Integration of Eda signaling with other signaling pathways in oral ectodermal organogenesis

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Academic dissertation

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“Nothing in biology makes sense except in the light of evolution”

- Theodosius Dobzhansky
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In addition, unpublished data is present.

Contributions:

I The author contributed in planning the experiments, conducting all experiments and data analysis and writing the manuscript.

II The author contributed in planning the experiments, conducting almost all experiments and all data analysis and writing the manuscript.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AE</td>
<td>Anterior end of molar region</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>DKK</td>
<td>Dickkopf</td>
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<td>DHH</td>
<td>Desert hedgehog</td>
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<td>DOWNLESS</td>
<td>Spontaneous Edar-mutant mouse strain</td>
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<tr>
<td>DSH</td>
<td>dishevelled</td>
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<tr>
<td>E</td>
<td>Embryonic day</td>
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<td>ED</td>
<td>Ectodermal dysplasia</td>
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<td>EDA</td>
<td>Ectodysplasin, Eda-A1</td>
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<tr>
<td>EDAR</td>
<td>Ectodysplasin A1 receptor</td>
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<tr>
<td>EDARADD</td>
<td>Edar-associated death domain</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>EM</td>
<td>Extra molar</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
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<tr>
<td>FVB</td>
<td>Mouse strain</td>
</tr>
<tr>
<td>FZD</td>
<td>Frizzled</td>
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<tr>
<td>GLI</td>
<td>Glioma-associated oncogene homolog</td>
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<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase β</td>
</tr>
<tr>
<td>HED</td>
<td>Hypohidrotic ectodermal dysplasia</td>
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<tr>
<td>HH</td>
<td>Hedgehog protein family</td>
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<tr>
<td>IHH</td>
<td>Indian hedgehog</td>
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<tr>
<td>IκB</td>
<td>Inhibitor of NF-κB</td>
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<tr>
<td>IKK</td>
<td>IκB kinase complex</td>
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<tr>
<td>ISH</td>
<td>In situ hybridization</td>
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<tr>
<td>JNK</td>
<td>cJun N-terminal kinase</td>
</tr>
<tr>
<td>K14</td>
<td>Keratin-14 promoter</td>
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<tr>
<td>LEF-1</td>
<td>Lymphoid enhancer-binding factor 1</td>
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<tr>
<td>LRP</td>
<td>Low-density lipoprotein receptor-related protein</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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NF-κB  Nuclear factor kappa-B
NMRI  Mouse strain
OMIM  Online Mendelian Inheritance in Man
PLCλ  Phospholipase C λ
PI3K  Phosphoinositide 3-kinase
PTC  Patched
PTHRP  Parathyroid hormone-related protein
RISH  Radioactive in situ hybridization
RTK  Tyrosine kinase receptor
SHH  Sonic hedgehog
SMG  Submandibular salivary gland
SMO  Smoothened
SOSTDC1  Sclerostin domain-containing 1
TABBY  Spontaneous Eda-null mouse strain
TGFβ  Transforming growth factor β
TNF  Tumor necrosis factor
TNFR  Tumor necrosis factor receptor
TRADD  TNF-receptor associated death domain
TRAF  TNF-receptor associated factor
XEDAR  Ectodysplasin A2 receptor
XL-HED  X-linked hypohidrotic ectodermal dysplasia
WNT  Wnt protein family
WT  Wild type
Summary

The salivary glands and the teeth are organs derived from the embryonic germ layer, ectoderm, and share a common early development. Ectodysplasin (Eda) is a signaling molecule belonging to the tumor necrosis factor (TNF) family and its function has been shown to be vital for the formation of the ectodermal organs in vertebrates from teleost fish to mammals. A mutation in Eda in human causes hypohidrotic ectodermal dysplasia (HED), an X-linked hereditary disease causing reduced salivation and missing or modified teeth, in addition to defects in other ectodermal organs. The spontaneous mutant mouse for Eda, called Tabby, shares similar defects and serves as a good model to study HED. In Tabby, teeth are smaller and modified in shape. While the mechanisms of Eda signaling are well known in teeth, they are not yet understood in salivary gland development. However, it is known that in Tabby, the salivary glands are smaller with reduced branching of the saliva-secreting epithelium. In both organs, beside Eda, other major signaling pathways including Wnt/β-catenin, Hh, BMP and Fgf, also operate simultaneously during development. Eda is thought to interact with these pathways; however, it is not known how Eda is integrated with these other pathways.

Here I have analyzed the role of Eda in salivary gland development and showed that branching of the epithelium, required to produce adequate surface area for saliva production, is dependent on Eda. In Tabby, branching of the epithelium and thus, the surface area, was reduced. The effect of Eda was largely mediated by the Hh pathway in the salivary gland. I also showed that the transcription factor NF-kB is required for Eda signaling and that the Wnt pathway induced Eda expression in the salivary gland. In tooth, Eda induced the Fgf pathway ligand Fgf20, which I identified as a novel regulator of tooth development. Using an Fgf20-null mouse crossed with either the Eda loss-of-function (Tabby) or the gain-of-function mice (K14-Eda), I showed that Fgf20 mediates many functions of Eda and was required for the regulation of tooth size and shape. Interestingly, loss of Fgf20 in the K14-Eda mouse supported the formation of an extra molar in the place of the ancestral premolar, a structure lost during rodent evolution 45 million years ago. I observed that reducing Fgf20 levels from normal to null in K14-Eda mouse mimics the shift from omnivorous to faunivorous type of rodent dentition, evoking a scenario that Fgf20 and Eda might be genes operating in the microevolution of dentition.
1. Review of the literature

1.1. Introduction

The process in which a fertilized egg gives rise to an adult individual is studied within the discipline of developmental biology. Starting from the fusion of two cells, the sperm and the egg into one, development must thereafter encompass cell number expansion and organization into specialized cells capable of forming functional tissues, organs and organ systems, ultimately forming a functional individual. Organ development, or organogenesis, requires the interaction of different cell types forming different tissues to be built up in a correct way. How cells are organized within tissues and organs into a functional unit is determined during development, and is largely based on cell signaling. The nature of this inductive signaling has been a long-standing interest in developmental biology (Saxen et al., 1964). This communication is mediated by signaling molecules, soluble proteins, lipids, ions or other molecules, which can bind the receptors in other cells. The other possible interactions between the cells may be mediated by the extracellular matrix and by physical forces and interactions. The outcome of this communication is a response in the receiving cell, which can lead to cell proliferation, apoptosis, differentiation or migration. The communication between cells is usually reciprocal, as the response must be fine-tuned and usually graded. A negative feedback is a common form of response, limiting the effect of the original signal. By applying these principles, functional organ systems within individuals can be formed from a single fertilized egg. If, however, cell signaling is disturbed, development may be disrupted, possibly leading to a developmental defect. In human, many known disorders are inherited, and the genetic determinants for these hereditary diseases have been intensively studied. The ultimate goal in understanding how these genetic factors lead to developmental defect, is to be able to use this knowledge in diagnosis and prevention of these diseases. Such approaches have been developed for clinical use for some developmental disorders.

1.2. Developmental biology linked to evolutionary studies

The symbiosis of developmental biology and evolution studies has been intimate for over a century. In taxonomy, a discipline classifying organisms in hierarchical groups, certain key characteristics are used to separate groups from others. The birds can be separated from other flying animals such as bats or dragonflies, by the existence of feathers, air sac system and specific bones for birds, for example. However, to use a character as a classification criterion, one must be sure that the structure is homologous, not analogous. Otherwise, in the example above, one could classify birds, bats and dragonflies into one group, based on the existence of wings. Wings, however, are analogous structures. To separate analogous from homologous structures, the knowledge of how they are formed during development is key. Analogous structures usually differ in development, whereas homologous structures share, at least to some extent, similar developmental patterns. The use of developmental biology, including comparative embryology and histology, has clarified many of the taxonomical puzzles and the data from comparative developmental biology have been used to define the phyla, among lower taxa. However, paradoxically,
after the discovery of genetic tools in evolutionary biology, the intimate link between evolution and developmental biology was nearly lost for some decades (Gilbert and Bolker, 2001, Gilbert, 2010).

The first signs of relevance of developmental genetics to evolutionary studies came with the discovery of the Hox gene family. The patterning of a body plan in segmented animals was used as one character in taxonomy, but very little was known how the segmentation is governed. Thus, the use of segmentation, without knowing how it is formed, as a classification criterion was poorly justified. The data from the Hox-genes and the other homeobox genes have shed light onto the origin of the key innovations in animal development: the origin of bilateral symmetry and triploblasty, the development of three germ layers (Garcia-Fernandez, 2005). The homeo-box-family is conserved throughout the animal kingdom, as homologous genes can be found from vertebrates to cnidarians (Garcia-Fernandez, 2005). The widespread expression of homeobox genes shows that at least some genes and developmental mechanisms are highly conserved. However, many characteristics have changed and new ones have been acquired during metazoan development. This leads to a question: how have these changes happened during evolution?

The combination of developmental biology/genetics, paleontology and evolutionary biology has now become a research field of its own, named evo-devo, for evolution and development. Developmental biology, by studying the molecular mechanism regulating organ formation, can clarify the framework in which evolutionary mechanisms can operate. The critical questions in evolutionary biology, such as how new structures emerge, or how structures are modified into a new function better adapted for a new environment, can be answered by studying the developmental mechanisms. Of particular importance are genetically modified animals. These models have shown, for example, that new structures can be developed not only by acquiring new genes, but also changing the activities of pre-existing genes. By combining the knowledge gained from genetically modified animal models with fossil data, the possible genetic mechanisms leading to certain forms of teeth from an ancestral one, for example, can be postulated. Computational models have been especially useful in determining the possible molecular mechanisms leading to certain forms found in fossil records (Salazar-Ciudad and Jernvall, 2010).

1.3. Ectodermal organs

In mammals, certain organs develop from the outer germ layer of the embryo, the ectoderm. These organs include teeth, hair, nails and several exocrine glands (e.g. salivary glands, mammary glands, sweat glands and sebaceous glands). Ectodermal organs share a similar early development. These organs develop through reciprocal interactions between two tissues, the epithelium and the mesenchyme. The tissue domains interact by soluble signaling proteins, extracellular matrix (ECM) including basement membrane interactions, and physical forces. The early development of the ectodermal organs is described as: i) thickening of the epithelium forming a placode which is underlain by a condensed mesenchyme; ii) the growth of the epithelium and invagination into the mesenchyme, followed by iii) organ-specific morphogenesis (Fig 1). The ectodermal organs are not specific to mammals. The feathers of birds as well as the scales of reptiles and teelost fish are ectodermal organs, developing similarly to mammalian ectodermal organs. Not only is morphogenesis similar among these ectodermal organs, but many of the responsible genes and the pathways are conserved among birds, reptiles, teleost fish, and mammals (Kollar, 1986, Wu et al., 2004).
Fig 1. Common early development of the ectodermal organs. (A) The epithelium and the mesenchyme are initially uniform. (B) Formation of the placode, a local thickening is formed, when epithelium invaginates to the mesenchyme and the mesenchyme adjacent to the placode is condensed. (C) Later morphogenesis is organ-specific, giving rise to, for example, hair follicle, tooth or the salivary gland.
1.4. Signaling pathways in ectodermal organ development

Tissue compartments, the epithelium and the mesenchyme, communicate throughout organogenesis. The signaling is reciprocal and inductive. The development of all multicellular animals (metazoan) is governed by tissue interactions. These interactions are therefore largely conserved throughout evolution. All of the signaling pathways regulating ectodermal organ formation share common features. First, the ligand is usually soluble and can pass through tissue boundaries, allowing long-range signaling. The ligand may, however, also function in an autocrine fashion. The receptors are localized in responsive cells, in which an intracellular signaling cascade leads to the activation of transcription factors, or to other modifications of cell function. Second, the pathways involve both positive and negative regulators, the latter usually induced by the activity of the same pathway, serving as negative feedback regulation. Third, the pathways in many cases induce or inhibit other pathways. Thus, the conserved pathways are usually linked, forming an integrated signaling network. Ultimately, the activity of the pathways regulates the cell-level responses: cell proliferation, migration, apoptosis and differentiation (Gilbert, 2010).

The common pathways in ectodermal organ development include Ectodysplasin, Fgf, Wnt, Hedgehog, and Tgf-β/BMP pathways. The importance of all of these pathways has been implicated in the development of the best studied ectodermal organs, teeth and hair (Mikkola, 2009, Tummers and Thesleff, 2009). As the Tgf-β/BMP pathway was not a subject of this study, it will not be described further.

1.4.1. Ectodysplasin signaling

In human, the Ectodysplasin (Eda) gene is located on the X-chromosome (Xq12-q13.1; length 423Kbp) and encodes two functional isoforms, Eda-A1 and Eda-A2. The difference between the produced proteins is two amino acids in length, in Eda-A1 (length 391 aa) inclusion of Glu 308 and Val 309 in the receptor binding site which causes a difference in the shape and charge of the site, and accounts for the binding specificity for the receptor: Edar for Eda-A1 and XEdar for Eda-A2 (Bayes et al., 1998, Yan et al., 2000, Schneider et al., 2001, Hymowitz et al., 2003). Both transcripts include exon 1, which encodes a short intracellular domain, a transmembrane domain, and 73 amino acids of extracellular protein sequence. Translated proteins are trimeric type II membrane proteins with an extracellular portion containing a collagen domain and a TNF-ligand motif in the C-terminal region (Mikkola et al., 1999). The Eda-A1/Edar signaling is functional in ectodermal organogenesis whereas Eda-A2/XEdar seems not to play a role in ectodermal organ development, but may affect skeletal muscle homeostasis (Newton et al., 2004, Mikkola, 2008). Therefore, only Eda-A1 will be discussed hereafter, and will be referred as Eda.

The ligand Ectodysplasin (Eda) is a member of tumor necrosis factor (TNF) family. In mammals, other members of the same family are mostly involved in the inflammatory responses, but Eda, however, is active mainly during development. In Drosophila melanogaster, Eiger and its receptor, Wengen, comprise a ligand-receptor pair corresponding to the mammalian TNF-system (Moreno et al., 2002). However, Eiger is required for both embryogenesis and immunity, whereas in mammals, usually separate TNF ligands regulate immunity and developmental functions, indicating functional divergence during evolution (Kanda and Miura, 2004).

The Eda pathway is composed of the ligand Eda and the TNF-receptor Edar which is linked to intracellular pathways via an adaptor molecule Edaradd (Cui and Schlessinger, 2006; Fig 2). The TNF ligand motif of the trimeric and membrane bound Eda protein must be cleaved to make it
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soluble and active, capable of binding the Edar (Elomaa et al., 2001). The solubility of Eda allows paracrine signaling. In the target cell, the membrane bound complex of Eda/Edar/Edaradd triggers a cytoplasmic signaling cascade, leading to the activation of at least nuclear factor-kappaB (NF-κB) transcription factor (Yan et al., 2000, Koppinen et al., 2001, Schmidt-Ullrich et al., 2001, Schmidt-Ullrich et al., 2006, Pispa et al., 2008). In an unstimulated cell, NF-κB is bound to the inhibitory protein IκB in the cytoplasm. The Eda ligand binds to Edar to induce the death domain adapter protein Edaradd to bind Edar inside a cell, which recruits cytoplasmic adaptor protein Traf6. TAK-1 binding protein (TAB2) binds Traf6 and links it to Tgf-β activated kinase (TAK1). The protein complex can then activate the IκB-kinase (IKK) complex, which leads to the phosphorylation of IκB by the IKK complex. The degradation of the inhibitory regulator of NF-κB, IκB, leads to the release of NF-κB, which can move to the nucleus to induce the expression of the target genes (Doffinger et al., 2001, Naito et al., 2002, Morlon et al., 2005, Mikkola, 2009) (Fig 2).

