

**The function of Bmps and Runx2 in normal tooth development and in  
the pathogenesis of cleidocranial dysplasia**

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## LIST OF ORIGINAL PUBLICATIONS

**I** Åberg, T., Wozney, J., and Thesleff, I. Expression patterns of bone morphogenetic proteins (Bmps) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. *Developmental Dynamics* 1997; 210: 383-396.

**II** D'Souza, R.N. \*, Åberg, T. \*, Gaikwad, J., Cavender, A., Owen, M., Karsenty, G., and Thesleff, I. *Cbfa1* is required for epithelial-mesenchymal interactions regulating tooth development in mice. *Development* 1999; 126: 2911-2920.

\* Equal contribution

**III** Åberg, T., Cavender, A., Gaikwad, J.S., Bronckers, A.L., Wang, X., Walimo-Siren, J., Thesleff, I., and D'Souza, R.N. Phenotypic changes in dentition of *Runx2* homozygote-null mutant mice. *Journal of Histochemistry & Cytochemistry* 2004; 52: 131-139.

**IV** Åberg, T. \*, Wang X. \*, Kim, J-H., Yamashiro, T., Bei, M., Rice, R., Ryoo, H-M., and Thesleff, I. *Runx2* mediates FGF signalling from epithelium to mesenchyme during tooth morphogenesis. *Developmental Biology*. 2004; 270: 76-93.

\* Equal contribution

## ABBREVIATIONS

AER	Apical ectodermal ridge
APC	Adenomatous polyposis coli
ActR	Activin receptor
Alk	Activin like receptor-like kinase
Bmp	Bone morphogenetic protein
Bmpr	Bone morphogenetic protein receptor
BSA	Bovine serum albumin
Cbfa	Core binding factor alpha
Cbfb	Core binding factor beta
CCD	Cleidocranial dysplasia
Dhh	Desert hedgehog
DNA	Deoxyribonucleic acid
Dkk1	Wnt inhibitor dickkopf
Dlx	Vertebrate homologue of <i>Drosophila distal-less gene</i>
Dspp	dentin sialophosphoprotein
E	Embryonic day
Eda	Tnf ligand ectodysplasin
Edar	Tnf receptor for ectodysplasin
Fgf	Fibroblast growth factor
Fgfr	Fibroblast growth factor receptor
Fox	Forkhead box
Gli	Vertebrate homologue of <i>Drosophila cubitus interruptus gene</i>
Gsk	Glycogen synthase kinase
HED	Hypohidrotic ectodermal dysplasia
Hip	Hh interacting protein
Hh	Hedgehog
Hox	Vertebrate homeodomain box gene
Hspg	Heparan sulphate proteoglycans
Ihh	Indian hedgehog
Jnk	Jun N-terminal kinase
Lef1	Lymphoid enhancer factor 1
L-fng	Lunatic fringe
Lhx	LIM homeodomain genes
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
Msx	Vertebrate homologue of <i>Drosophila muscle segment (msh) gene</i>
Mmp	Matrix metalloproteinase
Osf2	Osteoblast specific factor 2
Otlx	<i>Rieg/Pitx2</i> , gene causing Rieger syndrome
P	Post natal day
p21	21 kD cyclin dependent kinase interacting protein
Pax	Paired- like homeobox
PCR	Polymerase chain reaction
Pebp2	Polyoma enhancer binding protein
RT-PCR	Reverse transcription – polymerase chain reaction
Pitx2	Pituitary homeobox 2
PST	proline/serine/threonine
PTHrP	Parathyroid hormone-related protein
Ptc	Patched

Q/A	glutamine and alanine repeats
Rankl	Receptor activator of nuclear factor $\kappa$ B ligand
Rieg	<i>Otlx2/Pitx2</i> , gene causing Rieger syndrome
RNA	Ribonucleic acid
Runx	Vertebrate homologue of the <i>Drosophila runt</i> gene
Shh	Sonic hedgehog
Smad	Vertebrate homologue of the <i>Drosophila mother against decapentaplegic (MAD)</i>
Smo	Smoothened
Tcf	T-cell specific transcription factor
Tgf $\beta$	Transforming growth factor
Tle	Transducin-like enhancer of split
Tnf	Tumor necrosis factor
Tnfr	Tumor necrosis factor receptor
Vegf	Vascular endothelial growth factor
Wnt	Wnt-family member
ZPA	Zone of polarizing activity

Mouse genes are written in italics and proteins in non italics. Human genes are written in uppercase letters and italics.

## ABSTRACT

Teeth form as epithelial appendages such as hairs and glands. During development, reciprocal and sequential epithelial-mesenchymal interactions regulate processes such as proliferation, differentiation and morphogenesis. These interactions are mediated by conserved signaling pathways that are reiteratively used during development of all organs. Bone development begins with the formation of mesenchymal condensates. Most bones form through the endochondral ossification mechanism although in the facial area intramembranous ossification predominates. Both tooth and bone development is characterized by formation of mineralised tissues, which in the tooth include bone-like dentin and cementum as well as epithelially derived enamel.

Mutations in genes encoding molecules in the signaling pathways cause numerous abnormalities in craniofacial bones and teeth including missing or supernumerary teeth, and disturbances in formation of dentin and enamel. Cleidocranial dysplasia (CCD) is a congenital syndrome where both bone and tooth development is affected. The syndrome is characterized by short stature, missing or abnormal clavicles, general bone dysplasia, tooth eruption problems, and supernumerary teeth. CCD is caused by mutations in *RUNX2*, a transcription factor that is a key regulator of osteoblast differentiation and bone formation

The first aim of this study was to analyse the expression of a family of key signaling molecule *Bone morphogenetic protein (Bmp)* at different stages of tooth development. Bmps have a variety of functions and they were originally discovered as signals inducing ectopic bone formation. We performed a comparative *in situ* hybridisation analysis of the mRNA expression of *Bmp2*, -3, -4, -5, -6, and -7 from initiation of tooth development to differentiation and crown formation. *Bmp2*, -4, and -7 were frequently coexpressed and showed marked associations with epithelial-mesenchymal interactions. Their expression shifted between the epithelium and mesenchyme starting from the stage of tooth initiation and they were subsequently expressed in the enamel knot, the signaling center regulating morphogenesis. In addition, their expression domains prior to and during the differentiation of odontoblasts and ameloblasts were in line with functions in cell differentiation and/or secretory activities of the cells. The expression of *Bmp3* was confined to mesenchymal cells, in particular to the dental follicle cells that give rise to the cementoblasts. *Bmp5* was expressed only in the epithelial ameloblasts. *Bmp5* may be involved in the induction and formation of dentine and enamel, and *Bmp3* in the development of cementum, respectively. The remarkable overlaps in the expression domains of different *Bmp* genes may implicate functional redundancy and/or formation of active heterodimers between different Bmps.

The second aim was to study the role of *Runx2* during tooth development and thereby to gain better understanding of the pathogenesis of the tooth phenotype in CCD. To elucidate *Runx2* function we analysed the tooth phenotype of *Runx2* knockout mice. Molars of both wild-type and *Runx2* mutant mice were analysed using several methods including *in situ* hybridisation, tissue culture, bead implantation experiments, and epithelial-mesenchymal recombination studies. *In situ* hybridisation analysis of *Runx2* expression showed that it is restricted to dental mesenchyme between the bud and early bell stages of tooth development. Although

*Runx2* regulates osteoblast differentiation, it was downregulated in fully differentiated odontoblasts. However, it was expressed in ameloblasts during the maturation phase of enamel formation. Epithelial-mesenchymal recombinants demonstrated that the dental epithelium regulates mesenchymal *Runx2* expression during the bud and cap stages. This effect could be mimicked by Fgf but not by Bmp in bead implantation assays.

Phenotypic analysis of *Runx2* *-/-* mutant tooth development showed that teeth failed to advance beyond the bud stage and that mandibular molars were more severely affected than maxillary molars. *Runx2* *-/-* tooth explants, when transplanted beneath the kidney capsules of nude mice, failed to progress in development. Tooth epithelial-mesenchymal recombinations using wild-type and *Runx2* mutant tissues indicated that the defect in the mesenchyme cannot be rescued by normal dental epithelium.

We searched for downstream targets of *Runx2* by extensive *in situ* hybridisation analysis. The expression of *Fgf3* was downregulated in the mesenchyme of *Runx2* *-/-* teeth. FGF-soaked beads failed to induce *Fgf3* expression in *Runx2* *-/-* dental mesenchyme whereas in wild-type mesenchyme they induced *Fgf3* in all explants. *Fgf3* was induced by overexpression of *Runx2* in cultured *Runx2* *-/-* calvarial cells suggesting that *Fgf3* may be a direct target of *Runx2*. Furthermore, *Runx2* was downregulated in *Msx1* *-/-* tooth germs indicating that *Runx2* functions downstream of *Msx1* in the dental mesenchyme. *Shh* expression was absent from the enamel knot in the lower molars of *Runx2* *-/-* and reduced in the upper molars. However, neither FGF or *Shh* could rescue the expression of *Shh* in the enamel knot nor morphogenesis of the *Runx2* *-/-* molars. Other enamel knot markers were expressed normally in mutant upper molars, while reduced or missing in lower molars.

In conclusion, these studies showed that *Runx2* regulates key epithelial-mesenchymal interactions that control advancing tooth morphogenesis and histodifferentiation of the epithelial enamel organ, and that it mediates the functions of epithelial Fgf signals regulating the expression of mesenchymal *Fgf3* which may be a direct target gene of *Runx2*. These data indicate a non-redundant role for *Runx2* in tooth development that may be distinct from its role in bone formation. In addition, in the upper molars of *Runx2* mutants extra buddings occurred at the palatal side of the tooth bud which provided clues for the pathogenesis of CCD. We suggest that these extra buds occur at sites where the formation of the secondary teeth is normally prevented in mice and that *Runx2* acts as an inhibitor of successional tooth formation by preventing advancing development of the buds. Accordingly, we propose that *RUNX2* haploinsufficiency in humans cause incomplete inhibition of successional tooth formation resulting in supernumerary teeth.

## **REVIEW OF THE LITERATURE**

### **Regulation of embryonic development**

Embryonic development is regulated by the genetic code and epigenetic interaction. The development of an organism is accomplished mainly by proliferation, differentiation, patterning and morphogenesis. Embryonic development begins with a single cell, the fertilized egg, which divides mitotically to produce a multicellular organism in which each cell contains the same DNA. During development growth can be accomplished by proliferation, or increase of cell size or by extracellular matrix. Cells that have undergone proliferation usually continue to either proliferate or differentiate. Differentiation results from the expression of specific set of genes according to the type of cells. Patterning is providing the blueprint of the organism, defining where each tissue and organ should be in the three dimensional space. Morphogenesis regulates the modeling of each tissue and organ to produce the shape and function needed. Patterning and morphogenesis are closely linked processes and by morphogenesis the organism is patterned. During gastrulation of the embryo, three germ layers are produced, endoderm, mesoderm and ectoderm. Endoderm gives rise to the lining of the digestive tube and respiratory system. From mesoderm form the bones, muscle, heart, blood cells, kidneys and gonads. Ectoderm gives rise to epidermis, nervous system, pigment cells and, in teeth, the enamel producing ameloblasts. Signals and signaling pathways that regulate morphogenesis have been conserved between different organs, and even between invertebrates and vertebrates (reviewed by Gilbert, 2006).

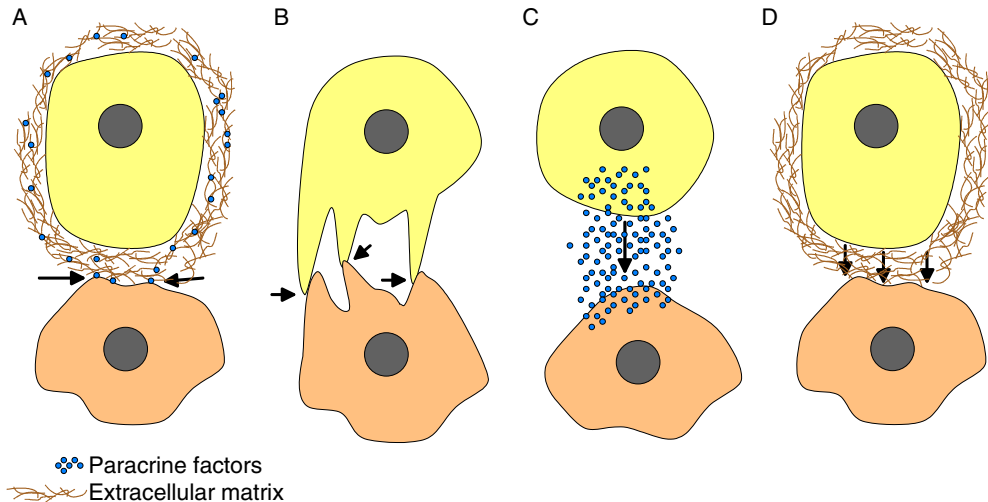
Congenital anomalies can be caused by genetic or environmental factors. Syndromes consist of sets of developmental abnormalities that are linked to each other. Organs that are linked in developmental syndromes share either a common origin or a common mechanism of formation. In recent years, the molecular etiology of several craniofacial developmental syndromes have been described, including CCD, hemifacial microsomia, Treacher Collins syndrome and many more. In addition, many mutations are known which cause dental abnormalities such as congenitally missing teeth, supernumerary teeth, and dentin and enamel formation disturbances. By finding the causative gene mutation in a developmental syndrome, the understanding of molecular pathogenesis can be further studied and eventually lead to a more complete etiological picture and thus targeted and more effective treatment (reviewed by Rice, 2005).

### **Cell and tissue interactions**

Inductive interactions involve an inducer that signals to a responding tissue, which, if competent, responds to the signal (Fig. 1). Throughout development, reciprocal and sequential inductive interactions take place between tissues. This mechanism ensures that the adjacent tissues are compatible with each other and that the timing is controlled. Cascades of inductive events are responsible for organ development. Most organs are formed of epithelial and mesenchymal tissues and interactions between these tissue layers regulate proliferation, differentiation and morphogene-

sis and are transduced by signaling molecules. Depending on developmental stage and organ system, either epithelium or mesenchyme may play an instructive role in organogenesis. In many organs the mesenchyme is the dominant tissue in early stages but later this capacity shifts to the epithelium by the epithelial-mesenchyme interactions.

### Signal transduction



**Fig. 1.** Inductive interactions are transmitted through either juxtacrine or paracrine mechanisms. Juxtacrine interactions take place between the cell membranes of adjacent cells or between a cell membrane and an extracellular matrix secreted by another cell (A, B). Paracrine interactions occur when a cell or tissue secretes soluble proteins that induce changes in neighbouring cells (C, D). Paracrine factors are proteins secreted by inducing cells that bind to cell membrane receptors in competent responding cells. In addition, some paracrine factors are thought to act as morphogens, which can diffuse over several cell layers, form concentration gradients and thus, specify distinct cell fates. Competent cells respond to paracrine factors through signal transduction pathways. Signal transduction pathways begin with a paracrine or juxtacrine factor (signaling molecule) binding to cell membrane receptor which leads to activation of the cytoplasmic domain of the receptor protein. This activity allows the receptor to phosphorylate other cytoplasmic proteins. Eventually, a cascade of such reactions activates a transcription factor (or set of factors) that activates or represses specific gene activity (reviewed by Gilbert, 2006).

### Transcription factors

There is cross talk between signal transduction pathways, which allows the cell to respond to multiple inputs simultaneously. The maintenance of the differentiated state can be accomplished by positive feedback loops involving transcription factors, or signaling molecules. Transcription factors are proteins that bind to enhan-

cer or promoter regions and interact to activate or repress the transcription of a particular gene. Transcription factors have three major domains. The first is a DNA-binding domain that recognizes a particular DNA sequence. The second is a trans-activating domain that activates or suppresses the transcription of the target gene to whose promoter or enhancer the factor has bound. The third is a protein-protein interaction domain that allows the transcription factor's activity to be modulated by other transcription factors.

