Ectodermal organ development: Regulation by Notch and Eda pathways

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We act as though comfort and luxury were the chief requirements of life, when all we need to make us happy is something to be enthousiastic about.

-Charles Kingsley

CONTENTS

ABBREVIATIONS

AER = apical ectodermal ridge
Bmp = Bone morphogenetic protein
BSA = Bovine serum albumin
BHLH = Basic helix-loop-helix motif
Ca = Calcium
Catnb = Catenin beta, b-catenin
Cdc10 = cell division cycle 10
DD = death domain
Dl = downless
E = embryonic day
Eda = Ectodysplasin
Edar = Ectodysplasin receptor
Edaradd = Edar (Ectodysplasin-A receptor) associated death domain
Erkl = Mapk3 = mitogen activated protein kinase 3
Erm = Etv5 = ets variant gene 5
Ets1 = E26 Avian leukemia associated oncogene 1
Fak = Focal adhesion kinase, also called Ptk2 protein tyrosine kinase 2
Fgf = Fibroblast growth factor
Fgfr = Fibroblast growth factor receptor
Frs2 = Fgf receptor substrate 2
HED = Human ectodermal dysplasia
Hh = hedgehog
Il17rd = Interleukin 17 receptor D
IkB = Inhibitor of Nfkb1
Ikk = IkB kinase
Ins 1,4,5 P3 = Inositol 1,4,5, trisphosphate
Jnk = Jun-aminoterminal kinase, current form: Mapk8, mitogen activated protein kinase 8
K14 = keratin 14
Lfng = Lunatic fringe
Nfkb1 = Nuclear factor kappa B (former symbol NF-kB)
Mapk = Mitogen activated protein kinase
Mfng = Manic fringe
NEMO synonym Ikbkg = Inhibitor of kappaB kinase gamma
Nik = Nfkb1-inducing kinase, current form Map3k14 = mitogen activated protein kinase kinase kinase 14
Nlk = Nemo like kinase
Nog1 = Noggin
OMIM = On-line Mendelian Inheritance in Man

OPA = Optic atrophy 1 homolog
Ptc1 = Patched-1
Pea1 = Pearly 1
PEST = Amino acid sequence rich in proline (P), glutamic acid (E), serine(S) and threonine (T)
Plcg1 former PLCg1 = Phospholipase C, gamma 1
Rfng = Radical fringe
Rbpjk = Recombination binding protein Jkappa, synonym Rbpsuh Recombining binding protein suppressor of hairless (Drosophila)
Src = Rous sarcoma oncogene
SH2 = Src homology region 2
SMG = Submandibular salivary gland
Su(H) = Suppressor of hairless
Tak1 current symbol Map3k7 = mitogen activate protein kinase kinase 7
Tnf = Tumor necrosis factor
Wnt = Wingless related MMTV integration site
Xflrt3 = Xenopus Leucine rich repeat transmembrane protein 3

Mouse gene symbols were written according to the current nomenclature of Jackson laboratories (http://www.informatics.jax.org/mgihome/nomen/). Upper case letters in gene names refer to Humans. Lower case letters refer to other animals. Italics are used for genes. Non italics are used for proteins. In this thesis, unless specifically stated otherwise, I shall refer to the mouse as a model for discussion.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals. Some unpublished data are also presented.

I **Harada, H., Kettunen, P., Jung, H.S., Mustonen, T., Wang, Y., A., Thesleff, I.** (1999). Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. J. Cell Biol. 147:105-120.

II **Mustonen, T., Tummers, M., Mikami, T., Itoh, N., Zhang, N., Gridley, T., Thesleff, I.** (2002). Lunatic Fringe, FGF, and BMP regulate the Notch pathway during epithelial morphogenesis of teeth. Dev. Biol. 248:281-293.

III **Mustonen, T., Pispa, J., Mikkola, M. L., Pummila, M., Kangas, A.T., Pakkasjärvi, L., Jaatinen, R., Thesleff, I.** (2003) Ectodysplasin stimulates ectodermal organ development. Dev. Biol. 259:123-136.

IV **Mustonen, T.*, Ilmonen, M.*, Pummila, M., Kangas, A., Jaatinen, R., Laurikkala, J., Pispa, J., Gaide, O., Schneider, P., Thesleff, I., Mikkola, M.** (2004) Ectodysplasin-A1 increases the size of ectodermal placodes by promoting placodal cell fate. Development, in press.

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ABSTRACT

To cope with the harsh environmental conditions wild animals need their ectodermal organs such as teeth and hair. In addition to wild animals, ectodermal organs are important in the wellbeing of humans and domesticated animals. Ectodermal organ development is regulated by molecular conversations between the ectoderm and the underlying mesenchyme, and also between the different cell groups, or compartments within the tissues. Signalling molecules regulate the cell fate, proliferation and differentiation, so that the development proceeds in an organised manner. Components of the molecular mechanisms in the developmental processes are strikingly similar in different organs, so what we learn about the molecules involved in the development of one ectodermal organ can often help us to reveal more general principles.

Notch pathway molecules are important in cell fate determination in the development of vertebrates and invertebrates. Notch-molecules and their ligands are cell membrane proteins, thus signalling between neighbouring cells. Protein cleavages and glycosylation events delicately regulate their interactions. In this work the expression of genes encoding Notch pathway molecules, and in particular, the Notch signalling modulator Lunatic Fringe, were studied in the bud-to-cap stage transition of developing molar and in the continuously growing postnatal mouse incisors. *Lunatic fringe* was expressed in a dynamic way in the forming cervical loops of the early molars. In culture experiments its expression was found to be dependent on mesenchymal signals, which could be mimicked by Fgfs. This positive regulation by Fgfs could be inhibited by Bmp4. Fgf10 was found to induce and maintain *Lunatic fringe* expression also in postnatal incisors in culture, and the expression patterns and results from tissue culture experiments led to a model of functional stem cell compartment in continuously growing incisors. Ectodysplasin, a Tnf family member, plays a key role in the development of ectodermal organs and its mutations in mice and men cause ectodermal defects. Typically, mutations in the genes encoding mouse Ectodysplasin signalling pathway proteins cause loss of the first hair placodes and thus the guard hairs, lack of third molars, changes in molar cusp number, reduction of molar size, defects in ectodermal glands, and kinked tails. Role of Ectodysplasin was further studied by overexpressing *Ectodysplasin* in the developing ectoderm of transgenic mice. Ectodysplasin overexpression under *Keratin-14* (K14) promoter resulted in stimulated development of ectodermal organs. The mice had supernumerary nipples, long nails, increased function of sweat glands, larger and abnormally oriented hair follicles, and extra teeth. The mice lacked the normal four distinct hair types, and instead, they had intermediary type of hairs. The dental enamel was defective or absent, causing loss of lower incisors due to wearing out. Therefore, the upper incisors lacked their usual opposite hard surface and grew abnormally long. Expression of Ectodysplasin receptor gene, *Edar,* was restricted to its normal placodal sites also in transgenic ectoderm, which suggests the extra organs formed because of increased signalling activity of placodal Edar. The early hair placodes were larger than normal, their morphological shapes were irregular, and they fused. The three separate waves of hair follicle development were lost and the hair follicles formed continuously. Extra placodes developed from the dental lamina and mammary line. Proliferation analysis of the wild type and K14-*Eda-A1* hair placodes showed that Eda-A1 promotes placodal cell fates without affecting proliferation rates of the cells. In conclusion, Notch and Eda signalling pathways play central roles in regulating the cell fate and processes of ectodermal organ development.

1.REVIEW OF THE LITERATURE

1.1. Ectodermal organ development

1.1.1. Origin of Ectoderm

In the beginning of its development, a multicellular animal such as a human or a mouse, is nothing but one cell. The fertilised oocyte is a totipotent cell containing all the information and capacity to develop to an animal capable of surviving in harsh environmental conditions and capable of producing offspring. Mouse fertilised egg develops as it travels along the oviduct towards the uterus. It divides to form a multicel-

lular morula, which develops further by means of cellular arrangements to a hollow ball of cells, the blastocyst. Blastocyst consists of the inner cell mass or epiblast, which later gives rise to the embryo proper, a fluid filled cavity i.e. the blastocoel, and outer cell layers i.e. trophectoderm. Trophectoderm gives rise to the chorion, one of the two important embryonic membranes. Amnion, the other membrane, is formed from the cells of the inner cell mass contacting the blastocoel, which is the primitive endoderm. The blastocyst hatches from the protective membrane zona pellucida and implants into the uterine wall. Following stage called gastrulation is characterised by regulated migration and proliferation of cells and turning of the cell groups so that they form three separable tissue layers, outermost of which is the ectoderm. Innermost tissue layer is the endoderm, later giving rise to e.g. inner lining of the digestive system of the animal and participating in the formation of many inner organs such as liver, lungs and pancreas. The middle layer between the endoderm and the ectoderm is mesoderm, giving rise to e.g. muscle tissue and most of the bones, and participating in the formation of several organs involving ectodermal part and in other cases endodermal part.

1.1.2. Common mechanisms of ectodermal organ development

Organs developing as ectodermal appendages include hair, feathers, scales, nails, teeth, and many exocrine glands such as sweat glands and mammary glands. Functions of ectodermal organs are as diverse as their appearance, and lead to multiform adaptations to environmental conditions. Some functions associate to protection against harsh environmental conditions, such as cold, wind, humidity, heat (hairs, feathers, sweat glands), hostile animals of the same or different species (teeth, claws, camouflage colours of the fur or feathers, specific glands of a skunk), and hard ground under the feet (hoofs of horses). Other functions are associated to feeding offspring (mammary glands) and mastication: breaking hard peels of a nut or fruit, catching prey and tearing animal meet (teeth, claws). However, lots of functions of ectodermal organs are social and/or associated to reproduction. To mention a few of them: apocrine glands secrete pheromones, colourful feathers or fur can ensure the successful breeding, and among humans a smile with healthy white teeth can be a sign of either good genes or a wealthy social status.

Organogenesis is typically regulated by series of interactions between morphologically distinct tissues, in the case of epithelial appendages those two are the ectoderm and the underlying mesoderm. One or two cell layer ectoderm (germinal ectoderm) is multipotent and its development depends on the signals from the underlying tissue, mesenchyme containing only mesodermal cells or containing also neural crest derived cells. The first molecular signal initiating ectodermal organ formation is believed to originate from the mesenchyme. Yet, the first morphological indication of ectodermal organ development is the formation of an epithelial thickening, a placode, which requires an inductive signal from the mesenchyme. Placode starts to express many signal molecules regulating condensation of the underlying mesenchyme and inhibiting the surrounding epithelium from gaining a similar fate. Reciprocally the condensed mesenchyme also expresses several signalling molecules. The placode grows into the underlying mesenchyme and in case of epithelial appendages, forms an epithelial bud. Other epithelial placodes such as olfactory, lens and otic placodes develop into a vesicle instead of an elongating bud. The further development of an epithelial appendage bud is already morphologically tissue specific. In the glands the epithelial bud elongates and forms branches, whereas in hair and tooth the tip of the bud stops growing and the edges grow downward to surround the mesenchymal domain called dermal or dental papilla, respectively.

1.1.3. Introduction to selected molecules with a central role in ectodermal organ development

1.1.3.1. Fgf, Bmp, Shh, and Wnt pathways

Communication between adjacent tissues is a fundamental phenomenon regulating the initiation and growth of most organs. During the development of ectodermal organs the interactions between epithelium and mesenchyme occur

largely by secreted signalling molecules that bind to their receptors in the adjacent tissue. Molecular cascades transduce the signals through the cell membranes and cytoplasm into the nuclei. Changes in the protein phosphorylation, protein-protein interactions, protein degradation and other types of signal transduction finally lead to changes in the transcriptional machinery in the nuclei of the receiving tissue cells. Transcriptional changes sometimes cause specific responses making the signalling reciprocal. Signalling molecules have different ways to achieve their target receptors. First, some signalling molecules are soluble and secreted to the extracellular space. Second, some of those secreted molecules bind to binding and transporting proteins in the intercellular space for their proper distribution. Third, some signalling molecules remain bound to cell membrane and thus signal between the neighbouring cells or to a far-away cell by means of the long cellular extensions, and thus, the signal must be spread from cell to cell. In many cases signalling activity requires specific cleavages of the proteins, and often ligand binding requires or is intensified by accessory molecules. In the mouse and human embryo, specification of tissue lineages requires cell-cell interactions that are influenced by cellular neighborhood and coordinated cell migration. The key signalling pathways like those mediated by Fgf, Tgfβ, Shh, and Wnt largely convey those interactions.

Fibroblast growth factor molecules, Fgfs, are a large family of secreted polypeptide growth factors and an important group of the signalling molecules involved in the conversation between epithelium and mesenchyme during the development of ectodermal organs (Kettunen et al. 1998). In mouse there are 22 Fgf encoding genes and four Fgf receptor tyrosine kinase receptor (Fgfr) encoding genes identified so far (Ornitz and Itoh, 2001). Location and timing of expression of *Fgf* genes vary remarkably, and different forms of Fgf proteins result from alternative transcription initiation and alternative mRNA splicing. Fgfs are small peptides of 155 to 268 amino acids that have high affinity to heparan sulfates that are essential for Fgf receptor binding and activation. Fgfs can be classified into subgroups, members of which share sequence similarity and biochemical and developmental functional properties (Ornitz and Itoh, 2001). Most Fgfs have aminoterminal signal peptide and are secreted from cells, some lack signal peptide but are still secreted. Some, namely Fgf1 and Fgf2, are not secreted except in case of a cellular damage, while Fgf11-Fgf14 mainly are not secreted and remain intracellular. Some Fgfs are known to contain nuclear localization signal, but the biological function of nuclear Fgfs is not fully understood. Nuclear localization of receptor bound Fgfs have been reported to possibly regulate the mitogenic response (Klint and Claesson-Welsh, 1999). Fgfs bind to single transmembrane receptors composed of three extracellular immunoglobulin domains and an intracellular tyrosine kinase domain. Ligand binding causes dimerisation of the receptors and phosphorylation of the monomers, probably *in trans* (Klint and Claesson-Welsh, 1999). Four Fgf receptor genes, Fgfr1, Fgfr2, Fgfr3, and Fgfr4 are known today. Alternative splicing of Fgf receptors specifies the ligand-binding properties. Exon IIIb of Fgfr2 gene is specifically expressed in epithelial lineages, and exon IIIc in mesenchymal lineages (Ornitz and Itoh, 2001). Epithelially expressed Fgfr2b can be activated by mesenchymal ligands, namely Fgf7 and Fgf10 (Ornitz and Itoh, 2001).

Fgfr signalling activates a number of signal transduction molecules, including Ras and phospholipase Cγ (Plcγ) pathways. Ras pathway branches out into several parallel signal transduction cascades. Downstream of Ras in the pathway, Fgf induces sustained activation of the Map kinases, Erk1 and Erk2. Sustained Erk activation has been implicated in differentiation, i.e. in neurite outgrowth (Klint and Claesson-Welsh, 1999). Tyrosine phosphorylation of Plcγ leads to hydrolysis of phosphatidylinositol 4,5 bisphosphate to inositol 1,4,5 trisphosphate (Ins 1,4,5 P3) and diacylglycerol. In turn, Ins 1,4,5 P3 generation leads to Ca^{2+} release from internal stores, whereas diacylglycerol accumulation activates members of the protein kinase C family (Klint and Claesson-Welsh, 1999). Other potential substrates for Fgf receptors include Src family, Fgf receptor substrate 2 (Frs2), SH2 domain-

containing phosphotyrosine phosphatase Shp2, and cytoplasmic tyrosine kinase Ptk2 (former symbol Fak, focal adhesion kinase). Members of the Sprouty, Il17rd (former symbol Sef), and mitogen-activated protein kinase phosphatase families are negative modulators of Fgf signalling, whereas positive factors that promote Fgf signalling include the Ets transcription factors Etv5 (former symbol Erm) and Etv4 (former symbol Pea3) and the transmembrane protein Xflrt3 (Klint and Claesson-Welsh, 1999).

Bone morphogenetic proteins (Bmps), including more than 30 known members today, are the largest family of secreted signalling molecules of Transforming growth factor beta (Tgfβ) superfamily. Bmps have numerous functions in controlling cell proliferation, differentiation, apoptosis, adhesion properties and migration in developing tissues (Botchkarev, 2002). Bmps are synthesized as precursor molecules, which are substrates to subtilisin-like proteases releasing a carboxyterminal monomer. Two disulphide-linked Bmp monomers form biologically active homo- or heterodimers. Bmps share a motif containing six cysteine residues forming a rigid cysteine knot at the base of every mature monomer. Like other signalling molecule actions, Bmp signalling activity is regulated on several levels: expression of the molecules, posttranslational processing of the ligands, the ligand-receptor interactions, the cytoplasmic signalling and the expression of the target genes.

Like the other Tgfβ family proteins Bmps bind to heterotetrameric receptors containing serine/threonine-kinase activity. Type II receptors are constitutively active as kinases. Upon ligand binding the receptor II can phosphorylate the receptor I, which binds and phosphorylates the downstream signalling molecules. The canonical Bmp signalling pathway starts by phosphorylation of Smad proteins, while molecules signalling via Map kinase pathway constitute the noncanonical pathway. In mammals Smad family consists of eight different proteins. Smad1, Smad5 and Smad8 are receptor activated Smads (R-Smads) and they bind to Smad4 (common-partner Smad, Co-Smad), and this complex translocates to the nucleus to regulate transcription of the Bmp target genes (Botchkarev, 2002). Smad2 and Smad3 are activated by Tgfβ/activin instead of Bmps, and Smad6 and Smad7 are inhibitory to the phosphorylation of Smad1, Smad5, and Smad8. Smad6 might also inhibit binding of Co-Smad, Smad4, to Smad1. Activation of Tak1 via recruitment of certain adaptor molecules starts the non-canonical Bmp signalling. Tak1 is a member of Mapk kinase kinase family, which activates NEMO-like kinase Nlk, which in turn inhibits the phosphorylation of Tcf/Lef1 transcription factors and downregulates Wnt/Catnb dependent transcription. Tak1 also activates p38 and Jnk pathways which are involved in Bmp-induced apoptosis (Botchkarev, 2002). Diffusible extracellular Bmp inhibitors such as Noggin, Chordin, Chordin-like, Follistatin, Fsrp and Dan/Cerberus protein families also have central roles in ectodermal organ development. Their affinity to the Bmps is higher than that of the normal Bmp receptors, thus they restrict Bmp signalling to the regions without the expression of the antagonists. Some of them, like Noggin, have affinity also to heparan sulfate proteoglycans of the extracellular matrix and cell surfaces, which affects their tissue distribution. Bmp can be released from the antagonist like Chordin by Bmp1 metalloprotease. Bmps are capable of inducing their antagonists like Noggin and initiate a negative feed-back loop limiting their activity. Some Bmp antagonists can also neutralize the activity of other growth factors. For instance, Dan/Cerberus interacts in the extracellular space with Bmps, Wnts and Nodal (Botchkarev, 2002). A pseudo-receptor Bambi is a membrane bound inhibitor of Bmp and Activin.