1.4.1.1. Disorders caused by Eda pathway mutations

The receptor Edar and the adaptor molecule Edaradd are encoded by autosomal chromosomes whereas Eda is encoded by the X-chromosome. Mutations in any of these three genes cause hypohidrotic ectodermal dysplasia (HED), a hereditary disease characterized by defects in the ectodermal organs, including: missing or malformed teeth, absent or reduced sweating, sparse hair and usually impaired saliva flow (Clarke et al., 1987, Kere et al., 1996, Monreal et al., 1999, Headon et al., 2001, Lexner et al., 2007). Depending on which component in the pathway is mutated, HED can be autosomal (dominant or recessive) or X-linked (MIM 224900, MIM 129490, MIM 305100). HED can be also caused by mutations in the signaling cascade downstream of Edar and Edaradd. Mutations in the IKBKG gene in human, encoding NEMO protein, an essential component of the IKK complex, leads to impaired NF-κB signaling and anhidrotic ectodermal dysplasia, beside a defective immune system (Zonana et al., 2000, Doffinger et al., 2001). A similar ectodermal phenotype is also observed when Traf6 is mutated in mouse (Naito et al., 2002). Both of these results suggest that disturbing NF-κB signaling results in abnormal ectodermal organ development in addition to defects in the immune system.

X-linked HED (XLHED) is the most common form of HED. Thus males are more severely affected and female carriers show variable degree of symptoms. Missing teeth in the primary dentition is the clinical predictor usually leading to the diagnosis of HED. Different teeth in humans are affected differentially: frequencies of the loss of tooth type in order of decreasing prevalence are: mandibular incisors, lateral maxillary incisors, central maxillary incisors, mandibular molars, and maxillary first molars. In general, mandibular teeth are more often absent than maxillary teeth (Barberia et al., 2006). Absence of teeth in both primary and permanent dentition may also occur. No differences in the dentition of the patients with XLHED or autosomal forms of HED have been found (Clauss et al., 2008, Clauss et al., 2010). Xerostomia, the sensation of dry mouth, is common to HED (Clarke, 1987). The reduced saliva flow has been reported in the patients with HED and the saliva composition is altered: increased concentration of inorganic ions but reduced amylase activity was observed both in affected males and female carriers (Nor-dgarden et al., 1998, Lexner et al., 2007).

Mouse strains with mutations in the Eda pathway have provided good models to study the developmental defects leading to HED. Spontaneous mutations exist for Eda and Edar: Downless for Edar and Tabby for Eda, and also non-spontaneous Crinkled mutant for Edaradd, all showing variable degrees of defects in the ectodermal organs (Shrivastava et al., 1997, Monreal et al., 1999, Headon et al., 2001). Of these, Tabby is probably the best studied. Teeth are affected in
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Fig 2. The Eda/Edar/NF-κB pathway. The binding of Eda to Edar triggers trimerization of Edar (not indicated), capable of binding adaptor molecule Edaradd in the cytoplasmic C-domain. Edaradd recruits TRAF6 which binds TAK1-binding protein 2 (TAB2), linking TRAF6 to TGF-B activated kinase 1 (TAK1). This leads to the activation of IKK complex which then phosphorylates IκB, leading to IκB dissociation from NF-κB, degradation of IκB and release of NF-κB. NF-κB can then go to the nucleus to activate transcription of the target genes.
Tabby mice (Grüneberg, 1966; Pispa et al., 1999). Wild type (WT) mice have in each half of the jaw one incisor, a toothless diastema region and three molars (m1-m3). Tabby mice have smaller teeth and reduced cusp numbers, particularly in the first molar. Tooth number can also vary in Tabby, as 17-55% of Tabby mandibular quadrants lack the third molar (m3) (Charles et al., 2009, Grüneberg, 1966, Pispa et al., 1999, Kangas et al., 2004).

The Eda/Edar/Edaradd system is conserved throughout vertebrates, as teleost fish medaka (Oryzias latipes) and zebrafish have components in the pathway which are homologous to the mammalian pathway (Kondo et al., 2001, Harris et al., 2008). Loss of Edar in the fish medaka leads to almost complete absence of scales, suggesting a conserved role for Eda signaling in skin appendage development (Kondo et al., 2001). Interestingly, spontaneous mutants for the Eda pathway have been found in several taxa beside medaka, in several mammals, including cattle and dogs (Drogemüller et al., 2001, Casal et al., 2005). Variation in the Edar gene have been found also in humans as Edar alleles have been shown to account for the variation in hair thickness and incisor shape in Asian populations (Fujimoto et al., 2008, Kimura et al., 2009).

Developmental defects caused by Eda mutations can be rescued in the animal models. When recombinant Eda protein is administered to pregnant Tabby mice, the offspring are permanently, though partially, rescued. Most of the characteristics are rescued, but not all: the third molar is still absent in half of the jaw quadrants and only two (compared to four in wild type) hair types exist (Gaide and Schneider, 2003). Also in the dog model of HED, neonatal treatment with recombinant Eda partially rescues the defective teeth and glands (Casal et al 2007., Mauldin et al., 2009).

1.4.2. Fgf signaling

The large family of Fibroblast growth factor (Fgf) ligands consists of 22 members in vertebrates falling into seven subfamilies according to their sequence homology and function (Itoh and Ornitz, 2008). The Fgf ligands can be divided according their function as intracrine, paracrine or endocrine Fgfs. Intracrine Fgfs act as intracellular ligands, paracrine Fgfs act across tissue domains and endocrines function in a hormone-like fashion through the circular system. The ligands, excluding intracellular Fgfs (iFgf 11-14), bind the Fgf-receptors (Fgfr). The Fgfrs are tyrosine kinase receptors containing an extracellular ligand-binding domain with three immunoglobulin-like domains (I, II and III), a transmembrane domain and endocrines function in a hormone-like fashion through the circular system. The ligands, excluding intracellular Fgfs (iFgf 11-14), bind the Fgf-receptors (Fgfr). The Fgfrs are tyrosine kinase receptors containing an extracellular ligand-binding domain with three immunoglobulin-like domains (I, II and III), a transmembrane domain and a split intracellular tyrosine kinase domain (Itoh and Ornitz, 2011). Fgfr1–Fgfr3 encode two major versions of immunoglobulin-like domain III (IIIb and IIIc) generated by alternative splicing that utilizes one of two unique exons. The immunoglobulin-like domain III is an essential determinant of ligand-binding specificity (Chellaiah et al., 1999, Bellosta et al., 2001).

Fgfr activation caused by ligand binding triggers several intracellular cascades, such as activation of the Ras/ERK pathway (associated with proliferation and differentiation), the Akt pathway (associated with cell survival) or the protein kinase C (PKC) pathways (involved in cell morphology and migration; reviewed in Dorey and Amaya, 2010; Fig 3.). The Fgf pathway is evolutionarily ancient and is conserved throughout metazoan evolution. However, the number of ligands has expanded in two phases during evolution, from two or three to the current 22 in vertebrates. Correspondingly, the number of receptors has increased to four in mammals, with further diversification through alternative splicing to different isoforms (Itoh and Ornitz, 2011). Four Sprouty proteins in mammals function as negative regulators of Fgf signaling and share a homologous structure to the single Sprouty protein in Drosophila. Sprouty proteins are encoded
by Sprouty \textit{(Spry)} genes and function as RTK inhibitors at least by preventing Ras activation (Gross et al., 2001), but also possibly by interacting also with Erk1/2 (Edwin et al., 2009). Fgf signaling in mammals regulates development in many steps, from early embryonic development to organ differentiation (Dorey and Amaya, 2010).

1.4.2.1 Fgf20 in development

Based on structural similarity, Fgf20 belongs to the Fgf9 subfamily, together with Fgf9 and Fgf16 (Itoh and Ornitz, 2011). Fgf20 has been shown to be involved in the differentiation of dopaminergic neurons and has been associated with Parkinson’s disease (Wider et al., 2010). The role of Fgf20 in development is currently poorly understood. In the inner ear, Fgf20 regulates the specification of the sensory and supporting cells in the cochlea via Fgfr1 and Fgf20/-/- mice are deaf because some cochllear cells remain undifferentiated (Hayashi et al., 2008, Huh et al., 2012). In contrast to inner ear, nephron progenitors were differentiated in the absence of Fgf20, and Fgf20 and Fgf9 together were shown to be necessary to maintain progenitor stemness (Barak et al., 2012). A spontaneous featherless chicken mutation named ‘scaleless’ was recently shown to be caused by a nonsense mutation in the Fgf20 gene (Wells et al., 2012). The regulation of Fgf20 expression is also poorly known, however, studies in Xenopus and in human cell cultures suggest that Wnt signaling regulates Fgf20 expression (Chamorro et al., 2005).

1.4.3. Wnt signaling

The Wnt family consists of 19 secreted glycoprotein ligands in mammals. Wnt ligands signal across cell membranes and tissue boundaries to activate the Frizzled receptors. The activation of the receptor can induce different intracellular pathways: a canonical pathway, also known as the Wnt/β-catenin pathway, Wnt/Ca^{2+} pathway involving protein kinase A, and the planar cell polarity pathway or the pathway involving protein kinase C (Liu and Millar, 2010). The most studied of these is the canonical pathway, which has been shown to be involved in

\textbf{Fig 3. Fgf pathway.} FGF signalling is initiated by ligand-dependent dimerization of the FGFR, which leads to the cross-phosphorylation of tyrosine residues in the intracellular domain of the receptor tyrosine kinase (RTK; not shown). This can trigger three different intracellular pathways: i) PLC\_γ, ii) PI3K/PKB or iii) Ras/ERK pathways, leading to corresponding response in the cell (arrowed). Sprouty proteins serve as inhibitors of the Ras/ERK pathway. Modified from Dorey&Amaya 2010.
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many developmental processes, including development of the ectodermal organs (Andl et al., 2002, Chu et al., 2004, Liu et al., 2008). In the absence of canonical Wnt signaling, cytoplasmic β-catenin is associated with adenomatous polyposis coli (APC) and Axin proteins, and is phosphorylated by glycogen synthase kinase 3β (GSK3β) and casein kinase I (CKI) in its N-terminal degradation box. This results in polyubiquitination by βTRCP1 or βTRCP2 complexes and targeting for protease-mediated degradation (Liu et al., 1999). The activation of the Wnt/β-catenin pathway prevents the degradation of β-catenin. Binding of a WNT ligand to a Frizzled (FZD) receptor and a low-density lipoprotein-related-receptor protein (LRP) 5 or 6 co-receptor, and subsequent interaction of FZD with the cytoplasmic protein Disheveled (DSH), results in the phosphorylation of cytoplasmic DSH by CKI and its binding to GSK3β with the help of Frequently Rearranged in Advanced T-cell lymphomas (FRAT) protein. R-spondins act as positive regulators of Wnt signaling and bind to the Lgr4/5/6 receptors (de Lau et al., 2012). These interactions cause inactivation of the Axin/APC/GSK3β/CKI complex and stabilization of cytoplasmic β-catenin. The stabilized β-catenin then can bind to TCF/LEF family transcription factors to initiate transcription of the target genes (reviewed in Liu & Millar 2010). The details of the pathway are shown in Figure 4. Beside activating the downstream targets that induce cellular functions such as proliferation and differentiation, the Wnt/β-catenin pathway induces its own repressor, Axin2 (Jho et al., 2002). The signal intensity may be balanced by this negative feedback loop.

Fig 4. Wnt/β-catenin pathway in a schematic and simplified form. (A) In the absence of a WNT ligand, cytoplasmic β-catenin is associated with APC and Axin, phosphorylated by GSK3β and CKI, and polyubiquitinated by the βTRCP complex, targeting it for proteosomal degradation. LEF/TCF transcription factors in the nucleus associate with transcriptional co-repressors, such as Groucho, and the transcription of Wnt target genes is repressed. (B) In the presence of a WNT ligand, phosphorylation and degradation of β-catenin are inhibited, allowing it to accumulate in the cytoplasm and translocate into the nucleus. Nuclear β-catenin interacts with LEF/TCF family transcription factors and several other transcriptional co-activators to initiate transcription of target genes. Abbreviations: FZD, Frizzled; LRP, low-density lipoprotein-receptor-related protein; APC, adenomatous polyposis coli; GSK, glycogen synthase kinase; CKI, casein kinase I; DSH, Disheveled; LEF/TCF, Lymphoid enhancer factor/T-cell factor. Modified from Liu & Millar 2010.
1.4.4. Hh signaling

In mammals, three Hh (Hedgehog) ligands exist: Shh (Sonic Hh), Ihh (Indian Hh) and Dhh (Desert Hh). The Hh signaling pathway is highly conserved with the single fruitfly *D. melanogaster*. Hh binds to the transmembrane receptor Patched (Ptc1, Ptc1). In the absence of the Hh ligand, Ptc represses the activity of the seven-pass transmembrane protein Smoothened (Smo), a member of the G-protein-coupled receptor (GPCR) superfamily. Upon Hh binding the Ptc, Ptc repression of Smo is blocked, resulting in Smo activation. The result of Smo activation is modulation of the repressor and activator forms of the Ci/Gli zinc-finger transcription factors (Ci in *Drosophila*; Gli1-3 in mammals). In the Smo-inhibited state of the pathway, Ci/Gli2/Gli3 is phosphorylated by protein kinase A (PKA), casein kinase I (CKI) and glycogen synthase kinase 3 (GSK3), targeting the proteins for proteasome-dependent processing. Smo activation inhibits Ci/Gli2/Gli3 proteolysis and might promote the formation of biochemically undefined Ci/Gli activators from the full-length proteins. Smo activation inhibits Ci/Gli2/Gli3 proteolysis and might promote the formation of biochemically undefined Ci/Gli activators from the full-length proteins (reviewed in Wilson & Chuang 2010).

In recent years, the importance of the primary cilia for Hh signaling has been highlighted. The primary cilium is a structure found basically in all cells and is composed of microtubules arranged in a circular fashion of nine pairs (9+0 structure). Primary cilia depend upon a microtubule-based transport system called intraflagellar transport (IFT) for maintenance of the axonemal structure and trafficking of proteins in and out of the cilium. The IFT utilizes kinesin-II-based motors for the transport of cargo in the anterograde direction (into the cilium) and dynein-based motors for retrograde transport (out of the cilium) (Tasouri and Tucker, 2011, Roy, 2012).

Fig 5. Hh pathway. (A) In the absence of Hh ligand, Ptc is found on the primary cilium and normally prevents Smo from translocating into the cilium to activate signaling. (B) After binding of Hh ligand to Ptc, the complex moves off the cilium, allowing Smo to move along the axoneme in a Kif3a- and β-arrestin2-dependent manner. Abbreviations: Arrb2, β-arrestin2; Hh, Hedgehog; Ptc/Ptc1, Patched; Smo, Smoothened. Modified from Wilson & Chuang 2010.
Functionally intact primary cilia are critical for the Hh pathway. In the absence of a ligand, Ptch1 is found at the base of primary cilia and Smo is not associated with the cilia (Corbit et al., 2005, Rohatgi and Scott, 2008). Upon exposure to a ligand, Ptch1 and its ligand move out of the cilium and become internalized in the cytoplasm (Fig 5). Smo then moves into the cilium (Corbit et al., 2005) and allows both for the production of an activated Gli2 and for the inhibition of the proteolytic production of the short repressor form of Gli3. Both Gli2 and Gli3 are also localized to the primary cilia, being concentrated at the distal ends (Haycraft et al., 2005). The rapid translocation of Gli2 and Gli3 to cilia upon ligand binding occurs within minutes (Wen et al., 2010). Thus, both the transcriptional activation of Gli2 and Gli3 as well as the proteolytic processing of Gli3 from a full-length isoform to a shorter-length transcriptional repressor are dependent upon ciliary function. The essential contribution of cilia to Hh pathways and the enormous importance of these pathways for developmental processes throughout the body have resulted in an explosion of papers linking primary cilia to organogenesis.

1.4.5. Integration of Eda signaling to other pathways

The conserved pathways mentioned above are linked, forming signaling networks. This may explain why the same pathways are used in different morphogenetic events in different organs: the integration of the pathways may be different. In vitro cultures of tooth and skin suggest that Edar is induced by Activin-bA, whereas Eda is induced by the Wnt pathway (Laurikkala et al., 2002). Moreover, Eda is downregulated in the developing tooth and hair placode of Lef1/- mice and Lef1 has been shown to bind Eda promoter and regulate its expression (Laurikkala et al., 2001, 2002, Durmowicz et al, 2002).

