D'Souza et al. 1999). FGFs from the enamel knot may regulate *Dkk1* in the mesenchyme through *Runx2*. *Runx2* would thus combine the FGF and Wnt pathways and mediate the reciprocal signaling between the epithelium and mesenchyme. An interesting question we have not been able to answer yet is: what is inhibiting *Dkk1* in the proximity of the dental epithelium during normal tooth development? If Dkk1 was not inhibited, its expression might expand closer to the epithelium leading to inhibition of Wnt signaling. Whether the possible downregulation of Wnt signaling in dental mesenchyme would lead to an opposite situation (i.e. increased tooth formation) as in the *Runx2* mutant, or not, is highly speculative and needs more investigation.

Wnt signaling and *Runx2* have both been connected with the primary tooth development and also with tooth replacement. The gene networks regulating tooth replacement are presumably complex, and more investigation is needed on determining the interactions between regulatory genes and on the possible role of other signaling pathways. In this transgenic mouse model it was not possible to address the role of *Runx2* in tooth replacement. However, it may be speculated that similar gene networks would regulate also the replacement tooth generation and thus point a role for *Runx2*, FGF and Wnt signaling also in tooth replacement.

4.2 Wnt signaling in tooth renewal (II and unpublished)

It has been previously shown that Wnt signaling is involved in tooth replacement in humans (Lammi et al. 2004). I therefore wanted to further study the role of Wnt signaling in tooth development by using transgenic mouse models. β-catenin was stabilized in oral- and dental epithelium by crossing *β-catenin-flox-ex3* mice to *K14cre* mice. The phenotype of the created *β-cat*$^{\Delta ex3fl/+}$ mice was analyzed by investigation of histological sections. It was noticed that the normal tooth morphogenesis was disrupted at an early stage. Instead of a transition to a cap stage tooth, the tooth bud at E14 showed irregular shape. At E16 the irregular shape was even more evident: there were multiple epithelial protrusions into the mesenchyme (II Fig. 1). Wnt activity was analyzed by crossing the *β-cat*$^{\Delta ex3fl/+}$ mice with Wnt reporter *BATgal* mice (see methods in II). In wild type tooth Wnt activity was detected by *LacZ* staining in the enamel knot, but in the triple mutant, multiple *LacZ* positive spots in the tooth forming area were observed (II Fig. 1 and unpublished). This suggested that even if β-catenin was stabilized throughout the epithelium, Wnt signaling was active only in specific spots. This may be due to the requirement of other Wnt pathway molecules, such as Lef1/Tcf1 transcription factors, for the activation of the pathway or due to inhibitory mechanisms. Extensive *in situ* hybridization analysis with multiple enamel knot markers were performed and it was

noticed that all analyzed enamel knot markers were expressed in the same spots as the Wnt activity seen in *β-cat$^{\Delta ex3fl/+}$;BATgal* mice. These markers included *Shh*, *Epiprofin*, *Edar*, *Wnt10a* and *Axin2* (II Fig. 2 and unpublished results). This suggested that multiple enamel knots were formed. Multiple enamel knots were found already at E13 and as the tooth bud expanded in size, new enamel knots were forming. *Sostdc1* (*Ectodin*) expression was surrounding these enamel knots similarly as in the wild type tooth. As *Shh* expressing dots were detected along the oral epithelium, I used *Fgf3* as a marker for tooth forming area (Kettunen et al. 2000). *Fgf3* was observed only in the tooth forming region indicating that the *Shh* dots in the oral epithelium marked ectopic ectodermal placodes of other epithelial appendages in addition to the teeth (II Fig. 2). Taken together, these results showed that stabilization of β-catenin leads to the formation of multiple enamel knots which exhibit active Wnt signaling. Enamel knots were formed only in the tooth forming oral region, which apparently had been specified at an earlier stage.