Hedgehog (hh) is one of the many genes encoding signalling molecules that was first characterised in *Drosophila* studies. In mammals there are three hedgehogs, Sonic, Indian and Desert hedgehog (Shh, Ihh and Dhh respectively). Lipid-modification is strikingly essential for Shh activity. Lipid-modification of Shh is catalysed by the carboxy-terminal portion of the precursor that is also cleaved, which results in an amino-terminal peptide responsible for all known signalling activity of Shh. The peptide is modified further by esterification at its C terminus to a cholesterol molecule (Hhnp). Furthermore, addition of palmitate to the N-terminus of mammalian

Shhnp protein is dependent upon prior cholesterol addition, and may enhance signalling activity of the protein. Interestingly, in *Drosophila* cholesterol modification seems to target the Hh signal to a delivery system that includes Dispatched, and Tout velu, an enzyme involved in heparan sulphate biosynthesis that is required for efficient transport of, and response to the Hh protein signal. In the signal-receiving cell Hh binding to Ptc within the Ptc/Smo complex releases Smo activity without dissociation of Ptc and Smo (Taipale and Beachy, 2001). Cytoplasmic proteins involved in *Drosophila* Hh signalling include the serine/threonine protein kinase Fused (Fu), Suppressor of Fused (Su(fu)), the kinesin-like protein Costal-2 (Cos2), and the transcription factor Cubitus interruptus (Ci; Gli in mammals). These proteins form a large cytoplasmic complex anchored to microtubules, apparently through Cos2 (Taipale and Beachy, 2001).

Wnt proteins are also a family of secreted, also lipid-modified glycoproteins, whose first homologue encoding gene *Wingless* was found in *Drosphila* genetic studies. Wnts can activate different intracellular pathways upon binding to certain members of the Frizzled family of Wnt receptors (Bhanot et al., 1996) and members of the low-density lipoprotein receptor-related protein (Lrp) (Pinson et al., 2000). Canonical Wnt signalling includes destabilisation of Axin molecules at the cell membrane, and resulting degradation of Axin enables *Drosophila* Armadillo, homologue of vertebrate Catnb, to accumulate and relocate to the nucleus (Tolwinski and Wieschaus, 2004). In the absence of Wnt pathway stimulation, Catnb protein is destabilized by a cytoplasmic complex containing the proteins Axin, adenomatous polyposis coli (Apc), and a serine-threonine protein kinase called glycogen synthase kinase-3 (Gsk3) (Bientz and Clevers, 2000). The action of this complex is antagonized by Dishevelled, a cytoplasmic protein that is activated by an unknown mechanism upon binding of Wnt to its receptor. Thus, Wnt signalling induces the stabilization of cytosolic Catnb, which then associates with high-mobility-group (HMG)-box transcription factors, lymphoid enhancer factor and T-cell factor (Lef/Tcf), to form a transcription complex that activates Wnt target genes in the nucleus (for a review see Nusse, 1997). Humans and mice have four *Tcf* genes with highly homologous DNA and Catnb interacting domains. Interaction of Lef/Tcf with Catnb creates a transcriptional repressor or activator depending on the context (for a review see Clevers and van de Wetering, 1997). Interestingly, all these components of the Wnt pathway possess other functions in addition to their signalling activity, interacting with multiple factors implicated in cellular control processes like cell adhesion and movement. Catnb like plakoglobin, is a protein that binds to cadherin associated protein complex and links adhesion complex to the cytoskeleton, and thus is required for the assembly of adherens junctions (for a review see Nusse, 1997). Some Wnts like Wnt4, Wnt5A or Wnt11 are able to elicit an intracellular release of calcium ions, which is sufficient to activate calcium sensitive enzymes like protein kinase C (Pkc), calcium-calmodulin dependent kinase II (CamKII) or calcineurin (CaCN). Like Bmps, Wnt family also has extracellular Wnt inhibitors that prevent ligand-receptor interactions that control Wnt activity.

1.1.3.2. Notch pathway

Notch signalling regulates cell fate and development of tissue compartments in metazoans (Artavanis-Tsakonas et al. 1999) and it is thought to be crucial in the regulation of stem cell populations (for a review see Pazianos et al., 2003). In mouse and human four Notch receptor proteins have been identified. Notch is a single-pass transmembrane cell surface receptor for transmembrane ligands Jagged1, Jagged2, Delta1, Delta3 and Delta4 (Artavanis-Tsakonas et al. 1983, Johansen et al. 1989, del Amo et al.1993, Dunwoodie et al. 1997, Mitsiadis et al., 1995, Sidow et al., 1997, Valsecchi et al., 1997, Kusumi et al., 1998, Shutter et al. 2000, Rao et al., 2000, Yoneya et al., 2001). Large extracellular domains of Notch contain tandem epidermal growth factor-like (Egf) repeats and membrane proximal cysteine-rich repeats typical to this receptor family. Notch1 contains 36 Egf repeats. Intracellular part of Notch contains cdc10/ankyrin repeats involved in protein-protein interactions. This motif is found also in ankyrin

protein and Nfkb1 transcription factor (Martinez-Arias, 2000). In addition to six tandem cdc10/ankyrin repeats, Notch proteins contain a glutamine rich domain (OPA) and a PEST sequence also critical to downstream signalling (Artavanis-Tsakonas, 1995).

Activation of Notch by its ligands does not generate the type of second messengers that amplify the signal in so many other signaling systems. Instead, full length Notch is cleaved at least three times before generating its intracellular signal transducting peptide activating its downstream targets. First, as part of the biosynthetic processing of Notch, a Furin-like protease in the Golgi converts nascent Notch proteins into heterodimers. This cleavage is necessary for cell-surface expression of Notch. Ligands binding to the Notch heterodimer then trigger proteolysis at two additional sites. The first ligand-dependent cleavage cuts off most of the Notch extracellular domain, leaving only 12 extracellular amino acids attached to the transmembrane domain. However, only the subsequent cleavage within the transmembrane domain releases the transcriptionally active intracellular domain (Krämer, 2000). Notch undergoes this essential intramembrane cleavage by presenilins, membrane-spanning novel aspartyl proteases that function within a macromolecular complex to mediate γ-secretase activity within the cell membrane (Li, 2001, Selkoe and Kopan, 2003).

Soluble intracellular fragment of Notch can translocate to the nucleus and in combination with Rbpj it acts as a transcription factor (Tamura et al. 1995). All four Notches of mammals bind to Rbpj. The amount of intracellular Notch fragment required for signalling is very small, in many cases just within the present limits of detection. Notch signalling is bi-directional, since the cleaved extracellular domain can be transendocytosed by the signalling cell together with the cell surface ligand (Figure 1.; Krämer, 2000).

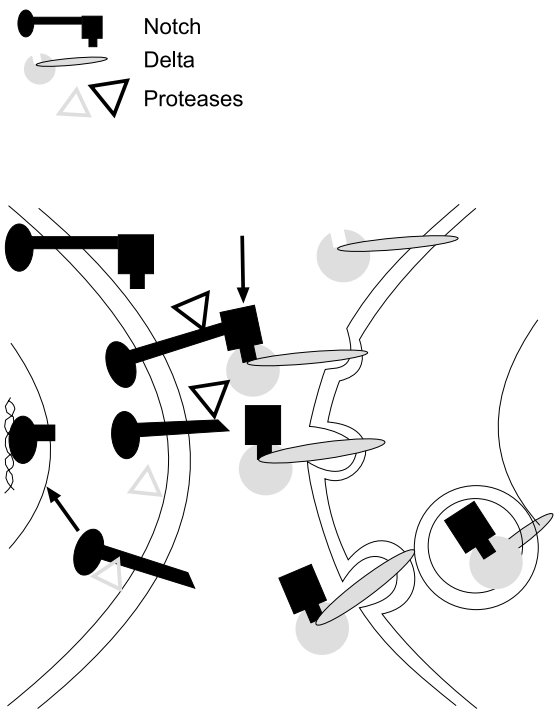

Figure 1. Notch signalling is bidirectional. Delta ligand binding induces cleavage of extracellular Notch that is transendocytosed by the ligand expressing cell, together with the ligand. The remaining Notch peptide is again cleaved releasing intracellular active Notch fragment that can translocate to the nucleus and participate in the regulation of transcription.

Regulation of Notch activity

Ligand binding and activation of Notch is regulated by *O*-fucosylation within the Egf repeats (Lei et al. 2003). Notch is modified in its Egf domains by the addition of fucose to serine or threonine residues. O-fucosylation is mediated by protein O-fucosyltransferase 1, and down-regulation of this enzyme prevents Notch-ligand binding in *Drosophila* (Okajima et al. 2003). Overexpression of this molecule in *Drosophila* increases Serrate-Notch binding but inhibits Delta-Notch binding. Effects of O-fucosyltransferase 1 overexpression or inactivation are opposite to, and are suppressed by, expression of the glycosyltransferase Fringe (Fng). There are three mammalian Fng molecules known today: Manic fringe (Mfng), Lunatic fringe (Lfng) and Radical fringe (Rfng) (Cohen et al. 1997, Laufer et al., 1997, Rodriguez-Esteban et al., 1997). Mouse Fngs are modulators of Notch activation and they specifically modify O-linked fucose moieties (Shao et al. 2003). Thus, Notch-ligand interactions are dependent upon both the presence and the type of O-fucose glycans. In *Drosophila*, Fng inhibits a cell's ability to respond to Serrate protein and potentiates its ability to respond to Delta protein (Panin et al., 1997). Fngs show a preference for O-fucose on some Egf repeats of Notches relative to others. This specificity appears to be encoded within the amino acid sequence of the individual Egf repeats. Mfng modifies O-fucose both at the ligand-binding site (Egf 12) and in the Abruptex region. Fng action on Notch receptors may influence both the affinity of Notch-ligand binding and cell-autonomous inhibition of Notch signalling by its ligand (Shao et al. 2003).

Separation of cells into compartments that do not intermix is a fundamental organising principle during development. In *Drosophila*, loss of Fng function causes dorsal wing cells to violate the dorsoventral (D-V) compartment boundary, and ectopic expression of Fng causes ventral cells to violate the compartment boundary (Rauskolb et al. 1999). Activation of Notch does not confer dorsal or ventral cell location, indicating that Fng can influence compartmentalisation within regions of ubiquitous Notch activation. Similarly to the *Drosphila* Fng function during the wing development (Irvine and Wieschaus, 1994), Rfng is important in boundary formation during the vertebrate limb development, as its ectopic expression in developing chicken limb buds disturbs AER formation at the limb dorsoventral boundary (Rodriquez-Esteban et al., 1997; Laufer et al., 1997). *Lfng* is expressed dynamically during the somitogenesis of mouse embryos forming a tissue compartmental boundary at the presomitic mesoderm (Cohen et al., 1997; Johnston et al., 1997). Null mutation of *Lfng* causes a shortened trunk with a rudimentary tail, malformed rib cages, and defects in the somite formation during the embryogenesis (Zhang and Gridley, 1998). Lfng inhibits Jagged1 (Jag1) mediated signalling and potentiates Delta1 mediated signalling through Notch1, but instead potentiates both Jag1 and Delta1 mediated signalling through Notch2 (Hicks et al. 2000). The *Rfng* mRNA expression was shown by Northern blotting analysis to be most abundant in the adult rat brain. By in situ hybridisation *Rfng* expression was shown in most neurons, but not glial cells, throughout the brain (Mikami et al., 2001). Rfng protein significantly inhibited expression of the Notch effector *Hes1* mRNA in primary neurons, which indicated that Rfng functions by inhibiting Notch signalling in postmitotic neurons of the brain (Mikami et al., 2001). In developing limb bud Fgf10 induces *Rfng* expression together with other AER markers (Ohuchi et al., 1999).

Feedback regulation by Notch and its ligands ensures spatial segregation of Notch receptors and ligands to neighbouring cells (Mitsiadis et al. 1998). Interestingly, instead of performing as a transcriptional activator Notch3 intracellular domain has been reported to act as a repressor of Notch1 signalling by blocking the ability of the intracellular Notch1 to activate expression of its target genes (Beatus et al., 1999). On the other hand, also intracellular Notch2 has been reported to antagonise the function of the intra cellular Notch1 and Notch3 (Shimizu et al., 2002).

Direct targets

The activated and cleaved intracellular Notch domain translocates to the nucleus, binds to Suppressor of Hairless (Su(H), Rbpj in mammals), and acts as a transactivator of *Enhancer of Split* (*Hes*

in mammals) gene expression. *Hes1* and *Hes5* encode basic helix-loop-helix transcriptional repressor proteins Hes1 and Hes5, respectively (Sasai et al., 1992; Takebayashi et al., 1995), which are related to the *Drosophila* Hairy and Enhancer of Split proteins. Hes1 is known to play a role in neuronal differentiation (Kageyama and Nakanishi, 1997) and inhibit differentiation of several cell types (Kageyama et al., 2000). Hes1 also inhibits cell proliferation in neuronal cells (Ishibashi et al., 1994; Castella et al., 2000) and in the absence of Hes1 the positive bHLH proteins like Mash1 cause the premature differentiation of cells (Kageyama et al. 2000). Thus Hes1 seems to have a dual role in the regulation of tissue differentiation. Moreover, constitutive expression of Hes1 prevents differentiation of adipocytes similarly to overexpression of Notch1 and Jag1, but on the other hand total block of *Hes1* expression also prevents adipogenesis, which suggests that some Hes1 is needed in the differentiation process (Ross et al., 2003). *Hes5* mRNA is present exclusively in the developing nervous system, but its level decreases as neural differentiation proceeds (Takebayashi et al., 1995). Notch targets include also genes encoding secreted factors such as *Drosphila Wingless* (Diaz-Benjumea and Cohen, 1995).

Interactions with other signalling pathways

Signalling pathways do not function isolated in a cell, but they interact with other signalling pathways thus forming a network with multiple nodal points. These interactions affect centrally the final interpretation of the signals in the nucleus at a certain time point. Notch pathway interacts with Fgf pathway at several levels. *Jag1* gene is induced by Fgf4 in dental mesenchyme explants, but not by Bmp, while the converse is true for *Notch* genes (Mitsiadis et al., 1997). In *Drosophila*, Notch is activated in tracheal cells by Fgf-like ligand Branchless (Ikeya and Hayashi, 1999) through stimulation of *Delta* expression. During the branching of *Drosophila* trachea, Notch is required to restrict activation of Map kinase to the tip of the branches, in part through negative regulation of Branchless expression (Ikeya and Hayashi, 1999). Tgfβ and Bmps induce *Delta1* expression in dental mesenchyme, but epithelial-mesenchymal interactions do not seem to play a role in the regulation of *Delta1* expression, unlike to the genes encoding Notch family receptors and Jag1 (Mitsiadis et al., 1997; Mitsiadis et al. 1998). Functional synergism in the regulation of Notch target protein Hes1 has been reported with Tgfβ signalling (Blokzijl et al., 2003). Intracellular domain of Notch interacts directly with Smad3, which is an intracellular transducer of Tgfβ signalling. Same type of synergism has been reported in mouse neuroepithelial cells where Bmp2 enhances Notch-induced transcriptional activation of Hes5 and Hesr1 (Takizawa et al., 2003). Bmp2 enhances Notch-induced *Hes5* expression so that Smad1, that is activated by Bmp2, facilitates recruitment of a coactivator p300 to form a complex with intracellular Notch (Takizawa et al., 2003). Notch pathway has been associated also to Nfkb1 signalling, and thus to numerous other molecular signalling networks via Nfkb1 (see the chapter 1.3.3.Tnfs). In keratinocyte monolayers soluble Notch1 ligand, Jag1 peptide, induced Ikkα mediated Nfkb1 activation and increased peroxisome proliferator activated receptor (Ppar) (Nickoloff et al., 2002). Intracellular Notch1 makes a complex with Rbpjκ, which is a repressor of Nfkb2 (p52), which thus might cause Nfkb2 promoter activation (Nakazawa et al., 2001). Furthermore, intracellular domain of Notch can function as a direct coactivator for Lef1 transcription factor (Ross and Kadesch, 2001) in cells where intracellular Notch levels are high.

1.1.3.3. Tnfs

Tumor necrosis factor (Tnf) was identified as cytotoxic product of lymphocytes and macrophages more than thirty years ago (Granger et al. 1969; Carwell et al. 1975). After that, other proteins of similar structure and with some amino acid sequence similarity were found with receptors sharing some sequence similarity as well (for a review see Locksley et al. 2001). In addition to the central roles in adaptive immunity, Tnf superfamily proteins are known communicators between cells orchestrating dynamic tissue remodelling involved in organogenesis e.g. of lymphoid organs, hair follicles and bone (Locksley et al. 2001). In bone Tnfs regulate osteoclast

differentiation and activation and hence the calcium storage and mobilisation.

Ligands of the Tnf superfamily are type II proteins (i.e. C-terminus outside of the cell when membrane bound) that can have both membrane bound and cleaved soluble forms (Locksley et al. 2001). Tnf receptors are type I transmembrane proteins (N-terminus out) with cysteine rich domains each defined by three intrachain disulphide bridges. Receptors can be cleaved also by Tnf converting enzyme (Tace) and other related proteases to generate soluble decoy receptors capable of inhibiting Tnfs, but this regulation mechanism is still largely lacking direct evidence.