In vitro evidence suggests that the Eda pathway modulates several major signaling pathways. In hair follicle development, follistatin, Shh and certain chemokines are triggered by Eda. In addition, Eda has the common targets Pthrp, Wnt10a and Wnt10b during mammary gland development and hair placode development (Zhang et al., 2009, Voutilainen et al., 2012). This suggests that some of the target genes induced by Eda are shared by different organs. The variety of genes and pathways downstream of Eda signaling also advocates for the role of Eda to be a signal integrator or balancer, modulating several pathways simultaneously (Lefebvre et al., 2012). The balancing role is especially shown in the hair follicle development, in which the Eda and the Wnt pathways act reciprocally and Eda induces the expression of both Wnt pathway activators, such as the ligand Wnt10a and also a repressor of the pathway, Dkk4 (Fliniaux et al., 2008, Zhang et al., 2009). Also in the skin, Eda induces Bmp4 and Bmp7 in culture, but exogenous Bmp4 inhibits Edar signaling (Mou et al., 2006).

Which genes mediate Eda signaling in the tooth is currently unknown. Tabby teeth are, however, partially rescued in vitro by Fgf10 protein, suggesting a link to the Fgf family (Pispa et al., 1999). Even though signaling during tissue development is in many cases considered to act hierarchically, the integration at multiple levels makes the signaling system more complex.

1.5. Patterning mechanisms by diffusing molecules

The patterning of the tissues during development, defining the shape and organization of tissues and organs, requires cell communication. This communication is mediated partially by molecules moving from cell to cell in a paracrine fashion and causing a response in the receiving cell. These different diffusable factors are required for patterning (Saxen and Toivonen, 1961, Saxen
Chemical reactions in the cells and in the intracellular space are local phenomena; patterns cannot be formed without the contribution of spatial and temporal variables. The simplest form of movement of the molecules, morphogens in a developmental context, is diffusion. The distance travelled by diffusion is proportional to $\sqrt{Dt}$, in which $D$ is a diffusion coefficient and $t$ is time (Howard et al., 2011). The square root proportionality means that the diffusion is effective only within short distances (<5 μm). For longer distances, active transport is more effective. Whether the rate of diffusion is high enough for patterning organs during development is debated (Howard et al., 2011). However, most likely a general answer cannot be given: the sufficient rate for diffusion depends on the rate of development.

One of the first and probably the most cited mathematical models explaining the formation of periodical biological patterns was proposed by Alan Turing. In his paper ‘The Chemical Basis of Morphogenesis’ (1952) he explained a simple model with two molecules, or morphogens, diffusing, interacting and reacting. The diffusion rates and the reaction constants are different for two molecules, X and Y in his paper as an example. These two parameters, the difference in the diffusion and in the reaction to induce the production of the morphogens, are required for the automated patterning of the tissues. The strength of the model is that it can explain the formation of patterns in a naïve state, meaning that no pre-patterning is required. The ground state can be homogenous or random from which the pattern is formed by the activity of two (or more) morphogens. By changing the reaction term and the wavelength of the ring wave formed, six different states could be formulated. Out of these, the most widely applied model to explain biological patterns is the stationary wave. This model, also referred to as Turing’s model, is maintained by a dynamic equilibrium of the system and the outcome is a stationary wave with a finite wavelength. At the peak values, in a distance of the wavelength, the periodic pattern is formed. However, the pattern generated by the model can be highly variable depending on the parameters set. The concept of the automated patterning based on Turing’s model has been later used to explain the development of the periodic patterns of feathers and the variable coloration of the skin in mammals and fish, among others (Kondo and Miura, 2010). A more general model explaining the pattern formation similar to, and largely based on, Turing’s model was formulated by Meinhardt and Gierer (Gierer and Meinhardt, 1972, Meinhardt and Gierer, 2000) who showed that a system needs only to include a network that combines “a short-range positive feedback with a long-range negative feedback” to generate a periodic pattern. The important addition to the model was the implementation of self-regulated activating events, such as induction and inhibition events, such as lateral inhibition to the model. Due to positive feedback of the activator, the patterning can be initiated if the baseline production of the activator (term iv in the Fig 6, upper equation) crosses a given threshold. This kind of system is not dependent on the source of the morphogens or the way they are travelling in space; the active transportation may be used as well as diffusion. The model by Meinhardt & Gierer is illustrated in Fig 6.

Even though the Turing model and its modifications are beautifully simple, the biological evidence for the models is difficult to obtain. Correlative evidence for model predictions and biological outcomes has been gained to explain the spot and stripe patterns in zebrafish and the regeneration of Hydra, but the molecules acting as activators or inhibitors have not been identified (Nakamasu et al., 2009., Turing 1952). In hair placode patterning, the Wnt/Dkk activator-inhibitor system has been proposed to follow Turing’s model (Sick et al., 2006); however the diffusion rates, the distance of action or autoregulation of the activator were not analyzed. To prove that a Turing mechanism underlies pattern formation, it would be important to show the connection between the activator and the inhibitor and to measure rates of diffusion and reaction and show that they can quantitatively account for the spatial and periodic dynamics of the patterns (Howard et al., 2011).
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1.6. Salivary gland development

The salivary glands are evolutionary old structures, found in almost all bilaterian taxa. The salivary glands have functionally diverged during evolution: for example, the venom of snakes is produced in venous glands which are considered to be modified salivary glands and some birds (Apodidae) use saliva to build their nests. Even in some mammals, both extinct and extant, the salivary glands produce venom with the duct of the gland opening to the canine tooth, linking teeth and salivary glands into a functional unit (Fox and Scott, 2005). The plasticity of the salivary glands during evolution, by changing the composition of the saliva, has been suggested to be a significant adaptive feature in mammalian adaptive radiation (Phillips et al., 1998).

In human, the major salivary glands include paired parotic- (PG), sublingual (SLG) and submandibular (SMG) salivary glands. In addition, there are estimated to be 600–1,000 minor salivary glands in humans and these are located in the buccal, labial, distal palatal, and lingual regions of the oral mucosal membrane (Eliasson and Carlen, 2010). In healthy adults, up to one and a half liters of saliva is produced daily (Furness et al., 2011). Reduction in the saliva flow or alteration in composition may result in a subjective sensation of dry mouth known as xerostomia. The causes can be variable. The most severe forms of xerostomia can be the result of irradiation for the treatment of head and neck cancers, Sjögren’s syndrome, an auto-immune disease causing loss of saliva producing cells, or a variety of other pathological stages or a hereditary disease, such as ectodermal dysplasia. The prevalence of xerostomia is estimated to be relatively high, approximately 20% in general population (Furness et al., 2011).

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Normal salivary excretion is made up of resting saliva and reflex saliva production. The resting saliva has the function of moistening the oral mucosa and maintaining oral health. The major and minor salivary glands take part in resting saliva production. The reflex saliva is produced in response to sensory stimulation associated with the smell and taste of food and chewing, mostly in the major salivary glands. Parasympathetic stimulus increases the production of liquid, or serosal, saliva, whereas sympathetic stimulus induces sticky, or mucosal, saliva production.
Serosal saliva is mainly produced in the parotic salivary glands, whereas sublingual and submandibular glands produce both, but mostly mucosal. The composition of the mucosal saliva is mainly due to mucin protein, a highly glycosylated protein with a lot of negatively charged (hydrophilic) groups, thus having ability to hold water and therefore forming a gel-like, sticky saliva. Serous saliva lack mucin, but contain amylase (Furness et al., 2011).

Saliva has several functions: it facilitates speech, acts to wash away food residue from around the teeth, neutralizes potentially damaging food acids by producing bicarbonate ion ($\text{HCO}_3^-$), enhances the ability to taste food, and generally lubricates the mouth. Saliva also acts to soften food, making it easier to chew and swallow. Some enzymes in saliva start the digestion of starch and fats, while other substances in saliva, such as epidermal growth factors, promote tissue growth, differentiation, and wound healing in the digestive tract. Antibacterial, antifungal, and antiviral agents in saliva balance oral flora and help to prevent oral infections and minerals in saliva are delivered to the tooth surface in order to maintain the enamel (Humphrey and Williamson, 2001). Thus, reduction of the saliva production in xerostomia may have a variety of adverse effects on health and wellbeing.

In functional major salivary glands, the maximal surface area of the saliva-producing and conducting epithelium is packed into a minimal organ size. The development of the glands must fulfill this requirement. In human and mouse, the three major salivary glands (PG, SLG, SMG) develop in a rather similar manner, but the development of the submandibular gland is best described in the literature. For this reason, I focus on the SMG here.

1.6.1. Salivary gland morphogenesis

The development of the SMG starts at E11-E12 when an epithelial thickening, or placode, is

![Diagram of SMG branching morphogenesis and the corresponding developmental stage (E=embryonic day in mouse).](image)
formed in the oral epithelium. The epithelium is derived from the ectoderm without endoderm contribution, as was recently shown with lineage tracing (Rothova et al., 2012). The placode grows down into the underlying mesenchyme, forming an epithelial bud. At E13, branching is initiated when three clefts are formed in the bud, separating three end buds. At E14.5, over 20 end buds are already formed and after this, with repetitive cleft formation and budding, branching continues throughout embryogenesis and even after birth. During branching, the epithelium is constantly proliferating whereas the mesenchyme is not, and epithelium grows into the mesenchyme. The first sign of epithelial differentiation is detected at the initiation of the branching when the main duct is formed and the duct epithelium differentiates into columnar epithelium, forming a lumen. The duct system thereafter develops and branches into the terminal end buds at around E15-E16, and the end buds start to differentiate at around E16 into secreting epithelium, forming saliva secreting acini, which can be serosal, mucosal or seromucosal. The acini are connected to the main duct by smaller and more branched ducts, called intercalated ducts and striated ducts. In rodents, granular ducts connect intercalated ducts to the striated ducts. Together with the developing epithelium and mesenchyme, the innervation and blood system is developed. During the acini differentiation, a layer of myoepithelium is developed and surrounds the acini, providing mechanical contraction to reinforce saliva secretion (Tucker, 2007). In the literature, the terms “prebud” (E11-E12), “initial bud” (E12.5), “pseudoglandular” (E13-E14), “canalicular” (E15-E16) and “terminal bud” (E17-E18) are used to refer to the developmental stages described above (Melnick et al., 2001). However, I find those subjective and thus, inaccurate and will not use them hereafter. A similar pattern of branching morphogenesis is shared with the development of the lung, the mammary glands and the kidney, although some morphological and molecular mechanisms are organ-specific (Tucker, 2007). The stages of the SMG morphogenesis are summarized in Fig 7.

1.6.2. Regulation of salivary gland development

1.6.2.1. Initiation

Relatively few factors have so far been shown to regulate the initiation of the salivary glands. Mutation in the gene encoding Fgf10 causes aplasia, the absence of glands (Ohuchi et al., 2000). Human syndromes causing aplasia of the lacrimal and salivary glands (ALSG; OMIM 180920 and OMIM 103420) was shown to be caused by mutations in the Fgf10 gene (Chapman et al., 2009). Fgf10 is expressed around the developing epithelium in mouse at E12. The receptor in the epithelium for Fgf10 is Fgfr2b and the loss of function mutants suffer from aplasia (De Moerlooze et al., 2000). However, more detailed analysis revealed that SMG development is initiated but is soon terminated, and by E13.5, SMG has disappeared (Jaskoll et al., 2005). In the Fgf8 mutants, the salivary glands initiate but degenerate before branching occurs (Jaskoll et al., 2004b).

1.6.2.2. Branching

As the epithelium grows constantly during branching, the factors regulating proliferation are important for morphogenesis. Classical tissue recombination studies by Grobstein (1953) and others suggest that the mesenchyme is the source for the growth and branching promoting signals. In his studies, he separated and recombined E13.5 salivary gland epithelium and mesenchyme with different non-salivary gland epithelium and mesenchyme. He observed that the mesenchyme has an instructive role: when SMG mesenchyme was combined with the epithelium of other organs, the epithelium developed into a branched structure resembling salivary gland epithelium, but salivary gland epithelium combined with non-salivary gland
mesenchyme did not induce branching morphogenesis. However, the cytodifferentiation in the epithelium appeared to be an innate character of the epithelium. More recent in vitro studies have shown that salivary gland epithelium can branch and differentiate without the mesenchyme. In this experiment, serum-containing media was supplemented with growth factors Egf or Fgf, and the isolated epithelium was cultured on matrigel containing ECM factors (Nogawa and Mizuno, 1981). This suggests that the soluble growth factors that induce epithelial proliferation normally produced in the mesenchyme and the ECM components are the only factors needed to induce the branching of the epithelium. During early branching, but not later in development, epithelial cells show high motility, which is not dependent on proliferation (Larsen et al., 2006). The function of this dynamic, although non-directional, movement is not known.

Cleft formation

Salivary gland cleft formation is a profound step in branching. The location of the site for new branching to occur is determined by the cleft formation, in which a groove or a furrow is formed at one site of the epithelium that thereafter deepens, mechanically dividing epithelium into two end buds which thereafter will grow separated from each other. Cleft initiation is a dynamic process as many clefts appear to form but later regress (Larsen et al., 2006). The initiation of the cleft is independent on cell proliferation (Nakanishi et al., 1987). The vital role for ECM molecules, especially in the basement membrane, and the modifiers of the ECM during cleft formation has been shown in many reports. These ECM proteins and their modifiers include fibronectin, collagen I, III and IV, and the matrix metalloproteinases and their activators, respectively, accumulate at the site of a forming cleft (Tucker, 2007). One example is MT2-MMP, a metalloprotease that cleaves the active part of collagen IV, the NC1 domain (Rebustini et al., 2009). Inhibition of either MT2-MMP or NC1 reduces cleft formation and branching. Fibronectin dynamics have been especially well documented during cleft formation. As the cleft deepens, fibronectin is continuously accumulated at the base of the cleft, moving as an arrow towards the interior of the gland (Larsen et al., 2006). Inhibition of fibronectin assembly reduces branching, whereas exogenous fibronectin induces extra cleft formation (Sakai et al., 2003). Fibronectin induces cell-matrix adhesions on cultured human salivary epithelial cells with a local loss of E-cadherin at cell-cell junctions. This function of fibronectin to inhibit cell-cell contact and to simultaneously induce cell-matrix adhesion is mediated via integrin receptors, and is crucial for cleft formation (Sakai et al., 2003).

The tension in the epithelium at the sites of cleft initiation may be an important factor during branch initiation, as inhibition of Rho-kinase (ROCK) and contraction of actin-myosin cytoskeleton is suggested to lead to reduced tension, inducing supernumerary cleft formation (Daley et al., 2012). This ROCK activity is required for basement membrane formation, specifically to the fibronectin and collagen IV accumulation to the cleft. ROCK1 also regulates epithelial cell polarity through Par1b in the columnar, outer epithelial layer. This regulation is independent of myosin, in contrast to the regulation of tension and basement membrane accumulation. The loss of polarity, together with the reduction in the adhesion, has been associated with cleft formation (Daley et al., 2009, Daley et al., 2012).

Local paracrine signaling in the clefts may also be important for cleft initiation and progression. Semaphorin 3A and 3C proteins induce branching in ex vivo SMG culture through neuropilin1, Plexin A2 and Plexin D2. They do not induce cell proliferation, but instead act on cleft formation (Chung et al., 2007). The specific analysis of the factors regulating cleft formation was achieved by microdissection of the cleft and bud regions separately, followed by microarray analysis and comparison of gene expression in these two regions (Musselmann et al., 2011).
With this experimental setting, Btbd7 was found to be a critical signaling factor in cleft formation (Onodera et al., 2010). *Btbd7* is induced by fibronectin and largely mediates its functions, leading to the loss of E-cadherin in the columnar epithelium. Btbd7 also induces the transcription factor Snail2, which has been associated with cell scattering. The loss of adhesion and promotion of scattering induced by Btbd7 is required for branching (Onodera et al., 2010).