Transcription factors can be grouped together in families based on similarities in structure and DNA-binding sites (reviewed by Gilbert, 2006). Such transcription factor families include Homeobox (including Msx, Dlx, Otx, Hox), basic-helix-loop-helix, Runx, Pax, Fox, Basic leucine zipper, Zinc finger, Nuclear hormone receptors and Sry-Sox families.

## **Families of signal molecules**

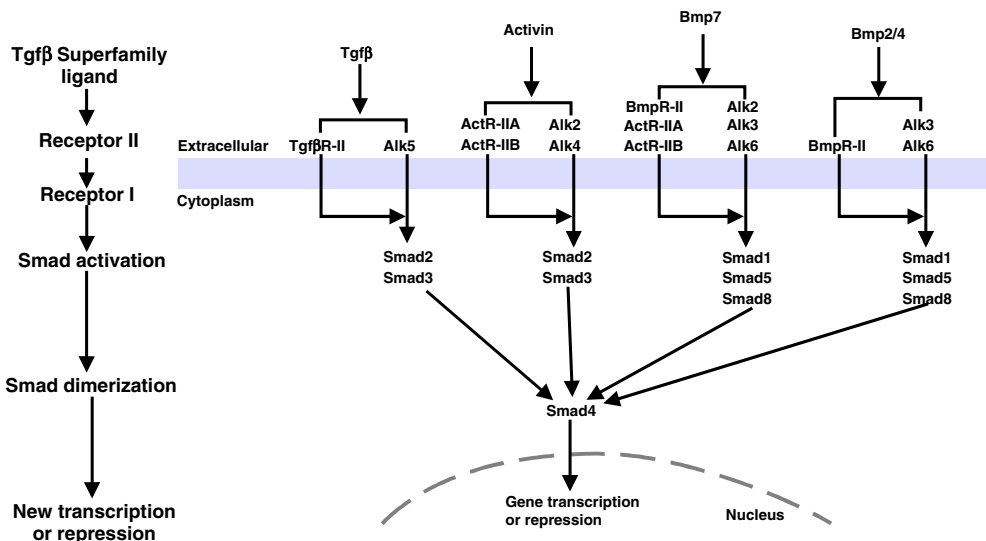
Structure and function of signaling molecule families involved in this study will be described in following section. Some details about possible functions during embryonic development are also mentioned. Involvement in tooth and bone development will also be briefly described. The stages of tooth development is described in Fig. 5 (page 25) and 6 (page 29). Tooth and bone development, including molecular regulation, is described in detail in a later section (pages 20-32).

### **Tgf $\beta$ superfamily**

#### Structure and function

The Tgf $\beta$  superfamily consists of more than 50 structurally related members with several subgroups including Tgf $\beta$ s, Bmps and Activin/Inhibin. Most of the molecules are signaling factors and their inhibitors (Massague, 2000; Balemans and Van Hul., 2002). As in many other signal transduction pathways the expression of both the signal, its inhibitor and the receptor is under intrinsic control. In the Tgf $\beta$  superfamily the signal transduction is fairly well known and studied. Members of the Tgf $\beta$  superfamily are synthesized as large precursor molecules that are proteolytically cleaved to yield biologically active dimers. Homodimer is the most common form but some members, e.g., Activins and Bmps, can also form heterodimers (Hogan, 1996).

The activated type I receptor propagates the signal downstream through the phosphorylation of the cytoplasmic Smad family transcription factors (R-smads, receptor regulated). R-smads form heteromeric complexes with Smad4 (common partner Smad, co-smad) mediating the signal into the nucleus for the activation of target genes (Peik et al., 1999; Massague and Wotton, 2000; Balemans et al., 2002). Tgf $\beta$  and Activin signals are mediated by R-smads Smad 2 and Smad 3, and Bmp signals by Smad1, -5 and -8, respectively (Fig. 2).



**Fig. 2.** Schematic presentation of the Tgfβ superfamily signal transduction. Tgfβ1-3, Bmp2-7 and Activin all bind to similar receptor complexes. The receptor complex consists of two transmembrane type I receptors (also known as Activin like receptor-like kinase, Alk) and two type II receptors with serine-threonine kinase activity (Piek et., 1999). Several different isoforms of type I and type II receptors exist and their affinity for the different signaling molecules in the Tgfβ family varies. Tgfβ binds to the TgfβR-II type II receptor, and Activin to the type II receptors Actr-IIA and -IIB. Bmp2/4 bind to the type II receptor Bmpr-II. Bmp7 binds both to the Bmpr-II and Actr-IIA and -IIB. Of the type I receptors Tgfβ binds to Alk5, Activin to Alk2, and -4, Bmp7 to Alk2, -4, and -6 and Bmp2/4 to Alk3 and -6. Upon ligand binding a ligand-receptor complex is formed and the type II receptor activates type I receptor by phosphorylation.

Regulation of the signal transduction is delicately controlled by inhibitory signals on all levels in the pathway. In the Bmp signaling pathway, inhibition takes place intracellularly by inhibitory Smads (I-Smads; Smad6 and -7). Smad6 appears to inhibit Bmp signaling by binding to the Smad1, and Smad7 inhibits Tgfβ signaling by binding to Smad2 and -3 and thus interfering with the complex formation with Smad4 (reviewed by Tsumaki and Yoshikawa, 2005). In addition, Smurfs inhibit Bmp and Tgfβ signaling intracellularly, by degrading Smads1 and -5 and by interacting with Smads6 and -7 to degrade Bmp and Tgfβ type I receptors (Tsumaki and Yoshikawa, 2005). Inhibition can also take place at the plasma membrane level by pseudoreceptor Bambi. At the extracellular level several modulators of the Bmp signaling are known including Noggin, Chordin, Chordin-like, Dan/Cerberus protein family, Sclerostin, Ectodin, Follistatin and Follistatin-related proteins (Balemans and Van Hul, 2002; Laurikkala et al., 2003). In addition, Bmp3 has been shown to inhibit Bmp signaling (Daluisi et al., 2001; Gamer et al., 2005). Bmp/Tgfβ signaling can also be transduced intracellularly by MAP kinase signaling pathways JNK, p38 and Erk that does not involve Smads (Derynck et al., 2003).

## Tgfb $\beta$ superfamily during embryonic development

Members of the Tgfb $\beta$  superfamily play diverse roles during embryogenesis, including morphogenesis and angiogenesis, and in the regulation of homeostasis in adult tissues. A wide spectrum of cellular functions during embryogenesis such as proliferation, apoptosis, differentiation, and migration are controlled by Tgfb $\beta$ s (Peik et al., 1999; Massague, 2000; Balemans et al., 2002). The role of Tgfb $\beta$ 1-3, Bmp2-7, Activin and Follistatin are probably best known and will be addressed in more detail as examples of function of the superfamily members.

### Tgfb $\beta$ 1-3

Three isoforms of Tgfb $\beta$  are known which have unique expression patterns, e.g. *Tgfb $\beta$ 1-3* (Pelton et al., 1991). Tgfb $\beta$ 1-3 are involved in proliferation and differentiation of osteoblasts, osteoclasts and chondroblasts (reviewed by Karsenty, 1998). *Tgfb $\beta$ 1* expression was the first to be shown to shift between epithelium and mesenchyme and to be associated with known inductive tissue interactions. In tooth development at bud stage, *Tgfb $\beta$ 1* is expressed in the epithelium and then shifts to the condensing mesenchyme. During cap stage it is expressed intensely in the cervical loop epithelium (Vaahtokari et al., 1991). *Tgfb $\beta$ 1-3* are expressed intensely during odontoblast and ameloblast differentiation (Pelton et al., 1990; Vaahtokari et al., 1991). Tissue recombination experiments showed that epithelial *Tgfb $\beta$ 1* is induced/maintained by mesenchymal signals during tooth development (Vaahtokari et al., 1991). *Tgfb $\beta$ 2* deficient mice exhibit a wide variety of defects including a small dysmorphic mandible, cleft palate and a reduction in cranial bone size and ossification (Sanford et al., 1997). *Tgfb $\beta$ 3* knockout mice have cleft palate (Proetzel et al., 1995). A role for Tgfb $\beta$  in suture development has also been demonstrated. *Tgfb $\beta$ 1* and -2 are associated with rat suture development and continue to be expressed later during suture closure while Tgfb $\beta$ 3 has a role in keeping calvarial sutures patent (Opperman et al., 1997; Roth et al., 1997, Lin et al., 1997).

### Bmp2-7

Bmps were found by their ability to induce ectopic bone and cartilage formation, and hence named bone morphogenetic proteins (Urist, 1965; Wozney et al., 1988). Bmps can initiate ectopic bone formation and they have been suggested to be of key importance during mesenchymal condensation (reviewed by Li and Cao, 2006). Bmps have been shown to have functions in chondrocyte and osteoblast differentiation mediated through induction of *Msx* genes (reviewed by Li and Cao, 2006). *Bmps* are expressed in the condensed mesenchyme of bone primordia and in the perichondrium and periosteum (*Bmp2*, -4, -5 and -7; Lyons et al., 1989; Kingsley, 1994), as well as in osteoblasts and hypertrophic cartilage (*Bmp6*; Gitelman et al., 1994, 1995). *Bmp5* inactivation causes the mouse mutation short ear, which is characterized by several skeletal defects (Kingsley, 1992). Apart from skeletal development, Bmps have also pleiotropic roles in the induction of ventral mesoderm, differentiation of neural tissues, and in controlling multiple organogenetic processes (reviewed by Hogan, 1996). Bmp signals have been shown to have

a wide range of biological activities and regulating cell growth, proliferation, differentiation, chemotaxis and apoptosis in various cell types. Several knockouts of *Bmps* have been made to elucidate their function during embryogenesis. *Bmp2/4* knockout mice are early embryonic lethal (Winnier et al., 1995; Zhang and Bradley, 1996). *Bmp3* knockout mice exhibit increased bone density (Daluiski et al., 2001). *Bmp6* null mutant mice have delayed sternum ossification (Solloway et al., 1998). *Bmp7* knockout mouse revealed that apart from few skeletal deformities several other defects were found including those in kidneys and eyes (Dudley et al., 1995; Luo et al., 1995, reviewed by Karsenty, 1998). *Bmp2* has been shown to possess functions during heart development and *Bmp4* in the development of lungs and testis (reviewed by Karsenty, 1998). *Bmp4* and *7* are involved in the patterning of the neural tube (reviewed by Hogan, 1996). *Bmp2*, *-4*, and *-7* are parts of the signaling networks that regulate patterning and outgrowth in the developing limbs (Francis et al., 1994). In the developing hair follicle *Bmp7* is expressed in the epithelial placode whereas *Bmp4* is expressed in the mesenchymal condensation beneath the placode (Chuong 1998; Millar 2002).

### *Bmps* in tooth development

The expression of several *Bmps* has been localized to the developing tooth at several stages (Vainio et al., 1993; reviewed by Thesleff and Mikkola, 2002). Of particular interest was the observation that *Bmp4* is associated with the shift of odontogenic potential from epithelium to mesenchyme (Vainio et al., 1993). *Bmps* have been shown to participate in epithelial mesenchymal signaling regulating tooth morphogenesis (Thesleff et al., 1995). *Bmp2*, *-4*, and *-7* were localized in a restricted epithelial cell population, the enamel knot together with other signaling molecules suggesting functions as signals regulating the shape of teeth (Jernvall et al., 1994; Vaahtokari et al., 1996; Jernvall et al., 1998). Several *Bmps* have been localized in odontoblasts and ameloblasts (Lianja et al., 1993; Begue-Kirn et al., 1994).

### Activin and Follistatin

Activins regulate growth and differentiation in many biological systems, including mesoderm induction, development of reproductive system, erythropoiesis, neural cell differentiation and bone remodelling (Thomsen et al., 1990, Woodruff, 1998; Maeshima et al., 2001; Chang et al., 2001; Lin et al., 2003). *Activin  $\beta$ A* is expressed in the dental mesenchyme since the initiation of tooth development. At bell stage, intense *Activin* transcripts are restricted to the cusp region of dental papilla mesenchyme (Heikinheimo et al., 1997; Ferguson et al., 1998). *Activin  $\beta$ A* null mutant mice die soon after birth with defects in whiskers, hard palate and teeth (Matzuk et al., 1995). *Activin  $\beta$ A* null mutant mandibular molars and incisors are arrested at the bud stage, but maxillary molars are unaffected (Ferguson et al., 1998). Activin acts on the dental epithelium and reciprocally induces the expression of its own inhibitor *Follistatin* (Ferguson et al., 1998)

Follistatin is an extracellular inhibitor of several members of the Tgfb superfamily proteins, including Activin, *Bmp2*, *-4*, and *-7* (reviewed by Li and Cao,

2006; reviewed by Rosen, 2006). It has been shown that Follistatin regulates tooth morphogenesis and tooth crown shape (Wang et al., 2004). Furthermore, Follistatin regulates enamel patterning in mouse incisors by asymmetrically inhibiting Bmp signaling and thus ameloblast differentiation (Wang et al., 2004).

## **Fgf family**

### Structure and function

Fgfs form a growth factor family of intercellular signaling molecules which in vertebrates consists of 22 members (Ornitz and Itoh, 2001). Fgfs are major regulators of embryonic development and affect a variety of cellular functions such as proliferation, survival, differentiation, adhesion and migration (Szebenyi and Fallon, 1999). Fgfs bind to cell surface receptors which are single transmembrane proteins with intracellular tyrosine kinase domains. Their extracellular domain consists of immunoglobulin-like motifs one of which (the Ig-3 loop) determines the ligand selectivity. There are four Fgf receptor genes in vertebrates, but in dental tissues only three *Fgfr* are expressed (*Fgfr1*, -2, and -3). Alternative splicing creates receptors with two different Ig-3 loops and hence *Fgfr1*, -2, and -3 exist in two isoforms with differences in ligand binding. The extracellular domain of Fgf receptors interacts with heparan sulphate proteoglycans (HSPG), including syndecan and perlecan, which regulates ligand binding (Zhu et al., 1991; Faham et al., 1996; Ornitz 2000; Ornitz and Itoh 2001). When a Fgf-cofactor complex binds to an *Fgfr*, the receptor either homo- or heterodimerizes, which in turn leads to phosphorylation and intracellular signal transduction (Givol and Yayon, 1992; Johnson and Williams, 1993; Faham et al., 1996). Numerous indirect and direct Fgf-regulated target genes have been identified including *Sprouty* genes encoding inhibitors of Fgf signaling (Casici et al., 1999; Maillieux et al., 2001).

### Fgfs during embryonic development

Most if not all Fgfs are required throughout embryonic development for the morphogenesis of various organs and tissues. *Fgfs3*, -4 and -5 are involved in early development both prior to and during gastrulation and neurulation (Wilkinson et al., 1988; Haub and Goldfarb, 1991; Niswander and Martin, 1992; Slack, 1994, reviewed by Crossley and Martin, 1995). *Fgfs1*, -2, -3, -4, -8, and -9 are involved in limb bud development (reviewed by Martin, 1998) and *Fgf8* is involved in brain development (Crossley et al., 1996). In the lung, the Fgf family member, *Fgf10* has the potential to induce bud formation and thus it stimulates branching morphogenesis (Bellusci et al., 1997). The role of Fgfs has also been analyzed extensively in the morphogenesis of a variety of organs developing as epithelial appendages such as hairs, glands and teeth (Chuong, 1998; Kettunen and Thesleff, 1998; Kettunen et al., 2000; Maillieux et al., 2001).