Both soluble and membrane bound forms of Tnfs act as trimers. The amino acid sequence similarity in the domains responsible for trimerisation is mostly 25-30%. Receptor binding domains responsible for receptor selectivity naturally show little similarity. After ligand binding the receptor cytoplasmic tails form a stoichiometri cally defined complex with 3-fold symmetry with signalling adaptor proteins. These adaptors are of two principal classes: Traf (Tnf receptor associated factors) superfamily and DD (death domain) molecules. Some Tnf receptors are known to self-assemble and ligand binding re-arranges the trimer. The receptor cytoplasmic tail has a binding motif for either of the two signalling molecule types, and signalling is extremely rapid and specific. The activation of downstream signalling is not totally clear, but p38 and Jnk kinases, and sphingomyelin and Ca^{2+} and other signalling molecules are involved. Traf6 can function as a nonproteolytic E3-like ubiquitin ligase implicated in Nfkb activation. At least six DD molecule-binding members of the Tnfr family (so-called death receptors) can stimulate apoptosis through activation of caspase family of proteases. Other Tnfrs can modulate the response to the death receptors. Tissue remodeling during development and immune responses require apoptotic or necrotic cell death pathways, and both apoptotic and necrotic pathways are induced by Tnf/Tnfr superfamily.

Nfkb (also used symbol NF-κB) represents a family of eukaryotic transcription factors that are located in cytoplasm in non-stimulated cells. Nfkb1 is normally a heterodimer of p50 and p65 (RelA), and must translocate into the nucleus to regulate gene expression. In mammals, five Rel family proteins are known to form heterodimers: RelA/p65, RelB, c-RelA, P50/p105 and p52/p100 (for e review see Chen and Greene, 2003). A family of inhibitory proteins, IkBs, binds to Nfkb1 and masks its nuclear localization signal domain and therefore controls the translocation of Nfkb1. IkB kinase complex (Ikk) is a signalsome containing Ikkα/Ikk1, Ikkβ/Ikk2 (Chen and Greene, 2003). Canonical signalling stimulates IkB kinase to phosphorylate IkB and release p50/RelA to translocate into the nucleus. Upon certain receptor activation, e.g. Tnfα or Il1, a protein complex containing Nik kinase is recruited to phosphorylate and activate IkB kinase (Ikk). In turn, IkB is phosphorylated and targeted to ubiquitin dependent degradation, releasing Nfkb1. This process frees Nfkb1 from the Nfkb1/IkB complexes and enables Nfkb1 to translocate to the nucleus where it regulates gene transcription. Many effector genes including those encoding cytokines and adhesion molecules are in turn regulated by Nfkb1. Noncanonical signalling starts by Nik kinase mediated phosphorylation of Nfkb2/p100 Rel leading to proteolytic processing producing p52/RelB heterodimers, which also translocate to the nucleus to regulate various genes (Chen and Greene, 2003).
Target genes activated by Nfkb1 include also negative regulators controlling its own activity. Classically, Nfkb1 activation is defined as a transient response initiated by the degradation of IkB inhibitor proteins leading to nuclear import of Nfkb1, and culminating with the resynthesis of IkBα. IkBα can shuttle in and out of nucleus and it can remove Nfkb1 from DNA, and thus inactivate the transcription factor. This type of regulation is considered the paradigm for Nfkb1 activation, but also other regulatory profiles are known to exist. By far the most common of these is chronic or persistent activation of Nfkb1. In addition, Tnf has been reported to induce Nfkb1 activation in a biphasic manner, although this is not so often documented and its biological significance remains poorly understood. In biphasic activation cascade the first activation phase is transient, being terminated

within 1 hour of cytokine addition, while the second phase persists for an additional 24-36 hours. Post-translational modifications, such as phosphorylation and acetylation, have recently been found to regulate Nfkb1 transcriptional activity and contribute to shaping the strength and duration of the Nfkb1 response. Both subunits of the heterodimer are acetylated in multiple lysine sites and this reversible acetylation functions as intranuclear switch to control Nfkb1 activation (Chen and Greene, 2003).

1.1.3.3.1.Ectodysplasin pathway

Ectodysplasin (Eda) and its receptor (Edar) were cloned as genes whose mutations cause hypohidrotic ectodermal dysplasias (HED) in human and mouse (see the database Online Mendelian Inheritance in Man, OMIM). HED patients have abnormal teeth, lack sweat glands, have defects in other exocrine glands and nails, have sparse hair and sometimes abnormal nipples. Genetic heterogeneity in HED is due to different mutations in the genes of the Eda signalling pathway. In spite of genetic heterogeneity, the phenotypes are almost, if not fully identical. *Eda* mutations cause X-linked HED, and *Tabby* phenotype in mouse (Kere et al., 1996). *Tabby* mice have a bald area behind the ears, kinked tail tips and they lack some of the sensory hairs of the face (Falconer, 1953). These mice lack the first wave of hair follicle formation, i.e. the primary hair follicles giving rise to guard hairs (Laurikkala et al. 2002). They don't have latest forming placodes for auchene and zigzag hairs either (Claxton, 1967) so their pelage consists of the intermediate hair types that origin from the hair placodes that are formed at E17 (Falconer, 1953). *Tabby* mice also lack ectodermal glands, among others sweat glands, and if the glands have developed, they appear reduced in size or changed in shape (Grüneberg, 1971; Blecher et al., 1983). *Edar* mutations are responsible for autosomal recessive and dominant forms, *downless* and *Sleek* phenotypes in mouse, respectively. Also mutations in the gene encoding adapter signalling protein Edaradd cause identical phenotype, *crinkled*. Thus, Edar pathway plays a key role role on the ectodermal differentiation. Eda is a type II membrane protein that is cleaved by furin-like protease for creating a soluble ligand. Unlike other Tnfs Eda has a collagen-like domain in its carboxyterminus. The Tnf domain and the collagen-like domain as well as the cleavage site are essential for the function of Eda (for a review see Thesleff and Mikkola, 2002). Several splice variants have been detected for Eda, but most important seem to be Eda-A1 and Eda-A2, which differ by two amino acids that are present in Eda-A1 but not in Eda-A2 (Srivastava et al., 1997; Bayés et al., 1998; Mikkola et al., 1999). This amino acid difference affects the receptor binding selectivity so that Eda-A1 binds to Edar and Eda-A2 signals via Xedar, another related, but distinct Tnfr (Yan et al., 2000). Edar ligand binding domain is closest in homology to the respective domain of an orphan Tnf receptor Taj (also known as Troy, or Tnfrsf19) whereas the death domain is most similar to that of p75 neurotrophin receptor.

Edar contains an intracellular DD-binding motif that is specifically recognised by Edaradd upon receptor activation (Thesleff and Mikkola, 2002). Tnfrs containing the DD-binding motif, the death receptors, activate two main pathways: a cell survival promoting cascade leading to the activation of Nfkb1 and Jnk, and a caspase cascade leading to apoptosis. Several lines of evidence stress the importance of Nfkb1 in the mediation of Edar signalling and in the development of ectodermal organs. Edar has been shown to also repress Lef1/Catnb-dependent transcription and this ability is defective in Edar mutants associated with anhidrotic ectodermal dysplasia. While Ikk1/Ikkα and Ikk2/Ikkβ are required for Edar-induced Nfkb1 activation, they are dispensable for its ability to repress Lef1/Catnb-dependent transcription. In contrast, Nik is not involved in Edar-induced Nfkb1 activation or Lef1/Catnb transcriptional repression. As Lef1/Catnb pathway controls the expression of Ectodysplasin-A (Eda), there seems to be a negative feedback regulation on the Eda-Edar axis (Shindo and Chaudhary, 2004), which is in line with their complementary expression patterns in developing mouse skin during the hair placode formation (Laurikkala et al., 2001). In hair and teeth Eda/Edar pathway mediates signalling within the ectoderm, as their dynamic expression patterns

appear in the ectodermal layer. Ectodysplasin is induced by Wnt signals, whereas Edar is up-regulated by ActivinβA (Laurikkala et al., 2001; Laurikkala et al., 2002).

1.1.4. Tooth development

Tooth enamel is the hardest tissue of animal body, and the proper function of teeth is crucial for the wellbeing of the animal. Differences in the dental pattern and structure have a central role in the evolution of animal species, as those differences allow the use of different diets. Mouse dentition consists of one monocuspid incisor and three multicuspid molars in each side of a jaw. Basically tooth development proceeds like other ectodermal organ development, where growth and patterning are regulated by molecular crosstalk between epithelium and mesenchyme. Odontogenesis has been extensively studied, and there is a vast amount of morphological and molecular data about the processes involved in it. But not all details are known yet.

In the following text I mention some factors that are interesting to me and that are present at distinct developmental stages of tooth development, but a more complete list can be found at the web site: http://bite-it.helsinki.fi/

Formation of the dental lamina and placode

The first morphological sign of tooth development is the formation of the epithelial dental lamina, a U-shaped ectodermal ridge in the mandible and maxilla, at embryonic day 10.0 (E10.0) (Figure 2). Teeth are known to form only at the area of dental lamina, and in addition to that normal location also in some teratomas (e.g. McGinnis et al., 1978; Konovalov et al., 1992), where most probably a similar laminar structure formation initiates the development. What are the exact mechanisms regulating the location of dental lamina and dental placodes at E11.0 is not known in detail. Not so much is known about the cellular actions during the placode formation either, although they could be such as increased proliferation, altered attachment properties and increased migration to the placodal area.

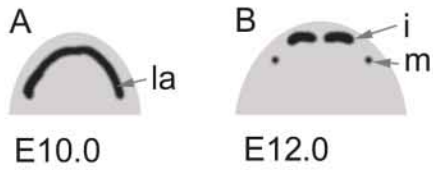

Figure 2. A schematic view from above the mouse embryonic lower jaws. U-shaped dental lamina (la) of the mouse E10.0 lower jaws (A) gives rise to the separated incisor placodes (i) and molar placodes (m) in the E12.0 jaws (B).

The classical tissue recombination experiments have produced information about which tissues contain the potential for tooth formation at speci fied developmental stages. Neural crest cells from the posterior midbrain migrate to populate the first branchial arch mesenchyme at E8.5-E9.0 (Imai et al. 1996). Only this mesenchyme containing neural crest derived cells has the capacity to form tooth, and the non-neural crest mesenchyme, such as limb mesenchyme cannot participate in tooth formation even if recombined with dental epithelium (Lumsden, 1988). The odontogenic potential resides in the epithelium at E9.0-E11.0, so when this epithelium is recombined with neural crest derived mesenchyme from non-dental regions it can induce tooth formation (Lumsden, 1988). The odontogenic potential is shifted to the underlying mesenchyme before E12.0 (Mina and Kollar, 1987, Lumsden, 1988).

Dental lamina gives rise to the epithelial thickenings, i.e. dental placodes (Figures 2B, 3). Specific locations of the dental placodes have been suggested to be patterned by the Fgf and Bmp signalling (Neubüser et al., 1997; Mandler and Neubüser, 2001). Dental placode secretes several signalling molecules, which are part of the reciprocal signalling between the dental epithelium and dental mesenchyme, leading to gradial morphogenetic growth and differentiation. These signalling molecules include Fgfs, Bmps, Wnts and Shh (Thesleff and Mikkola, 2002). Fgf10 expression can be detected in the

E10.0 presumptive dental epithelium and in the adjacent mesenchyme (Kettunen et al. 1999). After E10.0 Fgf10 is permanently downregulated in the dental epithelium and is very weak also in the mesenchyme next to the dental epithelium at E11.0-E12.0 (Kettunen et al. 1999). Fgf8 has been implicated as an early epithelial signal, which regulates mesenchymal expression of several key odontogenic transcription factors (Peters and Balling, 1999, Neubüser et al., 1997). Fgf8 as well as Fgf9 are expressed at E11.0 dental lamina (Kettunen and Thesleff, 1998). Fgfr2b is expressed throughout the dental and oral epithelium at E11.0, whereas Fgfr2c transcripts have not been detected at the early stages of tooth development (Kettunen et al., 1998). Fgfr1b has shown to be absent, but Fgfr1c was found in mandibular arch mesenchyme (Kettunen et al., 1998). Bmp2 is expressed in the dental placode, Bmp7 in the oral and dental epithelium, and Bmp4 in the buccal side of the underlying mesenchyme (Åberg et al., 1997).

CoSmad is expressed in the dental tissue through out the development and Smad1 and Smad5 are expressed first in dental lamina and later in the condensed dental mesenchyme (Xu et al., 2003), possibly reflecting the transmission of the odontogenic potential. *Nog1* mRNA was not seen in developing tooth (Laurikkala personal communication) but another Bmp inhibitor, Ectodin, is involved in epithelial signalling and seems to integrate several signalling pathways (Laurikkala et al., 2003). *Ectodin* is expressed throughout the surface of an E11 embryo, and at E12.0-E14.0 in the developing ectodermal organs, in both epithelium and mesenchyme. *Notch1*, *Notch2* and *Notch3* are expressed in the dental placode (Mitsiadis et al., 1995). At E11.0-E12.0 *Notch* is upregulated by retinoic acid in dental mesenchymal explants (Mitsiadis et al. 1995) but not by Fgf2. Notch ligand encoding gene *Delta1* is only weakly expressed in dental epithelium during tooth initiation (Mitsiadis et al., 1998).

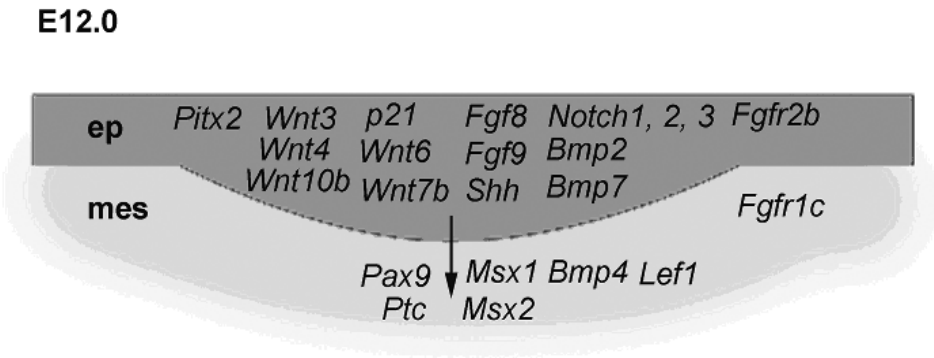

Figure 3. A schematic view of a frontal section of a mouse E12 dental placode. At E12.0 the developing tooth consists of the epithelial (ep) thickening and the condensed mesenchyme (mes). Epithelial thickening, i.e. dental placode functions as a signalling center secreting several growth factors such as Fgfs, Bmps, Shh and Wnts. One of the earliest marker for the dental epithelium is *Pitx2* mRNA expression in dental lamina, later also *p21* expression is upregulated in the placode. Also Notch encoding genes *Notch1*, *Notch2* and *Notch3* are transcriptionally active in the dental placode. *Fgfr2b* is expressed throughout the dental and oral epithelium. In the mesenchyme (mes) the epithelial signals induce expression of *Pax9, Ptc, Msx1, Msx2, Bmp4* and *Lef1. Fgfr1c* is expressed in the dental and oral mesenchyme.

Target genes of these signals encode factors that regulate the precise cell responses. They include transcription factor encoding genes such as *Lef1* (Kratochwil et al., 1996), *Pitx2* (St.Amand et al., 2000), *Msx1, Msx2* (Alappat et al. 2003), and *Pax9* (Peters et al., 1998). Lef/Tcf factors have been mentioned earlier in chapter 1.1.3.1. Fgf target and effector protein Pitx2 is a paired-related homeodomain transcription factor essential for craniofacial development, as well as for cardiac, pituitary gland, eye and limb organogenesis. Its mutation in humans causes haploinsufficient Rieger-syndrome with defects in dental, ocular and abdominal wall development. *Pitx2* is expressed in the dental epithelium throughout odontogenesis, and it has been reported to be a positive regulator of Fgf8 and a repressor of Bmp4 acitivity (St.Amand et al., 2000). It has a function in regulation of cell motility in craniofacial region of a developing embryo (Liu et al., 2003). Its mRNA has been reported to normally have very rapid turnover, but as positive regulation Wnt/Catnb signalling can stabilise *Pitx2* mRNA effectively, as well as other mRNAs such as *c-Jun*, *cyclin D1,* and *cyclin D2* (Briata et al., 2003).

Msx proteins are homeodomain transcription factors that function as transcriptional repressors. In the invaginating dental lamina of molars *Msx2* expression is detected asymmetrically on the buccal side. *Msx1* expression is restricted to the dental mesenchyme throughout the development (Alappat et al., 2003) and between E9.5 and E13.5 Msx1 shows broad distribution mesio-distally in both mandible and maxilla. It becomes localized to the condensing mesenchyme of the incisors and molars after the localization of *Bmp4* expression (Alappat et al. 2003). *Msx1* expression is controlled by retinoids, Nfkb1, Fgf2, Fgf4, Fgf8, Fgf9, Bmp2 and Bmp4, and a mesenchymal transcription factor from endothelin-signalling pathway, dHand. In turn, mesenchymal expression of *Bmp4*, *Fgf3*, *Dlx2*, *Syndecan1* and *Ptc* show dependence on *Msx1* expression (Alappat et al., 2003). Msx1 and Msx2 proteins differ by the characteristics of their N-terminal domains, causing higher affinity of Msx2 to DNA and characteristics that make Msx1 a more potent repressor. Bmp4, directly via Smad4 regulates Msx2 (Alappat et al. 2003). Pax9, a member of paired-box transcription factor family, is expressed in somites, pharyngeal pouches, mesenchyme involved in craniofacial, tooth, and limb development, as well as other sites during mouse embryogenesis (Peters et al., 1998). In developing somites Pax9 is induced by Shh and Nog1 secreted by notochordal cells (Goulding et al., 1994; Christ et al., 2000). Homozygous *Pax9* mutant mice die shortly after birth, most likely as a consequence of a cleft secondary palate. Furthermore, craniofacial and visceral skeletogenesis is disturbed, and all teeth are absent (Peters et al., 1998). Pax9 has thus been proven to be a central molecule regulating the proceeding of tooth development. In the mandibular arch mesenchyme, the expression of *Pax9* marks the prospective sites of tooth development prior to any morphological signs of odontogenesis and is maintained in the developing tooth mesenchyme thereafter. It has been reported to be regulated by Fgf signalling in prospective incisor and molar mesenchyme until E11.0 (Mandler and Neubüser, 2001). Weakening of *Pax9* expression at the dental lamina stage is the earliest difference between the tooth buds giving rise to molars and incisors and diastema buds that cease their development (Keränen et al. 1999).