**Growth promoting signals**

In addition to cleft formation, the epithelium must proliferate constantly to expand the surface area for saliva production and transportation. After the cleft has formed, the buds are separated due to the continuous growth of the epithelium. One of the first signaling cascades shown to affect SMG branching was Fgf signaling. Besides the role in the initiation of the SMG, Fgf10/Fgfr2b signaling has been shown to be crucial for branching. Fgfr2b full knockout mice do not develop SMG at all; also the heterozygous *Fgfr2b* mice exhibit reduced branching (Jaskoll et al., 2005). Several other Fgfs are also expressed during early branching morphogenesis at E13: Fgf1, Fgf2, Fgf3, Fgf4, Fgf7, and Fgf10 are expressed in the mesenchyme, while transcripts for Fgf1, Fgf8, and the Fgfr1b and Fgfr2b are found in the epithelium (Hoffman et al., 2002). Neutralizing antibodies to Fgf1, Fgf7 and Fgf10, or antisense oligonucleotides for Fgfr1 or Fgfr2 reduce *ex vivo* branching (Hoffman et al., 2002, Steinberg et al., 2005). Fgf7 and Fgf10 appear to have a slightly different role in promoting branching. Supplementation of Fgf7 in isolated wild type epithelium grown on matrigel induced epithelial budding but Fgf10 induced duct elongation. The latter induced proliferation at the tip of the ducts whereas Fgf7 induced widespread proliferation in the buds (Steinberg et al., 2005). Beside the role of Fgfs in promoting cell proliferation in the epithelium, Fgf signaling interacts with several ECM factors in cleft formation. A negative regulator for Fgf10-dependent proliferation was recently found to be a micro-RNA, miR-200c, which targets the very low density lipoprotein receptor (Vldlr) and its ligand reelin (Rebustini et al., 2012). In the tissue, the diffusion of the Fgf ligands is at least partially controlled by binding to heparan sulfates. This may be the mechanism for gradient formation in tissues and was shown to account for the different functions of Fgf10 and Fgf7 during the salivary gland branching (Makarenkova et al., 2009).

Egf signaling is a well-studied cascade in relation to the salivary gland branching morphogenesis. Egf protein was the first growth factor to be found and was isolated by Cohen et al. from the rat salivary gland (Cohen, 1965). The stimulatory effect of Egf on salivary gland branching was first reported by Nogawa & Takahashi (1991). The Egf ligand binds and activates the Egf-receptor (Egfr) in the epithelium and the mesenchyme. The role of Egf-Egfr paracrine signaling in branching morphogenesis was addressed by Kashimata and Gresik (1997), who observed in an *in vitro* study that Egf protein stimulated branching whereas Egfr inhibition decreased branching and suggested that Egf-Egfr system regulates the synthesis of a-2 integrin subunit. As mesenchyme-free culture of the salivary gland epithelium described by Nogawa & Takahashi (1991) required serum-containing media, Noguchi et al., (2006), isolated the factor in serum that is required along with Egf and ECM to stimulate branching. They identified the molecule as lysophosphatidic acid and it was found to fully substitute the serum and induce branching morphogenesis in cooperation with Egf. Koyama et al. (2008) compared the effects of Egf stimulation in comparison with Fgf stimulation for *in vitro* culture of the salivary gland epithelium and observed that Egf stimulated cleft formation mainly via Erk1/2 whereas Fgfs used PLCγ and PI3-kinase pathways, and also induced duct elongation.

Despite the longstanding interest of Egf-Egfr system for SMG branching, all abovementioned studies used *in vitro* cultures even though the *Egfr-/-* mouse was generated as early as 1995.
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(Miettinen et al., 1995). Häärä et al (2009) reported the salivary gland phenotype of the \textit{Egfr-/-} mouse. Branching morphogenesis of the salivary gland was defective in \textit{Egfr-/-} mouse when E13 SMG were cultured \textit{ex vivo} for 2 days and \textit{in vivo} until P0, when the mice die due to lung defects. Epithelial proliferation was reduced and mesenchymal apoptosis increased in the mutants, causing reduced epithelial growth and likely defective epithelium-mesenchyme crosstalk. Also the ratio of mesenchyme:epithelium was increased, likely due to upregulation of matrix modifiers, MMP2 and plasminogen activators in the case of Egfr inhibition. Epithelial differentiation was also dependent on Egfr since mutant mice exhibited impaired tubular and acinar differentiation (Häärä et al., 2009). Despite the fact that the Egf ligand has been used in all abovementioned studies to stimulate Egfr and branching, the physiologically active ligand for Egfr has not been identified. In fact, \textit{Egf} is not expressed in the SMG during the morphogenesis (Salivary gland molecular anatomy project, NIH: www.http://sgmap.nider.nih.gov/sgmap/sgexp.html). Instead, amphiregulin (AR), as shown for the mammary gland (Sternlicht et al., 2005), may be a potential ligand since it has been shown to stimulate branching similar to Egf (Häärä et al. 2009). In addition, heparin-binding Egf (HB-Egf) have been suggested to regulate branching via Egfr but it is mainly expressed in the epithelium (Umeda et al., 2001).

Beside Fgf and Egf pathways, a few other signaling cascades have been related to salivary gland branching morphogenesis. These include Shh and Bmp/Tgfβ. Addition of Shh protein in organ culture has been shown to stimulate branching (Jaskoll et al., 2004a). \textit{Bmp4} and \textit{Bmp7} are highly expressed during early SMG development and are regulated by Fgfr signaling (Hoffman et al., 2002). Related to Bmps, Activins have been also implicated in SMG morphogenesis. Activin-βA and Activin-βB are expressed during SMG development and have profound inhibitory effects on SMG branching morphogenesis. Exogenous Activin A added to \textit{ex vivo} SMG culture resulted in the disappearance of interlobular clefts, a fusion of epithelial buds, and inhibition of branching morphogenesis (Ritvos et al., 1995). Despite the profound and well-studied role of Wnt signaling in ectodermal organogenesis (Mikkola & Millar 2006, Liu & Millar, 2010), no reports indicating Wnt signaling in SMG development have been published so far.

1.6.2.3. Epithelial differentiation

Along with branching morphogenesis, epithelial differentiation leading to the formation of secreting acini and transporting ducts is required for the functional salivary gland to develop. The first signs of differentiation are detected already at E12.5 as the outer layer of the bud is organized into columnar epithelium whereas the inner layer remains a round-shaped, less organized cell population. The outer layer will later mainly give rise to the acini, whereas the inner layer will develop mainly into the ducts. During duct formation, the cells also form columnar epithelium and express E-Cadherin and polarize, expressing ZO1 in the apical side (Walker et al., 2008). The duct formation occurs before acini differentiation, and the first markers of cell differentiation in the main duct are expressed as early as E13.5, when keratin 7 (K7) positive cells line the forming duct. The main duct is organized into columnar epithelium by E15, expressing cytokeratin 8 (K8), a marker for cell differentiation, and K8-immunoreactivity proceeds to acini by E17. In the \textit{Egfr-/-} mice, the K8-positive cells emerge later in development and are reduced in new born mice, suggesting that Egfr signaling regulates the differentiation of the duct epithelium (Häärä et al., 2009). The steps initiating lumen formation are poorly understood. It has been suggested that TNF/TNFR/NF-κB mediate apoptosis to precede the canal and lumen formation (Jaskoll and Melnick, 1999, Jaskoll et al., 2003); however, in other reports, apoptosis has not been associated with the lumen formation (Kiukkonen et al., 2006, Häärä et al., 2009).

Together with differentiation, the innervation of the salivary gland is required to link the saliva-
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The innervation develops in parallel with the SMG morphogenesis and interestingly, also affects morphogenesis: the parasympathetic submandibular ganglion stimulates the cytokeratin 5 (K5) positive basal progenitor cells to proliferate through acetylcholine signaling via muscarinic M1 receptor and Egrf signaling (Knox et al., 2010). This stimulation also sustains the epithelial cells in an undifferentiated stage, thus promoting proliferation. In the adult SMG, parasympathetic submandibular ganglion supports SMG regeneration after injury (Proctor and Carpenter, 2007).

1.6.2.4. Stem cells and regeneration in the salivary glands

Salivary glands show exceptional capacity to regenerate in mammals, but the mechanism for this has not been identified. The stem cells in the adult SMG are thought to reside in the ducts and a subpopulation of cells expressing a stem cell marker c-Kit has been shown to contribute the SMG regeneration after irradiation (Lombaert et al., 2008). Another marker, Alsc3, is also localized to a subpopulation of the duct cells and Alsc1 positive cells have been shown by lineage tracing to form both acinar and alveolar structures. However, in the Alsc3 loss-of-function mice, functional SMG, albeit smaller, is still developed (Bullard et al., 2008). Moreover, deletion of the Alsc3 positive cells by Cre-activated expression of diphtheria toxin (DTA) in the Ascl3-expressing (Ascl3+) cell population resulted in smaller, but functional, salivary glands (Arany et al., 2011). The Wnt/b-catenin pathway has been also suggested to regulate the proliferation of the putative stem/progenitor cells in the intercalated ducts (Hai et al., 2010).

1.6.3. Ectodysplasin in the salivary gland morphogenesis

In the developing salivary gland, Eda is expressed in the mesenchyme and Edar in the epithelium (Pispa et al., 2003). The Tabby mouse has delayed postnatal development of SMG and ex vivo culture indicates reduced embryonic branching (Blecher et al., 1983, Jaskoll et al., 2003). Adult Tabby mice have reduced weight and less granular convoluted tubules in the adult, indicating permanent dysfunction in saliva production (Blecher et al., 1983). Also, Tabby SMG are reported to have less ductal structures, whereas Eda overexpressing K14-Eda mice show larger lumens (Nordgarden et al., 2004) while adult mice with enhanced Edar signaling activity were reported to have more elaborately branched salivary glands (Chang et al., 2009). Another study reported that Eda-deficient SMGs are hypoplastic whereas Edar-deficient SMGs are severely dysplastic, suggesting involvement of the Eda pathway in lumen formation (Jaskoll et al., 2003). Addition of recombinant Eda protein to salivary glands in culture has been shown to increase epithelial branching (Jaskoll et al., 2003). These reports indicate that Eda influences epithelial branching but there is no detailed understanding how Eda affects SMG development and especially, how the Tabby SMG phenotype is developed.

1.7. Evolution and development of the dentition

Food processing and vocalization are dependent on the functional dentition. The lack of teeth, due to a developmental defect, injury or aging has a variety of adverse effects on wellbeing, including nutritional related issues, communication and aesthetical considerations with facial alterations.
1.7.1. Trends in the evolution of dentition

Key innovations in evolution are characteristics that once emerged, have led to widespread adaptive radiation of the taxon. The first tooth-like structures evolved around 500 million years ago, in conjunction with the origin of the jaws. After this, a rapid radiation of the vertebrates with jaws and teeth occurred. This forms a superclass *Gnathostomata* which comprises almost all living vertebrates today. Although some groups of Gnathostomata have lost their teeth (such as most of the frogs), in most groups they are present. The high variation of tooth morphologies between and inside the taxa indicates a functional diversification of teeth in response to environmental constrains and may be the grounds for adaptive radiation of *Gnathostomata* (Zerina & Smith, 2005).

The evolutionary origin of teeth remains debated. A classical “outside-in” theory suggests that teeth are modified skin denticles called odontodes that invaded into the oral cavity (Huysseune et al., 2009). The evidence for the theory came from the morphological similarity of the skin denticles and teeth in fossils and in the present day Chondroichtyes (sharks, rays). According the outside-in theory, teeth were evolved in conjunction or later that jaws. Later on, another theory proposed that teeth originated before the jaws and evolved from endoderm-derived pharyngeal denticles, rather than ectodermal skin denticles. This hypothesis became known as “inside-out” hypothesis and was proposed by Smith & Coates (1998). Recently, modifications to these theories have been proposed. One such was introduced by Huysseune et al (2009) who formulated a revised outside-in hypothesis. According this hypothesis, teeth originated before the jaws, and were modified odontodes invading into the oral cavity through the mouth but also through the gill slits. Fraser (2010), however, concluded that skin denticles and teeth have evolved convergently. It seems, however, that evidence for all of these hypotheses is rather scattered.

The uptake of energy is perhaps the most vital thing for animals, and teeth are the first structures responsible for catching the food. The adaptation to the food sources available requires a modification of dentition and this modification should reflect the evolutionary history of the species. The first teeth were homodont, meaning all teeth were of similar shape. Such dentition is seen in most of the present day bony and cartilagous fish and in reptiles. Tooth morphology in the homodont species is often cone-shaped and is suitable for predatory behavior and defense. The more versatile dentition developed with morphological diversification of different regions in dentition; in the present day mammals the dentition is, with few exceptions, heterodont. This means that four kinds of teeth may be formed (from back to front): molars, premolars, canines, and incisors. The shape and size of these different kinds of teeth can vary and some may be lost during evolution: for example, mice (*Mus musculus*) have lost the canines and premolars whereas in human a “primitive” mammalian dentition with all tooth types is present. The functional diversification of teeth allows adaptation to different food sources, but also supports defense and acts on sexual selection (Dean and Beynon, 1991). The incisors are normally flat, chisel-shaped teeth that meet in an edge-to-edge bite. Their function is cutting, slicing, or gnawing food so that it fits into the mouth for further processing. The canines are immediately behind the incisors. In many mammals, the canines are pointed, tusk-shaped teeth, projecting beyond the level of the other teeth. In carnivores, the canines are primarily offensive weapons for catching prey and may act on sexual selection. In other mammals such as some primates, canines are used to split open hard surfaced food. The premolars and molars usually prepare pieces of food to be swallowed by grinding, shearing, or crushing, to process the food into smaller pieces making further processing more effective (Dean and Beynon, 1991).

Molars are morphologically the most variable and complex teeth. The occlusal surface of the
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molars is folded to form the cusps. The organization, number, size and shape of the cusps together with crests bring the variation to the molar shape in the occlusal surface. As the molars are mainly responsible for food chewing and processing, the variation in the shape of the occlusal surface is affected by the food type: the carnivorous animals usually have molars with less in number but more sharp and pronounced cusps whereas the herbivorous taxa have a trend towards higher complexity with more but smaller cusps (Evans et al., 2007, Kavanagh et al., 2007). The omnivores, such as pigs, human and bears, have intermediate complexity. Interestingly, the trend in tooth complexity is independent on the taxonomic position, and has evolved independently at least in carnivores and rodents, probably also in various other taxa (Evans et al., 2007). During evolution, the number of cusps has been increased, allowing more delicate specialization (Osborn, 1888). A normal mouse dentition and the nomenclature of the cusp pattern of the first molar are illustrated in the Figure 8.

1.7.2. Molar development

Molar tooth characteristics are determined during embryonic development (Fig 9). In mice, the first signs of dental development are detected at embryonic day 10 (E10), when the dental lamina is formed. It is a horseshoe-shaped epithelial thickening supported by neural crest derived mesenchyme, and dental lamina normally defines the position of tooth formation. At E11-E12, separate epithelial placodes for incisors and molars are formed in the dental lamina, which thereafter invaginates into the mesenchyme, forming a bud. The toothless diastema is located between the placodes for molars and incisors (Thesleff, 2006, Tummers and Thesleff, 2009). The diastema is a specialization of murine dentition and is caused by the loss of canines and premolars. The contribution of the cells in the first placodes and bud to the developing first molar has been debated. Although classically considered that the first placodes gives rise to the first bud, which thereafter folds into the cap of the first molar, an alternative view is also proposed. According to this, in the molar forming space, three epithelial buds arise sequentially from anterior to posterior (or mesial to distal) direction in the lower jaw. The first epithelial bud, called the mesial segment, regresses through apoptosis, and the next bud, a rudimentary bud, is formed at E13. These buds are thought to represent the primordia of ancestral premolars, which were lost during rodent evolution. The second bud has been suggested to fuse with the third bud forming at E14, which thereafter develops into m1 (Viriot et al., 2002, Prochazka et al., 2010). Whatever the origin of the m1, the bud of the first molar folds into a cap at around E14 and is surrounded by the mesenchymal dental papilla.