As the *β-cat$^{\Delta ex3fl/+}$* mice die for unknown reason before birth at around E18, I used a kidney capsule transplantation method to study the further development of the mutant tooth. When a normal wild type molar is cultured under kidney capsule for three weeks, all three mouse molars develop. However, when I cultured one *β-cat$^{\Delta ex3fl/+}$* mutant tooth germ for three weeks, 42 individual teeth formed (II Fig. 3). The teeth were inside a structure resembling a geode, with their cusps towards the inside and the roots pressed at the roof of the geode, towards the outside. The ends of the roots were seen as circles on the outer surface. The whole structure was almost half the size of the mouse kidney. Interestingly all teeth had differentiated tooth structures, enamel, dentin and the forming roots. However, most teeth were unicusped and conical and resembled more non-mammalian vertebrate than mouse teeth. The largest teeth were about the size of the mouse third molar. As it was not possible to follow the development of these teeth, but only the end result, I placed a mutant and a wild type E14 tooth germ into tissue culture conditions and followed the development under stereomicroscope for 11 days. The wild type tooth germ developed into the first and second molars. The mutant tooth germ formed ca. 10 individual small tooth buds in a circle. I dissected two of them into a subculture and followed similarly their development for about three weeks. New tooth buds formed from the pre-existing teeth. Altogether five additional new tooth germs formed sequentially (II Fig. 4). The mode of this tooth renewal resembled the generation of non-mammalian vertebrate replacement teeth. Activator-inhibitor mechanisms have been suggested to regulate the formation of enamel knots (Salazar-Ciudad et al. 2002). A mathematical model was used to predict the enamel knot pattern. The multiple enamel knot phenotype could be reproduced by only raising the intrinsic amount of the activator *β-catenin*. These results suggest that *β-catenin* is an upstream activator of the signals leading to the formation of enamel knots (II Fig. 5). In conclusion, continuous activation of β-catenin in dental epithelium leads to continuous tooth generation. Continuous tooth generation involves the iterative formation of ectopic signaling centers, enamel knots. Enamel knot activation and lateral inhibition, where the enamel knots inhibit each other, are the key mechanisms. The mode of tooth renewal in the *β-cat$^{\Delta ex3fl/+}$* mutant resembles the formation of teeth in non-mammalian vertebrates. It can be speculated that Wnt signaling was involved in evolution as the shape of mammalian teeth became more complex, and at the same time the tooth renewal capacity was reduced.

However, the phenotype of stimulated tooth renewal in *β-cat*$^{\Delta ex3fl/+}$ mice seemed contradictory to that of the human syndrome where heterozygotic loss of the Wnt inhibitor *Axin2* leads to tooth agenesis (Lammi et al. 2004). I asked why in the mouse activated Wnt signaling in the epithelium leads to more teeth and on the other hand activated Wnt signaling in humans leads to loss of teeth? The expression of *Axin2* was compared between *β-cat*$^{\Delta ex3fl/+}$ and wild type sections. *Axin2* expression is thought to indicate active Wnt signaling (Clevers 2006). On the other hand it is a Wnt antagonist. In the wild type *Axin2* is expressed in the enamel knot and in the mesenchyme (Lammi et al. 2004). In *β-cat*$^{\Delta ex3fl/+}$ there was strong expression in the enamel knots, but evidently downregulated or absent expression in the mesenchyme (unpublished results). This suggested that when Wnt signaling is activated in the epithelium, it may need to be downregulated in the mesenchyme in order to the new teeth to form. Thus there might be differential requirements of Wnt signaling in epithelium and mesenchyme. My hypothesis thus was that when Wnt signaling is activated in epithelium, tooth renewal is activated, but when Wnt is upregulated in mesenchyme, tooth renewal is inhibited. I tested the activation of Wnt signaling in dental mesenchyme by conditional stabilization of β-catenin in dental mesenchyme under the *Dermo1-Cre* promoter. The promoter starts to function at E9,5 in dental mesenchyme, therefore the construct has enough time to be activated in order to affect the initiation of tooth development at E11. However, *Dermo1Cre-Flox-ex3-β-catenin* mice died around E12,5 due to unknown reasons, and it was not possible to follow tooth development further *in vivo*. Also I noticed that at E12,5 the tissue started to loose its conformation and no dental placodes were detected in histological sections. Therefore I transplanted E11,5 dental tissue under the kidney capsule for three weeks. Both the wild type control and the mesenchymally Wnt activated tissue formed normal molars and incisors (unpublished results). I expected to see reduction of tooth development, but these results indicate that ectopic activation of Wnt signaling in dental mesenchyme does not reduce tooth development and the contradictory phenotypes of *β-cat*$^{\Delta ex3fl/+}$ mice and *Axin2* humans may be explained by other reasons. However, it is possible that the conditional construct *Dermo1Cre-Flox-ex3-β-catenin* may not function in every cell or that the construct itself has some unknown side effects. Also it cannot be ruled out that the molar development in mice is different from the permanent tooth development in human, and the molecular networks regulating these two events may be different. Also the molecular mechanism leading to continuous tooth renewal and tooth replacement may be different. Therefore I wanted to analyze the replacement tooth generation closer in other animal models.