Mutations in mouse and human *PAX9* cause defects in craniofacial regions and limbs. A missense mutation in the paired domain causes nonsyndromic oligodontia, that mainly involve posterior teeth, permanent molars (Lammi et al., 2003; Jumlongras et al., 2004). Mapping the mutations causing dental defects has given fundamental knowledge of the molecules regulating tooth development. To date, the human genes associated with the non-syndromic form of tooth agenesis are *MSX1* and *PAX9* (Lammi et al., 2003; Vastardis et al., Stockton et al., 2000). A mutation in WNT signalling regulator gene *AXIN2* also causes oligodontia with no other congenital malformations, but interestingly, this mutation is also associated to predisposition to colorectal cancer (Lammi et al., 2004).

Bud and cap stages of tooth development

The molar epithelium growing into the mesenchyme forms a bud at E13.0 (Figure 4.). Tip

of the bud differentiates to a signalling center secreting molecules of many families of factors, e.g. like at the previous developmental stage Bmps, Fgfs, Wnts and Shh. Shh has been shown to be important for the growth and shape determination of the tooth (Dassule et al. 2000). *Notch1* transcripts are absent from the basal epithelium of tooth bud, but *Notch1* is expressed in the stellate reticulum, suggesting that mesenchymal signals downregulate *Notch1* in the proximal epithelial cells (Mitsiadis et al., 1995). Bmp2 and Bmp7 are expressed at the tip of the molar bud, but Bmp4 is expressed in the condensed mesenchyme (Åberg et al., 1997). In Pax9-deficient embryos tooth development is arrested at the bud stage. At this stage, Pax9 is required for the mesenchymal expression of *Bmp4, Msx1*, and *Lef1*, suggesting a role for Pax9 in the establishment of the inductive capacity of the tooth mesenchyme (Peters et al., 1998).

At E14.0 the tip of the tooth bud has stopped growing, while the tissue surrounding it grows into the mesenchyme, forming the cervical loops. Due to the shape of the folded epithelial organ the E14 molar is said to be in the cap stage of the development (Figure 5.). In the center of the "cap" there are a group of non-dividing cells that form the developmental signalling center called enamel knot (Jernvall and Thesleff, 2000; Figure 5.). The enamel knot secretes signalling molecules belonging to several growth factor families. One of the factors expressed in the enamel knot at this point is the Fgf4 (Jernvall et al., 1994) and among other factors are Fgf9, Shh and members of Wnt and Bmp molecule families. *Fgf3* expression is weak in the tip of the epithelial bud and the underlying condensed mesenchyme, but during the cap stage the expression is upregulated in the enamel knot and the dental papilla (Kettunen et al., 1999). *Fgf10* expression is upregulated in the mesenchymal cells of the dental papilla and follicle specifically on the lingual side of the molar (Kettunen et al., 1999). *Msx2* expression is seen in the inner enamel epithelium and enamel organ (Alappat et al. 2003). At this developmental stage Bmp2, Bmp4 and Bmp7 are all expressed in the enamel knot, and Bmp4 expression is strong also in the dental mesenchyme (Åberg et al., 1997). Bmp signal

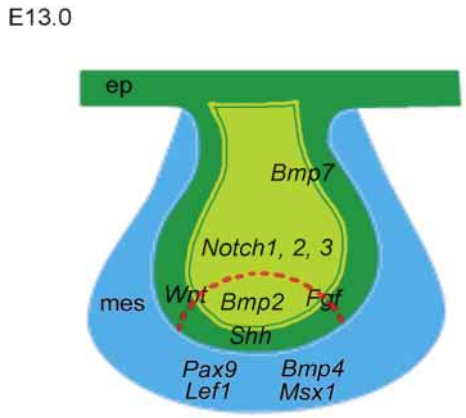

Figure 4. At E13.0 lower molar development is at bud stage, a schematic view of a frontal section. Epithelium (green and yellow) expresses several growth factors genes: *Bmps, Fgfs, Wnts* and *Shh.* Genes written inside the red lining, *Shh* and *Bmp2,* are expressed at the tip of the bud, so called pre-enamel knot. *Notch1* is expressed in the stellate reticulum of the epithelium (yellow). Dental mesenchyme (blue) expresses *Pax9*, *Msx1, Lef1* and particularly the buccal side of the mesenchyme expresses *Bmp4.*

transducers Smad1 and Smad5 as well as Smad2 and Smad3 are expressed in the inner enamel epithelium and dental mesenchyme. Also inhibitory factors Smad6 and Smad7 are expressed in the developing tooth (Xu et al., 2003).

Bell stage and differentiation of dental cells

When the cervical loops grow further, the molar progresses to the bell stage (Figure 6.). The signalling center either divides or new signalling centers arise i.e. the so-called secondary enamel knots appear (Jernvall et al. 2001). As more apoptosis has been reported to occur in the primary enamel knot (Vaahtokari et al., 1996), it is tempting to speculate that the primary enamel knot cells die and the new secondary enamel

knot cells are differentiated among the cells of the inner enamel epithelium. Signals, including molecules from the same families as in the earlier developmental phases, e.g. Fgf4 and Wnt10b, secreted from the enamel knot cells, specify the form of the molar by regulating the growth of the cusps. Smad1 and Smad5 are still expressed in the inner enamel epithelium and dental mesenchyme transmitting Bmp signalling, which is sent from secondary enamel knots (Bmp2) dental papilla (Bmp4) and inner enamel epithelium (Bmp7) (Åberg et al., 1997). Fgf3 that was expressed in the enamel knot is not found to be expressed in secondary enamel knots (Kettunen et al, 1999). Fgf10 expression is strong in the dental papilla cells but is downregulated in the follicle cells (Kettunen et al., 1999).

At E18 the differentiation of epithelial ameloblasts secreting enamel, and mesenchymal odontoblasts secreting dentin, begins. Differentiating inner enamel epithelium cells decline *Msx2* expression, whereas the odontoblasts and the subodontoblastic regions of dental papilla continue *Msx2* expression (Alappat et al. 2003). *Fgf3* and *Fgf10* are expressed in preodontoblasts and differentiating odontoblasts (Kettunen et al., 1999), whereas interestingly, while *Fgf7* is not expressed during molar development, it is expressed in ever-growing mouse incisor mesenchyme next to the outer dental epithelium and cervical loop (Kettunen et al., 1999). Other molecules known to be important for dental cell differentiation include for example Bmp2 (Nadiri et al. 2004), Fgf1, Fgf2, Tgfβ (Unda et al. 2001), Wnt3b (Millar et al. 2003) and Shh (Gritli-Linde et al., 2002). Delta1 expression is upregulated during cytodifferentiation of ameloblasts and odontoblasts (Mitsiadis et al. 1998).

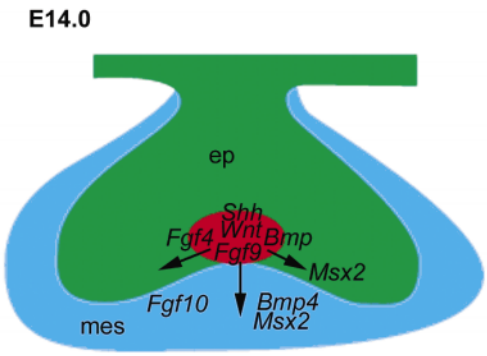

Figure 5. A schematic view of a cap stage tooth at E14.0. Enamel knot (red) contains cells that have stopped proliferating and differentiate into a signalling center cells secreting factors like Shh, Fgf4, Fgf9 and Bmps that control the growth of the surrounding tissue at the dental epithelium (ep, green) and the underlying dental mesenchyme (mes, blue). Dental mesenchyme secretes Fgf10 and Bmp4, and expresses *Msx2.*

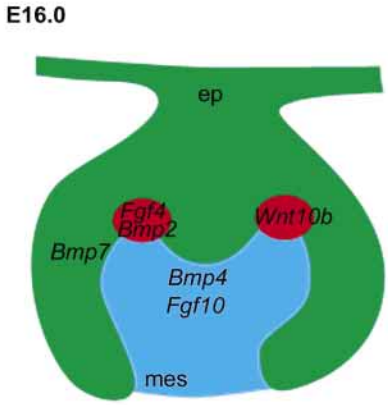

Figure 6. At E16.0 the molar tooth development has progressed to the so called bell stage. At bell stage the secondary enamel knots have formed (red) in the dental epithelium (ep, green). They express growth factor genes *Fgf4, Bmp2*, *Wnt10b* for example. Dental mesenchyme or dental papilla (mes, blue) also expresses cell differentiation regulating protein encoding genes such as *Bmp4* and *Fgf10.*

1.1.5. Hair, sebaceous gland, and nail development

Wild type mice have four different types of hair which develop in successive waves and which can be distinguished by their length and appearance (Fraser, 1951; Fig. 6A in III; Figure 7). The first follicles at E14 give rise to the longest guard hairs, and the second wave at E17 gives rise to straight awls which are shorter than 9 mm. Guard hairs (tylotrichs) are straight hairs of 9 mm or more in length (Mann, 1962). Bended hairs form the soft undercoat of the animal and they include auchenes with one constriction and zigzag hairs with several, typically 4 or 5 bends. Bended hairs start to bud at the time of birth.

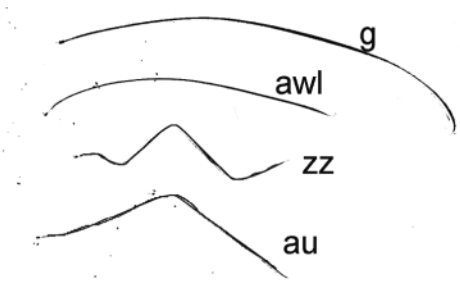

Figure 7. Hair types of the mouse pelage. Guard hairs (g) and awls form the outer coat of a mouse, and the bended hair types zigzags (zz) and auchenes (au) form the soft undercoat. The length of the hairs is usually between 5 and 9 mm, except the guard hairs that are longer than 9 mm.

Epithelial placode formation initiates the hair development in developing mouse skin at E14. The placodal cells are elongated compared to the adjacent interplacodal cells and they form an epithelial thickening. Placode formation is known to initiate in response to molecular signalling from the mesenchyme, including at least Bmps and their inhibitors, Wnts and Fgfs. Wnts are putative placode promoting factors signalling from the mesenchyme or within the epithelium, as overexpression of activated form of Catnb causes ectopic hair follicle formation (Gat et al., 1998). Furthermore, ubiquitously expressed *Catnb* mRNA is strongly increased in the epithelium of the newly formed placodes and K14-Cre mediated deletion of the *Catnb* gene in the embryonic ectoderm causes failure of placode development (Huelsken et al., 2001). Forming placodes express *Wnt10a* and *Wnt10b* (Reddy et al., 2001). Wnts are putative first (mesenchymal) signals that induce the expression of *Eda* that has a central role in the development of the first hair follicles in mouse skin (Laurikkala et al., 2001). The gene encoding Edar is induced at least by ActivinβA that is expressed in the mesenchyme underlying both hair and tooth placodes (Laurikkala et al., 2001). In early developing skin, the expression of both *Edar* and *Eda* is uniform. When hair formation is initiated, *Eda* expression becomes excluded from the placodal areas, while *Edar* expression is restricted to placodes (Laurikkala et al. 2001). Interestingly, the negative feed-back of Eda-Edar axis has been reported to be possible via Lef/Catnb, as Eda expression requires Lef/Catnb and Edar activity represses this transcriptional complex activity (Shindo and Chaudhary, 2004).

During the second signalling step the placodal epithelium secretes signalling molecules to the underlying mesenchyme causing clustering of mesenchymal cells to form a dermal condensate. One of the molecules involved in the second signalling step might be Shh that is not needed in the earliest steps of placode formation (Chiang et al., 1999). Shh is essential in the proliferative expansion of the hair follicle epithelium (St-Jacques et al., 1998). Second dermal message possibly includes Wnt5a that is induced by Shh in the dermal condensate (Reddy et al., 2001). Second dermal message causes the epithelial cells in the placode to proliferate and to form a bud invading into the dermis (see figure 8), eventually surrounding the dermal condensate which then forms the dermal papilla at the base of the follicle (Millar, 2002). The epithelial bud forms a pilosebaceous unit, which consists of a hair bulb, a bulge region and a sebaceous gland (see figure 8; Deplewski and Rosenfield, 2000). Bulge region is the site of attachment of the *arrector pili* muscle (see Figure 8) and according to many lines of evidence, it is

also the presumptive location of the hair stem cell niche (Alonso and Fuchs, 2003).

The early sebaceous gland forming cells are identical to the skin basal and hair piliary canal cells and they form a solid cord. Cells composing the cord are filled with sebum and eventually they rupture and form a channel, the first pilosebaceous canal. Fetal sebocytes are thus functional and probably contribute to the *vernix caseosa* (Deplewski and Rosenfield, 2000), which is the waxy substance that coats the fetus in the uterus, and is believed to protect the skin from exposure to amniotic fluid. Postnatal sebaceous glands secrete sebum via ducts into the bases of hair follicles. Sebum is a mixture of free fatty acids, qualene, waxes, stearic acid ester, free stearic acids, triglyceride, diglyceride and monoglyceride, that protect the skin and the hair from drying. Molecular regulation of sebaceous gland development is poorly understood. Hormones are certainly involved in regulating the development and function of sebaceous glands (Deplewski and Rosenfield, 2000). Interestingly, targeted overexpression of *c-Myc* in the basal layer of the epidermis results in an increase in sebaceous gland size and number at the expense of hair follicles, indicating that c-Myc, as a downstream target of Wnt pathway, is involved in these cell fate decisions (Koster et al., 2002). Administration of Fgf7 induce hypertrophy (Danilenko et al., 1995) and PLCdelta1 deficiency in transgenic mice causes hyperplasia of sebaceous glands (Nakamura et al., 2003). Transgenic mice with blocked or enhanced expression of *Shh* evidence an important role for Shh in sebaceous gland development. Inhibition of the Shh pathway selectively suppressed sebocyte development, while Shh pathway activation led to a striking increase both in size and number of sebaceous glands. Remarkably, ectopic Shh signaling also triggered the formation of sebaceous glands from footpad epidermis, in regions normally devoid of hair follicles and associated structures (Allen et al., 2003).

Mature hair follicle contains epithelial cell layers forming concentric cylinders known as root sheaths that surround the hair shaft produced by the bulb (Millar, 2002). Among other targets, Lef1 regulates keratin and cytokeratin encoding gene expression levels in differentiating hair follicle (Zhou et al., 1995; Dunn et al., 1998) and the gene encoding the cell-adhesion molecule E-cadherin. Microtubule associated protein (Map) is expressed in the innermost layer of outer root sheath, so-called companion layer with keratinisation and many circumvential cell-cell contacts, during growth phase (Hallman et al., 2002). Hair shaft differentiation is also regulated by Shh (Ellis et al., 2003). Overexpression of Shh in interfollicular basal cells causes suppression of hair follicle development between E14.0 and E19.0, resulting in a complete absence of guard, awl, and auchene hair fibres. Hair differentiation is regulated also by Notch and Bmps (Kulessa et al., 2000). Moreover, hair differentiation is affected by mutations of several other molecules, e.g. β1-integrin and laminin10, (Raghavan et al., 2000; Li et al., 2003).

The hair follicles undergo constant cycling regularly producing new hair shaft. Pituitary prolactin (Prl) regulates seasonal hair follicle growth cycles in many mammals. Transgenic mice with nonfunctional Prl receptor gene have shorter hair cycle than controls, and despite of this, slightly longer and coarser hair than controls. Prl has an inhibitory effect on murine hair cycle events. As Prl receptor is expressed in the hair follicles, the pituitary Prl regulation of hair follicle cycles observed in seasonally responsive mammals may be a result of pituitary Prl interacting with a local regulatory mechanism. (Craven et al., 2001). Dermal papilla plays a crucial part in the regulation of successive cycles of postnatal hair growth (Millar, 2002). The first growth phase of mouse pelage hairs takes cirka 21 days and the first hair cycle is quite synchronous (Alonso and Fuchs, 2003). During the growth phase, i. e. anagen, the epithelial part of the hair follicle grows in length penetrating to the underlying dermis. Shh is known to be essential for the progression of anagen (Wang et al., 2000). Expression patterns of transcription factor genes *Msx1* and *Msx2* can be used in defining the transition from anagen to the regression phase, catagen. They are both expressed in the matrix cells of hair bulb at anagen (Reginelli et al. 1995, Satokata et al., 2000; Ma et al., 2003).

When the growth phase ends, *Msx1* mRNA expression is downregulated in the bulb and the expression domain shifts upwards to the root sheath (Satokata et al., 2000) while *Msx2* mRNA expression continues in the bulb, which has reduced to an anchoring club during the onset of the regression phase, catagen. The shortened epithelial portion rests in the epidermis at this point. Catagen is an apoptosis-driven process accompanied by terminal differentiation, proteolysis, and matrix remodeling. By the end of catagen, the dermal papilla relocates near the bulge area of the outer root sheath, just beneath the sebaceous duct. Two Fgf5 splice variants have been shown to affect hair cycling, the short form antagonises the function of the long form during the anagen, while the function of the long form is to inhibit hair growth during anagen and promote the transition from anagen to catagen (Suzuki et al., 2000). Also Tgfβ has been suggested to control catagen (Foitzik et al., 2000). p53 protein is strongly expressed and co-localized with apoptotic markers in the regressing hair follicle compartments during catagen (Botchkarev et al., 2001).