A critical structure for later development, the primary enamel knot is formed during the bud to cap transition in the epithelium (at ~ E14). It is a signaling center composed of non-proliferating cells and expresses a set of signaling molecules for the regulation of tooth characters, including multiple signals in several families: Fgf ligands (-4, -9, -3, -15), Wnt ligands (-10a, -10b, -3, -6), Bmp ligands (-2,-3,-7) and Shh (Thesleff, 2006, Kettunen et al., 2011, Porntaveetus et al., 2011). The primary enamel knot induces the formation of secondary enamel knots from E15-E16. The secondary enamel knots regulate cusp formation so that each knot regulates the local proliferation and differentiation of the adjacent epithelium and mesenchyme which causes unequal growth leading to the folding of the epithelium and thus, cusp formation. The crown shape is fixed when the enamel and dentin matrices mineralize. Ameloblasts secreting enamel and odontoblast-secretion dentin eventually cause the tooth crown to be composed of hard, mineralized enamel on the surface and mineralized dentin facing the tooth pulp. As ameloblasts and the enamel epithelium are removed after the enamel is formed, the injuries with enamel defects cannot be repaired. Odontoblast precursors, though, remain viable and can differentiate into odontoblasts and secrete more dentin. After the crown shape is completed, the development
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of roots proceeds. In species with the capability of molar replacement, the stem cells are located in the cervical loops. Eventually roots are mineralized when the mesenchyme differentiates into odontoblast-secreting dentin and the follicular cells differentiate into cementoblasts, secreting cementum on the surface of the roots. The periodontal ligament embedded in the cementum ligates the roots to the adjacent alveolar bone (Diekwisch, 2001, Tummers and Thesleff, 2009). The key morphologies of molar development are summarized in Fig 9.

Fig 8. Mouse dentition and the nomenclature of the cusps. m1= first molar, m2= second molar, m3= third molar. Anterior to left, buccal to up.
1.7.3. Regulation of molar development and tooth number

1.7.3.1. Initiation and early morphogenesis

The odontogenic potential to form teeth appears to reside in the early epithelium. Tissue recombination studies have shown that the epithelium from the mandibular arch of E9-E11 mouse embryos combined with neural crest mesenchyme derived from the trunk induces tooth formation, but not vice versa. However, odontogenic potential shifts to the mesenchyme by E12 (Mina and Kollar, 1987). Bmp4 expression is also shifted from the epithelium to the mesenchyme at this stage (Vainio et al., 1993). The placodes for molars and incisors are positioned within the dental lamina and are active signaling centers expressing the signals of the major pathways. Inhibition of Fgf, Wnt, BMP or Hh, can lead to the failure of early tooth morphogenesis: removal of epithelial Fgf receptor, Fgfr2b, or inhibition of Wnt/β-catenin signaling by deletion of β-catenin or by ectopic expression of Wnt inhibitor Dkk1, over-expression of Bmp antagonist Noggin in the dental epithelium or deletion of the Hh pathway components Gli2/Gli3 together leads to the arrest of tooth development at placode/bud stage (Hardcastle et al., 1998, De Moerlooze et al., 2000, Liu et al., 2008, Wang et al., 2012). In contrast, when Wnt/β-catenin signaling is stabilized in the epithelium, multiple teeth are initiated (Järvinen et al., 2006). Also, if β-catenin degradation is disturbed in mice by loss-of-function mutation in the Apc gene, teeth can be formed outside of the dental lamina, although they develop close to the lamina (Wang et al., 2009). Genes expressed during tooth development can be searched in the Bite-it database (http://bite-it.helsinki.fi). Similar to SMG morphogenesis, innervation of the molar occurs during morphogenesis. During bud stage, first axons of the trigeminal ganglion neurons grow adjacent to the developing molar and interestingly, these nerves were lacking in the diastemal tooth primordia (Loes et al., 2002). The development of tooth innervation is controlled, at least partially, by the same factors that mediate the epithelial-mesenchymal interactions. In addition, the morphogenesis and innervation are integrated at least by Fgfr2b mediated signaling (Luukko et al., 2005, Kettunen et al., 2007).

The bud to cap transition occurs at E13-E14. This transition is an important step in tooth development and many mouse mutants, such as loss-of-function for Lef1, Msx1 or Runx2, fail to proceed into cap stage (Satokata and Maas, 1994, van Genderen et al., 1994, D’Souza et al., 1999). During the transition, the enamel knot is formed. The signals expressed in the primary enamel knot regulate the proliferation of the adjacent cells (Jernvall et al., 1994). The primary enamel knot regulates the formation of the secondary enamel knots which express the same genes as the primary enamel knot. The primary enamel knot is eventually reorganized and at least partially removed by apoptosis (Vaahktokari et al., 1996).

1.7.3.2. Late morphogenesis

Secondary enamel knots define the shape of the tooth crown. The spatial patterning of the secondary enamel knots in the forming molars pinpoints the position of the future cusps (Jernvall et al., 1994). The patterning of the secondary enamel knots is thus a crucial process, and altering the secondary enamel knot dynamics can produce variation in the cusp pattern. For example, by analyzing the prepatterning of the secondary enamel knots, differences in the cusp pattern between mouse and vole molars can be predicted (Jernvall et al., 2000). Several mouse models show defects in the cusp patterning: extra cusps can be gained by, for example, increasing Eda level (K14-Eda) or the receptor Edar (K14-Edar), removing Sostdc1 (Sostdc1-/-) or reducing Shh signaling (anti-Shh antibody injection) (Pispa et al., 2004, Kangas et al., 2004, Kassai et al., 2005, Cho et al., 2011). Significantly more cusps develop when several pathways are modulated
Introduction

Simultaneously, inhibition of Shh and activation of Activin A and Eda were used in combination in organ culture to stimulate cusp formation (Harjunmaa et al., 2012). Cusp number can be decreased in an opposite manner. Tabby mice show reduction in the cusp number: especially anterior cusp (anteroconids) and posterior cusp pairs (talonid) are affected, possibly due to incomplete separation of secondary enamel knots (Pispa et al., 1999).

The patterning by paracrine factors alone is not sufficient for morphogenesis. As noted already by Turing (1952), the mechanical forces causing pressure and other mechanical interactions are in many cases required to generate a shape. For tooth patterning, a model composed of an auto-regulated activator-inhibitor morphogen system and cellular variables for cell behavior was formulated by Salazar-Ciudad & Jernvall (Salazar-Ciudad and Jernvall, 2010). In this work they noticed that to reiterate real tooth shape, in that case a tooth of a ringed seal, the parameters of activator-inhibitor dynamics were most crucial: the parameters producing the most realistic variation were activator self-regulation, activator diffusion, inhibitor strength, inhibitor diffusion, and secondary signal threshold. These are the parameters that in real teeth are regulated by the action of the secondary enamel knots. The spacing of the secondary enamel knots is determined by the lateral inhibition from the pre-existing knots and by the activation, the first inducing proliferation and the latter inducing the differentiation of the epithelial cells (Salazar-Ciudad and Jernvall, 2002). The spacing of the cusps, predetermined by the spacing of the secondary enamel knots, appeared thus to be regulated by the activator-inhibitor dynamics. The molecules acting as activators or inhibitors have not been characterized, but putative candidates have been suggested. Currently, candidate molecules considered as activators include Bmps and Wnts, which induce differentiation markers in the dental epithelia and are associated with the cessation of mitosis in the knot. Putative inhibitors include fibroblast growth factors (Fgfs), Sostdc1 and Shh, all of which stimulate growth and survival of dental epithelia, mesenchyme, or both (Salazar-Ciudad and Jernvall, 2002, Salazar-Ciudad and Jernvall, 2010). In the newer model by Salazar-Ciudad and Jernvall (2010), the adjustment or tinkering with the cellular parameters systematically did not produce as realistic of an output in tooth shape as modulating the activator-inhibitor dynamics. Instead, however, tinkering with the cellular parameters, especially epithelial growth was required to formulate the morphologies and the changes observed in nature inside a tooth row, to model the relative size of each molar and cusp shape in the heterodontic tooth row. Another model suggests Wnt as activators, Sostdc1 as modulators and Shh as inhibitors during molar patterning (Cho et al., 2011).

The relative size of the molars within a tooth row has been shown to be regulated by the inhibitory cascade. One model describes the anterior to posterior size reduction of molars, proposing this reduction to be caused by the inhibition from the anterior molar to the adjacent posterior one, and indicating that varying the strength of the inhibition causes variation in the relative size (Kavanagh et al., 2007). According to the model by Salazar-Ciudad & Jernvall (2010), the inhibition is likely to affect the cellular parameters shown in the model to be mostly reiterating the natural variation. This work also demonstrated how few changes in parameters could produce significant morphological variation, a palette of tooth morphologies also observed in nature.

1.7.3.3 Tooth number

In human, the dental formula for upper and lower jaw is the same per quadrant, consisting of two incisors, one canine, two premolars and three molars in adult (2.1.2.3. pattern for incisors, canines, premolars and molars, respectively). Among mammals, a huge variation exists in the dental patterns. For different types of teeth, a complete loss, reduction or increase in number
has occurred during mammalian radiation. Also, dentition in the upper and lower jaw may be different, an example being the cow which has lower but not upper incisors and canines. In most mammals there are two sets of teeth: the deciduous (milk-) and permanent teeth. The deciduous teeth are the first to develop followed by the permanent teeth. The development of two sets of teeth allows the craniofacial growth without disrupting the tooth pattern or vice versa. The important exception to this rule is the taxon rodentia, including the mouse, which have only one set of teeth composed of molars and continuously growing incisors. The premolars and canines have been lost during evolution, leading to a toothless diastema between the incisors and the molars (Fig 8). However, some rodents such as squirrels have retained the premolar. The loss of the premolar P4 in the mouse-like rodents has been dated to have occurred around 45 million years ago which, in the evolutionary scale, can be considered as a rather recent phenomenon (Rodrigues et al., 2011).

It seems that mice have also retained the capacity to form the premolar. Despite the 45 million years for purifying selection to remove this capacity, several mouse models with altered gene expression can still develop a tooth at the site of ancestral premolar (Table 1). Even more importantly, a transient signaling center in the place of the ancestral premolar is also observed in WT mice (Kangas et al., 2004, Prochazka et al., 2010). This kind of evolutionary ‘throwback’ is called atavism. Increasing the activity in that signaling center by overexpressing *Eda* (*K14-Eda*), increasing Fgf signaling by removing the inhibitors (*Spry2-/-* and *Spry4-/-*), or removing a negative regulator for Bmp and Wnt signaling (*Sostdc1-/-*) can lead to the development of the teeth allows the craniofacial growth without disrupting the tooth pattern or vice versa. The important exception to this rule is the taxon rodentia, including the mouse, which have only one set of teeth composed of molars and continuously growing incisors. The premolars and canines have been lost during evolution, leading to a toothless diastema between the incisors and the molars (Fig 8). However, some rodents such as squirrels have retained the premolar. The loss of the premolar P4 in the mouse-like rodents has been dated to have occurred around 45 million years ago which, in the evolutionary scale, can be considered as a rather recent phenomenon (Rodrigues et al., 2011).

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extra molar in the place of the premolar (Kangas et al., 2004, Kassai et al., 2005, Klein et al., 2006). Whether the purifying selection is unable to remove the capacity due to the overlapping gene expression pattern in the premolar placodes and molar placodes, or whether the premolar placodes have some function in the WT mice remains speculative. The latter was suggested in the report by Prochazka et al. (2010), who argued that the placodes for the premolar, the rudimentary bud, is fused with the placodes forming the first molar and forms the most anterior cusp pair, the anteroconids of the first molar (Fig 8). Although slightly modified anteroconids are formed in the mice developing the extra molar (Spry mutants and in K14-Eda), the contribution of the premolar placodes to m1 development in WT mice cannot be excluded.

Supernumerary teeth are also observed in human. Although thought of as atavism, this view has been criticized (Rajab and Hamdan, 2002). The supernumerary teeth, defined by an addition to the normal dental formula, may occur in any region of the dental arch but have a particular predilection for the premaxilla, so called mesiodens. Supernumerary teeth may occur singly, multiply, unilaterally or bilaterally, and in one or both jaws. Classifications of supernumerary teeth are based on their location in the dental arches, or on their morphology. Haploinsufficiency in Runt-related gene 2 (Runx2) in human results in a cleido-cranial dysplasia (CCD), an autosomal hereditary disease characterized by supernumerary teeth (Goseki-Sone et al., 2001, Ryoo and Wang, 2006). These teeth exhibit a third dentition, in contrast to normal two in human, thus, mutations in Runx2 could unlock the capacity for continuous tooth replacement in human (Jensen and Kreiborg, 1990). A similar phenotype with supernumerary teeth is observed in humans with a missense mutation in the gene encoding interleukin 11 receptor alpha (ILR11a; Nieminen et al. 2011). It has been suggested that supernumerary teeth are formed as a result of local, independent, conditioned hyperactivity of the dental lamina (Jensen and Kreiborg, 1990). As the position and the shape of the supernumerary teeth is variable, it is probably not related to the extra premolar-like tooth found in the abovementioned mouse models, and therefore should not be referred to as atavism (Rajab and Hamdan, 2002).

Congenitally missing teeth are much more common than supernumerary teeth both in humans and in mouse models. Anodontia implies complete absence of teeth. Selective tooth agenesis (non-syndromic) can be divided into two types: hypodontia, the agenesis of fewer than 6 teeth, and oligodontia, the agenesis of 6 or more permanent teeth. These do not include the third molars (wisdom teeth). The prevalence of non-syndromic hypodontia in permanent dentition (OMIM 106600) is estimated to be 2.8%-7.4% and the frequency of non-syndromic oligodontia in permanent dentition (OMIM : 604625, 313500) is 0.1 to 0.3 % of the population (Polder et al., 2004). Some genetic determinants for non-syndromic hypodontia and oligodontia have been located. For example, a missense mutation in exon 2 of the transcription factor Pax9 has been found to underlie tooth agenesis in human and the Pax9/- mouse lack teeth completely, whereas partial reduction of Pax9 cause oligodontia (Peters et al., 1998, Boeira Junior and Echeverrigaray, 2012). Other genes found to underlie non-syndromic hypodontia and/or hypodontia include Mx1 and Axl2 (Vastardis et al., 1999, Lammi et al., 2004). Recently, Wnt10A mutations were found in about 50% of non syndromic oligodontia (van de Boogaard et al., 2012). Mutation in the Eda gene can also cause non-syndromic hypodontia (reviewed in Clauss et al, 2008). Teeth can be lost due to syndromic genetic diseases. As described above, hypohidrotic ectodermal dysplasia (HED) are well characterized examples in which mutations in the Eda pathway components, Eda, Edar or Edaradd lead to the HED syndromes with missing and/or malformed teeth, and the corresponding mouse models reflect the human syndrome (Mikkola, 2009). Also mutations in other pathways lead to reduced number or complete absence of teeth in mice. Altogether, as the mouse models with supernumerary teeth may not reflect the human syndromes, the models with tooth agenesis seem to have a good correspondence with congenital human syn-
dromes (Nieminien, 2009).

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Mutation/description</th>
<th>Frequency of premolar</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tabby +/-</td>
<td>Eda heterozygote</td>
<td>Rare</td>
<td>Grüneberg 1966; Peterková et al. 2005</td>
</tr>
<tr>
<td>K14-Eda</td>
<td>Eda gof.</td>
<td>~50%</td>
<td>Mustonen et al. 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kangas et al. 2004</td>
</tr>
<tr>
<td>Spry2/-</td>
<td>Epithelial Fgf inhibitor Sprouty2 lof</td>
<td>Common</td>
<td>Klein et al. 2006</td>
</tr>
<tr>
<td>Spry4/-</td>
<td>Mesenchymal Fgf inhibitor Sprouty4 lof</td>
<td>Common</td>
<td>Klein et al. 2006</td>
</tr>
<tr>
<td>Lrp4</td>
<td>Wnt inhibitor reduction</td>
<td>Common</td>
<td>Ohazama et al. 2008</td>
</tr>
<tr>
<td>Sostdc1/-</td>
<td>Wnt/Bmp antagonist Sostdc1 lof</td>
<td>Common</td>
<td>Kassai et al. 2008</td>
</tr>
<tr>
<td>Sostdc1\textsuperscript{+/−};Shh\textsuperscript{creERT}</td>
<td>Reduction of Sostdc1 and Shh</td>
<td>Common</td>
<td>Ahn et al. 2010</td>
</tr>
<tr>
<td>Ctnnb1\textsuperscript{ex3};Shh-CreERT</td>
<td>Activation of Wnt in Shh-expressing cells</td>
<td>Unknown</td>
<td>Ahn et al. 2010</td>
</tr>
<tr>
<td>Tg737\textsuperscript{orpk hypomorph}</td>
<td>Reduction of polaris causing increase in Shh in diastema</td>
<td>Common</td>
<td>Ohazama et al. 2009</td>
</tr>
<tr>
<td>Wnt1-Cre; polaris\textsuperscript{is\textsuperscript{lox/lox}}</td>
<td>Polaris lof in dental mesenchyme</td>
<td>Common</td>
<td>Ohazama et al. 2009</td>
</tr>
<tr>
<td>Gasl/-</td>
<td>Shh inhibitor Gas1 lof</td>
<td>Common</td>
<td>Ohazama et al. 2009</td>
</tr>
</tbody>
</table>

Table 1. Mouse models with an extra molar (premolar). Lof=loss of function, gof= gain of function.