## Hedgehog family

### Structure and function

Hedgehog (Hh) gene was discovered originally in *Drosophila* as a signal regulating segmental and imaginal disc patterning. *Drosophila* has only one *hedgehog* gene whereas mammals have three – *Sonic hedgehog*, *Indian hedgehog* and *Desert hedgehog* (*Shh*, *Ihh*, *Dhh*) (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993). They encode secreted proteins that are involved in signaling between cells. Hh signal transduction is mediated by transmembrane receptors Smoothed (Smo) and Patched, which has two genes in mammals (Ptc1 and -2). Ptc inhibits Smo but only when a ligand binds to Ptc this inhibition is prevented allowing signal transduction. Ptc itself is also regulated by Hhs establishing a negative feedback loop. Signal transduction through Smo activates intracellular targets including Gli family zinc finger transcription factors (Gli1, -2, -3) (Ingham, 1998). Ptc1 as well as Hh interacting protein (Hip) also restricts Shh signaling by sequestering Shh protein. Hip is a membrane bound protein that binds to all three Hhs (Chuang and McMahon, 1999; Cobourne and Sharpe, 2002).

### Hh in embryonic development

In vertebrates, Hhs are involved in cell fate specification, cell survival and cell proliferation in different developmental processes such as limb morphogenesis, myotome and sclerotome specification, cartilage differentiation, hair follicle development, regulation of left-right asymmetry, cranial neural crest survival, motor neuron induction and spermatogenesis (Hammerschmidt et al., 1997; reviewed by Ingham and McMahon, 2001).

Ihh has rather restricted functions during development, regulating mainly skeletal differentiation. In addition it is expressed in gut and in cartilage and is important for postnatal bone growth (Bitgood and McMahon, 1995; Bitgood et al., 1996). Dhh is involved in spermatogenesis and is expressed in Schwann cells and Sertoli cells of the testis. *Dhh* knockout mice exhibit defective spermatogenesis (Bitgood and McMahon, 1995; Bitgood et al., 1996; Clark et al. 2000). In contrast, Shh regulates a wide variety of developmental events such as left-right asymmetry and the patterning of the neural tube and limbs. Shh also regulates the morphology of several organs, predominantly epithelial structures dependent on epithelial-mesenchymal interactions in lung (branching), hair follicle, tooth and bladder. Shh is expressed in three regions that have key organiser activity in the embryo: notochord, floor plate and the zone of polarizing activity in the limb bud (McMahon, 2000; Martin, 1998; Vaahtokari et al., 1996). *Shh* deficient mice have several developmental abnormalities including defects in craniofacial structures, neural tube and limbs (Chiang et al., 1996).

## **Notch family**

### Structure and function

Notch was first identified in *Drosophila* as a large membrane receptor controlling cell fate decisions. In vertebrates four Notch homologs are known. In vertebrates the ligands to the Notch receptors are Jagged (*Drosophila* Serrate) and Delta-like molecules. In Notch signaling, both the ligand and receptor are transmembrane proteins and thus signal transduction requires intimate cell-cell contacts (Lewis, 1998; Bray, 1998; Artavanis-Tsakonas et al., 1999).

Ligand binding to the Notch receptor causes the release of the intracellular domain which translocates into the nucleus activating the target genes belonging to the Hes family of basic helix-loop-helix (bHLH) transcription factors, mainly *Hes1* and *Hes5* (Sasai et al., 1992; Takebayashi et al., 1995). Furthermore, activation of Notch in a given cell regulates production of Notch ligands by that cell (Lewis, 1998; Bray, 1998; Artavanis-Tsakonas et al., 1999).

Notch signaling activity is modified by Fringe molecules which are glycosyltransferases that affect the glycosylation of the molecules involved in the Notch signaling at the cell surface. There are three *Fringe* genes in mammals: *Lunatic Fringe*, *Radical Fringe*, and *Manic Fringe* (Wu and Rao, 1999). The modulation by Fringe is context-dependent and varies between different ligands. In *Drosophila*, Fringe inhibits a cell's ability to respond to Serrate protein (Jagged in vertebrates) and potentiates its ability to respond to Delta protein (Panin et al., 1997).

### Notch signaling during embryonic development

Notch signaling has been implicated in cell fate decisions and in the formation of tissue compartments during the development of insects and vertebrates (Artavanis-Tsakonas et al., 1999; Mustonen et al., 2002; Baron, 2003). Fringe functions in the establishment of tissue boundaries in *Drosophila*, and it has been implicated in border formation in the apical ectodermal ridge (AER) signaling centers in vertebrate limbs (Laufer et al., 1997). Interestingly, *Lunatic Fringe* (*L-Fng*) expression forms a sharp boundary with the enamel knot, which itself is devoid of *Fringe* expression. It is thus possible that L-Fng play a role in the formation of the enamel knot signaling center in the dental epithelium. However, tooth morphogenesis appears unaffected in *L-Fng* null mutant mice (Mustonen et al., 2002).

## **Wnt family**

### Structure and function

The vertebrate Wnt family contains over 20 secreted glycoproteins that are involved in a variety of developmental processes such as cell proliferation, migration and differentiation as well as in tumorigenesis (Wodarz et al., 1998; Sharpe et al., 2001). Wnts signal through the Frizzled family of receptors and to date more than ten receptors have been identified in vertebrates. Although extensive research, the ligand specificity and function of the receptors are still far from clear. Wnt function

is also extracellularly modulated by secreted Frizzled-related proteins, such as dickkopf, cerberus, and Wnt inhibitory factor-1. They are thought to have antagonist activity, although in some cases they may also act as Wnt agonists (Wang et al., 1997; Piccolo et al., 1999; Hsieh et al., 1999; Polakis, 2000; Zorn, 2001). Wnts have also a co-receptor, the low-density lipoprotein receptor-related protein (Lrp5/6) which bind the extracellular inhibitor Dickkopf (Huelsenken et al., 2001)

After ligand binding to the Frizzled receptor, several different signal transduction pathways can be triggered and at least three different signaling cascades are known. In the classical, canonical, pathway, Wnt signaling through Frizzled activates the intracellular Disheveled. This leads to the inactivation of the glycogen synthase kinase 3 enzyme (Gsk-3) and subsequently to  $\beta$ -catenin stabilisation. When Gsk-3 is active, it prevents  $\beta$ -catenin from dissociation from the adenomatous polyposis coli (APC) protein and thus targets  $\beta$ -catenin for degradation. However, when Wnts signal through Frizzled, activate Disheveled and thus inhibit Gsk-3,  $\beta$ -catenin can dissociate from the APC protein and translocate to the nucleus. In the nucleus  $\beta$ -catenin functions as a transcriptional coactivator by interacting with T cell factor/lymphoid enhancer binding factor (Tcf/Lef) transcription factors to mediate many of the effects of Wnts on gene transcription (reviewed by Krishnan et al., 2006).

Notably,  $\beta$ -catenin is also a structural adaptor protein linking cell-cell adhesion molecules cadherins to the actin cytoskeleton (Nelson and Nusse, 2004). In addition, APC functions as a tumour suppressor in adults and Gsk-3 also regulates glycogen metabolism (Korinek et al., 1997; He et al., 1998).

### Wnt gene family during embryonic development

The knowledge of the signaling and developmental functions of Wnts is based on the studies of the *Drosophila* wingless, the Wnt ortholog of the fly. Wingless is involved in segment polarity patterning and development of various organs. (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998; Huelsenken and Birchmeier, 2001). In *Xenopus* Wnts are also involved in axis formation. Wnts have different abilities to promote secondary axis formation in *Xenopus* and this correlates also with the oncogenic potential and thus Wnts are not functionally equivalent (Polakis, 2000; Kuhl et al., 2000). In humans, constitutive activation of the Wnt signal transduction can cause cancer (Lustig and Behrens, 2003). Mutations in *AXIN2*, part of the  $\beta$ -catenin degradation complex, can cause severe oligodontia and predispose to colorectal cancer (Lammi et al., 2004). In mice Wnts are involved in the development of most of the organs regulating cell proliferation, migration, differentiation and epithelial-mesenchymal interactions (Millar, 2002). In addition, Wnts are involved in skeletal development and bone remodelling (reviewed by Abe, 2006).

### Other signaling molecules

The tumour necrosis factor (TNF) family consists of more than 15 members, most of which are involved in regulating host defence, immunity, and inflammation (Baker and Reffy, 1998; Locksley et al., 2001). TNF family members are also involved in osteoclast survival and differentiation as well as terminal differentiation

of mammary gland alveolar buds (Filvaroff and Derynck, 1998; Fata et al., 2000). The identification of genes responsible for hypohidrotic (anhidrotic) ectodermal dysplasia (HED) syndromes has indicated that TNFs are also important regulators of ectodermal organogenesis (Kere et al., 1996; Headon and Overbeek, 1999; Mikkola et al., 1999). In ectodermal organ development two Tnf members have been implicated, *Ectodysplasin A-1 (EDA-A1)* and *EDA-A2*, which are splice variants of the same gene (Bayés et al., 1998; Yan et al., 2000). The receptor for EDA-A1 is EDAR (mouse Downless) and for EDA-A2 XEDAR (Headon and Overbeek, 1999; Yan et al., 2000). For a third receptor, TROY, the ligand is unknown (Hu et al., 1999; Eby et al., 2000; Kojima et al., 2000). *Troy* is coexpressed in teeth with *Edar* (Pispa et al., 2003).

### **Tooth as a model for ectodermal organ development**

The early embryonic ectoderm is divided into three major domains: neural crest, neural tube and the surface ectoderm. The neural crest gives rise to the peripheral nervous system, melanocytes, most craniofacial bones and cartilage and the mesenchymal component of the tooth. The neural tube gives rise to the brain and spinal cord. The main derivative of surface ectoderm is the epidermis of the skin but also several other structures like oral epithelium, olfactory epithelium, lens and the cornea develop from surface ectoderm. Several organs including hair, feather, tooth and glands are formed as appendages of the embryonic ectoderm. These structures are formed of the ectoderm and the underlying mesenchyme through sequential and reciprocal epithelial-mesenchymal interactions (Gilbert, 2006; Pispa and Thesleff, 2003).

During development epithelial appendages protrude out of the epithelium (hair, teeth, feathers) or invaginate into the surrounding mesenchyme (glands including sweat-, saliva- and mammary glands). All these structures share similar regulatory mechanisms and developmental stages, such as initiation, morphogenesis and differentiation. Accordingly, the developmental regulatory molecules are shared between these organs (Thesleff and Mikkola, 2002).

At the initiation stage, a thickening of the epithelium forms an ectodermal placode, which causes the condensation of underlying mesenchymal cells. The placode will subsequently invaginate into the mesenchyme and form an epithelial bud (teeth, hair, and glands) except in feather development where the bud will grow out of the mesenchyme. The bud will then undergo morphogenesis, by growing, folding and/or branching, and attain the final size and shape of the organ. Through differentiation cells gain their organ specific functions and form structures such as dentin and enamel of teeth and secretory cells of glands (Pispa and Thesleff, 2003).

### **Mammalian tooth development**

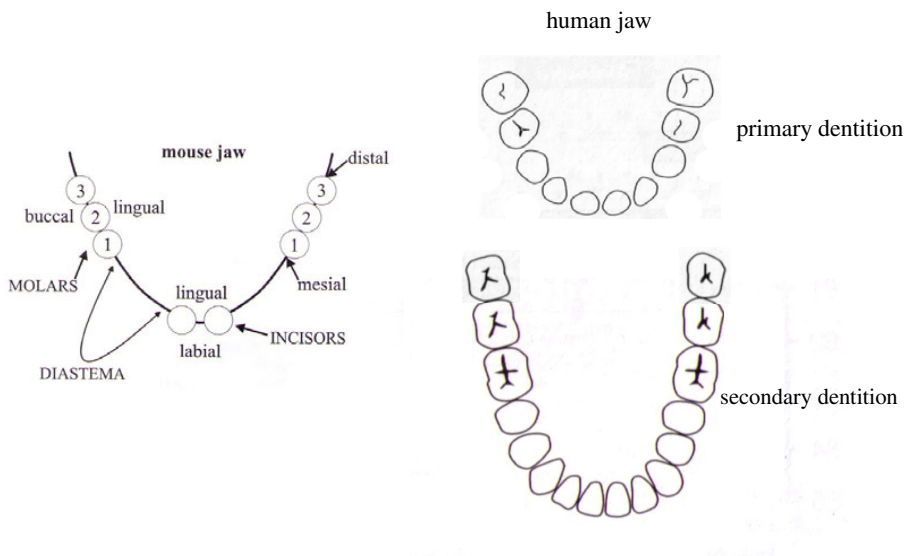
The development of mammalian dentition requires a sequence of events determining the dentition as a whole, subregions of tooth classes (e.g. incisor and molar regions), and morphogenesis of individual teeth within each tooth class (Jernvall and Thesleff, 2000). Mammals usually have three different tooth families: incisors, canines and molars (including premolars). Incisors have blade like crowns for cut-

ting the food whereas canines have a more cone-shaped crown suitable for piercing or tearing the food. Molar crowns consist of several cusps and are used to grind and masticate food (Fig 3; Berkovitz et al., 2002).

Tooth development is a good model for organ development and it has been studied extensively in mice. Although timing, number and final shapes of human teeth differ from mice, it is likely that the signal transduction pathways and molecules are very similar. Furthermore, the early stages of tooth development are morphologically quite similar in mice and humans. In the following description of tooth development we mention the mouse embryonic day for each stage.

### Differences between mouse and human tooth development

In rodents, only incisor and molar tooth families are present (Fig. 3). In the toothless diastema region in rodents, between the incisors and molars, rudimentary tooth germs develop which are arrested by the bud stage and eventually removed by apoptosis (Tureckova et al., 1995; Keränen et al., 1999). In each jaw quadrant rodents have one incisor and humans have two, and both have three molars (Fig. 3). The incisors of rodents are continuously erupting in contrast to human incisors. Furthermore, the rodent incisors are different in having enamel only on the labial surface.



**Fig. 3.** Schematic drawings of mouse and human dentitions

## Primary and secondary dentition

Rodents have only one dentition whereas most mammals have two, deciduous and permanent teeth (Fig. 3). The permanent incisors, canine and premolars form from the dental lamina at its deepest extremity. The proliferating activity in the dental lamina leads to the formation of another tooth bud on the lingual aspect of the deciduous tooth germ. The molars of the permanent dentition have no deciduous predecessors and thus are formed as a backward extension of the dental lamina forming the first, second and third molar (Ten Cate, 1998). Permanent dentition is predominantly affected in human hypodontia which can be diagnosed in up to 8 % of the population, if wisdom teeth are not included (Thesleff and Pirinen, 2004).

## Initiation of tooth development

Mammalian tooth development is initiated from the ectoderm covering the maxillary, frontonasal and mandibular processes, where the dental lamina is formed. The dental lamina can be distinguished in mouse at embryonic day 10.5 (E10.5) as a horseshoe-shaped epithelial thickening. The mesenchymal component of the maxilla and mandible is derived from the neural crest cells that have migrated from the posterior midbrain and anterior hindbrain (Imai et al., 1996; Chai et al., 2000; Zhang et al., 2003). Classical tissue recombination experiments have demonstrated that the early stage oral epithelium (E9-11) possesses the odontogenic potential. After initiation stage, at E12, the odontogenic potential shifts to the mesenchyme (Mina and Kollar, 1987; Lumsden, 1988).