At early anagen, dermal papilla cells are thought to activate the bulge stem cells to migrate down to the bulb and proliferate, and thus, to push the dermal papilla downwards again. When the progenitor cells reach the base of the epithelial follicle, they are pushed upwards from the hair matrix and they differentiate to form the new hair shaft. When the dermal papilla is away from the bulge area, the stem cell niche becomes quiescent again. During anagen the dermal papilla enlarges and develops extensive extracellular matrix (Deplewski and Rosenfield, 2000).

In addition to the eight different hair cell lineages, the hair stem cells can also give rise to the sebocytes and the interfollicular epidermal cells (Niemann and Watt, 2002). Catnb seems to be involved in stem cell regulation in skin, since cells rich in β1 integrins, which have been shown to be skin stem cells, have high levels of free Catnb in the cytosol. Furthermore, the expression of N-terminally truncated Catnb, incapable of binding to the cell membrane complexes, increases the proportion of putative stem cells in the culture (Zhu and Watt, 1999). In the absence of Catnb, stem cells fail to differentiate into follicular keratinocytes, but instead adopt an epidermal fate (Huelsken et al., 2001). A truncated *Lef1* expressed ectopically caused progressive hair loss with formation of dermal cysts with interfollicular epidermis markers at the base of the hair follicles (Niemann et al. 2002). In these cysts Catnb signaling was inhibited as evidenced by absence of cyclinD1 expression. The levels of Catnb signalling possibly determine whether keratinocytes differentiate into hair or interfollicular epidermis, and perturbation of the pathway can lead to tumour formation (Nieman et al. 2002).

Telogen is the resting phase of the hair cycle. At some point of the telogen the old club hair is shed away. This point of the cycle can be defined also as a separate phase, exogen (Paus et al., 1999). *Bcl2* is expressed in the epithelial portion of the cycling hair follicle during the anagen, but the expression levels decrease in catagen and disappear in telogen (Stenn et al., 1994).

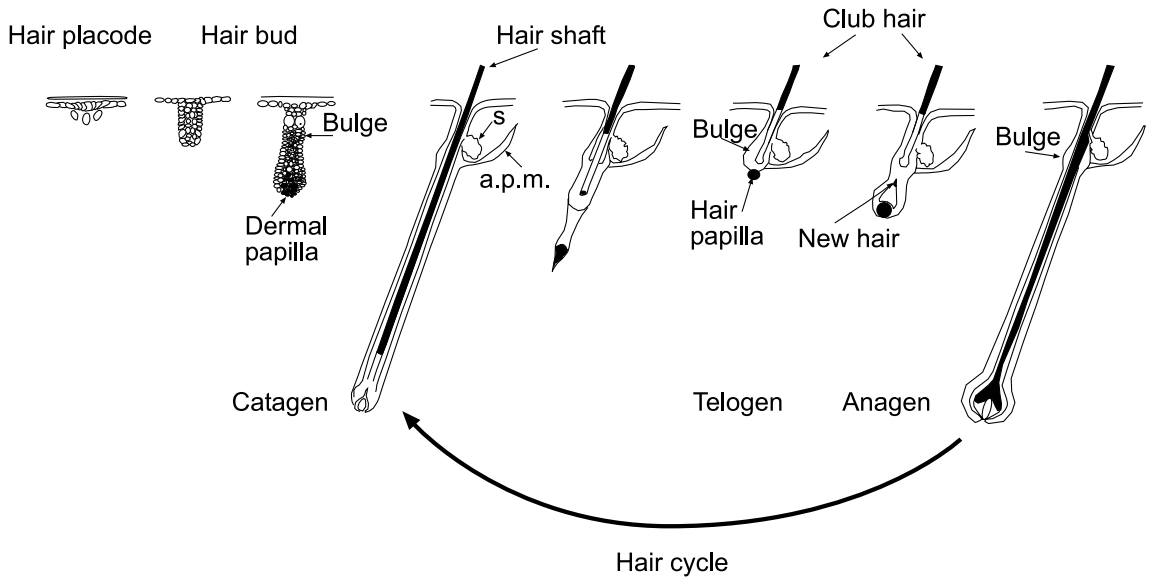

Figure 8. Hair development and cycling. Hair follicle development begins with the formation of the epithelial thickening, a hair placode. The placode grows into the mesenchyme and forms a hair bud. Growing epithelium forms then an elongated hair bud with dermal papilla and epithelial bulge. At cirka three weeks after birth a new hair follicle is complete with hair shaft protruding from the skin. Sebaceous gland (s) and arrector pili muscle (a.p.m.) have formed at the upper portion of the hair follicle. The three stages of follicular cycling are anagen the growth phase, catagen the regression phase of the completed hair follicle and the resting phase telogen. During the catagen the epithelial portion of the hair follicle regresses and involutes towards the skin surface. During the telogen the dermal papilla is located near the epithelial bulge region with putative stem cell niche of the hair follicle, resembling the situation at the late bud stage of the hair follicle development. Dermal signalling induces the epithelial growth and formation of new hair when the new anagen phase begins. The old club hair is shed away when the new hair forms.

Notch is expressed in developing or differentiating epidermis and hairs, and its activation inhibits the terminal differentiation of the epidermis, and also regulates hair differentiation. In hair follicles Notch is found in matrix cells not in contact with dermal papilla, in the similar way to dental epithelial matrix (Lin and Kopan, 2003; Mitsiadis et al., 1995). Inhibiting Notch signalling by conditionally disrupting the mouse *Rbpj* gene in a mosaic pattern to avoid embryonic lethality of Rbpj-deficiency, leads to hair loss, epidermal hyperkeratinization, and epidermal cyst formation. (Yamamoto et al., 2003). Activated Notch protein has been localised strictly to the suprabasal epidermis, and nail matrix (Lin et al., 2003). In skin keratinocytes Notch induces differentiation by activating *p21* (Rangarajan et al., 2001).

The nail grows continuously through the adulthood in mouse and man, at a rate that reflects balance between cell proliferation, terminal differentiation and wear-and-tear (Lin and Kopan, 2003). Like hair follicle, nail is composed of keratinised stratified epithelium and support-

ing mesenchyme. The nail fold is a specialised epithelial transition zone at the base of the nail organ. Nail fold is followed by nail matrix containing proliferating cells. As in the hair follicle, the proliferating cells are not restricted to the cells in contact with the basement mebrane (Lin and Kopan, 2003). The hard protective nail plate, above the nail matrix, forms by keratinisation and flattening of postmitotic matrix cells, and by their nuclear loss and cytoplasmic condensation (Baden and Kvedar, 1993). Nail plates express a subset of hair hard keratins (Heid et al., 1988; Lynch et al., 1986, Moll et al., 1988) and like in growing hair companion layer, Map is expressed in the corresponding domain in nail upper matrix (Hallman et al., 2002). Under the nail plate and distal to matrix is nail bed, a zone of mitotically inactive cells (Baden and Kvedar, 1993). The epithelium connecting the nail bed with the ventral epidermis of the digit is called hyponychium. Mutation analyses have revealed that the molecules involved in nail patterning are among the factors conducting the patterning of the limb. Loss of functional *Wnt7a* causes ventralisation of the limb resulting in nail truncation and appearance of ventral structures to the dorsal side (Cygan et al. 1997). Ectopic Engrailed1 (En1) dorsalises the limb causing extra nails on the ventral side of digits (Cygan et al., 1997). In the mesenchyme, Lim-homeodomain protein Lmx1B acting downstream of *Wnt7a*, is required for nail development (Chen et al., 1998). Msx1 is required for formation of normal nail plate (Jumlongras et al., 2001), whereas Shh is not needed (Chiang et al., 2001). In the absence of HoxC13 and Hairless, long curled and continuously growing nails form (Ahmad et al., 1998, Godwin and Capecchi, 1998; Potter et al., 2001). Ectopically activated Notch cause also lengthening of the nails (Lin and Kopan, 2003) due to increase of proliferation in the nail matrix and an expanded keratogenous zone (Lin and Kopan, 2003). This effect might be transduced by Wnt signalling.

1.1.6. Mammary, sweat, and salivary gland development

Mammals have a unique feature of feeding their offspring by the milk secreted by their specialised mammary glands. Mouse mammary gland development begins at E10.5 by formation of two embryonic ectodermal thickenings called mammary lines or milk lines on the sides of the early embryo. There is actually controversy about whether the mammary lines are anatomical structures (Veltmaat et al., 2003), in rat, human and rabbit the mammary line can be detected as an elevated ectodermal ridge. In mouse embryos no elevated ectodermal ridge is formed, but the thoracic buds are joined by a thickened line of epithelium. In mouse, five pairs of ectodermal placodes appear at E11.5 at reproducibly precise positions along both of the presumptive milk lines. A genetic reporter of Wnt signalling *TOP-GAL* has revealed a thin line of cells with active Wnt signalling connecting the buds 2, 3, 4, and 5, while bud 1 seems to be a separate structure. The placodes appear in the order of pair 3, then 4, followed by 5 and 1, and finally pair 2 (Veltmaat et al., 2003). Cellular mechanisms involved in the formation of mammary lines and the following mammary gland placodes have been studied and in rabbit and mouse it is suggested, that proliferation is not the major mechanism in the early phases (Propper, 1978, Veltmaat et al., 2003).

According to regulative signalling between the mammary epithelium and the mesenchyme the placodes grow to buds at E12.0-E13.5, which are surrounded by primary mammary mesenchyme at E14.5 (for a review see Veltmaat et al., 2003). In male mouse embryos these buds degenerate at E13.5-E15.5. Cell proliferation intensifies at the tip of the bud causing sprouting into the mesenchyme, the prospective mammary fat pad at E16.0 onwards. The fat pad forms the stroma of the adult mammary gland. Later development leads to the formation of nipple and the branching morphogenesis of premature mammary glands. Maturation occurs only after hormonal stimuli during puberty, pregnancy, and birth of the pups (Hennighausen and Robinson, 2001).

In the mammary epithelial/mesenchymal signalling key roles are suggested for Wnt, Fgf and parathyroid hormone related peptide (Pthrp) (for a review see Veltmaat et al., 2003). Pthrp is important in the elongation of the primary sprout and the mutants lacking Pthrp or Pthrp receptor fail to undergo the initial branching growth

(Wysolmerski et al. 1998). Catnb together with Lef/Tcf factors act as a switch to determine cell fate and promote cell survival and proliferation at several stages during mammary gland development (Hatsell et al., 2003). Mice overexpressing the negative regulator of Wnt/Catnb signalling Dickkopf (Dkk) under the control of K14 promoter fail to form mammary buds, and mice lacking Lef1 show an early arrest in mammary gland development at E13.5. Stabilized Catnb initiates precocious alveologenesis during pubertal development, and negative regulators of endogenous Catnb signalling suppress normal alveologenesis during pregnancy. Stabilized Catnb induces hyperplasia and mammary tumors in mice. Each of the Catnb-induced phenotypes is accompanied by upregulation of the target genes *cyclinD1* and *c-Myc*. CyclinD1, however, is dispensable for tumor formation and the initiation of alveologenesis but is essential for later alveolar expansion (Hatsell et al., 2003). Lef1, p63, Msx1 and Msx2 are important intracellular factors in the early development of mammary glands (van Genderen et al. 1994, Mills et al. 1999). Both Shh and Ihh are expressed in the developing embryonic mouse mammary rudiment as early as E12.5, and lack of functional Shh is most probably compensated by *Ihh* since the null mutation of *Shh* does not cause mammary gland defects (Michno et al., 2003).

Sweat glands have their own openings to the skin, sweat pores, so they are not appendages of the hair follicle. Sweat glands have been suggested to be formed by progenitors from the multipotent epidermal stem cells. Under some conditions cells from the sweat glands can reconstitute a stratified squamous epithelium (Niemann and Watt, 2002). There are two types of sweat glands: apocrine and eccrine glands. Eccrine glands secrete sweat, watery perspiration in response to heat. Apocrine glands are deeper and they release thicker secretion containing pheromones in response to stress, like sex attraction. Sweat glands have a critical role in human thermostasis, but in mice this role is probably not so important, as the mice have sweat glands only in the skin of the palms of their feet. Thus the fur and the skin blood circulation have the major roles in mouse thermostasis.

Submandibular salivary glands (SMGs) bud at E11 from the oral epithelium to the mesenchyme, and after elongation of the bud the epithelium branches repeatedly to form a compound branched structure (Jaskoll and Melnick, 1999). Growth of the bud and branching is regulated by epithelial-mesenchymal interactions and signalling molecules involved are largely similar to the other ectodermal organs. The role of Eda/Edar signalling in mouse SMG development has been studied in detail recently. *Edar* is expressed already at E12-E14 in the budding and branching SMG epithelium (Pispa et al., 2003). At later developmental stages both proteins can be immunolocalized to apical surfaces of lumen bounding epithelium (Jaskoll et al., 2003). Postnatal *Tabby* SMGs are hypoplastic. Eda-A1 supplementation to E14 SMG in vitro results in increased branching whereas inhibition of Eda/Edar signalling resulted in the reduction of branch number (Jaskoll et al., 2003). Epithelial basement membrane components such as laminin and proteoglycans are also required for salivary gland branching (Sakai et al. 2003). Also type III collagen and fibronectin are essential, and it has been proposed that epithelial cell fibronectin regulates branching by converting cell-cell adhesions to cell-matrix adhesions (Sakai et al. 2003). Branching morphogenesis is initiated by the formation of shallow clefts in a globular bud, which deepen to generate new buds. The basal proportions of the buds then elongate to form cylindrical stalks. These steps are repeated successively to generate progressively complex branched structure (Sakai et al. 2003).

1.2. Stem Cells

Some adult tissues can replace lost cells by activating a quiescent cell stock, the stem cell population, to divide and some of their daughter cells to differentiate appropriately. Although the stem cell concept is under continuous debate, it is obvious that many tissues of animals have pools of cells with potential to self-renew and to give rise to differentiated cell populations forming the functional tissues. This phenomenon is essential for the tissues that undergo continuous renewal, for example for the function of skin, as well as for the continuosly erupting ro-

dent incisor. Skin forms a protective barrier by keratinisation and death of skin cells in a regulated manner, and the lost cells are substituted by new ones from the basal layer of the skin (Niemann and Watt, 2002). Rodent incisors are evergrowing, meaning that the teeth produce new enamel and dentin secreting cells during the whole life span of the animal, as the tips of the teeth wear out. Rodent incisors grow at the basal ends and are worn at the apical ends, as the animal uses them as powerful tools to break obstacles and to eat hard food materials. So, it is thought that in the rodent incisor, the basal end apex resting in the jaw is the source of stem cells (see upper picture in figure 10, page 59). However, also a thin epithelial cell layer underlying the polarised cells, the stratum intermedium has been suggested to contain stem cells. In the labial side of the epithelial apex, the basal epithelial cells contacting the basal lamina and the mesenchyme surround the stellate reticulum cells and this structure is called the cervical loop. The cells in the apex divide rapidly, and there is a gradient of differentiated more polarised cells towards the incisal direction.

Tissue regeneration after tissue damage is an interesting biological phenomenon, and in spite of wealth of study it still largely remains a mystery and an unreached target. It has been speculated that the hematopoietic tissue could provide a supply of stem cells circulating in the blood stream, that could be recruited to different parts of the body and induced to differentiate in the new host tissue accordingly (Grove et al., 2004). There is some evidence that support this view in e.g. myocyte cultures, regenerating muscle and brain (Muguruma et al., 2003; Dreyfus et al., 2004; Charge and Rudnicki, 2004; Nakano et al., 2001). Bone marrow-derived cells have been shown to have capasity to differentiate into a multitude of tissues in *in vitro* cultures (for a review see Alison et al., 2003). Moreover, in addition to bone marrow-derived stem cells wide plasticity has been suggested to adult, e.g. neural, stem cells, when injected into a blastocyst or regenerating adult muscle (for a review see Vescovi et al., 2002). However, recent evidence indicate that hematopoietic stem cells previously shown to promote regeneration of heart muscle (Rafii et al., 2002) may not differentiate into cardiac myocytes but rather formed vascular tissue, which may have contributed to healing by increasing the blood supply (Murry et al., 2004). Also hematopoietic stem cells transplanted to irradiated mice contributed mainly to the brain parenchymal microglial cells and perivascular cells (Hess et al., 2004). Also in pancreas regeneration process bone marrow-derived endothelial cells facilitated pancreas recovery by providing neovascularization, but did not contribute to the insulin-expressing cells (Mathews et al., 2004). In line with these results, in adult pancreas the pre-existing beta-cells have been reported to be the source of the new beta-cells (Dor et al., 2004). So, there is controversy about the origin and plasticity of the stem cells in adult tissues (Frisen J, 2002; Alison et al., 2003) and several possibilities remain for tissue regeneration. For example in liver the hepatocytes are capable of proliferation after tissue damage. Nevertheless, in case of more severe tissue damage the potential stem cell compartment in liver is still suggested to contribute, and also bone marrow-derived stem cells are suggested to contribute under a strong positive selection pressure (Alison et al., 2004).