1.7.4. Eda and Fgfs in molar development

Eda and Edar are expressed in the dental epithelium throughout molar morphogenesis in a complementary pattern and importantly, Edar is confined to both the primary and the secondary enamel knots (Laurikkala et al., 2001). Loss of Eda in Tabby causes several defects in teeth: molars are smaller, cusp number is reduced, enamel is defective and m3 is lacking in 17-55% of the jaw quadrants (Grüneberg 1966, Pispa et al., 1999, Kristenova-Cermakova et al., 2002). During
development, Tabby molars are smaller and abnormal in shape from E14 onward. The size of the primary enamel knot is reduced and the first secondary enamel knots are fused (Pispa et al., 1999). The Tabby phenotype was partially rescued by exogenous Fgf10 protein in culture. The mouse mutant for Edar; downless, also suffers from defective morphogenesis and the cusps are flattened or fused in the adult mouse (Tucker et al., 2000). When Eda signaling is over-activated in the epithelial cells in vivo by ectopic expression of Eda under the Keratin-14 promoter (K14-Eda), extra cusps are formed and, on average, in 50% of the jaw quadrants an extra molar is developed in the place of the ancestral premolar (Mustonen et al., 2003, Kangas et al., 2004). Misexpression of Edar under the K14-promoter (K14-Edar) leads to a phenotype similar to Tabby with smaller molars, but variable in terms of cusp patterning: either extra cusps or fused cusps are developed, depending on the strength of the transgene expression (Pispa et al., 2004, Tucker et al., 2004). One extra molar was formed in one individual of K14-Edar, and rare occurrences of an extra molar is detected also in Tabby heterozygotes (Pispa et al., 2004, Peterkova et al., 2005). However, both Tabby and K14-Eda tooth features are polymorphic, suggesting that a shift from normal Eda level may destabilize the development (Kangas et al., 2005).

Fgfs regulate the morphogenesis of teeth and typically epithelial and mesenchymal Fgfs function in signaling loops across tissues, and often regulate proliferation (Kettunen et al., 2000; Sun et al., 2000; Klein et al., 2008; Ornitz and Yin, 2012). Different Fgf ligands and Fgf receptors are expressed in the epithelium and the mesenchyme. During molar development, epithelial Fgfs, expressed in tooth placodes and/or enamel knots, include Fgf3, Fgf4, Fgf9 and Fgf15 (Åberg et al., 2004; Kettunen and Thesleff, 1998; Porntaveetus et al., 2011, Kettunen et al., 2011). Mesenchymally-expressed Fgfs include Fgf3 and Fgf10, which act redundantly to support the growth and patterning of molars mainly via epithelial Fgf receptor 2b (Fgfr2b) (Wang et al., 2007; De Moerlooze et al., 2000). The Fgfr1 IIIb isoform is expressed in the epithelium, whereas the Fgfr IIIC isoform is expressed both in the epithelium and the mesenchyme. Fgfr2 IIC and Fgfr3 are both expressed in the mesenchyme (Kettunen et al., 1998). Fgf4 was the first Fgf to be discovered in the enamel knot (Niswander and Martin, 1992) and suggested to regulate cusp formation (Jernvall et al., 1994). Deletion of Fgf3 and Fgf10 in combination, or deletion of Fgfr2b, arrests tooth development prior to bud stage and Fgf3-/- molars show aberrant cusp shape (De Moerlooze et al., 2000, Wang et al., 2007). The inhibition of Fgf signaling may keep the diastema toothless, as deletion of genes encoding the Fgf inhibitors, Spry2 or Spry4, cause the formation of an extra anterior molar (Klein et al., 2006). The abovementioned results together suggest a vital role for Fgf signaling in different stages of tooth development.

### 1.7.5. Development shedding light on evolution

If a structure has changed during evolution, the developmental code for the organogenesis must have been altered. Developmental genetics have shown that relatively small changes in the activities of even single genes can lead to robust phenotypic changes: for example, cusp pattern or tooth number can be changed by modulating the activity of only one gene, such as Eda or Sostdc1 (Kangas et al., 2004, Kassai et al., 2005). This challenges one major principle of Charles Darwin’s idea of evolutionary theory: that of gradualism. The possibility of inducing large morphological variation with simple genetic alteration favors the idea of rapid evolutionary change and supports the theory of punctuated equilibrium (Kutschera and Niklas, 2004). Developmental biology can thus indicate what kind of genetic changes are required for the phenotypic changes observed in fossil records. The variation within species, including the genetically modified mouse models, can reveal the genetic framework for morphological evolution. As the tooth enamel is hard, with low-solubility, it is well preserved material and is
commonly found in fossil records, a rather good picture of tooth morphological evolution in mammals have been reconstructed, and the data can be correlated with the mouse models with known alterations in the underlying molecular mechanism. Vestigial structures, such as premolar P4, can be observed during development even though they were not detected in adult phenotype. The genetic requirements for gaining or loosing structures can be experimentally tested. The *in vitro* culture of mouse teeth have turned out to be a potential model for analyzing constrains and molecular bases of morphological change. For example, the dependency of tuning multiple parallel pathways simultaneously to reiterate the evolutionary trend with increased complexity was shown with *in vitro* cultured teeth (Harjunmaa et al., 2012). Even though the statement ‘ontogeny recapitulates phylogeny’, as proposed by Ernst Haeckel is not considered to be uniformly true, by studying the molecular basis and mechanisms of development, the evolutionary constraints and possible mechanisms for morphological evolution can be uncovered. Thus, ontogeny may give rationale for phylogeny.
2. Aims of the study

When this work was initiated the role of Eda signaling in the salivary gland was poorly known, even though reduced saliva flow is observed in several different types of ectodermal dysplasia. The aim of this study was to characterize the function of Eda signaling, during early development and the morphogenesis of the submandibular salivary gland. Particularly important issues were the integration of Eda signaling to other signaling pathways in SMG development. As Shh was previously shown to affect SMG morphogenesis (Jaskoll et al., 2004a) and was induced by Eda in hair development (Pummila et al., 2007), it was reasonable to assume that Shh signaling could be one potential downstream factor. Wnt and Eda are integrated in hair development, but practically nothing was known about Wnt/b-catenin signaling in the SMG. The aims were:

1) To characterize of SMG phenotype in Tabby and correlation of Eda levels to branching
2) To investigate the integration of Eda to other pathways
3) To validate the importance of Wnt signaling in the SMG early morphogenesis

In molar tooth development, the function of Eda signaling has been better characterized. However, the downstream mediators were not known. As Fgf20 appeared to colocalize with Edar during molar development, and was upregulated in vitro upon Eda stimulus (Lefebvre et al., 2012), it was a potential downstream gene of Eda in tooth. The aim of this part of the work was:

1) To characterize the Eda-Fgf20 connection
2) To analyze the role of Fgf20 during tooth development
3. Materials and methods

### Mouse strains

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### Proteins and inhibitors

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### Methods

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**Statistical analysis**

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**Unpublished data**

The SMG were dissected at E13 and cultured for 20hr as described in (I). BIO (Sigma-Aldrich) was used at 5µM concentration. *DermoCre; b-cat^fl/fl* SMG was dissected at E12.5 and cultured for 2-days as described in (I).
4. Results and discussion

4.1. Eda/NF-κB activity is required for the salivary gland development (I)

Sensation of dry mouth, xerostomia, is associated with several types of ED, including HED (Nordgarden et al., 2003, Lexner et al., 2007). However, only a few reports exist for the function of Eda in SMG development. Blecher et al. (1983) report Tabby SMG to be smaller than WT in adult mice with a lower number of tubular structures in Tabby. Jaskoll et al. (2003) reported that Eda signaling regulates the differentiation of the acini and the ducts and that Tabby mice are hypoplastic, indicated by smaller acini and ducts and reduced number of ducts. They suggested that Eda/Edar signaling regulates lumen formation in the ducts and histodifferentiation in the ducts and acini. Jaskoll et al. (2003) also reported that Eda or Edar are not found in the early stages of SMG development, but becomes expressed during late pseudoglandular - early canalicular stage. This stage is detected at around E15. These few reports suggested that Eda has a role in SMG development, but do not imply the mechanism of how Eda regulates the SMG development. Importantly, the present data are insufficient to uncover the pathogenic mechanism leading to xerostomia in patients with HED.

To address the molecular mechanisms leading to impaired function of salivary glands in HED patients, we analyzed submandibular salivary gland development in Eda mutant mice, both loss- and gain-of-function, followed by in vitro morphometric and functional studies. We used RISH to localize the expression of Eda and Edar in the SMG at E14. We detected Edar expression in the epithelium similar to the other ectodermal organs, in the tooth, hair or mammary placodes. However, in the SMG, Eda was expressed in the mesenchyme, unlike in hair and teeth, but similar to the mammary gland, another branched organ (Pispa et al., 2003, Voutilainen et al., 2012 and this study). To study the effect of Eda signaling on branching morphogenesis of the salivary glands, we focused on the early stages of branching at E13-E15, and used a well-established ex vivo culture system for submandibular salivary glands (SMG) which is known to recapitulate the branching pattern that occurs in vivo and allows an accurate quantification of forming branches (Borghese, 1950; Patel et al., 2006). We cultured SMG of Tabby, WT and K14-Eda mice, followed by quantitation of branching and statistical analysis (number of end buds). We showed that the salivary gland branching is dependent on Eda levels ex vivo: in Tabby, branching was reduced (65% of WT) whereas in K14-Eda, the branching was significantly augmented (180% of WT).

In developing teeth and hair follicles, the effects of Eda are mediated largely by transcription factor nuclear factor-kappaB (NF-κB) (Schmidt-Ullrich et al., 2001; Pispa et al., 2008). Inhibition of NF-κB in vitro by cell-permeable peptide SN50 was reported to inhibit SMG branching morphogenesis (Melnick et al., 2001). However, more recent studies by the same group (Melnick et al., 2009) suggested that NF-κB activity is not essential for Eda signaling in the salivary gland. To study this obvious discrepancy, we first analyzed NF-κB activity with a NF-κB LacZ reporter mouse (Bhakar et al., 2002; Pispa et al., 2008) in the developing salivary gland. At all stages studied (E12-E17), we observed strong NF-κB signaling activity in the epithelium where Edar was also expressed (Pispa et al., 2003). However, NF-κB activity was not distributed equally throughout the epithelium, but was more intense in the outermost cell layer adjacent to the mesenchymal source of Eda (Pispa et al., 2003). Our observation of early NF-κB
activity in the epithelium at E12-E15, and lack of NF-κB activity in the ducts at E15-E17, contrast with the previous assumption by Jaskoll et al., (2003) that Eda signaling is not active before E15 and that Eda thereafter mediates duct differentiation. Perhaps the defect in duct differentiation observed by Jaskoll et al. (2003) is a secondary effect.

We next studied whether Eda signaling is dependent on NF-κB activity. The activity of NF-κB correlated spatially with Edar expression, and was dependent on Eda signaling: no NF-κB expression was observed in Tabby, and K14-Eda showed increased activity compared to WT (all crossed with NF-κB LacZ REP). However, NF-κB reporter activity was rescued in Tabby when treated with Eda protein. The effect of inhibition of NF-κB activity was studied in IκBαΔN mouse, which ubiquitously express the transdominant super-repressor IκBαΔN (Schmidt-Ullrich et al., 2001). This inhibition of NF-κB led to a similar branching defect as observed in Tabby mouse, and was independent of Eda levels: WT and K14-Eda crossed with IκBαΔN did not differ in number of end buds. These results are in contrast to the previous report of Eda pathway in the SMG (Melnick et al., 2009), but in line with several reports in other systems (Doffinger et al., 2001, Schmidt-Ullrich et al., 2001, Koppenen et al., 2001, Schmidt-Ullrich et al., 2006, Voutilainen et al., 2012). The independence of NF-κB and Edar signaling reported by Melnick et al. was based on in vitro inhibition of NF-κB activity. They, however, did not test the efficacy or specificity of the treatment. From our in vivo and ex vivo results, we can conclude that Eda-signaling is completely dependent on the transcription factor NF-κB in the SMG development.

The branching defect we observed in Tabby mouse, used as a mouse model for HED, may give light to the defective development of the salivary glands in human patients with HED. The reduction in the total saliva flow is a characteristic feature of X-linked form of HED. The total protein composition of the saliva, except for amylase, is not changed, suggesting a reduction in the capacity to produce all components of the saliva (Nordgarden et al., 2003, Lexner et al., 2007). This could be achieved by reducing the total secretion area of the glands that affects all cell types equally, or by defects in cell differentiation of secreting cells. The latter, however, should affect each cell type differentially and cause altered protein composition in the saliva. In this clinical investigation, the authors did not specify the mutation underlying the HED in the patients, but the phenotype resembled Eda-null mutation. So far, the developmental mechanisms leading to reduced saliva flow in HED is unknown. An especially important question remains: is the impairment caused by reduced secretory capacity through defective branching, or by a defect in cell differentiation leading to a lower number of secreting cells in the glands, or both?

The reduction in the branching of the epithelium, leading to reduced epithelial area and thus, secretory capacity, appeared to be the most striking difference in the Tabby phenotype compared to the WT. The lower number of granular convoluted tubules in the Tabby SMG reported by Blecher et al. (1983) is most likely due to reduced branching morphogenesis, leading to lower number of tubular structures. Reduced growth in Tabby mice was not rescued later in the development, since adult Tabby salivary glands were smaller than in the WT (our unpublished observation and Blecher et al 1983).

To conclude, we showed that Eda/Edar/NF-κB signaling is essential for SMG branching morphogenesis. In the Tabby mouse, the reduction in branching was permanent. From this analysis of the mouse model for HED, we can hypothesize a developmental mechanism leading to HED in human. The impaired production of saliva in the X-linked form of HED is most likely caused by reduced branching morphogenesis and growth leading to smaller surface area for saliva production, although a possibility that acinar cell differentiation is secondarily disturbed due to Eda mutation cannot be excluded. In addition to defects in the major salivary glands in the Tabby mouse, the minor salivary glands are absent in Tabby (Wells et al., 2011). As the minor
salivary glands take part in the production of the resting saliva, the reduction in the resting saliva flow in HED may be affected, in addition to impaired major salivary glands and especially SMG, by the possible defective minor salivary glands. However, no reports of the defects in the minor salivary glands in human patients have been published.

4.2. Eda is integrated with Wnt and Hh pathways to support SMG branching (I)

We next analyzed the downstream signaling of Eda in the SMG. Shh has been shown to be involved in SMG development (Jaskoll et al., 2004a), and there is evidence that Shh may be a direct downstream target of Eda in hair follicles (Cui et al., 2006a; Schmidt-Ullrich et al., 2006; Pummila et al., 2007). Therefore, we analyzed the connection between Eda and Shh in the developing salivary gland. We first used qRT-PCR analysis to study if Shh expression is dependent on Eda, by comparing Shh levels in Tabby, WT and K14-Eda SMG at E14. We observed that Shh levels correlated linearly with in vivo Eda levels: the lowest levels were in Tabby, while the highest were in K14-Eda. Then we wanted to test whether Hh signaling is required for Eda-mediated branching. Suppression of Hh signaling with cyclopamine in the Tabby, WT and K14-Eda lead to a most dramatic reduction in K14-Eda, and least dramatic in Tabby, suggesting a vital role for Hh signaling in mediating Eda effects in the SMG development. Supplementation of Shh protein in culture led to more increased branching in Tabby and had no effect on K14-Eda, further supporting the idea of Hh signaling as the main downstream effector of Eda.