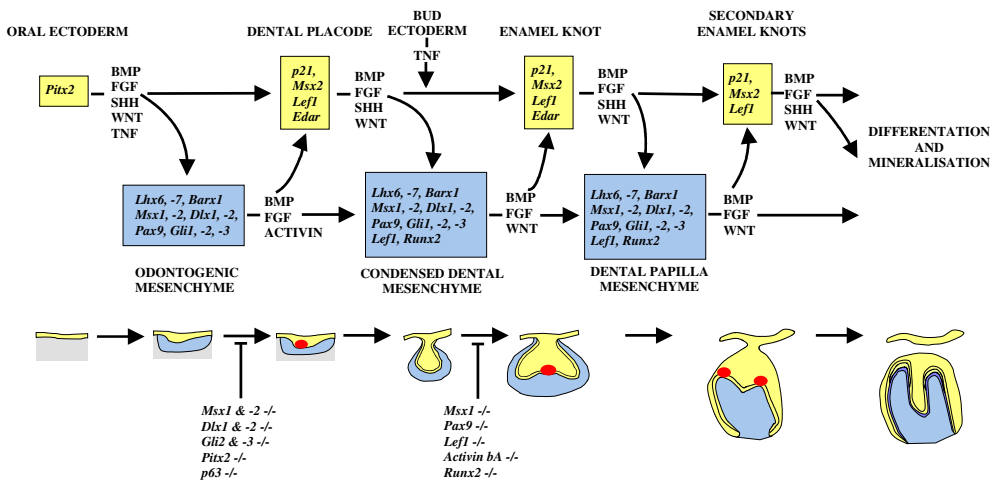
The complete picture of the signaling network leading to tooth induction is not known yet although extensive research has been conducted (Fig. 4). Antagonistic signaling between Bmps and Fgfs regulates various developmental processes in limbs and facial processes (Niswander and Martin, 1993; Buckland et al., 1998). *Fgf8* is expressed in the oral ectoderm and may be involved in the regional specification of the oral side of the maxillary and mandibular arch by inducing and regulating mesenchymally expressed transcription factors *Lhx6* and *Lhx7* (Grigoriou et al., 1998).

In addition, *Fgf8* stimulates and *Bmp2* and *Bmp4* inhibits *Pax9* expression in the mouse E10 branchial arch mesenchyme, which has also been suggested to determine the correct positions of individual tooth buds within the dental lamina (Neubuser et al., 1997).

*Pax-9* is a paired box transcription factor and its expression is restricted to the future tooth mesenchyme prior to any morphological signs of tooth development (E10.5). However, in *Pax9* deficient mice, the tooth buds do form in the dental lamina at the right locations, although tooth development does not proceed further (Peters et al., 1998). This indicates that other factors also contribute to the localisation of the tooth buds within the dental lamina. Indeed, other genes are more restricted to the dental lamina, such as the transcription factor *Pitx2* (*Otlx2*, *Rieg*) (Mucchielli et al., 1997). In mice deficient of *Pitx2*, the development of maxillary teeth is arrested at placodal stage and mandibular teeth are arrested at bud stage. In addition, in humans, haploinsufficiency of *PITX2* is associated with Rieger syn-

drome characterized by several missing teeth (oligodontia) (Semina et al., 1996; Flomen et al., 1998; Lin et al., 1999).

Antagonistic signaling between Shh and Wnt has been demonstrated to be involved in the definition of boundaries of developing tooth germs. *Shh* expression is restricted to the dental lamina of future incisor and molar regions at early stage and is later confined to the tips of the tooth buds (E11.5-14.5). In contrast, *Wnt7b* is expressed throughout the oral epithelium but is absent in the *Shh* expressing tooth forming regions (Sarkar et al., 2000; Hardcastle et al., 1998, Keränen et al., 1999). It has been suggested that *Wnt7b* restricts *Shh* expression to act locally in specific tooth forming regions where Shh stimulates cell proliferation required for the tooth bud formation (Gritli-Linde et al., 2001; Hardcastle et al., 1998; Sarkar et al., 2000).



**Fig. 4.** Schematic representation of the signals and transcription factors mediating the reciprocal signaling between epithelium and mesenchyme during advancing tooth development.

### Determination of tooth identity

It has been suggested that neural crest cells may be specified first as odontogenic lineage and later further regionally specified as maxilla/mandible/molar/incisor (Weiss et al., 1998; Tucker and Sharpe, 1999). Tissue recombination experiments have given an indication that the early dental epithelium and not the neural crest determines the tooth type (Kollar and Mina, 1991) but more recently early neural crest in mouse was shown to induce teeth in chick (Mitsiadis et al., 2003). *Fgf8* from the oral ectoderm induces the transcription factor *Barx1* in the molar region whereas *Bmp4* inhibits the expression in the incisor region. The epithelial *Bmp4* induces *Msx1* in the incisor area. After inhibiting *Bmp4* in the incisor region with

Noggin at E9-E10, the *Barx1* expression extended to the incisor area leading to the transition from incisor to molar (Tucker et al., 1998). This supports the theory that the oral ectoderm determines the tooth phenotype.

It has been suggested that dynamic patterns of signal molecules in the early facial process result in differential activation of key transcription factors. For instance, specific combinations of homeobox containing transcription factors might determine the identity of teeth (Tucker and Sharpe, 1999). There are a number of homeobox containing genes with overlapping and region-specific expression patterns in the facial ectomesenchyme, such as *Alx3*, *Barx1*, *Dlx1*, -2, -3, -5, -6, -7, *Pitx2*, *Msx1*, -2, *Lhx7*, -7 and *Gsc*. They are expressed prior to the first morphological signs of tooth development but also during later stages (Cobourne and Sharpe, 2003).

A great number of transcription factors have been identified as targets for early epithelial signals in branchial arch mesenchyme. Fgfs induce the expression of homeobox genes *Lhx6*, -7, *Msx1*, -2 and *Dlx1*, -2, Bmps upregulate *Msx1*, -2 and *Dlx1*, -2 whereas Shh induces *Gli1*, -2, and -3 (Bei and Maas, 1998; Hardcastle et al., 1998; Tucker et al., 1999).

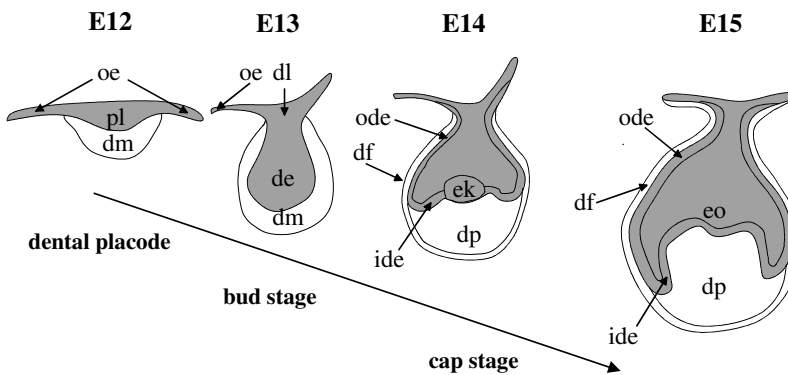
Several of the above mentioned molecules have been shown to be essential for tooth development at early stage, because deletion of their function in knockout mice results in an arrest of tooth development at the dental lamina stage. However, only double knockouts where functions of two genes have been deleted, such as both *Msx1* and *Msx2* cause an arrest of development at this stage. Similarly, *Gli2* and -3 double knockouts arrest also at the dental lamina stage except for the upper incisors that grow to bud stage. In addition, a double knockout of both *Dlx1* and -2 results in similar arrest at dental lamina stage except that it only affects maxillary molars (Thomas et al., 1997; Bei and Maas, 1998; Hardcastle et al., 1998). Therefore *Msx1*-2, *Gli2*-3 and *Dlx1*-2 transcription factors are probably functionally redundant because only when both genes are knocked out, an early tooth development arrest phenotype is seen. Although *Dlx1* and -2 are expressed both in the upper and lower molar regions, *Dlx5* and -6 are present only in the lower molars (Thomas et al., 1997; Weiss et al., 1998). This suggests that the latter genes compensate for the loss of *Dlx1* and 2 in the lower jaw resulting in the phenotype only in the upper jaw.

### **Dental placode - the early signaling center**

After a specific tooth type has been specified at a regionally specific location within the dental lamina, a thickening of the epithelium takes place (E11). The local thickening will then grow and protrude into the mesenchyme as an epithelial bud. Before the bud stage, epithelial signals induce mesenchymal signals that then act reciprocally on the dental epithelium to form the signaling center, also called a dental placode (Fig. 5). Other ectodermal organs have similar structures that share the morphological and molecular similarities with the dental placode (Pispa and Thesleff, 2003). The placode expresses locally several genes, including *Bmp2*, *Shh*, *Wnt10a*, *p21*, *Msx2* and *Lef1* (Jernvall and Thesleff, 2000) (Fig. 4). The early signaling center in tooth is similar to the apical ectodermal ridge in the limb bud and isthmus in central nervous system, as they regulate the behaviour of surrounding cells. At this stage, E11.5-E12, also the potential to instruct tooth development

shifts from the epithelium to mesenchyme (Mina and Kollar, 1987). Furthermore, it has been suggested that the mesenchyme may acquire the full competence to induce tooth development only after signals are received from the dental placode (Jernvall and Thesleff, 2000).

*Bmp4* and Activin  $\beta A$  have been proposed to be the key signals from the mesenchyme to induce the epithelial signaling center and subsequent budding of the tooth (Jernvall and Thesleff, 2000). Epithelial *Bmp4* induces the mesenchymal *Bmp4* expression via *Msx1* and this shift of expression from epithelium to mesenchyme (E11.5) coincides with the shift of the potential to instruct tooth development (Mina and Kollar, 1987; Vainio et al., 1993). Simultaneously, *Msx1* expression that have been widely expressed throughout the facial mesenchyme, becomes restricted to the tooth bud regions in the mesenchyme (Cobourne and Sharpe, 2003). In addition, *Bmp4* induces *p21* in the dental placode. *p21* is associated with the stop of cell proliferation and is expressed in several other signaling centers (Jernvall et al., 1998). Transgenic mice with deleted function of *Activin  $\beta A$* , which is expressed in the mesenchyme and induced by epithelial *Fgf8*, show a phenotype opposite to the *Dlx1-2* double knockout and all teeth except upper molars are arrested at early bud stage (Ferguson et al., 1998). In addition, exogenous Activin  $\beta A$  protein can rescue tooth development in the mutants but only when implemented at E11.5 and not later, which suggests that it has a key function in the initiation of the epithelial budding (Ferguson et al., 1998). Furthermore, in mice lacking *p63*, all ectodermal organs fail to develop, including tooth and hair. The dental lamina is formed in the *p63*  $-/-$  mouse but no tooth buds are formed (Laurikkala et al., 2006).



**Fig. 5.** Schematic representation of the stages of early morphogenesis of first molar tooth. oe, oral epithelium; dl, dental lamina; pl, dental placode; dm, dental mesenchyme; de, dental epithelium; ode, outer dental epithelium; df, dental follicle; ek, enamel knot; ide, inner dental epithelium; dp, dental papilla; eo, enamel organ.

## Enamel knot and bud to cap transition

After the dental placode has formed, the epithelial bud grows into the mesenchyme and at E13 it has reached its full vertical height. Subsequently the tip of the bud flattens due to the growth of the width of the bud. Simultaneously a new signaling center is formed, the primary enamel knot (E13.5-E14), at the tip of the widened bud (Fig. 5). The primary enamel knot is a non-proliferating transient structure that is thought to regulate the growth of the flanking epithelial cervical loops which invaginate and form a cap-shaped structure, surrounding the mesenchymal dental papilla (cap stage of tooth development) (Jernvall et al., 1998).

The transition from bud to cap stage is a critical step in tooth development and requires reciprocal and sequential signaling between the epithelium and mesenchyme. Interruption of this signaling leads to an arrest of tooth development at this stage shown in several knockout mice such as *Msx1*, *Lef1*, *Pax9*, and *Activin  $\beta$ A* (Jervall and Thesleff, 2000) (Fig. 4). In all these mutants, the formation of the enamel knot has been impaired which suggests that it is a prerequisite for the tooth bud to develop into cap stage. Interestingly, a common feature of the knockouts of transcription factors *Msx1* and *Pax9* is that *Bmp4* is missing from the dental mesenchyme. This indicates that *Bmp4* is a good candidate for the mesenchymal signal inducing the transition from bud to cap stage (Jernvall and Thesleff, 2000). This is also supported by experiments in which addition of exogenous *Bmp4* can rescue the arrest at bud stage of *Msx1* mutant embryos (Bei et al., 2000).

The primary enamel knot expresses, when formed at E13.5-E14, mRNA of several genes such as *Fgf9*, *Bmp2*, *Bmp7*, *Shh*, *Wnt10a*, *Msx2*, *Edar*, *p21* and *Follicle-statin* (Vaahtokari et al., 1996; Kettunen and Thesleff, 1998; Dassule and McMahon, 1998; Laurikkala et al., 2001; Jernvall et al., 1998; Heikinheimo et al., 1997). In addition, at cap stage (E14-E14.5), when the enamel knot is histologically distinguishable as a cluster of condensed cells at the tip of the tooth germ, also some additional genes are expressed including *Fgf3*, *Fgf4*, *Bmp4*, *Wnt3* and *Wnt10a* (Kettunen and Thesleff, 1998; Sarkar and Sharpe, 1999; Jernvall et al., 1998). Signal pathways of *Bmp*, *Fgf*, *Shh* and *Wnt* families have been shown to be integrated at different levels in tooth development and they partly activate the same transcriptional targets. Both *Fgfs* and *Bmp4* activate *Msx1* and *Dlx2*, although *Msx2* is only activated by *Bmp* and *Dlx1* by *Fgf* (Vainio et al., 1993; Bei and Maas, 1998; Kettunen and Thesleff, 1998). Furthermore, it has been suggested that *Lef1* may integrate *Wnt* and *Bmp* signaling as both can induce *Lef1* in the mesenchyme and *Lef1* interacts intracellularly with  $\beta$ -catenin, which in turn can regulate cell adhesion with E-cadherin (Dassule and Macmahon, 1998). *Shh* has been shown to repress *Wnt10b* expression in the dental epithelium and *Shh* receptor *Ptc*, which is regulated by *Shh*, require *Msx1* in the mesenchyme (Dassule and McMahon, 1998; Zhang et al., 1999).

*Fgf* have been suggested to be involved in the proliferation of the cervical loops and subsequent growth. All studied *Fgfs* function as mitogens in cultured dental tissues but epithelial *Fgf4* and *Fgf9* can stimulate proliferation of both epithelium and mesenchyme, whereas mesenchymal *Fgf10* can only stimulate epithelial cells (Jernvall and Thesleff, 2000). Abundance of *Fgf* receptors in the cervical loops and dental papilla suggest that these are target tissues, and this is in line with the distri-

bution of dividing cells in the tooth germ (Jernvall and Thesleff, 2000). The enamel knot expresses several growth stimulating signals, including *Fgf4*, *Fgf3* and *Fgf9*, while its cells remain non-proliferating themselves. This is probably due to the lack of Fgf receptors in the enamel knot itself (Jernvall et al., 1998; Kettunen and Thesleff, 1998). *p21* is also expressed in the enamel knot and is thought to be involved in the differentiation and withdrawal from the cell cycle (Jernvall et al., 1998). Interestingly, *p21* is also expressed in AER which is another signaling center expressing *Fgfs* while remaining itself non-proliferative (Parker et al., 1995). The reciprocal Fgf signaling between epithelium and mesenchyme regulating gene expression within the same signal family is similar as in limb development (Xu et al., 1998). It has been shown that *Lef1* is a critical survival factor for the dental epithelium during tooth morphogenesis (Sasaki et al., 2005). In *Lef1* mutants *Fgf4* is missing in enamel knot and *Fgf3* in dental mesenchyme and *Fgfs* can rescue the arrest of tooth development in *Lef1* mutants (Kratochwil et al., 1996, 2003).

### **Secondary enamel knots and cusp formation**

The primary enamel knot is a transient structure that is removed by apoptosis beginning from the distal/posterior end and proceeding until only the anterior portion remains. After primary enamel knot removal in teeth with more than one cusp (e.g. molars), new, secondary enamel knots form at the sites of future cusp tips. In this so-called bell stage, from E15 onwards, cervical loops grow downwards into the mesenchyme. Secondary enamel knots are non-proliferative, express *Fgf4* and are removed by apoptosis, similar to primary enamel knots (Vaahtokari et al., 1996; Coin et al., 1999b). The apoptosis in the enamel knot is associated with the expression of *Bmp4* which precedes the apoptosis of the knot cells. Apoptosis has been suggested to be a mechanism controlling the duration of the signaling, similar to the removal of the AER in the limb bud (Pizette and Niswander, 1999). *Bmp4* regulates also apoptosis in other events during development, e.g. in rhombomeres and formation of digits (Smith and Graham et al., 2001; Guha et al., 2002).