Stem cells require specific environmental conditions that maintain their immature stage, multipotency, and dictates the differentiation pathways selected by multipotent stem-cell progeny, the transit-amplifying cells. Certain environmental cues are needed to recruit some of the cells to proliferate and commit to the transit-amplifying pool of progenitors that subsequently give rise to differentiated cells of the tissue. The molecular mechanisms governing the maintenance, division and commitment of stem cells and differentiation of their progenitors are largely unknown. Diffusible factors, cell-cell contact, extracellular matrix proteins, the availability of oxygen and nutrients, and even mechanical stimuli such as stretch forces are all important in stem cell supporting microenvironments, so-called stem cell niches, and they affect the differentiation pathways selected by stem cell progeny (Niemann and Watt, 2000). Classical developmental signals and morphogens like Notch, Wnts, Bmps, Noggin, and Shh have

been reported to play important roles in maintaining adult stem cell niches. Skin epithelial stem cells have been described to express β1 and α6 integrins, and to downregulate expression of transferrin receptor, 14-3-3σ, a nuclear export protein and c-Myc (Alonso and Fuchs, 2003). Activation of Myc transcription factor stimulates epidermal proliferation without depleting putative stem cells, while blocking the Catnb signalling causes loss of label retaining cells, i.e. putative stem cells, through proliferation (Braun et al., 2003). This indicates that Catnb is required for the stem cell maintenance in skin. Also transcription factor p63 is required for skin stem cell maintenance (Mills et al. 1999, Yang et al. 1999). Dermal papilla has been suggested to activate stem cells residing in the hair follicle bulge region by Wnt signalling, while Shh signalling has been reported to maintain postnatal somitic stem cells in a quiescent state (Taipale and Beachy, 2001). Notch/Rbpj signalling has been suggested to regulate the epidermal and hair cell fate determination of hair follicular stem cells at the bulge region (Yamamoto et al., 2003). Notch and its ligand Jag1 are expressed in the regions of brain suggested to contain the stem cell niche (Stump et al., 2002). Notch has been suggested to suppress neuronal differentiation and decrease proliferation, thus creating a more quiescent cell (Alvarez-Byulla and Lim, 2004) but these characteristics of Notch signalling have been suggested to be essential in the maintenance, but not in the generation of the neural stem cells (Hitoshi et al., 2002). Also in quickly selfrenewing gut tissue the earliest cell fate decisions appear to be regulated by the Notch signaling pathway (Schonhoff et al., 2004). Notch is inactive in endocrine precursor cells, but differentiating precursor cells activate Notch in neighboring cells to switch off expression of proendocrine factors and inhibit endocrine differentiation. Shh has been reported to maintain the stem cell population, and regulate their proliferation, but there is another theory suggesting that Shh may actually promote proliferation or support survival of the transit-amplifying cells in brain stem cell niches (Alvarez-Byulla and Lim, 2004). In the stem cell niche regions of the adult brains *Shh* and *Bmp* expression is overlapping. Bmp inhibitor Noggin also participates in the regulation of stem cell compartment in brain (Alvarez-Byulla and Lim, 2004).

2. AIMS OF THE STUDY

Notch pathway is an essential cell-cell signalling system already partially characterised in the developing tooth. The first aim of this work was to combine the knowledge about Notch molecules to the new model system of stem cell maintenance, the continuously growing rodent incisor (I). Then the expression patterns of the Notch pathway molecules, and especially the role of Fng molecules regulating Notch pathway was studied in the first dental epithelial compartmentalisation stage, i.e. the bud-to-cap stage transition of developing molars (II). Also regulation of Lfng gene expression at this stage was analysed to reveal which factors affect the fast upregulation of Lfng in cervical loops of the late bud stage molars.

Eda pathway is known to be crucial for the first forming hair follicles and also to be important in tooth and other ectodermal organ development, as evidenced by the mutations in the *Eda* pathway genes that cause ectodermal defects such as *Tabby* phenotype. The aim of the third part of the work, the creation of the transgenic mice overexpressing Eda in the ectoderm, was done to possibly enhance the ectodermal development, and to learn about the developmental mechanisms and the roles of Eda signalling in those (III). Fourth, as the transgenic mice provided excellent model with enhanced ectodermal development, the aim of the last part of the work was to study the early ectodermal development, the placode formation, in detail (IV).

3. MATERIALS AND METHODS

Preparation of dental tissues was described in (Åberg et al. 1997) and *in vitro* cultures of dental tissues were described in (Kettunen et al. 2000, I, II). The embryonic day was counted starting from the plug day, so that day was E0. For more exact staging we used embryonic morphology according to Kaufmann, 1994.

In situ hybridisation was performed as described in Wilkinson and Green, 1990, with following modifications: in articles III and IV para-

formaldehyde fixation time of the tissues was prolonged to 48 h, and proteinase K treatment was perfomed in double concentration or for double time compared to the earlier protocols.

Whole mount *in situ* hybridisation was described in (Kettunen et al. 1998, II). In situ Pro machine was used in whole mount *in situ* hybridisations of the work in III and IV.

For the *Jag2* probe we made a sequence comparison of EST sequence database with *Jag1* sequence and found a new homologue, that was cloned by others also (Valsecchi et al., 1997). We ordered the plasmid pT7T3D with clone number 717B16, Image ID number 317487, and used EcoRI, NotI restriction enzymes and T7, T3 polymerase for antisense and sense probes respectively.

Other probes were described in the following articles: *Catbn* (Laurikkala et al., 2002), *Bmp4* (Vainio et al. 1993), *Eda* (Laurikkala et al. 2001), *Edar* (Laurikkala et al. 2001), *Fgf3*, *Fgf10* (I), *Hes1*, *Hes5* (Sasai et al. 1992), *Lfng* (I), *Ptc1* (Kim et al. 1998), *Lef1* (Travis et al. 1991), *Notch1*, *Notch2*, *Notch3* (Lardelli et al. 1994, Larsson et al. 1994), *p21* (Jernvall et al. 1998), *Wnt10a* (Wang and Shackleford, 1996, Dassule and McMahon 1998).

Generation and maintenance of K14-*Eda-A1* and K14-*Eda-A2* mice was described in detail in article (III).

Sweat test protocol was described in (III) and originally in (Wada and Takagaki, 1948).

For hair analysis the hairs were plucked and their overall form and length were studied under a stereomicroscope and the fine structure was photographed under a light microscope (III, IV).

For tooth analysis photographs were taken under stereomicroscope (III). Standard microscopy was used, placing the lower jaws on soft material (blue tack or agar).

Mammary gland staining was done as described in (III).

For scanning electron microscopy the tissues were washed in phophate buffer and treated further using standard protocols (III).

In vivo cell proliferation assay was performed as described in (IV).

4. RESULTS AND DISCUSSION

4.1. Notch pathway molecules are associated with stem cell regulation in the postnatal mouse incisor (I and unpublished data)

Mouse incisor, growing through the whole life period of the animal, produces new ameloblasts continuously, which means that in adult animals there has to be a supply of progenitor cells from a stem cell pool. The epithelial tissue at the labial aspect of the incisor apex forms the cervical loop, which consists of the morphologically less densely packed stellate reticulum cells surrounded by polarised basal epithelial cells contacting the dental mesenchyme (Figure 1 in I). Epithelial cells in the mesial apex proliferate faster than the cells in more incisal positions and there is a gradient of cell differentiation in the basal epithelium of the incisor from the apex towards the incisal direction. However, a pool of slowly dividing *Notch1*-positive cells was identified in the cervical loop of the incisor epithelium by DiI labeling experiment, radioactive in situ hybridisation analysis, and a BrdU incorporation experiment (I).

Notch pathway is known to be important in cell fate determination in invertebrates and in the regulation of stem cell and differentiated cell fates in vertebrates. Localisation of *Notch1* in the stellate reticulum indicates that Notch1 might be involved in the regulation of cell fates in the stellate reticulum and supports the idea of cervical loop possibly being the site hosting more immature undifferentiated cells, among which also could be the quiescent stem cells (I). The cells facing the inner enamel epithelium had most intense signal for *Notch1* mRNA, and those cells possibly are the cells that are recruited from the stem cell pool, increase their proliferation rates, make the commitment to the ameloblast cell fate, and have been speculated to intercalate to the inner enamel epithelium. We showed the increase in the proliferation rate (I) but the actual intercalation of the stellate reticulum cells between the polarised cells of basal enamel epithelium has not been studied yet. *Notch2* was expressed in the outer enamel epithelium and the underlying stellate reticulum, whereas *Notch3* expression was not detected.

Notch pathway signals between neighbouring cells, since both the receptor and the lig-

ands are cell membrane spanning molecules (Artavanis-Tsakonas et al., 1983). Expression patterns of Notch ligand genes were studied to determine the tissue domains where Notch signalling could be active. *Jag1* mRNA was not detected in the cervical loop (Fig.6 in I) but instead it was noticed to be a differentiation marker for ameloblasts. *Delta1* expression was undetectable, while *Jag2* was expressed throughout the dental epithelium (unpublished observations). Thus, Jag2 is a putative ligand for Notch receptors in postnatal incisor cervical loops. However, low levels of *Jag1* or *Delta1* expression cannot be outruled by these data. It is known that very small amount of the ligand is sufficient to activate Notch pathway, and on the other hand, large amounts of ligand can shut down the Notch signalling (Martinez-Arias, 2000). Thus, strong expression of *Jag1* in the differentiating ameloblasts might indicate that the ligand levels are high enough to inhibit Notch signalling, but this aspect could be studied by using the antibody recognising the activated Notch (Lin and Kopan, 2003).

A slowly cycling *Notch1*-positive cell population was located next to the *Lfng* positive basal cells, which were suggested to be transit-amplifying progenitor cells (I). Thus it is suggested that these *Notch1* positive cells are the slowly cycling stem cell population in the stellate reticulum. We suggest that some of the *Notch*-positive cells are recruited to proliferate, intercalate into and differentiate in the basal enamel epithelium (Figure 9). We also suggest that Lfng modified Notch signalling has a role in this recruitment (I).

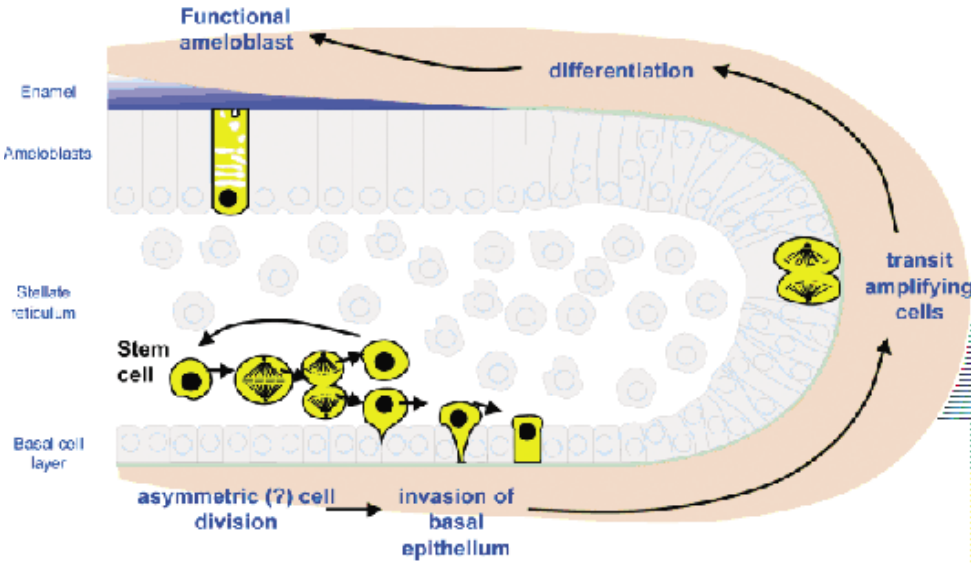

Figure 9. A schematic drawing of our model of the stem cell commitment, proliferation and differentiation in mouse incisor epithelium. Stem cells reside in the loosely organised stellate reticulum, where they remain quiescent or proliferate slowly. Some progeny is recruited to intercalate into the basal epithelium, and to contribute to the pool of transit-amplifying cells that divide faster. While moving towards the incisal end of the incisor the cells polarise and differentiate to enamel-secreting ameloblasts. (Courtesy of Mark Tummers and Irma Thesleff).

Drosophila and mammalian Fng proteins have glycosyltransferase sequence homology and activity (Moloney et al., 2000). Fng proteins have been proposed to be secreted signalling proteins due to their signal peptide near the amino terminus (Yuan et al., 1997). However, Fng has been localised to the Golgi apparatus and the complex between Notch and Fringe has been shown to form intracellularly (Ju et al., 2000; Munro and Freeman, 2000). If mouse Lfng functions only in the Golgi, it would modify Notch receptors cell-autonomously in the same cells that express *Lfng*, thus fine tuning their responses to Notch ligands. This could only indirectly affect the signalling response from *Lfng* expressing cells to the cells being recruited from the stem cell pool, unless the glycosylation of Notch affects the bidirectional signalling of Notch (Krämer, 2000). Instead, if Lfng was actually secreted and active extracellularly, it could modify the Notch signalling in the cells of the stellate reticulum, as was suggested in (I). This would affect the response of the stellate reticulum cells to Notch ligands, causing the recruitment of some cells to the transit-amplifying cell population. Fng has been reported to inhibit signalling by Serrate, and enhance Delta signalling in developing *Drosophila* wing (Panin et al., 1997). In vertebrates Lfng inhibits Jag1 mediated signalling and potentiates Delta1 mediated signalling through Notch1, but it potentiates both Jag1 and Delta1 mediated signalling through Notch2 (Hicks et al. 2000). Thus, it seems possible that in the incisor Lfng inhibits signalling through Jag2 cell-autonomously in the transit-amplifying cells carrying glycomodulated Notch1 on their surfaces. As Notch has been associated in many contexts to the inhibition of differentiation, this inhibition of Notch signalling might allow these cells to commit to the ameloblast cell fate and to start their differentiation process. Cells with uninhibited Notch1 signalling activation would remain in the more primitive cellular compartment in the stellate reticulum. But again, it would be tempting to analyse the location of Notch1 activation directly by using the antibody recognising specifically the activated form of intracellular Notch1 (Lin and Kopan, 2003).
It has been suggested that also the multipotent stem cells circulating in blood stream have striking plasticity and could be recruited by different target tissues and induced to proliferate in the host tissue and differentiate accordingly (Grove et al., 2004). Our data favours the hypothesis that the cervical loop stellate reticulum of the incisors is the source of the new ameloblasts, but it does not outrule the possibility that some stem cells are also recruited from the blood circulation. In *in vitro* conditions the postnatal mouse incisor grows without the cell supply from the blood circulation, so at least it can be concluded that those cells are not crucial for the continuous growth. The third possibility, that the stem cells reside in basal epithelium or in the stratum intermedium layer below the basal layer of the enamel epithelium, does not seem probable in the light of the experiments described in (I). Only the cervical loop region was capable of growing for long time periods in culture (I). Furthermore, the BrdU label was retained in the cervical loop after a long chase, indicating that the slowly dividing quiescent stem cells reside in the cervical loop instead of the basal epithelial layer.

4.2. Fgfs regulate stem cell compartment and *Lfng* expression in postnatal mouse incisor (I)

As mesenchymal signals have been shown in tissue recombination experiments to regulate and maintain the ectodermal epithelium (Vaahtokari et al., 1996), and as we suggested a central role for Notch pathway molecules in the maintenance of continuously growing tooth, candidate mesenchymal signals were analysed for regulation of Notch modulating *Lfng* expression in the cervical loop of the incisor. In isolated epithelial explant *Lfng* expression was downregulated, whereas in co-culture with dental mesenchyme *Lfng* expression was induced in the proximal dental epithelium (I). *Fgf10* was expressed in the incisor mesenchyme next to the basal epithelium expressing *Lfng*, whereas the gene encoding its receptor Fgfr2b was expressed in the epithelium (I). Incisor epithelium cultured with control beads soaked in BSA did not express *Lfng*, which is in line with earlier finding that *Lfng* expression in the epithelium is dependent on signals from the mesenchyme and is downregulated in the epithelium cultured in isolation. In contrast, the

beads releasing Fgf10 induced *Lfng* expression in the epithelium next to the bead (I). These results suggest that *Lfng* expression in incisor epithelium depends on mesenchymal signals and these signals can be mimicked by Fgf10. Dr. Harada has later studied the cervical loop morphology in the *Fgf10* knockout mice. The incisors of *Fgf10*-null mice proceeded to cap stage normally, probably due to Fgf3 signalling rescuing the lack of Fgf10 as they signal synergistically through the same the receptor Fgfr2b. But at later stages of development the cervical loops of the incisors were not formed (Harada et al., 2002). Thus it can be concluded, that Fgf10 is one of the central mesenchymal factors maintaining the continuously growing incisor epithelium (Figure 10). The molecules of our special interest in the work (I) are summarised in table 1.

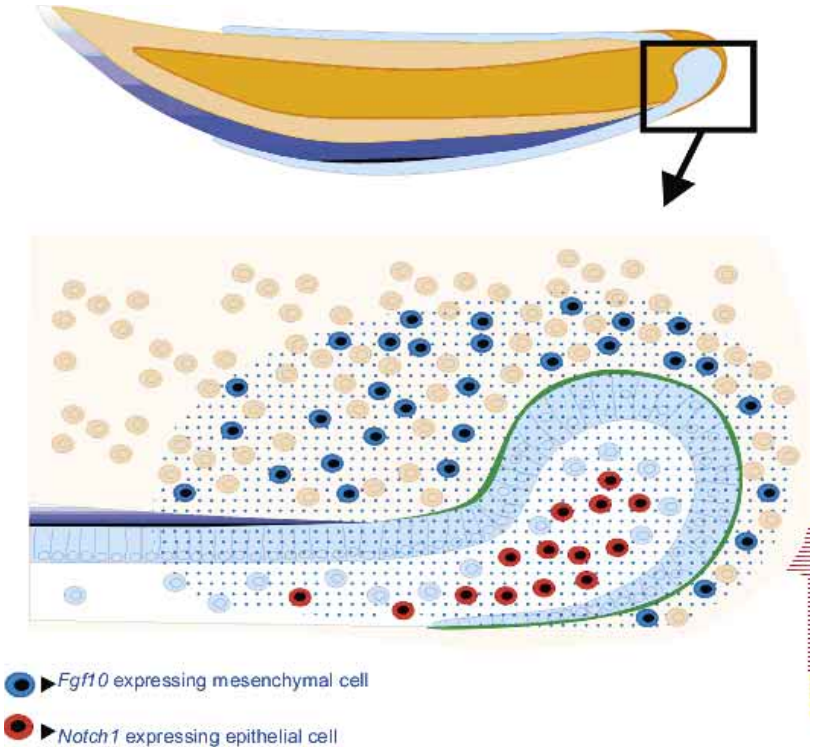

Figure 10. *Notch1* positive stem cell compartment of the continuously growing mouse incisor is maintained by mesenchymal Fgfs. (Courtesy of Mark Tummers and Irma Thesleff).