4.3 The shrew *Sorex araneus* as a model for evolutionary loss of tooth replacement (III)

We decided to use *Sorex araneus*, the common shrew, as a model to study the evolutionary loss of tooth replacement. Previously it has been shown that *Sorex araneus* has non-functional tooth replacement in some antemolar tooth positions (Ärnbäck Christie-Linde 1912; Kindahl 1959). Shrew embryos were obtained by catching pregnant females in the wild and they were staged by the length (Methods III). Embryos ranging from 7.5 mm to 12mm were attained representing a series covering five embryonic stages where development of all tooth positions was observed. In the limb buds of the 7.5mm embryo

the early separation of digits was detected, and the appearance of the embryo resembled the mouse E13 stage. Histological and molecular analysis showed that only the primary premolar 4 (dP4) from the deciduous dentition reached the cap stage. No traits of other deciduous teeth in incisor, canine or premolar tooth loci were detected. The deciduous dP4 was in an early cap stage at the earliest analyzed stage at 7.5 mm. At 9.0 mm dP4 was in a cap stage, but already at 12mm it had diminished in size and only a small spot was left. dP4 expressed *Shh* in the enamel knot and in the inner enamel epithelium at all stages. *Shh* expression was persisting even after dP4 had started to diminish in size and was detected still at the last stage at 12mm, when the *Shh* expression domain covered most of the remaining dP4 epithelia. The first indication of P4 was at the 9 mm stage, when a lingual epithelial protrusion into the mesenchyme was detected. Already at 7.5 mm dP4 bud was not symmetrical and the lingual cervical loop could be distinguished from the dP4 priori, by the differential orientation of cells. The true initiation time point for P4 thus was somewhere between 7,5 and 9,0 mm. The permanent premolar 4 (P4) was initiated before differentiation of dP4 cervical loop. At 9.0 mm the tip of P4 bud showed weak *Shh* expression in the early enamel knot. At later stages *Shh* was detected in the enamel knot and in the inner enamel epithelium of P4 (III Fig 1, 2, Figure 5.).

These results showed that there is non-functional replacement in the *Sorex* premolar 4 and that the development of the primary tooth is somehow suppressed. We asked, what is the mechanism of dP4 suppression? We compared the relative growth of teeth and asked whether these produce a "double-wedge" pattern of replacement used to infer competitive replacement (Krause 1986). There are two possible modes, internal suppression and suppression by replacement. Internal suppression takes place when the replacement tooth is initiated after the deciduous tooth has started to diminish in size. Suppression by replacement takes place when the replacement tooth is initiated coincidentally with the onset of deciduous tooth size reduction. We calculated the relative growth rates of dP4, P4 and M1 by measuring the size of the tooth from six embryonic stages. dP4 was growing until the stage 7.5 mm, and then suddenly started to diminish in size. At the same time P4 was initiated. The growth of P4 was very fast. These results indicate that the replacement tooth starts to inhibit dP4 as soon as it is initiated. As the growth rates of both permanent teeth, P4 and M1 were similar, the disappearing dP4 did not interfere with P4 growth rate. However, when the dynamics of the *Shh* expression domains were similarly calculated, it was noticed that relative expansion of

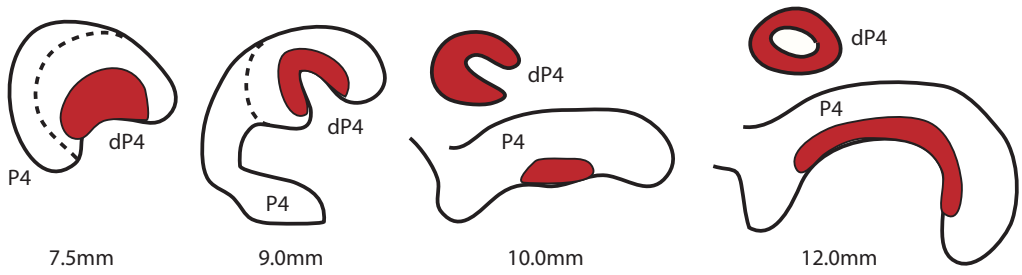

Figure 5. Schematic representation of the deciduous and replacement tooth development in the shrew. *Shh* expression is shown in red.