*Fgf4* expression is a good marker for secondary enamel knots and is strictly limited to the cusp tips whereas *Shh*, *p21*, *Fgf9* and mesenchymal *Bmp4* have more diffuse expression domains (Jernvall and Thesleff, 2000). Comparison between two mammalian species with different molar morphology suggested that the cusp patterning begins as early as E15, after removal of primary enamel knot, before any cusp development is evident morphologically (Keranen et al., 1998).

Secondary enamel knots form in a species-specific pattern and will dictate the future cusp pattern (Jernvall et al., 2000). The spacing of the secondary enamel knots must be accurately controlled as this determines the correct cusp position and size resulting in a functional, specific tooth shape in each species (Jernvall and Thesleff, 2000).

Bmps have been suggested to play a role in formation of periodic patterns by inhibiting the spreading of Fgf signaling (Jung et al., 1998). It has been suggested that in tooth, *Fgf4* functions as cusp activator while Bmps and possibly *Shh* could function as inhibitors regulating the distance between the cusps (Jernvall and Thesleff, 2000). *Shh* signaling was shown to be essential for asymmetrical growth of the enamel organ (Dassule et al., 2000). However, it has been suggested that the

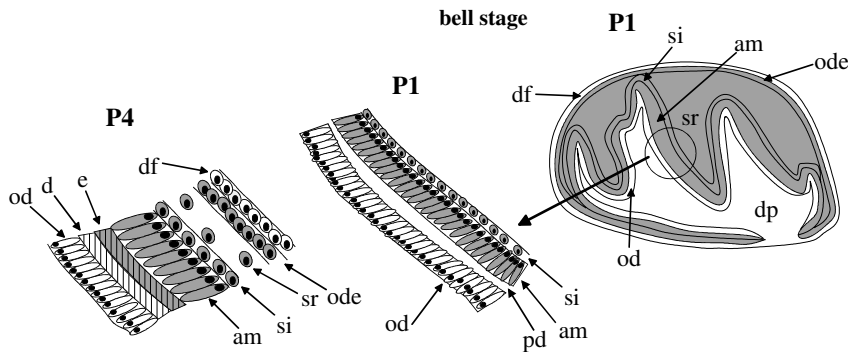
action of Shh on the dental epithelium is indirect and acts on the dental mesenchyme that reciprocally regulates growth of the epithelial cervical loops (Gritli-Linde et al., 2002).

More insight in cusp formation and patterning has been attained from studies of *Tabby* mutant mice with defects in cusp patterning. *Tabby* gene is the mouse homologue of the HED gene *EDA* and encodes a protein called Ectodysplasin, which is a member of the Tnf ligand superfamily (Mikkola et al., 1999; Pispas and Thesleff, 2003). Both HED patients and *Tabby* mice are characterized by abnormal development of epithelial appendages such as teeth, hair, and sweat glands. The cusp pattern in *Tabby* mice molars is compressed as the tips of the cusps are close or united to each other. Furthermore, the last developing cusps as well as the third molar are often missing (Jernvall and Thesleff, 2000). It has been proposed that the cause of the tooth phenotype of *Tabby* mice is the small size of the tooth germ and subsequently small primary enamel knot. Supporting this is the observation that the *Tabby* enamel knot express all the knot signals analyzed but is greatly reduced in size (Pispas et al., 1999).

In addition, the central role of Tgf $\beta$ /Bmp signaling in cusp patterning has also become evident from the aberrant shapes of molars in the Follistatin transgenic mice. Imbalance between the amount of Activins/Bmps and their inhibitor Follistatin has been suggested to cause abnormal proportion of activators and inhibitors of normal enamel knot formation and thus abnormal cusp patterning (Wang et al., 2004). Computer modelling of the function of activators and inhibitors indicates that the cusp pattern of different animal species can be mimicked by fine-tuning of their activities (Salazar-Ciudad and Jernvall, 2002). Further evidence of the importance of the correct ratio between activators and inhibitors have been shown in studies of mice with deleted Ectodin function (Kassai et al., 2005). Ectodin is a Bmp inhibitor and also a Wnt signaling modulator and these mice have severe cusp patterning defects (Kassai et al., 2005).

### **Cell differentiation and mineralisation**

During tooth cusp morphogenesis, the cells at the border between the inner enamel epithelium and the dental papilla mesenchyme begin to differentiate to ameloblasts and odontoblasts. The differentiation process begins in mouse at E16 and continues in the cervical direction until the root starts to develop. The differentiation of odontoblasts and ameloblasts is regulated by reciprocal epithelial-mesenchymal interactions, and the same signal molecules associated with morphogenetic regulation have also been linked with dental cell differentiation.



**Fig. 6.** Schematic representation of cell differentiation during first molar tooth development. od, odontoblast; d, dentin; e, enamel; am, ameloblast; si, stratum intermedium; sr, stellate reticulum; ode, outer dental epithelium; df, dental follicle; dp, dental papilla.

### Odontoblasts

Preodontoblasts are initially cylinder-shaped and organised as a single layer adjacent to the inner enamel epithelium. During differentiation, the preodontoblasts elongate and polarize becoming odontoblasts that are tall columnar cells (Fig. 6). Odontoblasts secrete dentin matrix consisting of mainly type I collagen but also non-collagenous proteins including proteoglycans, glycoproteins and dentin sialophosphoprotein (DSPP) (D'Souza et al., 1997).

Odontoblast differentiation is regulated by the inner dental epithelium (Fig. 5) (Thesleff and Hurmerinta, 1981; Ruch et al., 1995). Since odontoblast differentiation begins in the mesenchymal cells adjacent to the secondary enamel knots, it has been proposed that signals from the secondary enamel knot regulate the initiation of terminal differentiation of odontoblasts (Jernvall and Thesleff, 2000). Signals in the Tgf $\beta$  and FGF families have been implicated in odontoblast differentiation. *In vitro* experiments have shown that Tgf $\beta$ 1 and -3 and Bmp2, -4 and -6 can induce polarization of preodontoblasts and stimulate dentin matrix secretion (Begue-Kirn et al., 1992; Ruch et al., 1998).

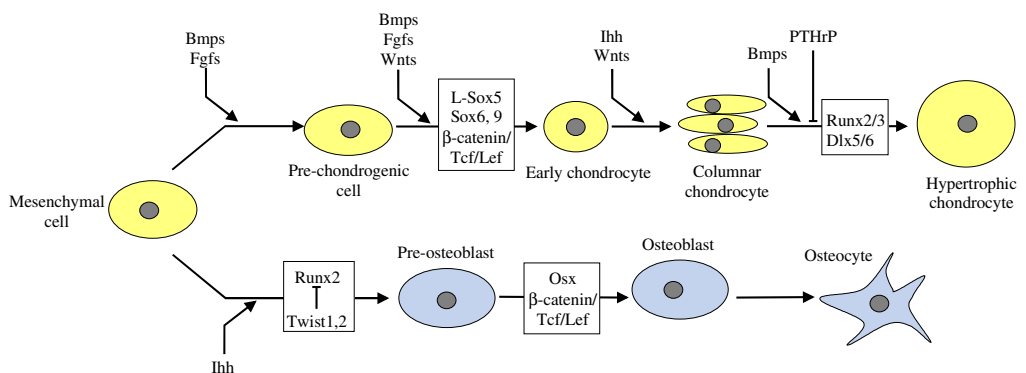
### Ameloblasts

Preameloblasts are derived from precursor cells of the inner dental epithelium of the enamel organ and during differentiation into secretory ameloblasts they become highly columnar and polarized with oval-shaped nuclei elongated along the apical-basal axis (Fig. 6) (Ten Cate, 1998). Functional ameloblasts secrete a number of enamel matrix proteins, including amelogenin, ameloblastin, enamelin, tuftelin, DSPP, laminin 5 as well as proteolytic enzymes (Robinson et al., 1998). When the enamel matrix deposition is complete, the secretory ameloblasts shrink in size. During maturation even 25 % undergo apoptosis and the remaining cells, together with the outer enamel epithelium, form a protective layer on the enamel until the tooth has erupted (Joseph et al., 1999).

Again, reciprocal epithelial-mesenchymal interactions regulate ameloblast differentiation and presence of functional odontoblasts and/or pre-dentin is required. Secretion of enamel matrix is only initiated when dentin matrix begins to mineralize (Coin et al., 1999a). Ameloblast and odontoblast cytodifferentiation can be induced by Tgf $\beta$ 1, Bmp2 and Bmp4 (Begue-Kirn et al., 1992,1994; Wang et al., 2004). It was recently shown that the main signal from odontoblasts inducing ameloblast differentiation is Bmp4, and that Activin emanating from the dental follicle antagonizes the effect of Bmp4 (Wang et al., 2004). In addition, overexpression of *Smad2* in the epidermis under *keratin 14* promoter resulted in abnormal enamel structure (Ito et al., 2001). It has also been shown that Shh is necessary for regulating cell proliferation within the dental epithelium and controlling cytodifferentiation of preameloblasts (Gritli-Linde et al., 2002).

## Bone development

Most of the craniofacial skeleton is of neural crest origin, except for the otic and occipital region and the parietal bones that are derived from cephalic and somitic mesoderm (Couly et al., 1993; Chai et al., 2000). Bone development begins with the formation of mesenchymal condensations at sites of future bones, consisting of cells that can differentiate into either chondroblasts or osteoblasts (reviewed by Hall and Miyake, 2000). In mouse, condensations appear between E10.5-E12.5 and subsequently bone formation begins by intramembranous or endochondral mechanism (reviewed by Karsenty, 1998). Most bones form through endochondral ossification but in the facial area intramembranous bone formation predominates. Mutations in the genes encoding paracrine factors, their receptors and transcription factors that regulate these ossification processes, cause numerous craniofacial developmental abnormalities (reviewed by Rice, 2005).



**Fig. 7.** Schematic representation of molecular regulation of chondroblast and osteoblast differentiation (modified from Kobayashi and Kronenberg, 2005). Transcription factors important for certain stages are indicated by a box. Signal molecules are indicated with an arrow.

## **Endochondral ossification**

First stage of endochondral ossification is the commitment of mesenchymal cells to become cartilage cells. This is caused by paracrine factors inducing two transcription factors, Pax1 and Scleraxis, that are thought to activate cartilage specific genes (Cserjesi et al., 1995; Sasic et al., 1997). Subsequently, the committed mesenchymal cells condensate into compact nodules and differentiate to chondrocytes. It has been suggested that N-cadherin is involved in the initiation of these condensations, and that N-CAM is critical for maintaining them (Oberlender and Tuan, 1994; Hall and Miyake, 2000). Sox9 is required for cartilage differentiation and expression of chondrocyte-specific genes that encode cartilage extracellular matrix components, including collagen types II, IX, and XI, and aggrecan (Åberg et al., 2005). The stages of chondrocyte differentiation are regulated by a complex series of signaling molecules and transcription factors in addition to Sox9 and Runx2/3 (Fig. 7). Signaling molecules involved in chondrocyte differentiation include Bmps, Fgfs, Ihh and Wnts. Transcription factors involved include L-Sox5, Sox6, Dlx5 and Dlx6 (Fig. 7) (reviewed by Kobayashi and Kroneberg, 2005). During the next phase, chondrocytes proliferate to form the cartilage model for the bone and simultaneously secrete a cartilage specific extracellular matrix. In the next phase, the chondrocytes increase their volume and become hypertrophic chondrocytes and altered matrix production enables calcification of the cartilage. Runx2 and Runx3 are the major transcription factors regulating chondrocyte hypertrophy (Fig. 7) (Kobayashi and Kroneberg, 2005). The chondrocytes are eventually removed by apoptosis (Hatori et al., 1995). In the last phase blood vessels invade the cartilage model and induce the differentiation of osteoblasts. Paracrine factors induce the expression of *Runx2* which is essential for the osteoblast differentiation (Otto et al., 1997). Hypertrophic chondrocytes also secrete the angiogenesis factor, Vegf, which stimulates vascularization (Gerber et al., 1999; Haigh et al., 2000). Transcription factors required for osteoblast differentiation are Runx2 and Osterix, which is a downstream target of Runx2 (Kobayashi and Kronenberg, 2005). Recently  $\beta$ -catenin, which is part of canonical Wnt signaling, has been shown to be important for various stages of osteoblast differentiation (reviewed by Kobayashi and Kronenberg, 2005). In addition, Dlx5, Msx1, Twist, Alx4 and androgen and estrogen receptors have been shown to affect the osteoblast differentiation (reviewed by Kobayashi and Kronenberg, 2005). The osteoblasts begin secreting bone matrix constructing a bone collar around the cartilage. Eventually, the cartilage is replaced by bone. Bone formation takes place also at the surface of the bone, in the periosteum, where no cartilaginous model exists. The shapes of bones are modified by this intramembranous-like process (reviewed by Gilbert, 2006).

## **Intramembranous ossification**

In the skull, the calvarial, maxillary, mandibular, and facial bones are formed by intramembranous ossification. In addition, also a large part of the clavicle is formed by the same mechanism. In intramembranous ossification, the mesenchymal cells condense into compact nodules, and the central cells differentiate into osteoblasts which secrete bone extracellular matrix and form ossification centers.

Intramembranous bone formation is regulated by transcription factors Runx2 and Osterix similarly as osteoblast differentiation in endochondral bone formation (Fig. 7) (reviewed by Kobayashi and Kroneberg, 2005). In addition, signaling molecules in the Bmp and Fgf families regulate formation of intramembranous calvarial bones and suture fate (reviewed by Rice, 2005). Those osteoblasts that become embedded in the calcified matrix become osteocytes. A single bone may have several ossification centers that fuse to form the final bone (reviewed by Kaufmann and Bard, 1999). Surrounding the bone is the periosteum that is a membrane of compact mesenchymal cells. Cells at the inner surface of the periosteum differentiate into osteoblasts and subsequently growth is achieved. Similarly to endochondral ossification, induction of Runx2 is essential for osteoblast differentiation and subsequent bone formation (Otto et al., 1997).

## Cleidocranial dysplasia and Runx2

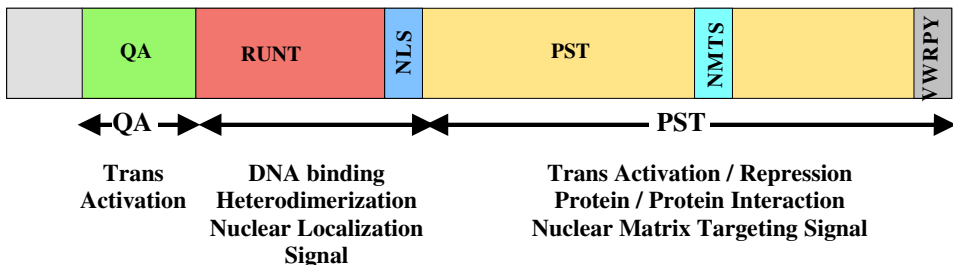
### Structure and function of Runx genes

The Runx gene family of transcription factors comprise a small group of genes characterized by a DNA-binding Runt domain that is highly conserved among different species. In *Drosophila*, two runt domain genes are known, Runt and Looz-enge and additionally two more have been found that are still functionally uncharacterized (reviewed by Coffman, 2003). The mammalian Runx genes are homologs of the Runt domain transcription factor family in *Drosophila*. In mammals, three *Runx* genes are known; *Runx1* (*Cbfa2/Pebp2aB/Aml1*), *Runx2* (*Cbfa1/Pebp2aA/Aml3*) and *Runx3* (*Cbfa3/Pebp2aC/Aml2*). The three *Runx* genes are very similar with respect to their genomic organisation and DNA sequence (Bangsow et al., 2001; Levanon et al., 2001; Thirunavukkarasu et al., 1998). Mammalian *Runx* genes exhibit two alternative promoters (P1 and P2) and subsequently two different Runx isoforms can be produced with possibly distinct functions (Bangsow et al., 2001; Coffman, 2003). In *Runx2*, type II isoform transcription is initiated at the distal P1 promoter, while type I isoform transcription is initiated at the proximal P2 promoter (reviewed by Stock and Otto, 2005). Type I isoform (pebp2aA) was originally cloned as a T-cell-specific factor but is also expressed in other non-osseous tissues and in osteoblasts, and type II isoform (Osf2, till1) as a osteoblast specific factor (reviewed by Stock and Otto, 2005). Also a third potential N-terminal isoform in mouse has been described (Ducy et al., 1997). In addition, the primary type I and II transcripts are processed into several alternatively spliced mRNA isoforms that are differentially expressed in various cell types and at different developmental stages. The role and biological relevance of these different isoforms are still unknown (reviewed by Stock and Otto, 2005). Also other mammalian Runx genes can generate alternatively spliced transcripts with possibly different roles during embryogenesis (Levanon et al., 1996; Bangsow et al., 2001). The functional transcription factor is a heterodimer of two subunits,  $\alpha$ -subunit encoded by the *Runx* genes in mammals, and the  $\beta$ -subunit (*CBF $\beta$* ) which is encoded by only one gene but appears in the mouse as three alternatively spliced products (Nagata and Werner, 2001). CBF $\beta$  does not bind to DNA itself but can enhance the DNA binding affinity of Runx proteins and also possibly stabilize

Runx from degradation (Coffman, 2003; reviewed by Ito and Miyazono, 2003). The CBF $\beta$  subunit is essential for the function of Runx proteins (Huang et al., 2001).