Table 1. Brief summary of mouse genes studied at 2d postnatal incisor.

Gene	Location of expression	Putative function
Notch1	Cervical loop stellate reticulum	Cell fate regulation, inhibition of proliferation
Notch2	Outer enamel epithelium, stellate reticulum	Cell fate regulation
Lfng	Basal epithelium	Modulation of Notch signalling, allows faster proliferation and beginning of differentiation processes
Fgf10	Dental mesenchyme	Maintenance of the epithelial growth and structures, induction of Lfng

4.3. Notch pathway at the bud-to-cap stage transition of developing molar (II)

In developing tissues different cellular compartments can have distinct differentiation states. Some parts differentiate terminally earlier e.g. to signalling centers that govern the growth of the other tissue compartments. In mouse molar, the developmental stage E13.0-E14.0, i.e. transition from the bud stage to cap stage with mature enamel knot, seems very critical because several mouse mutants undergo a cessation in tooth development at this specific stage (e.g. Peters et al., 1998; Kratochwil et al., 1996). Central event at this stage is the formation of the dental epithelial compartments and specifically the formation and maturation of the signalling center, the enamel knot (Jernvall and Thesleff, 2000). Some compartments of the tissue remain more primitive, as seems to be the case with the loosely packed stellate reticulum cells in developing molars. This corresponds to the epithelial compartments in the continuously growing incisors, where the stellate reticulum of the cervical loop might be the niche for the multipotent primitive stem cells as suggested in (I).

At E13 *Notch1* and *Notch2* transcripts were detected by *in situ* hybridisation in the dental epithelium, but the expression was excluded from the basal layer. Both *Notch* genes had stronger expression at mRNA level in the buccal side of the stellate reticulum at E13.5. At E14 *Notch1* positive domain was larger than that of *Notch2*, and buccal side of the molar still had stronger expression than the lingual side (Fig.2. in II). Like in postnatal incisor cervical loop, *Lfng* was also expressed in developing molar cervical loops. In *Drosophila* development *Fng* expression boundaries have been described to be of importance in defining tissue compartments with cell populations that do not intermix (Rauskolb et al., 1999). In vertebrates, *Rfng* and *Lfng* expression margins have been reported to locate at developmental signalling centers (Rodrigues-Esteban et al., 1997; Cohen et al., 1997; Poyet and Mitsiadis, 2000; Zeltzer et al., 2001). In the molar we visualised with whole mount and radioactive *in situ* hybridisation the *Lfng* expression boundary next to the enamel knot at E14.0, suggesting a role in defining the cellular compartments also in developing molar (II). *Lfng* expression was dynamically upregulated at E13.5 starting from the lingual side of the molar (II). Interestingly, the expression of Notch ligand encoding gene *Jag1* was transiently upregulated in the enamel knot at E13.5 (II). In these differentiating cells the strong transient expression of *Jag1* might actually inhibit or otherwise change Notch signalling allowing a further differentiation step in order to separate this compartment from the neighbouring cell populations. If Lfng had inhibitory effect on Notch1 signalling, it would have created a boundary in the lingual side of the molar stellate reticulum, the lingual border of the forming enamel knot. Expression pattern of *Hes1*, a gene encoding a Notch target molecule, did not show downregulation in that region. In contrast, its expression at mRNA level seems highest near the margin of the *Lfng* expression, which makes it tempting to suggest that in developing tooth the effect of Lfng might be stimulatory on Notch1 signalling. Notch signalling responses in cells are very sensitive, i.e. extremely small amount of Notch signalling is needed for the biological effect (Martinez-Arias, 2000). However, gene mutation experiments in fruit fly suggest that Hes-homologue encoding *Enhancer of split* activity is only essential for a subset of developmental processes involving *Notch* function (de Celis et al., 1996). Thus it is possible that some other marker for Notch activity would show more clearly whether there are changes in Notch signalling responses at *Lfng* expression margins.

Fast upregulation of *Lfng* mRNA at E13.5 lingual compartment of mouse molar suggests a powerful but delicately regulated molecular network in the control of *Lfng* expression. As in the postnatal incisor we showed that in E13.0 molar explants the mesenchyme is needed for *Lfng* expression in the developing dental epithelium (II). As

in article I, we were able to mimick this effect of the mesenchyme by Fgf10, and also by Fgf4. Both of those Fgfs are expressed in the bud and cap stage developing molar, Fgf10 is expressed in the mesenchyme (Kettunen et al., 2000) and Fgf4 at the tip of the bud, and subsequently it is upregulated at the enamel knot (Kettunen and Thesleff, 1998). Thus it seems possible the Fgfs affect Notch signalling through upregulation of *Lfng* in dental epithelium. Whether this effect is positive or negative on Notch activation, depends on the ligands and possibly other factors as well. Our result that ectopic Lfng protein could induce *Hes1* expression in dental cultures indicates that the effect of Lfng on Notch signalling is positive in this context (II). Interestingly, in development of pancreas Fgf10 has been reported to maintain Notch activation (Hart et al. 2003).

Bmp4 has a central role in dental development (Bei et al., 2000) and it is expressed in the dental mesenchyme with a buccal bias, and in the epithelium at E14.0 in the enamel knot (Åberg et al., 1997; Jernvall et al. 1998). Bmp4 activates Lef1 in developing tooth (Kratochwil et al., 1996) and in turn, Lef1 has been reported to be able to form a nuclear complex with intracellular domain of Notch1 (Ross and Kadesch, 2001). In vitro cultures showed that Bmp4 did not induce *Lfng* expression, but instead inhibited the induction by Fgf10 (II). As the antagonism between these signalling pathways has been reported several times in dental tissue (Neubüser et al., 1997; Laurikkala et al., 2003) this effect is probably at least partially direct, so that Bmp signalling prevents Fgf signalling through some common cytoplasmic signal transducer. Thus, the dynamics of the *Lfng* expression possibly results from the balance of Fgf signalling from the mesenchyme and enamel knot, and the Bmp4 inhibitory signalling from the buccal mesenchyme and the enamel knot. Bmp4 is known to induce its own inhibitors. Of these, *Nog1* expression has not been detected in the developing molars, whereas *Ectodin* has been reported to be expressed at all stages of tooth development (Laurikkala et al., 2003). Fgf and Shh signals from the enamel knot have been suggested to counteract the effect of Bmp in inducing its antagonist Ectodin (Laurikkala et al., 2003). At E12 the *Ectodin* expression was intense at the buccal side of the dental mesenchyme. At E13 the *Ectodin* expression continued in the mesenchyme surrounding the epithelial bud. In addition, transcripts were present in the bud and cap stage epithelium, but the enamel knot signalling center was negative. Thus, Ectodin is a potential factor allowing *Lfng* expression to be induced by Fgfs and to be spread to the cervical loops. The molecules studied in our work (II) in the bud-to-cap stage transition stage are summarised in table 2.

Table 2. Brief summary of mouse genes studied in the bud-to-cap stage mouse molar.

Gene	Location of expression	Putative function
Notch1, Notch2	Stellate reticulum of dental epithelium	Cell fate regulation
Lfng	Cervical loops of dental epithelium	Notch activity modulation, allows faster proliferation
Hes1	Dental epithelium	Notch target gene activation
Fgf10	Dental mesenchyme	Growth regulation, induction of Lfng
Fgf4	Enamel knot	Growth regulation, induction of Lfng
Bmp4	Dental mesenchyme, enamel knot (Åberg et al., 1998)	Regulation of differentiation, limits Lfng, synergistic signalling with Notch

4.4. *Hes5* is upregulated in the isolated epithelium (II and unpublished data)

Expression patterns of following Notch signalling pathway genes were analysed in II: *Notch1*, *Notch2*, *Notch3*, *Notch4*, *Lfng*, *Mfng*, *Rfng, Jag1, Jag2, Delta1, Hes1* and *Hes5*. Expression patterns of some of these molecules suggested that those molecules were not relevant to the regulation of dental cell fates. *Notch4* was expressed in blood vessels as described in litterature (Yoneya et al., 2001). *Delta1*, *Mfng* or *Rfng* transcripts were not detected in tooth at this stage. *Hes5* was found to be expressed outside the dental mesenchyme in a single cell, a putative neuron (Figure 11). This expression pattern was verified with serial sections.

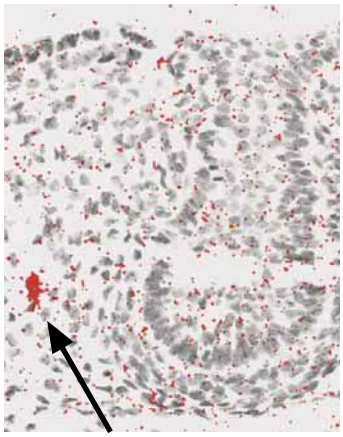

Figure 11. Expression of *Hes5* was detected in one cell (arrow) residing next to the dental mesenchyme.

Interestingly, *Hes5* expression was upregulated in the E13.0 dental epithelium cultured in isolation (Figure 12.). Mesenchymal signals were needed to inhibit *Hes5* expression in dental epithelium, and again the mesenchymal signals could be mimicked by Fgf10, as the Fgf10 protein applied in heparin beads could prevent *Hes5* upregulation.

In the neural tissue the expression of *Hes5* has been reported to coincide with the majority of *Notch1* expression and it was detected in the cerebral cortex, cerebellum and putative germinal zones (Stump et al., 2002). Functionally Hes5, in contrast to Hes1, did not seem to promote the astrocyte differentiation, but it inhibited both astrocyte and oligodendrocyte differentiation (Wu et al., 2003). Also in developing inner ear Hes5, in this context together with Hes1, has been reported to act as a negative regulator of hair cell differentiation. (Zine and Ribaupierre, 2002). Also in mouse retina Hes5 has been shown to be involved in cell fate decisions, as *Hes5* was specifically expressed by differentiating Muller glial cells and misexpression of *Hes5* with recombinant retrovirus significantly increased the population of glial cells at the expense of neurons. Hes5-deficient retina showed 30-40% decrease of Muller glial cell number without affecting cell survival (Hojo et al., 2000). In olfactory epithelium the expression of *Hes5* was restricted to clusters of neural progenitor cells but surprisingly, in this tissue, a mutation in *Hes5* did not produce any apparent defect without simultaneous mutation in *Hes1* (Cau et al.,

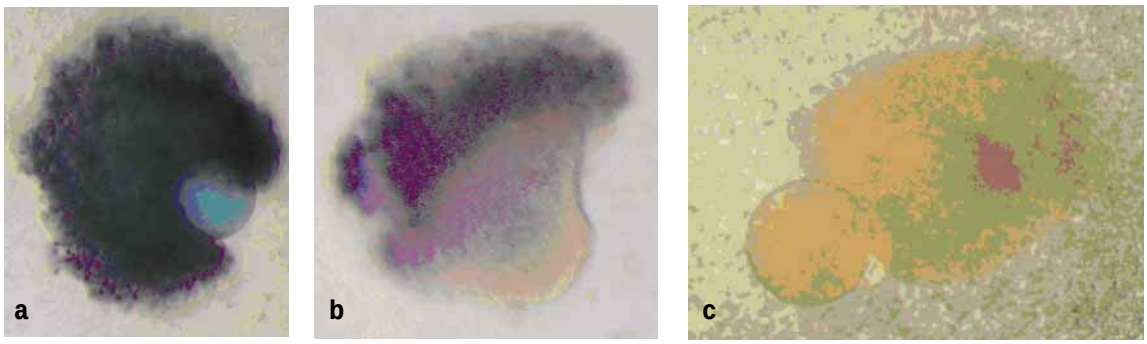

Figure 12. (a) In isolated E13 epithelium cultured with BSA control beads *Hes5* expression was strongly upregulated during overnight culture. (b and c) In epithelial explants cultured with Fgf10 soaked beads *Hes5* expression was prevented partially or completely.

2000). Ectopic activated form of Notch has been shown to induce the endogenous *Hes1* and *Hes5* expression and to inhibit neuronal differentiation in the wild-type, *Hes1*-null and *Hes5*-null backgrounds, but not in the *Hes1-Hes5* double-null background (Ohtsuka et al., 1999). Our finding about the upregulation of *Hes5* in isolated dental epithelium could mean that all cells change their fate and adopt a default pathway including *Hes5* upregulation. On the other hand, the isolated epithelium is known not to survive for long in culture conditions without mesenchymal signals (Vaahtokari et al., 1996). Why is *Hes5* upregulated in epithelial tissue separated from mesenchymal signals essential for its survival? Interestingly, Hes5 and Hes1 have been reported to directly promote Stat3 association to Jak2, causing Stat3 phosphorylation and activation (Kamakura et al., 2004). Stat 3 is an effector of various cytokines and growth factors such as interleukins and Egf. Furthermore, Stat3 is associated to cell survival and to both promotion and inhibition of apoptosis, depending on transcriptional activation of genes that encode proteins that participate in the cell death process, such as Bcl2-like1, caspases, Fas and Trail as well as those that regulate cell cycle progression, such as p21. Interestingly, Stat proteins may also regulate apoptosis through a non-transcriptional mechanism by inhibiting the anti-apoptotic protein Nfkb1 (for a review see Battle and Frank, 2002). Thus, it seems probable that upregulation of *Hes5* in isolated epithelium is associated with withdrawal from cell cycle and cellular preparation for tissue degeneration.

Interestingly, in contrast to the results seen in *in vitro* dental explants, where Fgf10 downregulated *Hes5* expression (Figure 12.), Fgf has proven to be crucial for *Hes5* expression in neural tissue (Ito et al., 2003; Alsina et al., 2004). It is possible that the other molecular components and transcriptional states of the cells in those tissues lead to the differences in the Fgf effects. The levels of *Hes1* and *Hes5* gene expression are known to be determined by various factors, such as the type and combination of the Notch receptors that confer the downstream signals and the expression level of Rbpjκ. (Shimizu et al., 2002).

4.5. Overexpression of *Eda-A2* (III)

Eda gene is expressed as many splice variants, two major of these are *Eda-A1* and *Eda-A2* which differ by short sequences encoding only two amino acids. The two amino acid difference affects the resulting proteins so that they bind to distinct receptors (Yan et al., 2000). Eda-A2 binds to the receptor Xedar and activates Traf6 (Yan et al., 2000) probably contributing to the activation of Ikk complex, which in turn activates Nfkb1 (Newton et al., 2004). The roles of Eda-A2 signalling during animal development and adult homeostasis are not known. To overexpress *Eda-A2* in the ectoderm we cloned a construct in which K14 promoter drives the expression of *Eda-A2*. In the southern blot analysis we found two founder animals. Progeny of these animals were used to collect tissue samples for *in situ* hybridisation, which showed expression of *Eda* in ectopic regions driven by K14 promoter. Despite this ectopic *Eda-A2* expression, no morphological defects were found in the transgenic embryos or adult animals. The histology of the ectodermal tissues was unaltered; the mice had all four hair types and seemed otherwise normal. Xedar null mutation mice have no obvious phenotype either (Newton et al., 2004). Overexpression of *Eda-A2* in the mouse muscles caused myodegeneration, suggesting a role in skeletal muscle homeostasis instead of ectodermal development (Newton et al., 2004).

4.6. Overexpression of *Eda-A1* (III, IV)

Eda-A1 is two amino acids longer than Eda-A2, and it binds to receptor Edar (Yan et al., 2000). Activated Edar signals via cytoplasmic adaptor protein Edaradd activating IKK complex, which leads to Nfkb nuclear localisation. As mutations in *Edar* and *Edaradd* produce ectodermal dysplasias in humans and mice, Eda-A1/Edar pathway has shown to be essential for the development of epidermal appendages, while the roles of Eda-A2/Xedar signalling remain obscure. For overexpression of *Eda-A1* in the developing ectoderm, transgenic mice with K14 promoter driving the expression of *Eda-A1* cDNA were produced. By PCR and southern blotting we identified 12 transgenic K14-*Eda-A1* founder mice. Ten of these showed clear and consistent

phenotype and were bred to see that the phenotype was inheritable. Subsequently 5 lines were bred for detailed analysis of the phenotype. Overexpression of *Eda* mRNA was verified by whole mount *in situ* hybridisation of E10.0 embryos and radioactive *in situ* at E15 (fig. 1. in III). Expression in the simple ectoderm was markedly stronger in K14-*Eda-A1* embryos than in wild type, and later the increased or ectopic expression was seen in the basal layer of the skin, outer root sheath of hair follicles, oral epithelium, and dental epithelium including polarized ameloblasts. Genomic copy number of transgene integrations was not analysed. This decision was based on the fact that not all integrations produce active transgenes, and the strong overexpression could be easily detected by *in situ* hybridisations.

4.6.1. Characteristics in the adult phenotype

The pelage of K14-*Eda-A1* transgenic mice looked ruffled up. Two lines had curly hairs and whiskers while other lines were straight haired. The length of straight hairs was analysed in wild type and transgenic mice. Transgenic hairs were of all lengths between 5 and 13 mm, while wild type hairs have distinct length groups (Dry, 1926). The proportion of the hairs longer than 9 mm, classified as guard hairs, was increased dramatically in the transgenics. Furthermore, the transgenic mice had longer hairs in the dorsal side of the feet, including toes, than wild type controls (III). We showed that the growth phase (anagen) of the first hair cycle was prolonged in the transgenic mice. As the length of hairs is usually proportional to the duration of the anagen phase (Muller-Rover et al., 2001), we suggest that the longer hairs in the transgenic mice resulted from a defect in hair cycling. The length was increased possibly also because the identities of the hairs were more often guard-hair-like. However, it is also possible that hair formation was stimulated by alterations in the proliferation or differentiation of hair matrix cells as shown in *Wnt3* overexpressing mice (Millar et al., 1999) since both *Edar* and the transgene were expressed in the hair bulb (Laurikkala, 2002).