Next we wanted to identify the Hh ligand that is active in SMG, by analyzing the expression levels of Shh, Dhh and Ihh. Together with Shh, Dhh but not Ihh, was expressed in the WT SMG, but only Shh levels correlated with the Eda levels. Dhh, however, has not been reported in SMG before this and we cannot exclude the possibility that Dhh could take part in Eda-mediated branching. Since, however, Shh turned out to be the best candidate to mediate Eda signaling, we next tested whether Shh protein could rescue the Tabby phenotype. We observed that Shh protein in culture rescued Tabby branching defect similar to Eda, but Eda was unable to rescue the observed branching defect in Shh+/- SMG, whereas Shh rescued that defect completely. From these experiments, we could conclude that Shh is a key downstream mediator of Eda signaling in SMG. We, however, observed some Shh expression in Tabby with qRT-PCR analysis, suggesting that factors other than Eda signaling can regulate Shh expression in SMG. While our paper was under review, Wells et al (2010) reported that Shh, but not Fgf8, was able to rescue the branching defect in Downless SMG. Shh and Fgf8 were chosen because these genes were shown to be downregulated in Tabby SMG compared to WT (Melnick et al. 2009). The result of Shh as a mediator of Eda signaling is in line with our report. During later stages when ducts develop lumens, Hh signaling regulates cell polarization in lumen formation (Hashizume and Hieda, 2006, Fiaschi et al., 2011). This function is most likely independent of Eda/Edar/NF-κB signaling, as NF-κB activity was not detected in the ducts.

In many ectodermal appendages, canonical Wnt activity colocalizes with Edar/NF-κB activity in the epithelium and studies on hair follicles have shown that the Wnt and Eda pathways regulate partially the same epithelial target genes (Fliniaux et al., 2008; Zhang et al., 2009). Moreover, in hair follicles the Wnt/β-catenin pathway has been placed upstream of Edar, which in turn is thought to regulate expression of Wnt ligands (Zhang et al., 2009). To address whether a similar interplay between the two pathways operates in developing salivary glands, we analyzed the localization of Wnt activity using the Axin2-LacZ reporter mice (Lustig et al., 2002). At
Results and Discussion

E13-E14, Axin2-LacZ activity was entirely confined to the mesenchyme surrounding the branching epithelium and co-localized with *Eda* expression.

Next we tested whether inhibition of mesenchymal Wnt/β-catenin activity affects *Eda* expression. We used *DermoCre*+; *β-catenin*^Flx/Flox^ mouse to inhibit mesenchymal Wnt/β-catenin activity. We observed reduced *Eda* expression in the mutant when quantified from the sections of *Eda* RISH and compared to controls. Edar expressed in the hair follicle epithelium was shown to be a direct target of Wnt/β-catenin signaling (Fliniaux et al., 2008, Zhang et al., 2009). However, in the SMG, our data indicated that Wnt targeted *Eda* in the mesenchyme, suggesting changes in the regulatory regions during evolution to drive different expression patterns in different organs.

As *Shh* mediated most of the effects of *Eda* during branching, could Shh protein be used to treat patients with X-linked form of HED? I think the answer is no, for two reasons. First, Eda protein has been shown to cure the developmental defects of the *Eda*-deficiency efficiently when administered to pregnant mice (Gaide and Schneider, 2003). Therefore there is no reason to use any other molecule, even though Shh rescued the SMG branching similar to *Eda* in *Tabby* mice. Secondly, over-activated Hh signaling has been shown to correlate with poor prognosis in several forms of cancers (reviewed in Lauth and Toftgard, 2007). Thus, administration of Shh to treat *Eda*-deficiency in SMG development might lead to tumor development and progression. Therefore, our results cannot be used directly to design new treatment for X-linked form of HED, but instead, the finding that Shh mediates Eda signaling in SMG might help to understand the potential risks related to Eda treatment for HED patients: too high concentrations of *Eda* may reflect the *K14-Eda* phenotype and, according our results, lead to overgrowth of the SMG epithelial area and to over-activation of Hh signaling, possibly increasing the risk for tumor development. The main results of *Eda* and its integration to Wnt and Hh pathways are illustrated in the Fig 10.

![Fig 10. An overview of the integration of Eda signaling to other pathways during SMG morphogenesis.](image)

In conclusion, we showed that the Eda/Edar/NF-κB pathway is a significant regulator of SMG morphogenesis, and promotes branching and growth of the SMG epithelium through *Shh* and that *Eda* is induced at least partially by Wnts. These results suggest that reduced branching caused by loss of Eda signaling in *Tabby*, and probably in the HED patients, leads to impaired
salivary gland function. Our results uncovered two poorly studied signaling cascades in SMG development, Eda, and Hh, to be required for SMG branching similar to well-studied Fgf and Egf signaling cascades.

4.3. Dual role of Wnt pathway in the salivary gland development (I, unpublished)

Wnt signaling has a central role in the development of the tooth and hair (Mikkola et al., 2009), but before our report, nothing was known about the Wnt/β-catenin in the mammalian embryonic salivary gland development. First, we mapped Wnt activity in the developing SMG. Several Wnt ligands were expressed during SMG development: we detected Wnt4 and Wnt6 initially in the mesenchyme (E13), and a day later in the epithelium. In addition, Wnt11 was expressed in the mesenchyme at E13 and E14. Wnt activity (Bat-Gal, Axin2 LacZ/LacZ) was mesenchymal at early stages of branching (E12-E14.5), but became exclusively epithelial at later stages (E16 onwards).

To study the effect of the mesenchymal Wnt signaling, we used the DermoCre+; β-cat[−/−]mouse model with inhibited mesenchymal β-cat activity. Removal of Wnt activity in the mesenchyme led to reduced growth and branching of the epithelium, and was associated with reduced Eda levels. We observed that Wnt activity appeared to be absent or reduced in the mesenchyme near the clefts (Fig 11 A). When β-catenin activity was inhibited in the SMG mesenchyme in vivo (DermoCre+; β-cat[−/−]), the branching was reduced to less than 50% of the controls and inhibition of β-catenin activity in vitro with XAV939 or CKI7 led also to reduced branching. Patel et al. (2011) reported increased branching after inhibition of Wnt/β-cat in vitro. This result contrasts our observation. They used dkk1 protein for Wnt inhibition, whereas we used XAV939, CKI7 and in vivo deletion of mesenchymal Wnt activity. We did not observe any effect of Dkk protein, neither on Wnt activity (Axin2lacZ/LacZ) nor on branching, in a similar culture system as shown in Patel et al. (2011), probably due to low penetration of Dkk protein in the tissue (unpublished). Even though different in vitro methods appear to give different and contradictory results, the observed reduction in the SMG growth and branching in vivo after the inhibition of mesenchymal Wnt/β-cat signaling strongly suggests that Wnt signaling is required for branching.

We next analyzed the effect of Wnt/β-cat activation on SMG branching. GSK3β, a protein targeting β-catenin to degradation, was shown to be localized in the buds and in the mesenchyme adjacent to the clefts, but was reduced at the base of the clefts (Musselmann et al. 2011). We used BIO to inhibit GSK3β (5µM) for E13 SMG cultured for 20h. I observed a complete arrest of cleft formation and even the disappearance of the pre-existing clefts (Fig 11 B-E). The observed reduction of the number of clefts after Wnt/β-cat upregulation was also reported by Patel et al. (2011), although Musselmann et al (2011) observed increased number of clefts after 6hr of treatment with BIO, but reduced branching thereafter. Next we used an in vivo model to study the effect of Wnt activation in the mesenchyme. When Wnt/β-cat signaling was stabilized in the mesenchyme using the DermoCre;β-cat[+/+/ex3] mouse, unlike in the controls, SMG were neither developed by E12.5, nor developed in 2-day culture (n=6, Fig 11F). Our in vivo and in vitro results of Wnt activation, however, suggest that local regulation of Wnt activity must take place during SMG development.

Wnt signaling was recently reported to also regulate lumen formation in the ducts (Patel et al.,
In line with this observation, we also detected a localization of Wnt activity in the ducts from E15. Wnts might also have a potential impact on stem cell maintenance in the salivary glands, as transient activation of Wnt/β-catenin pathway prevented radiation damage and the Wnt/β-catenin pathway has also been implicated in postnatal normal development and in the regeneration of mouse salivary glands. These putative stem cells are thought to reside in the intercalated ducts where we also observed Wnt activity (Hai et al., 2010).

In conclusion from our data and other reports, Wnt/β-catenin signaling regulates the SMG development from the first cleft formation to postnatal stages and again in the regeneration. Our paper was the first one to report the involvement of Wnt/β-catenin in the embryonic SMG development. Several open questions remain. Especially, which are the most relevant ligands in each step of development and how do Wnts coordinate the cleft and the duct formation and branching? Further studies are required to answer these questions.

**Fig 11. Activation of Wnt pathway in the SMG.**
(A) Wnt/β-catenin activity is localized to the mesenchyme but is absent in the cell layer closest to the clefting epithelium and the site of cleft formation (arrowed). (B-E) When Wnt/β-catenin pathway was activated in vitro with GSK-3 inhibitor BIO, branching was prevented and the pre-existing clefts were de-stabilized. In Axin2/ConductinLacZ/LacZ (Axin2LacZ/LacZ) mice. When E13 WT salivary glands were cultured in the presence of 5µM BIO (GSK-3 inhibitor) (C, D-E), the pre-existing clefts disappeared and the number of end buds were reduced to 64% from the start of the culture. In control with no BIO (B), the number of end buds increased 2.7-fold, and cleft formation was normal. D: mean number of end buds in control and BIO-treated salivary glands at 2hr and 20hr culture. E: fold increase in end buds (no. of end buds at 20hr/2hr), +SE of mean. (F) When β-catenin was stabilized in the mesenchyme (DermoCre+; β-catenin +/+ex3fl), no SMG were detected at E12.5 or after 2d culture, whereas in the control, normal SMG was present at E12.5, which branched extensively after two days in culture. (G) Suggested dual role of Wnt signaling in the SMG branching: mesenchymal Wnt activity is required for branching but is needed to be downregulated at the immediate vicinity of the clefts. **P< 0.01, ***P< 0.001.
4.4. Fgf20 is part of Fgf signaling loop and regulates several aspects of tooth development downstream of the Eda signaling (II)

The mechanisms whereby Eda regulates tooth size, shape and number can be understood by identifying signaling pathways that function downstream of Eda. Microarray analysis revealed that Fgf20 is one of the most rapidly induced genes by Eda in hair placodes and thus is a putative transcriptional target of Eda (Fliniaux et al., 2008; Lefèvre et al., 2012). To test whether Eda regulates Fgf20 in developing teeth, we treated E13 Tabby molar buds with recombinant Eda protein (250ng/ml) for 4 hours and analyzed the Fgf20 expression level by qRT-PCR. We observed that Eda protein rapidly induced Fgf20 expression and the steady state levels of Fgf20 correlated with Eda level in vivo: in Tabby, Fgf20 expression was reduced and in K14-Eda it was increased. This was observed by whole-mount in situ hybridization with Fgf20 probe (E12, E13 and E14), and by crossing Tabby, WT and K14-Eda mice with Fgf20+/− βGal (Fgf20-β-galactosidase (βGal) knock-in allele; Huh et al., 2012) reporter (E14, E15 and E16). Fgf20 co-localized with Edar in the molar epithelium: in the placode, the bud and in the enamel knots. This pattern of Fgf20 expression in the molar epithelium was also reported by Kettunen et al (2011) and Porntaveetus et al (2011). Fgf20 deletion in the mouse led to smaller teeth (reduced crown area) with subtly reduced anteroconids and in culture the talonids, resembling the Tabby tooth phenotype. In Fgf20−/−, the anteroconid cusps were narrower, shaping the anterior part of the tooth crown more compact and smaller, similar to Tabby (Grüneberg, 1966, Pispa et al., 1999, Cristenova-Kermankova et al., 2002). The abovementioned results suggest that Fgf20 expression is regulated by Eda signaling.

Until now, the downstream targets of Eda signaling were not known in tooth. Here we showed that in tooth development, Fgf20 lies downstream of Eda signaling and mediates many functions of Eda (II). In hair development, Wnt10a, Wnt10b, Dkk4, Shh, follistatin and chemokines were suggested to mediate the effects of Eda (Pummalila et al., 2007, Fliniaux et al., 2008, Zhang et al., 2009, Lefèvre et al., 2012). Out of those, Wnt10a and Wnt10b are also potential targets in the mammary glands, beside Pthrp (Voutilainen et al., 2012). Wnt10a and Wnt10b co-localize with Edar in the tooth placode and in the enamel knots (bite-it.helsinki.fi) and could mediate the effects of Eda also in the tooth. Shh turned out to be the most crucial downstream factor in the SMG morphogenesis, as discussed above, whereas Fgf20 expression was not detected in the salivary gland (data not shown). Thus it seems that there are both common and organ-specific targets for Eda signaling. It is likely then, taking into account the number of pathway suggested to be regulated by Eda signaling, that the Eda/Edar/NF-κB system acts as balancer between the pathways. Such a balancing role has been partially found in hair development, in which Eda pathway induces both Wnt ligands, the pathway activators, and Dkk, the inhibitors (Zhang et al., 2009, Lefèvre et al., 2012). How organ-specific regulation of Eda targets is achieved remains to be uncovered. Eda, however, is not the only pathway regulating Fgf20 expression: Wnt/β-catenin signaling also induces Fgf20 (Chamorro et al., 2005). This indicates that Fgf20 has Eda-independent functions as well; most likely also vice versa, Eda signaling has Fgf20-independent functions in tooth. The latter is supported by the Fgf20 phenotype being ‘mild-Tabby’, lacking some features of the Tabby phenotype which are probably induced by other target genes of Eda.

Another reason for the mild phenotype in the Fgf20−/− may be the redundancy of Fgf20 with other Fgfs. Epithelially expressed Fgfs (Fgf3, Fgf4, Fgf9, Fgf15 and Fgf20) and mesenchymally expressed Fgfs (Fgf3 and Fgf10) form a loop during molar development in which epithelial Fgfs induce the expression of mesenchymal Fgfs which act on the epithelium and regulate the growth of both tissue compartments (Jernvall et al., 1994, Kettunen and Thesleff, 1998, Kettunen et al., 1998, Kettunen et al., 2000., Åberg et al., 2004, Porntaveetus et al., 2011, Kettunen et al., 2011; Fig 12). We showed that Fgf20 functions at least partially redundantly with Fgf4 and Fgf9, as it
was capable of inducing mesenchymal proliferation and the expression of Fgf3 and Runx2 in the mesenchyme, as previously shown for Fgf4/Fgf9 (Jernvall et al., 1994, Kettunen et al., 1998, Åberg et al., 2004). Also, combined deletion of Fgf9 and Fgf20 (Fgf9-/-;Fgf20-/-) caused additive reduction in the enamel knot size (Shh expression) in vivo, further suggesting a redundancy between these Fgfs. In Tabby, the enamel knot is also reduced (Pispa et al, 1999), which supports the idea that Eda signaling can regulate enamel knot dynamics through Fgf20. Fgf20 also induced the expression of the negative regulators of Fgf signaling, Spry2 and Spry4. This implies that Fgf20 can balance the Fgf signaling loop between the mesenchyme and epithelium and coordinate the controlled growth in both compartments. The importance of the Fgf signaling loop was shown by deleting epithelial Fgf-receptor (Fgfr2b-/-) when molars were arrested at the bud stage (De Moerlooze et al., 2000). Partial reduction of epithelial Fgf signaling by reducing the dose of its ligands Fgf3 and Fgf10 (Fgf3-/-;Fgf10+/+) led to smaller teeth (Wang et al., 2007). Our observation with similar phenotype in Fgf20-/- suggests that reducing either epithelial or mesenchymal Fgf signaling can lead to similar retardation in growth, underlining the idea that Fgfs act in concert to regulate the growth of the molars. The contribution of Eda to this Fgf loop was a novel finding, although Fgf10 was earlier shown to rescue the Tabby phenotype partially (Pispa et al., 1999).