The  $\alpha$ -subunit (Runx) has the DNA binding domain Runt, which is a 128 amino acid motif (Fig. 8). The runt domain directs DNA binding of Runx proteins, and also contributes to protein-protein interactions, ATP binding, and nuclear localization (reviewed by Stock and Otto, 2005). Furthermore, the N-terminal region is rich in glutamine and alanine repeats (Q/A) (Fig. 8). In the C-terminal region is a proline/serine/threonine rich region (PST), which is necessary for nuclear matrix targeting, transcriptional activation or repression of target genes, and also contains MAP kinase phosphorylation sites (reviewed by Stock and Otto, 2005). In addition, most Runx genes terminate with a common pentapeptide, VWRPY, which can recruit the Groucho/TLE family of corepressors (Fig. 8) (Levanon et al., 1998; Javed et al., 2001)

Runx transcription factors bind to several DNA motifs, such as PyGPyGGT, on a number of enhancers and promoters, including murine leukemia virus and polyomavirus enhancer (Ducy et al., 1997; Karsenty and Wagner, 2002). In addition, Runx2 has been shown to bind to the OSE2 DNA motif AACAC within the 5' regulatory regions of genes (Ducy et al., 1997). Binding partners for Runx2 include DNA binding partners, chromatin remodelling factors (histone deacetylase 5) and mediators of signal transduction pathways (YES-associated protein) (Westendorf, 2006).



**Fig. 8.** Schematic representation of the Runx2 protein. Runx2 protein is composed of a glutamine/alanine-rich region (Q/A) in the N-terminal region, a centrally located DNA binding domain (Runt domain) and nuclear localization signal (NLS), and the C-terminal proline/serine/threonine-rich PST region containing transactivation and repression domains and a nuclear matrix targeting signal domain.

## Runx genes in embryonic development

Runt and Lozenge control a large number of developmental processes in *Drosophila*. Runt is part of a network of signals that control pattern formation in the embryo. In the central nervous system, Runt function is necessary for the development of a subset of neurons. In addition, Runt is also a key regulator of sex determination. Lozenge controls pre-patterning and cell-fate choices in the development of the visual system and is also required in haematopoiesis for the specification of a *Drosophila* blood cell lineage (Coffman et al., 2003).

In humans RUNX1 is essential for angiogenesis and mature haematopoiesis and is most frequently involved in acute leukemia (Otto et al., 2003; reviewed by Ito, 2004). *Runx1* knockout mice exhibit early embryonic lethality with massive haemorrhage due to lack of definitive haematopoietic stem cells and defective angiogenesis (reviewed by Ito, 2004). Also the  $\beta$ -subunit, CBF $\beta$ , is frequently involved in chromosome rearrangements associated with human leukemia (reviewed by Ito, 2004). Runx3 is involved in neurogenesis, thymopoiesis and in addition, a possible candidate to be a gastric cancer tumour suppressor (reviewed by Ito, 2004). The function of Runx proteins during development was first discovered when several research groups confirmed that Runx2 is a key regulator of osteoblast differentiation and bone formation (Ducy et al., 1997; Mundlos et al., 1997; Otto et al., 1997; Komori et al., 1997).

*Runx2* knockout mice die soon after birth and show no osteoblast differentiation and hence completely lack bone (Komori et al., 1997; Otto et al., 1997). Runx2 activity is needed for osteoblast differentiation in both intramembranous and endochondral bone formation where it also has a positive role in the differentiation of hypertrophic chondrocytes (reviewed by Kobayashi and Kroneberg, 2005). Runx2 is a transcriptional activator of osteoblasts and activates most osteoblastic markers, including osteocalcin, type I collagen and collagenase (Ducy et al., 1997); *Runx2* expression is both necessary and sufficient for osteoblast differentiation (Ducy et al., 1997). *RUNX2* haploinsufficiency is the cause of CCD in humans and inactivation of one *Runx2* allele in mice causes a similar skeletal phenotype (Mundlos et al., 1997; Otto et al., 1997). In addition, a mouse deficient for type II isoform of Runx2 have been generated by targeted disruption of the P1 promoter. The phenotype showed disturbances mainly in endochondral ossification in contrast to total *Runx2* heterozygous knockout mice that exhibit disturbance in intramembranous ossification. (reviewed Stock and Otto, 2005). CBF $\beta$  is required for Runx2 function during skeletal development and thus CBF $\beta$  mutations can also cause CCD-type phenotype (reviewed by Rice, 2005).

### Regulation of Runx2

Bmps that are inducers of osteoblast differentiation and bone formation upregulate *Runx2* expression in osteoblasts (Ducy et al., 1997). Runx2 mediates Bmp-induced osteogenic signaling pathway and is essential for Bmp signaling and regulation of downstream target genes (Afzal et al., 2005;). The activity of Runx2 can also be altered by direct interactions with other transcription factors such as Smads and AP1 factors (reviewed by Stock and Otto, 2005). Tgf $\beta$  has also been shown to re-

press Runx2 function through Smad3 (Kang et al., 2005). Runx2 can form a complex with Smads and function synergistically to regulate the target gene (Ito and Miyazono et al., 2003). Furthermore,  $\beta$ -catenin/TCF1 regulates directly *Runx2* in stimulation of bone formation (Gaur et al., 2005). There is recent evidence that a complex interaction between Wnt/ $\beta$ -catenin and Bmp/Tgf $\beta$  signal transduction occurs in the development of several tissues, including the skeleton and it has been proposed that these pathways converge in Runx2 to promote osteoblast differentiation (Gaur et al., 2005).

In addition, *Runx* genes can bind to the same DNA motifs and interact with a common transcriptional modulator (Ito, 1999). Furthermore, all *Runx* genes contain Runx binding sites in their promoter region. Hence it is possible that both positive and negative cross-regulation exists among Runx proteins (reviewed by Otto et al., 2003). Furthermore the transcription factors Msx2, Bapx1, Hox1-2 and PPAR $\gamma$ 2 have been shown to regulate *Runx2* expression (reviewed by Otto et al., 2003).

Runx2 is also regulated by Stat1 that interacts with Runx2 latent form in the cytoplasm, inhibiting nuclear localization and thus its nuclear transcriptional activity. Consequently, *Stat1*-deficient mice exhibit accelerated osteoblast differentiation which results in increased bone mass (Kim et al., 2003). Runx2 is inhibited by Twist, which is expressed in osteoprogenitor cells but not in mature osteoblasts and has been suggested to act as a negative regulator of osteogenesis (Rice et al., 2000; Yousfi et al., 2002). Loss-of-function mutations in *Twist* result in craniosynostosis, a condition with excessive osteogenesis of the calvarial bones. In addition, double heterozygotes for *Twist1* and *Runx2* deletion show a partial rescue of the widened calvarial sutures seen in *Runx2* heterozygous mice (Bialek et al., 2004).

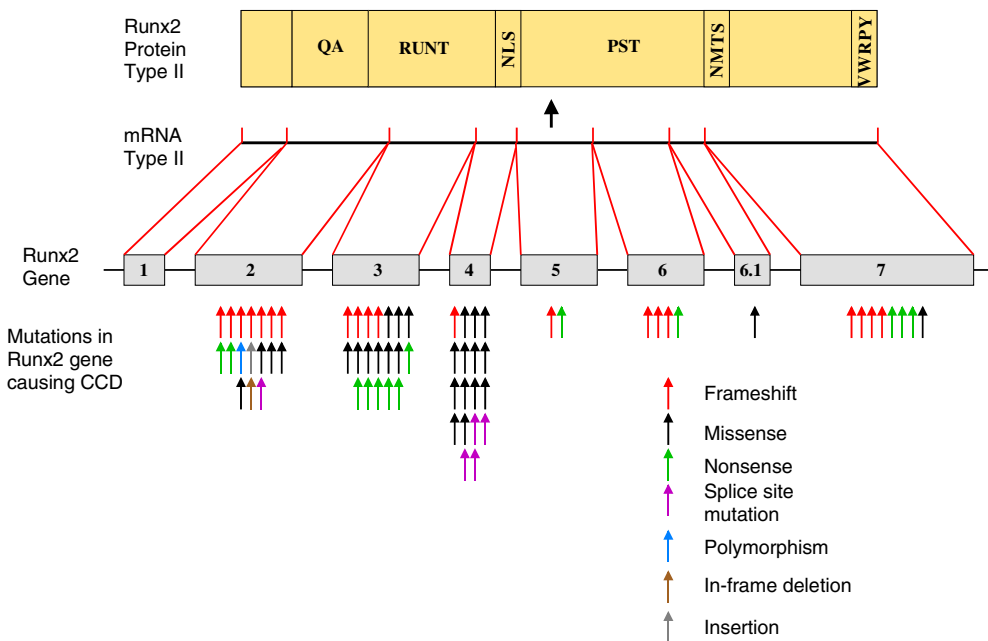
Runx2 is phosphorylated and can be activated by the mitogen-activated protein kinase (MAPK) pathway, stimulated either by extracellular matrix binding integrin on the cell surface or Fgf2. Parathyroid hormone/parathyroid hormone-related peptide is also able to phosphorylate and activate Runx2 protein (Thirunavukkarasu et al., 1998; reviewed by Franceschi et al., 2003). All three *Runx* genes are also transcriptionally controlled by retinoid/vitamin D nuclear receptors with positive regulatory effect on *Runx1* and 3 but negative for *Runx2* (Drissi et al., 2002).

At least Osterix, a zinc finger containing transcription factor, has been shown to act downstream of Runx2 during osteoblast differentiation. In *Osterix* *-/-* mice, both the endochondral and intramembranous bone formation is absent but Runx2 expression is normal and no tooth defects are present (Nakashima et al., 2002).

*Runx2* heterozygous mice have delayed tooth eruption and reduction of osteoclast recruitment to the jaws (Yoda et al., 2004). Rankl, a member of the Tnf family, is produced by osteoblasts and acts as a differentiation and activation factor for osteoclasts (Yasuda et al., 1998). *Rankl* expression is severely diminished and osteoclastogenesis abolished in *Runx2* *-/-* mice and by adding soluble Rankl protein, partial rescue of the osteoclastogenesis can be achieved (Enomoto et al., 2003).

## Cleidocranial dysplasia syndrome

Cleidocranial dysplasia is a rare bone dysplasia caused by mutations in *Runx2* gene with autosomal dominant inheritance. The condition is characterized by generalized bone dysplasia including delayed ossification of calvarial sutures and fontanelles, supernumerary and unerupted teeth, hypoplastic or missing clavicles and short stature (Gorlin, 2001). *RUNX2* haploinsufficiency is the cause of CCD in humans and mice (Mundlos et al., 1997; Otto et al., 1997). Mutations in *RUNX2* causing haploinsufficiency include chromosomal translocations, insertions, deletions, nonsense, missense and frameshift mutations (Otto et al., 2002) (Fig. 9). It has been suggested that 20%-40% represent new mutations (Gorlin, 2001).



**Fig. 9.** Schematic representation of localization and type of mutations in *RUNX2* causing CCD (based on review by Otto et al., 2002). *RUNX2* gene is located on chromosome 6p21 and contains eight exons.

The general appearance of CCD patients is characterized by short stature, pronounced frontal and parietal bossing, hypertelorism, hypoplastic maxilla and zygoma, broad nose at the base and narrow shoulders (Gorlin, 2001). The cranium is large but shows short biparietal bossing and the cranial base has a short sagittal diameter (Richardsson et al., 1994). In addition, the mandibular length is increased, maxilla is short vertically, paranasal sinuses and mastoids may be underdeveloped or absent and closure of the anterior fontanelle and sagittal and metopic sutures is delayed (Gorlin, 2001). Secondary centers of ossification may form in the sutures and give rise to Wormian bones, especially in lambdoid suture (Gorlin, 2001).

The clavicles are usually hypoplastic and the defect is more frequent at the acromial end. In some cases the clavicle has a central gap where fibrous connective tissue has replaced the bone. The clavicles are absent unilaterally or bilaterally in about 10% of the patients (Gorlin et al., 2003). Also other bones of non intramembranous origin are affected in CCD patients, including pubic symphysis with delayed closure, cone-shaped thorax, lumbar spondylosis, short broad thumbs, short middle phalanges and metatarsals (Gorlin, 2001).

CCD patients have several oral manifestations such as highly arched palate, delayed union at the mandibular symphysis, cleft palate, deficient ossification of the hyoid bone and poor development of premaxilla. The most characteristic features are, however, the dental anomalies including multiple supernumerary teeth but also multiple crown and root abnormalities, ectopic localization of teeth, and lack of eruption of permanent teeth. Some patients may even have a complete tertiary dentition (Jensen and Kreiborg, 1990). Based on the observation that epithelial remnants were associated with extracted teeth of CCD patients it was suggested that the supernumerary teeth may have resulted from lack of resorption of dental lamina epithelium (Lukinmaa et al., 1995). It has also been reported that cellular cementum in the roots of CCD patients' permanent teeth is absent and acellular cementum is partially hyperplastic (Jensen, 1990; Seow and Hertzberg, 1995; Lukinmaa et al., 1995). It has been proposed that insufficient *Runx2* activity may affect the differentiation of both osteoblasts and osteoclasts thus leading to diminished bone resorption of bone surfaces and delayed resorption of the primary teeth and subsequent delayed eruption of permanent teeth (Kreiborg et al., 1999).

Supernumerary teeth appear most often in the mandibular premolar and maxillary incisor region and the average number of extra teeth is five (Gorlin, 2001). Jensen and Kreiborg carried out a thorough study of 20 patients and showed that the primary teeth and permanent tooth crowns were normal. Primary teeth did also erupt normally but the permanent teeth did not, except for the first molars and occasionally other teeth too. Interestingly, they showed that during permanent tooth crown development the dental lamina was reactivated to form supernumerary teeth. Hence, supernumerary teeth are usually morphologically similar to their predecessors (Jensen and Kreiborg, 1990).