Shh expression domain in P4 was slower than in M1, suggesting that indeed, dP4 might inhibit P4 development. On the other hand, the onset of decrease in dP4 *Shh* expression domain happened as soon as P4 *Shh* expression was first upregulated. This supports the notion that dP4 is suppressed by its replacement tooth (Figure 6.).

I showed previously that tooth renewal requires activating signals (II). Moreover, it has been shown that adjacent molars inhibit the initiation and size of more posterior molars (Kavanagh et al. 2007). Thus, both activating signals and inhibition mechanisms may play a role in the tooth replacement in the *Sorex* premolar 4 locus. However, the fast appearance of P4 begins to inhibit dP4 that then fails to proceed beyond the cap stage. Thus the evolutionary suppression of deciduous teeth may involve early activation of replacement teeth, which in turn begin to suppress their deciduous predecessors. Our results indicate that *Shh* may not be the molecule responsible for triggering the suppression of dP4, but it is required for the growth and survival of developing teeth. The molecular networks responsible for tooth replacement and its evolutionary reduction remain to be determined.

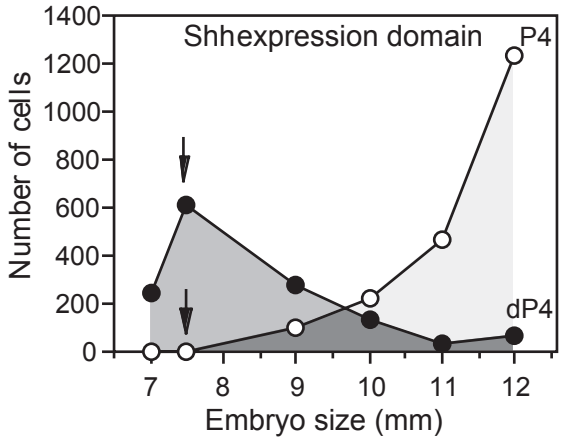

Figure 6. Growth rates of the dP4 and P4 suggest that P4 inhibits the further development of dP4.

4.4 The ferret *Mustela putorius furo* as a model animal for mammalian tooth replacement (IV and unpublished)

Since neither the mouse nor the shrew was an optimal model for studying the mechanisms of tooth replacement, they were investigated in *Mustela putorius furo,* the ferret. Although tooth replacement has been studied earlier in ferrets as well as many other mammalian species (Berkovitz 1973; Leche 1895; Luckett 1993), the developmental mechanisms have stayed unclear. Ferret samples were collected at embryonic stages E34, E35 and E37 and at PN2 (length of the gestation is 42 days). We used serial histological sections and 3D imaging techniques to analyze the physiological mechanisms of replacement tooth generation. In premolars the deciduous tooth reaches the bell stage before the replacement tooth is initiated. The first sign of replacement tooth initiation is the detachment of the successional dental lamina from the lingual cervical loop of the deciduous tooth. In frontal histological sections this is seen as a small bud (IV Fig 2), which apparently has lead to the common misunderstanding that the replacement tooth would bud off the primary tooth. However, I followed the process carefully by analyzing serial sections and showed that the bud is actually a wall of successional dental lamina detaching from the primary tooth. Only later, at stage PN2 this detached dental lamina starts to develop the replacement tooth. This process was illustrated in 3D reconstructions of serial histological sections (IV Fig 3). I conclude that the replacement tooth generation is a two step process, involving the detachment

and growth of the dental lamina and the budding of the newly formed free space on the lamina. However, in the canine (C) tooth position, the deciduous tooth stays connected with the dental lamina until both generations of teeth reach the differentiation stage (IV Fig. 2). This shows that the detachment of the primary tooth is not a prerequisite for the initiation of the replacement tooth and that different tooth families show different modes of development. I analyzed the expression of several molecular markers. *Shh* expression was similar in both generations of teeth and in all tooth loci, suggesting that the molecular regulation of tooth morphogenesis in different tooth families and both tooth generations are similar. This has been shown earlier also in the shrew (III), (Yamanaka et al. 2007). The Wnt/BMP inhibitor *Sostdc1* null allele mice have extra incisors and molars, and the molars show malformed shapes (Kassai et al. 2005). *Sostdc1* is expressed in mouse molar mesenchyme and epithelium (Laurikkala et al. 2003). However, in the ferret it was expressed in the intersection of the detaching dental lamina and the cervical loop of the deciduous tooth, and on the buccal aspect of the cervical loop in inter-tooth sections. The restricted expression pattern of *Sostdc1* in the ferret suggests that *Sostdc1* may play a role in the process of detachment. No apoptosis was detected in the area of detachment, suggesting that it is merely a process of separation and growth that leads to the final detachment of the primary tooth. Of special interest was the expression pattern of *Axin2*, as *Axin2* has been shown to be involved in tooth replacement in human (Lammi et al. 2004), but no tooth phenotype was detected in mouse (unpublished results). Mice do not have tooth replacement and it was anticipated that the generation of primary and replacement tooth generation may show differences in relation to *Axin2* expression. Thus ferret specific *Axin2* was cloned and the expression pattern was analyzed (unpublished results). Ferret-*Axin2* was expressed in dental mesenchyme and enamel knot in both generations of teeth. There was also expression in the mesenchyme surrounding the dental lamina in inter-tooth regions, supporting the hypothesis that Wnt signaling needs to be suppressed in the mesenchyme to enable tooth formation. However, the preliminary results from the expression patterns do not indicate a role for *Axin2* in the detachment or the budding process of the replacement teeth and the regulatory role of *Axin2* in tooth replacement remains to be investigated.