The only difference in the cusp pattern between Fgf20-/- and WT mice was the reduction in the anteroconids. To our knowledge, Fgf20-/- mice are the only mouse model published so far with a specific alteration in one cusp pair only. The subtle defect in the anteroconid patterning is interesting since the developmental origin of these cusps is debated. Prochazka et al. (2010) suggested that anteroconids are developed from the rudimentary bud observed at E13-E14 anterior to the m1 enamel knot. During the development, according to them, the rudimentary bud is fused with the anterior part of the m1-forming tooth space. The same rudiment is observed in the mouse mutants in which the extra molar is developed (suggested to be the ancestral premolar, also called P4 or dP4). Some mutants, however, such as Spry2-/- and Spry4-/- develop both normal or near-normal anteroconids and the extra molar (Klein et al., 2006), suggesting that the rudimentary bud is not the only source for the anteroconids. Despite the contradictory evidence, it is rather likely that when taking into account the close proximity of the rudimentary bud signaling (Shh expression) and the m1 anterior end during development, that the rudimentary bud somehow affects the development of the anteroconids. The effect could be via paracrine signaling, through contribution of the rudiment cells to the anteroconids, or both. Our data also support the interaction between the rudimentary bud and the anteroconids. The anterior end Shh expression, also referred to as rudimentary bud signaling, was stabilized in the Fgf20+/+ and Fgf20-/- jaws independent of the levels of Eda signaling (Fig 13). This part was also retained in the surface of the oral epithelium anterior to m1, and either disappeared later through apoptosis or fused with m1, or both (II). If part of the cells normally contributing to the anteroconids were lost through apoptosis, the number of cells forming anteroconids would be lower, causing smaller anteroconids. This observation during development, the stabilization of the rudimentary bud signaling, would support the rudimentary bud hypothesis suggested by Prochazka et al. (2010) and would explain the observed diminishing of the anteroconids.

In conclusion, Fgf20 was found to be a link between Eda signaling and the Fgf signaling loop in the molar epithelial-mesenchymal interaction and we showed that Eda balances this complex loop via Fgf20 (Fig 12). The epitheliaally-derived Fgfs function in concert and partially redundantly, to trigger mesenchymal Fgfs and cell proliferation, and to activate the negative regulators, as Fgf20 induced also the expression of Spry2 and Spry4. The mesenchymally-derived Fgfs induce epithelial cell proliferation under the control of Sprouty proteins. The signaling across tissue layers coordinates the controlled growth of the compartments, allowing isometric growth of the tooth.
4.5. *Fgf20* as an activator in the inhibitory cascade regulating the size of the posterior molars and crown complexity (II)

It has been suggested that activator-inhibitor dynamics determine the position of the cusps and tooth size within a tooth row. Increasing activation causes equal molar size within a tooth row and a more complex cusp pattern (Salazar-Ciudad & Jernvall, 2002, Kavanagh et al., 2007). As the *Fgf20*-/− phenotype was subtle, and because *Eda* has been suggested to take part in activator-inhibitor dynamics, we generated compound *K14-Eda;Fgf20*+/− mutants to assess the role of *Fgf20* in the phenotype of *K14-Eda* mice. To analyze whether *Fgf20* plays a role in the activator-inhibitor balance, we first measured the crown areas of all molars (m1, m2, m3) in the *K14-Eda*, *K14-Eda;Fgf20*+/− and *K14-Eda;Fgf20*-/− mice as an indication of molar size. We observed an overall reduction in the molar size of all molars after *Fgf20* deletion, as expected from the phenotype of the single mutant (*Fgf20*-/−). More importantly, a striking feature of the *K14-Eda;Fgf20*-/− mouse was the reduction of cusps and the relative reduction in size towards the posterior molars within a tooth row. When the relative size of molars within a tooth row was measured and m3/m1 and m2/m1 ratios were compared between *K14-Eda* and *K14-Eda;Fgf20*-/−, a significant reduction in both parameters was observed in *K14-Eda;Fgf20*-/− (Fig 13). The reduction in size towards posterior molars was evident also when absolute molar size was compared between the genotypes. The third molar was most strongly affected: it was either highly reduced or was lacking completely.

According to the inhibitory cascade hypothesis, molar size within a tooth row is determined by the strength of the inhibition from the anterior molar (Kavanagh et al., 2007). The stronger the inhibition, the later the molars are initiated and the smaller they grow. The molecular factors in the inhibitory cascade are, however, poorly known. So far, Activin A and Bmp4 have been shown to accelerate the development of m2 in tooth explant culture, but the contribution of these factors to the inhibitory cascade *in vivo* have not been reported (Salazar-Ciudad and Jernvall, 2002, Kavanagh et al., 2007, Harjunmaa et al., 2012). In fact, *in vivo* data in which the manipulation of the activity of one gene causes alteration in the relative size of the molars within a tooth row, as predicted by the inhibitory cascade model is, to my knowledge, lacking before our report. Our data, with the linear reduction in the posterior molars, suggests that *Fgf20* acts as an
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In line with the idea that Fgf20 acts as an activator, we observed that the removal of Fgf20 signaling in the K14-Eda mice led to a reduced number of cusps, in addition to the decrease in tooth size toward posterior molars (Fig 13, II). Partial removal of Fgf20 (K14-Eda;Fgf20+/-) appeared to inhibit extra cusp formation observed in K14-Eda (Kangas et al., 2004), normalizing the phenotype. The decreased cusp number indicates lower crown complexity. The Meinhardt & Gierer model (Fig 6) has been adapted and modified to explain the action of the secondary enamel knots regulating crown shape (Salazar-Ciudad and Jernvall, 2010), and the balance between the activators and inhibitors that regulate the spacing of the cusps. Specifically, the activators induce the differentiation of the cells and inhibit proliferation. The molecules considered as activators include bone morphogenetic proteins (Bmps), and Wnts, inducing cell differentiation in the enamel knot. Putative inhibitors include fibroblast growth factors (Fgfs), Sostdc1 and Shh, all of which stimulate growth and survival of dental epithelia, mesenchyme, or both (Salazar-Ciudad and Jernvall, 2002, Cho et al. 2011, Salazar-Ciudad and Jernvall, 2010). Activin A treatment, at least in the molar explant culture, increase the cusp number and act as an activator also in the cusp formation (Harjunmaa et al., 2012). Activin A and Fgf20 are currently the only genes shown to mediate activation in both the cusp spacing and in the inhibitory cascade, but Fgf20 is the only activator shown in vivo to affect both inhibitory cascade and cusp spacing. However, whether these two, Activin A and Fgf20, act similarly as the activators proposed by the Meinhardt & Gierer’s model, inducing their own expression, the expression of the inhibitor, and diffusing slower than the inhibitors, remains to be tested. The role of Fgf20

Fig 13. Fgf20 in the inhibitory cascade model. (A) Experimental data, (B) model prediction of the inhibitory cascade model and the ecological correlation (modified from Kavannagh et al., 2007): increased inhibition cause less equal sized molar and reduced crown complexity.
as an activator is surprising, as the activators are thought to inhibit proliferation whereas Fgf20 induces proliferation, at least in the mesenchyme in vitro. However, we observed that Fgf20 protein induced Spry2 and Spry4 expression in vitro, in isolated epithelium and mesenchyme, respectively. As Fgf20 appears to induce both the other Fgfs, but also the inhibitors, Spry2 and Spry4, the outcome of the Fgf20-deletion reflects these secondary impacts as well. Thus the increased inhibition caused by the removal of Fgf20 is the sum of all downstream effects after Fgf20 removal and may be caused by the destabilization of the Fgf signaling loop between the epithelium and the mesenchyme (Fig 13).

4.6. Ancient premolar can be recovered by stabilizing its embryonic rudiment (II)

K14-Eda mice develop an extra molar in the place of ancient premolar in 50% of the jaw quadrants, and many other mouse mutants also develop a similar anterior extra molar (Kangas et al., 2004; see Table1). To address whether removal of Fgf20 in K14-Eda affects the extra molar development, we systematically analyzed the frequency of EM occurrence in K14-Eda, K14-Eda:Fgf20+/- and K14-Eda:Fgf20-/- mandible jaws. We observed that absence of Fgf20 (K14-Eda:Fgf20-/-) or partial reduction of Fgf20 signaling (K14-Eda:Fgf20+/-) in K14-Eda mice lead to increased frequency of the extra molar in the place of ancestral premolars (Fig 14). To study the embryonic mechanism for this, we analyzed Shh expression in the E13 and E14 lower jaws, as anterior Shh expression has been associated with EM formation (Kangas et al., 2004). We showed that Fgf20 partial or complete deletion was sufficient to stabilize the anterior end Shh expression: the increased frequency of anterior end (AE) Shh expression was observed in Fgf20+/- and Fgf20-/- mice at E13 and E14 compared to WT (Fig 14). This suggests that the observed increase in EM frequency in K14-Eda:Fgf20+/- and K14-Eda:Fgf20-/- compared to K14-Eda was due to the additive effect of high Eda and low Fgf20 activities (Fig 14). Loss of Fgf20 activity, however, was not sufficient to stabilize EM, since when we analyzed LacZ stained Fgf20+/- and Fgf20-/- E15.5 jaws, we observed that the anterior end did not grow down at E15.5 to the mesenchyme to form a complete tooth. The molecular basis for the stabilization of the AE Shh expression may come from the observation that the Fgf20 protein induced Spry2 and Spry4 expression and more importantly both Spry genes were downregulated in the AE of K14-Eda:Fgf20-/- compared to K14-Eda. The loss of Spry2 or Spry4 in mouse induces AE Shh signaling and EM formation (Klein et al., 2006). As we showed that Fgf20 can, at least in vitro, induce both Fgf ligands and Fgf inhibitors, the net effect remained unclear. For this, we analyzed Etv5 expression in the AE of E14.5 molars in K14-Eda and in K14-Eda:Fgf20-/- mice. Etv5 is a direct target of Fgf signaling and was used to report Fgf signaling activity (Roehl et al., 2001). We detected high Etv5 expression in the epithelium but reduced or lost expression in the mesenchyme in K14-Eda:Fgf20-/- compared to K14-Eda. This suggests an imbalance in Fgf signaling between epithelium and mesenchyme.

So, how is EM development regulated? As tinkering with Hh, BMP, Wnt, Eda or Fgf signaling can induce EM formation, it is likely that the premolar rudiment is a rather unstable structure and even a slight modification in signaling can stabilize the EM formation from the rudiment. Not all individuals of the abovementioned mouse models have EM: for example, only 50% of K14-Eda jaw quadrants develop EM, even though AE Shh expression is detected in a higher frequency at E13-E14 (Kangas et al., 2004). Moreover, one half of the mandible can have EM while the other does not. The variation amongst the mouse lines and even within the individuals highly supports the idea of the unstable nature of the EM development. The fate of the EM
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appears stochastic to some extent. However, a clear correlation exists between the presence of the embryonic Shh expression (AE signaling, rudimentary bud signaling) in the AE of the molar forming region and the presence/frequency of the EM (Kangas et al., 2004, Kassai et al., 2005, Klein et al., 2006; Fig 14). We showed that the stabilization of the AE Shh expression is not sufficient to develop the EM: The deletion of Fgf20 expression led to the stabilization of AE Shh expression, but did not lead to EM formation in any of the jaw quadrants studied (n=18). The downgrowth of the AE followed by the growth and separation of the developing EM and m1 was supported by high Eda levels and was required for EM formation. We also showed that the effects of Fgf20 deletion and Eda upregulation are additive, suggesting partially parallel functions for these pathways (Fig 14). The decision to form an EM appears to require two steps: 1) the stabilization of AE Shh expression, and 2) the downgrowth and separation of the EM. In WT mice, the AE expresses Shh at E13, but usually at E14, the expression is lost, probably through apoptosis (Viriot et al., 2002). The survival of AE Shh signaling might be influenced by the relative size of the AE vs. m1 signaling: at least in the K14-Eda:Fgf20/- mice, the relative Shh expression in AE/m1, quantified from images, was on average higher compared to K14-Eda (unpublished).
I observed that another possible checkpoint in the EM development, the downturn of the developing EM, is timed at around E16 in development. At this stage, some of the K14-Eda;Fgf20 +/- jaws did not have a developing EM but instead, had an anterior extension of the molar similar to Fgf20-/- jaws (unpublished observation). Since Fgf20 activity appears to inhibit the formation of the extra molar, other Eda targets should stimulate it. The suggested Eda-induced genes include modulators of Bmp, Wnt, and Hh pathways (Mikkola, 2009; Lefebvre et al., 2012) all of which have been linked to EM stabilization (Table 1 and Tummers and Thesleff, 2009). It is likely that the balance between the different Eda targets, those that promote and those that inhibit extra molar formation, ultimately determine the frequency of its appearance.

Since multiple pathways can trigger EM, it is also possible that a mutation in a single signaling cascade was not responsible for the premolar loss during rodent evolution. However, our data suggest that Fgf20 activity might be a factor to keep the diastema toothless at least in the place of the premolars, by preventing the AE Shh signaling center. A comparative gene expression analysis for Fgf20 in the species in which the premolar is retained might give support for this hypothesis. Another interesting point from the evolutionary aspect is the high similarity between K14-Eda;Fgf20-/- mice and the faunivorous rodents and the fact that tinkering with the Fgf20 activity can shift the tooth pattern from omnivorous to faunivorous rodent species (Fig 13 and Kavanagh et al., 2007). This shift is reflected in both the cusp complexity and in the relative size of the molars. Now, as the adaptive evolution of gene expression through cis-regulation has been widely documented (Fraser et al., 2011), my observations evoke an interesting scenario that Fgf20 and Eda activities might be under selective pressure when dietary changes have occurred during rodent evolution. A comparative study of gene activities and gene regulation in tooth in different dietary groups might give light to this issue.
5. Concluding remarks

I have analyzed here the development of two ectodermal organs and the role of Eda signaling in their organogenesis. Particularly, I focused on the downstream signaling of the Eda pathway and found that Shh is among the most critical gene triggered by Eda/Edar/NF-κB pathway in the submandibular salivary gland development, and that Fgf20 is an important downstream mediator of the Eda pathway in the molar tooth development. Fgf20 was not expressed in the salivary gland, suggesting, at least partially, organ-specific downstream genes for Eda signaling. I analyzed the effect of these factors quantitatively and systematically for several phenotypic characteristics, and therefore could assess the importance of these downstream genes of Eda signaling for the development of the two organs, the molar tooth and the submandibular salivary gland.

Based on my results I suggest that the pathogenic mechanism causing reduced saliva flow in X-linked HED patients may be the reduced Shh signaling resulting in impaired branching morphogenesis of the salivary glands, and leading to smaller area of the saliva-secreting epithelium. I also found Wnt/β-cat signaling to be a novel regulator of the salivary gland morphogenesis and showed that it triggers the Eda pathway. Eda, Wnt and Shh pathways are poorly characterized in salivary gland morphogenesis and our studies shed light on their functions. The correct level of signaling seems to be critical for at least Wnts since both increased and reduced activity led to impaired branching. My data suggests that Wnts act on two important phases of SMG morphogenesis: in the stabilization of the clefts and in the promotion of epithelial growth and further branching.

I characterized a novel factor in tooth development, Fgf20, and showed that it is controlled by Eda and takes part in tooth growth and shaping, but also regulates tooth number. The role of Fgf20 in the Fgf signaling loop regulating molar development is to stabilize the loop by inducing proliferation and expression of mesenchymal Fgf ligands and Fgf inhibitors. I showed that Fgf20 functions as an activator and, for the first time, I characterized the activator in the activator-inhibitor balance in vivo. Interestingly, shifting Fgf20 levels from normal to null, the dentition changed from the omnivorous-type to resembling the faunivorous-type. These results provoke an interesting hypothesis that balance of Eda and Fgf20 might have been under selective pressures when adaptations to new food sources occurred during evolution.

Since the discovery of inductive signaling in embryogenesis and organogenesis by Hans Spemann and others, the research has focused on identifying these signals. Now, as hundreds of these genes and proteins in development have been uncovered, and with the genetically modified mouse models available and the functions of individual genes characterized, the next step is to understand the in vivo integration of these signals. These integrated networks ultimately trigger cell specification and organization into differentiated tissues and organs. As progresses in dedifferentiation of the cells into pluripotent stem cells have been made in recent years giving grounds to the regenerative medicine (reviewed in Gurdon and Melton 2008, Yamanaka and Blau 2010), next step would be to understand how these pluripotent cells can be differentiated back into specific cell types by the same mechanisms that occur during development, involving integrated signaling networks. Linking signaling networks, cellular parameters and physical forces into one model explaining the variation in morphogenesis and differentiation would give a comprehensive understanding of developmental phenomena behind genetic diseases and morphological evolution.
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