Several studies have tried to establish a correlation between the genotype and phenotype in CCD patients by mutational analysis of *RUNX2* (Yoshida et al., 2002; Quack et al., 1999; Zhou et al., 1999). It has been shown that mutations affecting the runt domain and resulting in haploinsufficiency yields a classic CCD while mutations outside this domain may result in hypomorphic states with wide clinical variety (Zhou et al., 1999). Accordingly, mutated protein associated with mild CCD, including isolated dental anomalies, have been shown to exhibit normal DNA binding and not causing haploinsufficiency but to be hypomorphic in nature, probably due to disturbance of Runx2 interaction with other modulators (Zhou et al., 1999). In addition, also a missense mutation in the very 3' end of the coding region caused an isolated dental anomaly consisting of supernumerary teeth with no other CCD features (Quack et al., 1999). In this mutation the VWRPY motif that has been suggested to interact with the TLE transcriptional corepressor family is affected (Quack et al., 1999). A mutation in *RUNX2* from a CCD patient has been described where *RUNX2* protein failed to interact and respond to Smads

(Zhang et al., 1999). A correlation between short stature and supernumerary teeth has also been made and from the number of supernumerary teeth predictions of final height could be made, although the generality remains to be established (Yoshida et al., 2002). It has also been suggested that the intramembranous bone formation is more sensitive to *RUNX2* gene-dosage than endochondral bone formation thus explaining why almost always some cleidocranial abnormality is present but not always skeletal abnormalities (Yoshida et al., 2002). Interestingly, the dental and skeletal abnormalities correlate but also isolated dental abnormalities can occur. This could reflect the different functional components of Runx2. It seems that Runx2 is developmentally differentially regulated in bone and tooth with different upstream regulators and downstream targets (James et al., 2006).

## AIMS OF THE STUDY

The aim of this study was to investigate the pathogenesis of CCD, in particular the functions of Runx2 and Bmps in tooth development. The specific aims were:

1. to perform a thorough comparative analysis of the expression of *Bmps2* through 7 during mouse tooth development in order to elucidate possible functions
2. to examine in detail the tooth phenotype of *Runx2* mutant mice
3. to analyze the expression pattern of *Runx2* during mouse tooth development as a basis for functional studies
4. to identify the possible upstream regulators and downstream targets of Runx2 during tooth development in order to understand CCD pathogenesis

## MATERIALS AND METHODS

Materials and methods used in the thesis are listed in following three tables; Mouse strains, Probes, and Methods. A detailed description of each method can be found in the original publications.

**Table 1.** Mouse strains used in articles I-IV

Mouse strain	Used in article	Described in article	Purpose
CBAT6T6 x NMRI	I, II, III, IV	-	Expression pattern analyzes of mRNA and proteins
<i>Runx2</i> null mutant mice	II, III, IV	Originally; Otto et al, 1997. Maintenance; III.	Analysis of phenotype and role in tooth development
<i>Msx1</i> null mutant mice	III	Satokata and Maas, 1994; Bei and Maas, 1998.	<i>Runx2</i> upstream regulation
<i>Lef1</i> null mutant mice	III	van Genderen et al., 1994	<i>Runx2</i> upstream regulation
<i>Tabby</i> null mutant mice	III	Pispa et al., 1999	<i>Runx2</i> upstream regulation
Nude mice	III, IV	Kratochwil et al., 1996	Kidney capsule transplantation of explants

**Table 2.** Probes used for *in situ* hybridisation in articles I-IV

Probe	Description or reference	Used in publication
<i>Activin <math>\beta</math>A</i>	Erämaa et al., 1992	IV
<i>Ameloblastin</i>	Lee et al., 1996	III
<i>Bmp2 and Bmp4</i>	Vainio et al., 1993	I, IV
<i>Bmp3, -5, -6, and -7</i>	Åberg et al., 1997 (III)	I, IV
<i>Proa1(I) collagen</i>	Metsäranta et al., 1991	II, III
<i>Dan</i>	Dionne et al., 2001	IV
<i>Dspp</i>	D'Souza et al., 1997	II, III
<i>Dentin matrix protein-1</i>	D'Souza et al., 1997	III
<i>Ectodin</i>	Åberg et al., 2004 (IV)	IV
<i>Eda and Edar</i>	Laurikkala et al., 2001	IV
<i>Fgf3 and Fgf10</i>	Kettunen et al., 2000	IV
<i>Fgf4</i>	Jernvall et al., 1994	IV
<i>Fgfr1</i>	Trokovic et al., 2003	IV
<i>Hairless</i>	Åberg et al., 2004 (IV)	IV
<i>Hes1 and Hes5</i>	Mustonen et al., 2002	IV
<i>Lef1</i>	Travis et al., 1991	IV
<i>Lunatic Fringe</i>	Mustonen et al., 2002	IV
<i>Mmp2</i>	Sahlberg et al., 1999	IV
<i>Msx1 and Msx2</i>	Jowett et al., 1993	IV

<i>Osteocalcin</i>	Ducy et al., 1997	III
<i>p21</i>	Jernvall et al., 1998	IV
<i>Pax9</i>	Neubuser et al., 1997	IV
<i>Runx1 and Runx3</i>	Yamashiro et al., 2002	IV
<i>Runx2</i>	D'Souza et al., 1999	II, IV
<i>Shh</i>	Vahtokari et al., 1996	IV
<i>Sprouty1, -2, and -4</i>	Zhang et al., 2001	IV
<i>Tgfb1</i>	Vahtokari et al., 1991	IV
<i>Timp1 and Timp3</i>	Sahlberg et al., 1999	IV
<i>Twist</i>	Rice et al., 2000	IV
<i>Wnt5a, -10a, -10b, and -11a</i>	Dassule and McMahon, 1998; Sarkar and Sharpe, 1999	IV

**Table 3.** Methods used and described in articles I-IV

<b>Method</b>	<b>Described in</b>	<b>Used in article</b>
Processing of tissues	I	I, II, III, IV
Tissue culture and bead implantation assays	II, III, IV	II, III, IV
Rescue experiments	IV	IV
Kidney capsule implantation	III, IV	III, IV
Genotyping by PCR	II, IV	II, III, IV
Radioactive <i>in situ</i> hybridisation of sections	I, II	I, II, III, IV
Whole mount <i>in situ</i> hybridisation	II	II, IV
Immunohistochemistry	IV	IV
RT-PCR	II, III	II, III
Cell transfection and analysis of gene induction	IV	IV
3D reconstruction	I	I

## RESULTS AND DISCUSSION

### Expression of *Bmps* during tooth development (I)

We performed a careful comparative analysis of the expression of *Bmp2*, -3, -4, -5, -6, and -7 from the time of initiation of mouse tooth development to the stage of mineralization of the dentin and enamel matrices. Morphological details of the stages of tooth development which were analyzed is shown in Figs. 5 and 6 (pages 25 and 29) and a summary of the expression patterns in Fig. 10 (page 45)(based on publication I, Figs. 3-5).

*Bmp4* expression was detected in the oral epithelium at the time of tooth initiation at E10 and subsequently, at E11-12, expression was detected transiently both in the epithelium and mesenchyme. However, before the dental placode is formed, it shifted completely to the mesenchyme. *Bmp2* transcripts were detected in those areas of the dental lamina where it started to form a bud, including the dental placode. *Bmp7* transcripts were detected throughout the oral epithelium at the time of the formation of the dental lamina, at E10 (data not shown).

At early bud stage *Bmp2* was detected throughout the bud but it became subsequently concentrated at the tip of the bud during bud stage. Weak expression of *Bmp3* transcripts was first detected at early bud stage and localized at the lingual aspect of the dental lamina in the area of tooth bud. *Bmp4* was detected in the condensed dental mesenchyme around the epithelial bud with somewhat more intense expression at the buccal side of the tooth. Weak expression of *Bmp6* was first detected at the early bud stage in the condensed mesenchyme surrounding the bud. *Bmp7* transcripts became localized at early bud stage in the tip of the bud. The expression pattern resembled that of *Bmp2* except that the expression was present also in the oral epithelium.

The expression of *Bmp2* was intense at E14 and restricted to the epithelial enamel knot. As the enamel knot started to disappear during late cap stage (E15), *Bmp2* expression spread to the neighbouring inner dental epithelium. At cap stage expression of *Bmp3* was seen in the dental papilla mesenchyme underlying the enamel knot. *Bmp3* expression was also seen in the dental follicle mesenchyme and intense expression was seen in lingual side of the outer dental epithelium. A high level of *Bmp4* expression continued in the dental papilla mesenchyme, and it reappeared in the epithelium in the distal part of the enamel knot. *Bmp6* was expressed in the dental mesenchyme underlying the lingual epithelial cervical loop. Expression of *Bmp7* was localized to the enamel knot extending somewhat outside the area. When the enamel knot started to disappear (E15), the expression of *Bmp7* continued in the inner dental epithelium.

At the early bell stage, *Bmp2* expression had disappeared from the epithelium (E16) and had shifted to the central cells of the dental papilla mesenchyme (E17). Subsequently (E18), expression extended coronally in the dental papilla and became prominent in the preodontoblasts. Expression of *Bmp3* continued in the dental follicle. The expression of *Bmp4* disappeared from the dental epithelium with the removal of the enamel knot, and became intense in the cuspal area of the dental papilla including the preodontoblastic layer. The central cells of the dental papilla showed very weak or no expression. The expression of *Bmp5* was seen for the first time in the differentiating preameloblasts (E18). The expression of *Bmp7* continued throughout the inner enamel epithelium/preameloblasts (E18) except in its cervical parts. It shifted to the preodontoblasts as they started to secrete predentin matrix (E19).

Expression of *Bmp2* continued in the odontoblasts when predentine secretion began (P1). During the first postnatal days, transcripts disappeared from the most advanced odontoblasts in the cuspal areas, and *Bmp2* expression was subsequently detected in functional ameloblasts. At P4, transcripts were seen mainly in the apical parts of the crown where differentiation continued. Some expression continued in the central cells of the dental papilla. Intense expression of *Bmp3* continued in the dental follicle cells. Expression of *Bmp4* was detected in the odontoblast and ameloblast cell layers. Expression in the odontoblasts decreased with advancing development whereas it persisted in the ameloblasts. *Bmp5* expression continued intensely in the ameloblast cell lineage during all stages of their differentiation and matrix secretion. The expression pattern of *Bmp7* was similar to that of *Bmp2*.

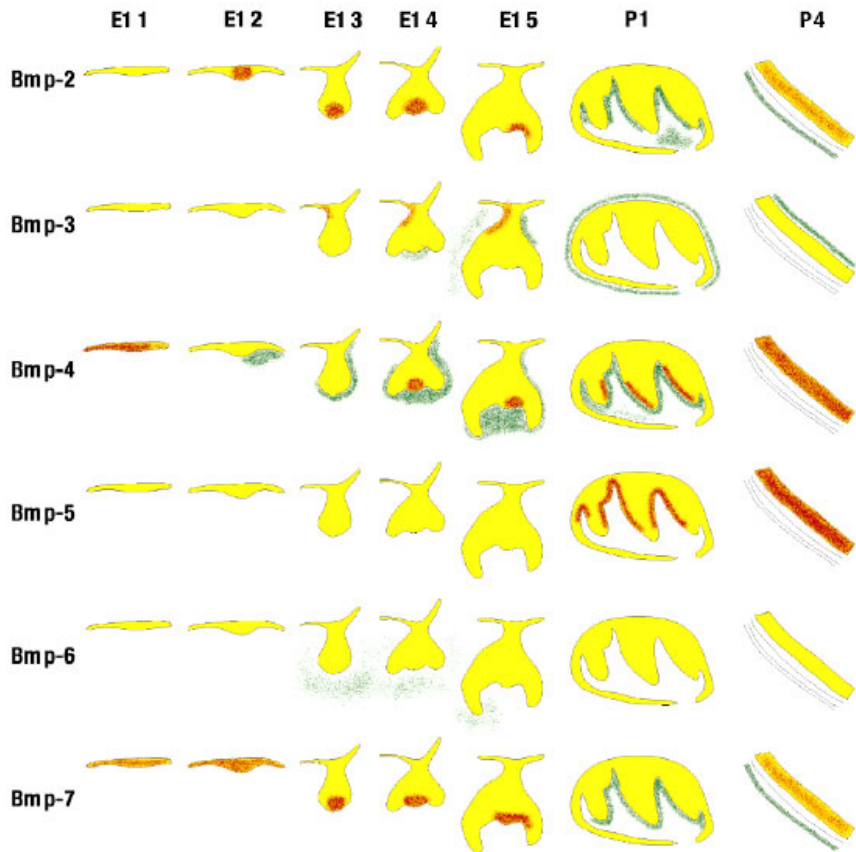
### **The function of Bmps in the regulation of tooth morphogenesis**

Bmps regulate most aspects of embryonic development and they are used repeatedly during the morphogenesis of various organs. The identification of signaling centres, i.e. enamel knots, in the developing tooth has greatly advanced the understanding of the interactions involved in tooth development. At the time of our expression pattern analysis there was very little functional evidence of Bmp involvement in tooth development. It is obvious that the expression does not implicate function of any gene product, but the function of some of the Bmps in tooth have been demonstrated after 1997. Our expression pattern analysis has later proven to be a valuable resource of information in these studies.

Knockout studies showed that null mutants of *Bmp2* and *-4* are early embryonic lethal. *Bmp7* knockout lacked a tooth phenotype, perhaps due to redundancy with *Bmp2*. In addition, a Cre-mediated deletion of the gene encoding Bmp receptor 1A in the surface epithelium and its derivativ causes arrest of tooth morphogenesis. The tooth buds, although significantly smaller, are formed but then regress (Andl et al., 2004). Furthermore, it has been shown that attenuation of *Smad2* results in an advancement of tooth development whereas attenuation of *Smad7* inhibits tooth development (Ito et al., 2001).

During the initiation of tooth development, epithelial signals induce mesenchymal factors that then reciprocally act on the dental epithelium to form the signaling center, also called dental placode. *Bmp4* and Activin  $\beta A$  have been proposed to be the key signals from the mesenchyme to induce the epithelial signaling center and subsequent budding of the tooth (Jernvall and Thesleff, 2000). Epithelial *Bmp4* induces the mesenchymal *Bmp4* expression via *Msx1* and this shift of expression from epithelium to mesenchyme (E11.5) coincides with the shift of the potential to instruct tooth development (Vainio et al., 1993; Mina and Kollar, 1987). Simultaneously, *Msx1* expression becomes restricted to the tooth bud regions in the mesenchyme (Coburne and Sharpe, 2003). *Bmp4* induces *p21* in the dental placode. *p21* is associated with the stop of cell proliferation and has been shown to be expressed in several signaling centers (Jernvall et al., 1998).

Later, during the cap stage, *Bmp4* is expressed in the enamel knot as well as in the mesenchymal dental papilla. *Bmp7* is coexpressed with *Bmp2* during early tooth development and also expressed in all signaling centers, if not as specifically as *Bmp2*. Better understanding of Bmp functions has been gathered from knockouts of other genes involved in tooth development. A common feature of the knockout of transcription factors *Msx1* and *Pax9* is that expression of *Bmp4* is missing from the dental mesenchyme. In all of these mutants, the formation of the enamel knot has been impaired which suggests that it is a prerequisite for the tooth bud to develop into cap stage. This indicates that *Bmp4* is a good candidate for a mesenchymal signal inducing the transition from bud to cap. Accordingly, in the *Msx1* knockout, tooth development is arrested at bud stage but can be rescued by adding exogenous *Bmp4* protein. Bmp signaling requires also the receptors in the target tissues to be functional, expression of *Bmps* as such does not indicate if it is functional or redundant. Recently, presence of *Bmpr1A* and -II has been shown by immunostaining at bud stage in the epithelium and of *Bmpr1B* and -II in the mesenchyme (Nadiri et al., 2006). At cap stage, *Bmpr1B* but not -II was shown to be present in the epithelium and at later cap stage both *Bmpr1A* and -II were present in the enamel knot, simultaneously with *Bmp4* (Nadiri et al., 2006). These results coincide with the *Bmp* expression and suggesting that there is functional signaling possible.



**Fig. 10.** Summary of the expression of *Bmp2-7* during tooth development. Dental epithelium is shown in yellow. Gene expressions in the epithelium are shown in red and in the mesenchyme in green.