Mechanisms of molar development were also investigated in ferret embryos. We noticed that M1 develops from a deep dental lamina, posterior to dP4. The first sign of M1 is a buccal bud on the dental lamina. The enamel knot forms on the dental lamina and the buccal bud grows deeper into the mesenchyme and forms the buccal cervical loop. The dental lamina becomes the lingual cervical loop (IV Fig. 2, 3).

The origin of the dental lamina and the development of deciduous teeth were investigated. I analyzed the primary epithelial band in oral epithelium, and noticed that it grows together with the developing tooth buds into the underlying mesenchyme. Only later, when this structure is seen deeper in the mesenchyme and is detaching from the primary teeth, I call this structure the dental lamina. [This distinction between the primary epithelial band and the dental lamina was first introduced already in the 19th century (Leche 1895), but has been forgotten since.] As molecular markers are now available, *Shh* expression was analyzed in the developing ferret jaws at stages E22, E24 and E25 to identify the initial tooth buds. *Shh* expression was seen in three distinct spots (IV Fig. 1). These were identified as incisor, canine and premolar and named tooth-

family placodes. These initial tooth-family placodes then bud new tooth buds both in anterio and posterio directions (IV Fig. 1, 5 and Figure 7.).

In conclusion, there are three modes of tooth generation. The primary teeth form from the primary epithelial band of oral epithelium. Replacement teeth form from the dental lamina which detaches from the lingual cervical loop of the deciduous teeth. And molars develop by budding from the posterior dental lamina. The molecular mechanisms regulating the morphogenesis in all three developmental modes seem to be similar based on the gene expression data. However, the molecular networks regulating these events remain to be revealed. There is evidence from my results and previous work (Kavanagh et al, 2007) that activator -inhibitor mechanisms regulate the initiation of the new tooth buds. It is intriguing to speculate that the role of activation of Wnt signaling in the epithelium and possibly the inhibition of Wnt signaling in the mesenchyme would play a pivotal role in the activation of new teeth. However, the optimal method to address this question would be to reproduce the *Axin2* mutation in an animal model with replacement tooth generation, by creating e.g. transgenic ferrets for *Axin2*. However, this may prove to be too challenging, and therefore *in vitro* studies with transgenic mouse tissues and expression analysis may prove to be more suitable methods. Also the potential role of other signaling pathways, such as Hh, FGF and BMP remains to be tested.