In addition to hair length, hair types can also be distinguished based on the internal structure of their medulla which differs in the number of longitudinal rows of air-cells seen under light microscope (Falconer et al., 1951; Sundberg, 1994). Guard hairs have two rows of air cells whereas awls have two, three or even four of them. Transgenic straight hairs looked relatively similar regardless of their length but differed from normal guard and awl hairs (III) thus leaving their identity unclear. No auchenes or zigzags were found in transgenic animals (III), so they lacked the undercoat, which may have caused the shaggy appearance of transgenic mice, together with abnormal orientation of the hair follicles (III). It is not known how the production of the hair shaft differs in the separate waves of hair follicles in wild type mouse embryos, but our results suggest that the levels of activities of various signalling molecules including Eda may regulate this process. Loss of Eda signalling in mice also causes loss of distinct hair types. However, the intermediate type of hair in *Crinkled* mice, with mainly one row of medullar air cells, differs from the intermediate hair type of *Eda-A1* overexpression mouse that is more guard-hair-like with two rows of air cells (Falconer et al., 1951; III). The null mutants of Eda, *Tabby* mice, lack variably the third molar or incisors, and heterozygous animals sometimes have altered cusp pattern (Grüneberg, 1966; Sofaer, 1969). Overexpression of Eda caused development of extra molars in front of the first molar. In all embryonic samples studied the extra tooth bud was found in front of the first molar, but in adult samples the fully formed extra tooth was found in approximately 60% of the jaws (III). Cusp pattern was largely normal, except that the cusps were wider than in wild type and there were extra pair of cusps in the distal end of the first molar (III). Mechanisms of the formation of extra molars in K14-*Eda-A1* transgenic mice and the lack of the third molar in *Tabby* mice will be discussed in the next chapter (4.6.2.).

In addition to teeth and hairs, overexpression of *Eda-A1* in developing ectoderm stimulated formation and growth of other ectodermal organs. Mammary gland development was affected, as supernumerary nipples were found regularly in K14-*Eda-A1* transgenic females (III). This is the first time we know a transgene causes supernumerary nipples. In mice, nipple deficiencies are

more common than the supernumerary organs (Veltmaat et al., 2003). Most commonly the deficiencies are found in the thoracic region, most often observed loss is that of the gland 3. Interestingly, an inbred strain A/J has similar characteristics to our transgenic mice, and the features are inherited in a single autosomal recessive locus, called *scaramanga* (ska) (Howard and Gusterson, 2000). Ska is suggested to be needed early in development, at E11.5 or earlier, when the mammary gland anlage is forming. The third nipple is sometimes missing or misplaced. Supernumerary nipples occur laterally to the 4th nipple, occasionally between the 2nd and third nipples, or below the third nipple along the milk line. Extra nipples in K14-*Eda-A1* mice were located between the third and fourth nipple, below the third nipple along the milk line as in some of the *scaramanga* mutants. It appears that the third and fourth glands are primarily affected by developmental thresholds that have been suggested to modulate development in teeth also (chapter 4.6.2.; Thesleff, 1996). Morphogenetic factors most likely interact with epigenetic mechanisms to reach a critical stage of activity in a very restricted area and somehow make these regions more susceptible to changes than other regions (Veltmaat et al. 2003). The placode 3 is the first to be formed and apparently the other placodes develop normally when the formation of placode 3 is disturbed, while the pair 4 is most resistant to regression due to genetic defects, like mutations of *Lef1*, *Fgf10* or *Fgfr2b* (Veltmaat et al., 2003). Loss of function mutations of *Fgf10* or *Fgfr2b* lead to the lack of induction of all placodes, except the mentioned pair 4, which develops until E16 and then undergoes apoptosis (Veltmaat et al., 2003). It can be concluded that induction of pair 4 is independent of induction of pair 3, and in general, unique identity to for each mammary rudiment has been suggested (Veltmaat et al., 2003). *Tbx3* gene mutations cause variable mammary phenotype in humans (Bamshad et al. 1997). Interestingly, this ulnar-mammary syndrome also causes defects in teeth, limbs, apocrine glands, and genital development. In conclusion, the intricate developmental tresholds seem to function along the milk line as well as along the dental lamina.

Other stimulated ectodermal organs in K14-*Eda-A1* transgenic mice include nails and sweat glands. Nails of the adult K14-*Eda-A1* mice were longer than in wild type (fig. 2 in III). Whether the increase in length is due to hardening of the nails with changes in the keratin and other molecular composition, or due to the increased cellular proliferation and differentiation in the nail matrix remains to be seen. Sweat gland staining with iodine, castor oil and starch was more intense in the transgenic paws than in the feet of the wild type mice (III) which also can be due to different reasons. First, sweat glands could be larger in size, or second, sweat glands could be functionally more active. Transgenic sebaceous glands of the hair follicles were larger in size than the wild type glands (III). The branching morphogenesis was upregulated in the transgenic submandibular salivary glands and the lumens of the ducts were larger (Nordgarden et al., in press).

4.6.2. Ectodermal placode formation

Dynamics of tooth formation along the molar placode seems to follow certain rules. When placode has extra signalling from the Edar, an extra tooth is formed mesially, i.e. from the mesial end of the molar placode (see Figure 2 (m)). When Edar signalling is weak, the third distalmost molar is not formed anymore. This kind of regulation ensures that the tooth row does not extend towards the jaw hinge, and the molecular basis controlling it would be extremely interesting to study. As pure speculation, the distal end of the dental placode might have shortage of or lack of some essential factor for dental organogenesis, which prevents the formation of any extra molars to that end. There might be a gradient of some factor mesiodistally (Figure 13.)

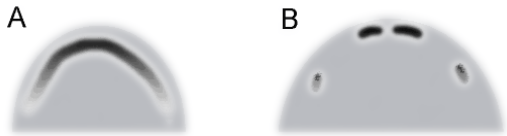

Figure 13. Speculated gradient of an unknown gene activity, with stronger expression mesially and weaker expression distally. E10.0 dental lamina is shown in A, E12 molar and incisor placodes are shown in B.

Thus the delicately regulated developmental thresholds (Thesleff, 1996) possibly determine the fate of the cells at the ends of the placode. At E13.0 the extra tooth placode visualised by *Shh* expression can be seen in both wild type and transgenic lower molars (IV), but it is regressed in the wild types subsequently. This suggests that the threshold needed for the formation of extra molar might be set relatively late, at the time when the wild type mesial "extra" placode regresses. However, in the formation of diastema buds of the upper jaws the threshold first appears as differences in the expression levels of *Pax9* (Keränen et al., 1998) but probably the threshold is set already before that difference in the dental mesenchyme by an unknown gene(s). At later stages similar tresholds seem to affect the cusp formation via activities of enamel knots, as *Tabby* mice have decreased number of molar cusps and K14-*Eda-A1* second molars have extra cusps.

The early K14-*Eda-A1* hair follicles were larger than wild type follicles as visualised by whole mount in situ hybridisation with Catnb probe. The *Edar* expression was restricted normally to the forming placodes of transgenic embryos, the expression domain being slightly larger than in the wild type follicles. The normal round follicle form was disrupted and the follicles tended to fuse. The *in situ* hybridisation on tissue sections with placode markers also showed larger follicles and follicles abnormally close to each other (IV). The placodes grew bigger in a concentration dependent *manner in vitro* skin explants cultured with Eda protein (IV).

Placode promoting factors and placode formation inhibiting factors probably compete in the early epithelium and the underlying mesenchyme, and the delicate balance of factors is disrupted by slight differences sensed by the cell membrane receptors. These differences can be rapidly potentiated by feedback mechanisms described in lateral-inhibition model of Notch signalling (for a review see Bray, 1998), where cells receiving more receptor activation further reduce their ligand expression, and ligand expressing cells with less receptor activation reduce receptor expression, thus increasing the subtle differences. The observations of K14-*Eda-A1* transgenic mice as well as *in vitro* experiments with recombinant Eda-A1 protein indicate that Edar signalling acts as a positive regulator of placode formation. The molecules like Eda, promoting placode formation, can be thought to be activators and some other molecules, such as Bmps, negative regulators or inhibitors of hair placode formation. Bmp4 releasing beads placed on embryonic skin explants have been shown to inhibit hair placode formation (Botchkarev et al., 1999, IV). Placodes secrete Bmps but they also express Bmp inhibitors such as Nog1, suggesting that Nog1 restricts Bmp placode formation inhibiting activity to the interplacodal areas (Botchkarev et al., 1999). The uniform expression patterns of many molecules in the primitive ectoderm become restricted upon development either into the organ placodes or in the interplacodal regions. This restriction might start by subtle differences in the levels of certain molecules between the cells. These differences are sensed so that the signalling autoregulates itself efficiently. The increase is faster in the originally a slightly more expressing cell, and it rapidly starts to signal to the other cell to reduce and stop expressing this molecule, which might occur by antagonists such as Wnt and Bmp signalling antagonists Dickkopf and Nog1 or Ectodin, respectively. Notch pathway molecules have been shown to regulate the cell fates, such as neural fates, by lateral inhibition so that cells expressing more receptor express even more receptor and downregulate their ligand expression, while cells having less Notch, express and upregulate the ligand expression. Thus the differences between the cells are amplified (Artavanis-Tsakonas et al., 1999). Edar signalling might have a negative effect on Eda expression, as in the E14 skin their expression patterns are complementary. Eda is expressed in the interfollicular epithelium, while Edar expression is restricted to the hair follicles (Laurikkala et al., 2001). Interestingly, Edar signalling has been reported to repress Lef1 activated transcription, and Eda expression is thought to depend on Lef1 signalling (Shindo and Chaudhary, 2003). In the case of overexpression this kind of regulation is destroyed, but naturally, Edar signalling occurs only in the cells expressing Edar. It seems that Eda-Edar signalling increases *Edar* expression only slightly, as the ex-

pression of *Edar* in K14-*Eda-A1* transgenic skin is still restricted to the placodes and downregulated in interplacodal skin (III). Thus it does not autoregulate its own expression very efficiently, but some of the normally occurring lateral inhibition in hair follicle development is certainly lost by administration of ectopic Eda. The Catnb positive placodal regions grew markedly larger and even fused to each other in transgenic skin, and in *in vitro* cultures the placodes grew bigger in a concentration dependent manner (IV). Some molecules that could be involved in the specification of placode size are summarised in table 3.

Table 3. Summary of mouse genes involved in patterning of hair placodes at E14

Gene	Location of expression	Putative function
Notch1	Inner cells of hair placode (Powell et al., 1998), interplacodal ectoderm	Cell fate regulation: inhibits differentiation and proliferation of inner cells of hair placodes, in interplacodal cells with Lef1and without Edar signalling promotes/maintains interplacodal cell fate
Eda	Interplacodal skin (Laurikkala et al., 2001)	
Edar	Hair placodes (Laurikkala et al., 2001)	Inhibition of Notch-Lef1 signalling, promotion of placodal cell fate specification
Bmp4	Condensed mesenchyme	Synergism via Smads (Takizawa et al., 2003) with Notch signalling in inhibition of placodal cell fate?
Lef1	Ectoderm including the placodes, condensed mesenchyme	Wnt signal transducer with Catnb, co-activator of transcription with intracellular Notch (Ross and Kadesch, 2001).

Molecular mechanisms regulate cellular functions, such as proliferation, migration and cellular adhesion properties. Mesenchymal signals instruct the overlying epithelium to thicken and specify it to dental or hair placode, and thus, the effects of Edar signalling on placode formation could be employed via the underlying mesenchyme. In that case, Edar signalling would induce a signalling molecule in the epithelium to influence the underlying mesenchyme, which would signal back to the epithelium. Recent report about Edar signalling counteracting Lef1/Catnb signalling (Shindo and Chaudhary, 2004) indicates that Edar function in cell fate specification could occur at least partially via Notch pathway signalling modulation, as activated intracellular Notch has been reported to be a co-activator of gene expression together with Lef1 (Ross and Kadesch, 2001). The exact mechanisms regulating the location of dental lamina and dental placodes at E11.0, mammary lamina and placodes at E10.5, hair placodes at E14.0, and general cellular actions leading to the placode formation have not been described to detail in the literature. Stimulated placode formation and activity in the ectoderm of K14-*Eda-A1* mice provided us a tool to investigate the mechanism of placode formation. We counted the numbers of BrdU incoporating S-phase cells, and did not find difference between the mitotic indices of transgenic and wild type placodes (IV). In contrast, the interplacodal regions contained more BrdU positive nuclei than the placodal regions in both transgenic and wild type skin. These results are in line with data from Wessells and Roessner (1965) who studied ^{3}H -thymidine incorporation and mitotic figures of hair follicle cells. They found that primary hair and vibrissae placodes were almost devoid of label, and the incorporation of label continued only after the placode had invaginated in the

mesenchyme. Similar results were obtained during feather development (Wessells, 1965). Knowing that it is not the increased proliferation, then what are the cellular mechanisms of placode formation and are those the same mechanisms that make the K14-*Eda-A1* placodes grow bigger than the wild type placodes, remain to be studied in detail.

CONCLUDING REMARKS

Now that the molecular tools have evolved to produce vast amounts of molecular data, details of molecular networks involved in mammalian development are revealed in a speed not seen before. New information is generated every day, and the overall picture is getting clearer as we find proper locations for each piece of the puzzle. The cellular responses to the molecular signals depend on the innate state of the cell, i.e. the signal transducing molecules and their regulation, the genomic regulatory switches that can silence and activate genomic regions, other transcription factors available, regulation of molecular traffic through the membranes of the nucleus and the cell, and cell cytoskeletal properties etc. The same molecules have different roles in different tissues and at different developmental stages of the embryos, but often the roles can be predicted and then tested if we understand the function of the specific molecule in a distinct situation.

Different stem cell supplies can function to ensure the proper recovery of a damaged tissue *in vivo*. Tissues contain undifferentiated quiescent progenitors, and more primitive multipotent stem cells, and they also receive migratory hematopoietic stem cells from the bone marrow. In some cases even the differentiated cells of the tissue can adopt proliferative state and provide new cells. These different ways to provide cells to an adult tissue to replace dead or damaged cells is probably an essential result of evolution, as quick healing certainly enhances survival and "fitness" of an animal. Also, if different stem cell populations are regulated by slightly different set of genes, they provide even better back-up mechanisms to each other, and that also makes the healing processes less susceptible to mutations caused by environmental hazards. These naturally occurring factors can include for example free radical molecules and other chemically active compounds, radioactivity, UV -radiation etc. Moreover, regarding this aspect it makes sense that the "ultimate stem cells", the hematopoietic stem cells with high plasticity reside in a fairly well protected niche, inside the thickest bones of the body. Furthermore, there seems be synergy between the different stem cell types in healing processes. For example, it has been noted that vascularization enhances other healing processes in a regenerating tissue. In this work, the Notch pathway molecules were associated to the epithelial stem cell maintenance and regulation by mesenchymal Fgfs in the continuously erupting mouse incisor. In long-term culture conditions the stem cell supply from the blood circulation cannot play a significant role. Thus we conclude that the stem cell population residing in the cervical loop of the mouse incisor in a well protected location in the jaw, is essential and sufficient for the continuous growth of the tooth.

Regulation of *Lfng*, a Notch modulator encoding gene, was studied at the bud-to-cap transition of mouse molar. Fgfs and Bmps are known to have antagonistic effects in the development of some organs such as teeth (Neubüser et al., 1998; Laurikkala et al., 2003). Based on our *in vitro* experiments we noted that *Lfng* expression was induced and maintained by Fgfs, whereas the Bmps prevented the Fgf dependent induction. Thus, Bmps can influence Notch signalling in several ways, at least via Smad effects on Lef1 function (Hussein et al., 2003), and via *Lfng* expression. How Lfng affects Notch signalling, cell-autonomously or non cell-autonomously, affecting the bidirectional Notch signalling, or inhibiting Notch activation by its ligands or by enhancing Notch activation by some of its ligands? The cell-autonomous effects seem probable as long as we don't get more evidence of the secretion and extracellular activity of Fng molecules. We could see induction of *Hes1* by Lfng protein administrated on dental tissue (II), but we could not exclude the possibility that the large amounts of ectopic protein were maybe endocytosed by the cells and subsequently recycled to the Golgi apparatus. There is data about Fng activity inhibiting Serrate and its vertebrate

homologue Jag signalling via Notch (Panin et al., 1997; Hicks et al., 2000), so we can speculate that in the incisor, the Notch signalling induced by Jag2 is inhibited in the transit-amplifying cells expressing Lfng, allowing these cells to begin their differentiation processes.

Eda pathway is essential for proper formation of ectodermal organs in mouse and human, as shown by many studies of the ectodermal defects caused by Eda signalling deficiency. In this work enhanced Eda pathway signalling activity was shown to stimulate the ectodermal organ development, as evidenced by the larger placodes leading to bigger and extra organs in the transgenic mice overexpressing *Eda-A1* in their ectoderm. Proliferation was outruled as a mechanism of placode formation, leaving cellular attachment properties and migration aspects open for future studies.

Hair placodes form normally well separated from each other, the secondary placodes appearing in between the first ones in a hexagonal pattern. Molecular mechanisms in placode formation include the activators such as Eda, Wnts and Fgfs and inhibitors such as Bmps. These activators and inhibitors seem to interact in a way resembling the lateral inhibition model associated to Notch signalling in certain developmental systems. In addition to similarity of the mechanistic models between Notch function in *Drosphila* (Artavanis-Zakonas et al., 1999) and regulation of cell fates in mouse hair placode formation, Notch pathway probably is actually involved in the placode formation and patterning. First, the Notch pathway molecules are expressed in forming hairs (Favier et al., 2000). Second, Notch pathway has been associated to Nfkb1 signalling, and thus to numerous other molecular signalling networks via Nfkb1. Furthermore, intracellular domain of Notch can function as a direct coactivator for Lef1 transcription factor (Ross and Kadesch, 2001). Hence, conceivably the signals through Edar and Notch are integrated at some levels of pathways, so that the outcome of their interactions is that Edar signalling promotes the placodal cell fate. As pure speculation, this might be the result of Notch functioning together with Lef1 (Ross and Kadesch, 2001) in promoting interplacodal skin fate, and this signalling can possibly be switched off or modulated to lead to other cell fate directions by Edar signalling in the placodes.

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David Hume: Truth springs from argument amongst friends.

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