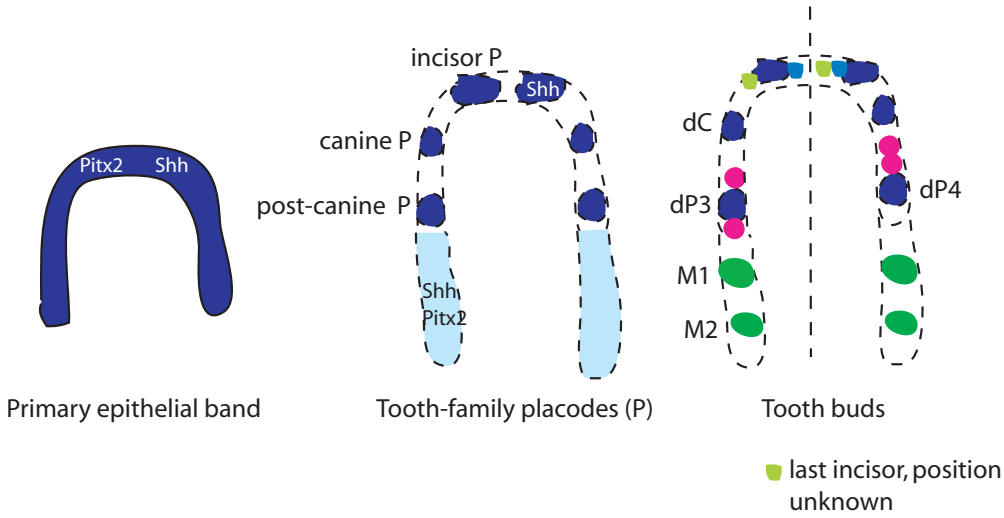

Figure 7. Formation of tooth family placodes and tooth buds on the primary epithelial band.

5. CONCLUDING REMARKS

This thesis work has brought new insight into the mechanisms and molecules regulating tooth renewal and tooth replacement. I have showed that Wnt signaling stimulates enamel knot formation and that it regulates tooth renewal as part of a lateral inhibition mechanism (II). I have showed in the ferret that the primary tooth detaches from the dental lamina, prior to the development of the replacement tooth from the successional dental lamina (IV). Similar features were detected in the *in vitro* cultures of the *β-cat*$^{\Delta ex3fl/+}$ teeth (II), when the developing teeth were separated from the previously formed teeth, as new additional teeth were generated. These examples further support the observations that lateral inhibition is one of the key mechanisms in new tooth formation. Moreover, as it has been shown in molar development (Kavanagh et al. 2007), and as I showed for the repression of tooth replacement in the shrew, the pre-existing teeth suppress the formation of new teeth.

The molecular mechanism whereby the successional dental lamina is activated to form the replacement tooth is not known at present. It can be speculated that the dental lamina has an intrinsic and lasting ability to form new teeth when the inhibition (the pre-existing tooth) is removed or that there is an activating signal, either in the dental lamina, in the primary tooth or in the surrounding mesenchyme. The suggestion that it would be merely a release of an inhibition, may not be true, as similar free dental lamina can be seen above the molar teeth in the ferret, and molars do not have successors. In addition, I showed that β-catenin acts as an activator of the signaling leading to the formation of enamel knots and of new tooth generation, indicating that activating systems may be needed. The molecular signal networks involved in the activator-inhibitor models remain to be revealed. The contradictory phenotypes of *Axin2* human tooth agenesis and the *β-cat*$^{\Delta ex3fl/+}$ transgenic mouse supernumerary teeth indicate that there may be differential regulation in epithelial and mesenchymal tissues. In human it is not known in which tissue *Axin2* function is required, but from the mouse studies it can be concluded that when Wnt signaling is activated in dental epithelium, new teeth are formed. This suggests that to gain the opposite effect in human, *Axin2* function should be missing in the mesenchyme thus leading to activated Wnt signaling in the mesenchyme. However, our mouse studies with activated Wnt signaling in the mesenchyme did not support this view, but the conditional transgenic approach may have some drawbacks and this question should thus be addressed more thoroughly by molecular and genetic analyses as other factors may also be involved. However, as mice do not normally replace teeth, it is not a good model for replacement tooth studies. My results on the ferret tooth development showed that there are distinct differences between the physiological mechanisms of tooth replacement, the formation of the first generation teeth on primary epithelial band and the sequential formation of molar teeth. However, my gene expression studies and others have shown that the same signaling molecule families are involved in all these modes (Yamanaka et al. 2007; Miyado et al. 2007). However, these signals may possibly be under differential regulation. Also it has to be noted that tooth *renewal* and tooth *replacement* might not be regulated similarly thus leaving the question of the role of Wnt signaling in tooth replacement open for future studies. There is no experimental evidence on the connection between *Runx2* and *Axin2* in tooth development and it

should thus be further investigated. In the future the results from the different animal models and human syndromes need to be combined and the molecular networks of tooth replacement and renewal mechanisms revealed. This may require new transgenic mouse models and further genetic studies on those model animals that replace teeth. The information gained from the studies on tooth development brings new insight into our understanding on how organisms are generated and how evolution occurs. Moreover, the knowledge on the regulation of embryonic tooth development and the mechanisms of tooth replacement may be of use in regenerative medicine, in tissue engineering of organs and in understanding the genetic mechanisms behind heritable diseases, possibly leading to medical applications.

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