### **Later function of BMPs in the differentiation of odontoblasts and ameloblasts and in dentin and enamel deposition**

The dentin-forming odontoblasts and the enamel-forming ameloblast differentiate during bell stage at the interface between the dental epithelium and mesenchyme, and there is abundant experimental evidence indicating that the differentiation of these cells is regulated by interactions between the two cell lineages (Thesleff and Hurmerinta, 1981; Ruch et al., 1995). The expression of *Bmp2*, *-4*, and *-7* showed apparent associations with the differentiation of odontoblasts and ameloblasts. In line with this, *in vitro* experiments have shown that *Bmp2*, *-4*, and *-6* can induce polarization of preodontoblasts and stimulate dentin matrix secretion (Begue-Kirn et al., 1994; Ruch et al., 1998). In addition, inductive roles of Bmps on odontoblasts differentiations have also been demonstrated in numerous transplantation

studies in which implantation of Bmps into the dental pulp induced *de novo* dentinogenesis (Lianja et al., 1993; Nakashima, 1994; Nakashima and Akamine, 2005).

As in odontoblast differentiation, ameloblast cytodifferentiation has also been shown to be induced by Tgf $\beta$ 1 and Bmp2 (Begue-Kirn et al., 1992, 1994). Our localization of *Bmp* transcripts show that they are present at the right time and location to act as mesenchymal signals on the differentiation of the inner enamel epithelium into ameloblasts. In addition, subsequent studies from our laboratory has shown that of the *Bmps* expressed by odontoblasts Bmp4 is most important (Wang et al., 2004). The Bmp receptors are also present in the ameloblasts and odontoblasts indicating that functional signaling is possible (Nadir et al., 2006).

The *Bmp3* expression pattern suggests that it may be involved in the formation of cementum. However, as the dental follicle regulates bone formation around the developing tooth, it is possible that Bmp3 is involved in the regulation of osteoblast functions in the alveolar bone. Recently, Bmp3 has been shown to have effects opposite to Bmp2, -4 and Activin and to function as a Bmp inhibitor (reviewed by Li and Cao, 2006). Indeed, the *Bmp3* expression at the lingual side of the bud and cap stage tooth could indicate an inhibitory function in successive tooth formation. Bmp5 apparently has no function in the regulation of morphogenesis, but is rather associated with cell differentiation. The function of the transient expression of *Bmp6* at bud and cap stage remains unclear and we could not detect it in later stages.

It has become more and more evident that signals of the Tgf $\beta$  family must be tightly regulated, and therefore finely tuned antagonistic effects between Tgf $\beta$  superfamily signals and their inhibitors are critical for normal tooth development. Mice with deleted *Ectodin* function (Ectodin is a Bmp inhibitor) have severe cusp patterning defects which show the importance of the correct ratio between activators and inhibitors (Laurikkala et al., 2003; Kassai et al., 2005). In addition, Follistatin regulates enamel patterning in mouse incisors by asymmetrically inhibiting Bmp signaling and thus ameloblast differentiation (Wang et al., 2004).

In conclusion, our findings are in line with the concept that Bmps have several different functions during tooth development. It is conceivable that they signal between the epithelial and mesenchymal tissues during initiation and morphogenesis of tooth development, as well as during the differentiation of odontoblasts and ameloblasts. Furthermore, they are also part of the signaling networks whereby the enamel knot regulates the patterning of tooth cusps. In addition, they are involved in the regulation of the deposition of the matrices of the dental hard tissues.

## **The role of Runx2 during tooth development (II, III, IV)**

### ***Runx2* expression during tooth development (II)**

*In situ* hybridisation analysis did not reveal any *Runx2* expression in mouse craniofacial tissue at E11. At E12, *Runx2* expression became evident in areas of future osteogenesis. In addition, *Runx2* expression was detected also in the mesenchyme underlying the dental epithelium, although the signal was very weak as compared to the expression in bone (II Fig. 2A-B). At E13, the *Runx2* expression continued in the preosteogenic mesenchyme and spread also to the nasal capsule. Intense

expression was observed in the mesenchyme surrounding the tip of the epithelial tooth bud at E13 (II Fig. 2C-D). At the cap stage (E14), high level of expression was evident in the dental papilla and follicle as well as in the osteogenic zones, clearly demarcating these regions from the surrounding tissues (II Fig. 2E-F). By E16, the early bell stage, *Runx2* expression was markedly downregulated in cuspal regions of the dental papilla mesenchyme (II Fig. 2G-H). At the late bell stage (E18), *Runx2* mRNA was downregulated throughout the dental papilla, however, intense expression remained in the dental follicle and in osteoblasts within the alveolar bone (II Fig. 2I-J). At the newborn stage (NB) and at 4 dpn *Runx2* expression was intense in all osteoblasts and in the dental follicle that will give rise to cementoblasts and periodontal ligament (II Fig. 3A-B). Newly differentiated odontoblasts and dental pulp cells near the cervical loop showed weak levels of *Runx2* expression while mature odontoblasts were negative (II Fig. 3B). At 7dpn, intense expression of *Runx2* remained in the dental follicle. Unlike secretory ameloblasts that appeared negative, maturation stage ameloblasts appeared strongly positive for *Runx2* (II Fig. 3E-F). In addition, we showed that *Runx2* expression is downregulated in differentiated odontoblasts prior to the expression of dentin matrix genes (II Fig. 3G-H).

The pattern of *Runx2* mRNA in the dental mesenchyme correlates with key developmental events. The inductive potential of the odontogenic epithelium shifts to the mesenchyme at E12, when *Runx2* expression also begins in the presumptive tooth mesenchyme (Jernvall and Thesleff, 2000). Hence, the onset of the expression in the dental papilla mesenchyme follows its acquisition of the odontogenic potential. Because *Runx2* expression is downregulated at the late cap stage, it seems that it is not involved in the formation of secondary enamel knots and subsequent cuspal development. However, we do not know the *Runx2* protein half life and can not predict when the protein activity is lost after the downregulation of the mRNA. Accordingly, we can not be certain that *Runx2* is not involved in odontoblast differentiation based on the downregulation of the mRNA.

In addition, the developmental profiles of *Runx2* expression in odontoblasts and osteoblasts appear different suggesting that the gene may be differentially regulated in these cells and that *Runx2* may not have a similar function as differentiation inducer in odontoblasts as it does in osteoblasts.

Furthermore, expression pattern analysis of three *Runx2* isoforms was performed in newborn mouse incisors which showed that all isoforms were expressed in both teeth and bone although with different relative levels (Chen et al., 2002). Although several different isoforms exists, it is not known if they have different specific functions or if they are redundant.

### **The dental phenotype of *Runx2* null mutant mice (II-III)**

To elucidate *Runx2* function during tooth development, we examined the phenotype of teeth in *Runx2* null mutant mice. These mice die at birth and we started by a histological analysis of *Runx2* *-/-* jaws. Frontal sections of the molar region of *Runx2* *-/-* embryos and their wild-type littermates were examined at developmental stages of E12, E13, E14, E16, and E18 (E12-13: data not shown, E14-E18: II Fig. 4 and III Fig. 1).

At E12, the wild-type and *Runx2* *-/-* tooth germs were morphologically similar and the first difference was observed during the early bud stage, when molar tooth buds of *Runx2* *-/-* were slightly delayed in development (data not shown). By cap stage (E14), the developmental arrest was remarkable in both upper and lower molars (III Fig. 1B-C). Although the dental mesenchymal condensate appeared normal in *Runx2* *-/-* molars, the enamel knot was not visible as a morphologically distinct entity within the ectodermal compartment. A characteristic feature of the upper molar epithelium at E14 was the presence of an extra budding at the palatal side of the bud (III Fig. 1C). The morphogenesis of *Runx2* *-/-* molars did not proceed during subsequent embryonic development. In addition, absence of *Runx2* affected mandibular molars more severely than maxillary molars (II Fig. 4C-D, III Fig. 1E). At E18, the lower molar tooth bud of *Runx2* *-/-* could not be distinguished from the highly proliferative dental lamina in contrast to the upper molar where the arrest at bud stage still was visible (III Fig. 1H-I).

We had predicted that *Runx2* *-/-* mice would have numerous supernumerary teeth because of the phenotype seen in CCD patients. To our surprise, no extra teeth were found but instead, a complete arrest at bud stage was evident. Mice exhibit only one dentition which has been suggested to represent the primary dentition in humans (Kreiborg et al., 1999) and the palatal bud observed in the *Runx2* *-/-* upper molars could represent the site of formation of successional dentition. In conclusion, it appears that *Runx2* is required for the tooth morphogenesis in the transition from bud to cap stage, and that it inhibits extra epithelial bud protrusion and subsequent formation of successional dentition. Support for the latter has come from a later study of *Shh* involvement in the *Runx2* signaling (Wang et al., 2005).

### **Regulation of *Runx2* expression in embryonic mesenchyme (II, IV)**

There is evidence that bone formation in the embryonic facial processes is dependent on signals from the epithelium (Hall, 1978). By culturing E11 mandibular arch mesenchyme with or without the presence of epithelium and analyzing *Runx2* expression, we could conclude that epithelium was not needed for the induction or maintenance of *Runx2* expression in mandibular mesenchyme (II Fig. 5A-C). However, similar cultures of tooth mesenchyme, with or without epithelium showed that epithelium is needed for the onset and maintenance of *Runx2* (II Fig. 5D-N). When tooth mesenchyme from E11-14 teeth was cultured alone, no expression of *Runx2* was seen (II Fig. 5J-K). When the mesenchyme was recombined with epithelium, *Runx2* expression was induced in the mesenchyme adjacent to the epithelium from E13 onwards (II Fig. 5G-I). The conclusion from these cultures was that dental epithelium is capable of stimulating *Runx2* in dental mesenchyme during the bud stage.

Because Fgfs and Bmps are known epithelial signals in the regulation of tooth morphogenesis, we examined whether these signals could be responsible for the epithelial induction and maintenance of *Runx2* in the mesenchyme. In addition, Bmps were shown to induce *Runx2* in bone development (reviewed by Otto et al., 2003). Hence, it would be conceivable that Bmps are involved in the regulation of *Runx2* during tooth development. However, this was not the case. We did bead implantation experiments on E11-E14 lower molar mesenchymes using *Bmp2*, -4

and -7 proteins and analyzed the explants for *Runx2* expression by whole mount *in situ* hybridisation. Our results showed, surprisingly, that *Bmp2*, -4, and -7 could not induce *Runx2* expression in the tooth mesenchyme at any stage between E11-14 (II Fig. 6A-G). However, similar bead implantation experiments using *Fgf8* protein on E11-12 dental mesenchyme and *Fgf4* protein on E13-14 explants showed that *Fgf4* induced the expression of *Runx2* in isolated dental mesenchyme from E13 onwards (II Fig. 7A-H).

Our results show that dental epithelium regulates mesenchymal *Runx2* expression and that these effects can be mimicked by the FGFs but not by the BMPs. We conclude from these results that *Runx2* regulates the expression of molecules in the mesenchyme that act reciprocally on the epithelium to control its morphogenesis. In addition, the role of *Runx2* in tooth development may be distinct from its role in bone development. Indeed, a recent study has shown that *Runx2* has different downstream targets in the bone and tooth (James et al., 2006).

Tooth development is arrested in several mouse mutants and to gain more insights into the upstream regulation of *Runx2*, we analyzed its expression in the arrested tooth buds of mouse embryos lacking the function of *Msx1*, *Lef1*, and *Ectodysplasin (Eda)* (IV Fig. 1B-G).

*In situ* hybridisation analysis of serial sections of E14 *Msx1* *-/-* heads indicated that *Runx2* expression was downregulated in the mesenchyme around the tooth bud, although intense expression was apparent in the area of forming mandibular bone. This finding indicates that *Msx1* is needed for the expression of *Runx2* in the dental but not osteogenic mesenchyme (IV Fig. 1E).

In *Lef1* null mutant teeth, the defect has been localized to the dental epithelium where *Lef1* is required for the expression of *Fgf4* and for subsequent induction of mesenchymal *Fgf* signals (Kratochwil et al., 1996; 2002). However, *Runx2* expression was apparently normal in the dental and osteogenic mesenchyme of *Lef1* mutant embryos, indicating that *Lef1* is not needed for *Runx2* expression. However, we cannot rule out the possibility that *Wnt* signals could regulate *Runx2* via other transcription factors (IV Fig. 1F).

*Runx2* was intensely expressed throughout dental and osteogenic mesenchyme in the E14 *Eda* null mutants. Since the defect in *Eda* mutants is in the enamel knot, *Runx2* could not be a direct target, but our results indicate that *Runx2* expression is not even secondarily affected by *Eda*-*Edar* signaling (IV Fig. 1G).

#### **Search for downstream targets of *Runx2* (IV)**

To position *Runx2* in the known signaling pathways regulating the bud to cap stage transition during tooth development, we examined the expression of potential downstream target genes of *Runx2*, by using *in situ* hybridisation. We compared E14 *Runx2* *-/-* tooth germs with both E13 (bud) and E14 (cap) wild-type teeth, since remarkable changes take place in gene expressions between bud and cap stages and *Runx2* *-/-* teeth are arrested between them.

The expression patterns of altogether 37 genes were compared between the teeth of *Runx2* *-/-* and their wild-type or heterozygous littermates. We described the patterns of the following genes in *Runx2* *-/-* and wild-type teeth: *Fgf3*, *Fgf4*, *Fgf10*,

*Fgfr1*, *Sprouty1*, -2, and -4, *Bmp2* and -4, *Lef1*, *Tgfb1*, *Msx1* and -2 (data not shown), *Shh*, *Activin*  $\beta$ A, *p21*, *Pax9*, *Edar*, *Twist* (data not shown), *Wnt5a*, -10b, *Runx1*, *Runx3*, and *Tenascin*. In addition, we analyzed the expression of the following genes and proteins but no apparent differences between the wild-type and *Runx2* *-/-* was found: The *Notch* pathway genes *Hes1*, *Hes5*, and *L-fng*; Bmp inhibitors *Dan* and *Ectodin*, *Wnt10a*, *Wnt11a*, *Eda*; the matrix metalloproteinase *Mmp2*; and the *Mmp* inhibitors *Timp2* and *Timp3*, *hairless*, and the basement membrane molecule laminin1 (data not shown).

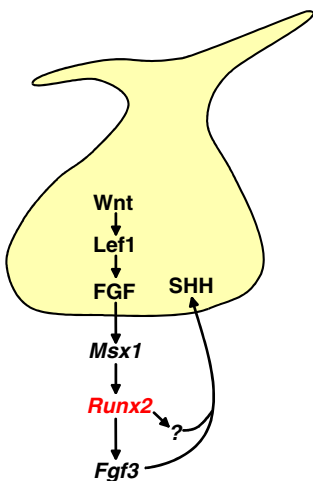
Most genes in the mesenchymal condensate were normally expressed in *Runx2* *-/-* teeth. We did not detect any significant changes in the expression of *Msx1*, *Bmp4*, *Pax9*, *Lef1*, *Tgfb1*, *Wnt5a*, and *Tenascin* in *Runx2* *-/-* molars (IV Fig. 2A-T). However, *Activin*  $\beta$ A expression was markedly reduced in both upper and lower molars of *Runx2* mutants, especially in the mesenchymal cells immediately underneath the epithelial bud (IV Fig. 2R). This suggests that *Activin*  $\beta$ A may be downstream of *Runx2* in this restricted cell population.

Fgfs act as both epithelial and mesenchymal signals and regulate tooth development at all stages and we have shown that *Runx2* is a target of Fgf signaling in the tooth. Therefore, we analyzed the expression patterns of several genes in the Fgf signaling pathway in *Runx2* *-/-* teeth. Since *Fgf3* and *Fgf10* are coexpressed with *Runx2* in the wild-type dental mesenchyme, they were candidates to be direct downstream targets. Interestingly, *Fgf3* expression was virtually absent from the *Runx2* mutant tooth germs and only occasionally was faint signal seen in mutant upper molar (IV Fig. 3C). No significant difference was found in expression patterns between *Runx2* *-/-* and wild-type teeth of following genes: *Fgf10*, *Fgfr1*, and *Sprouty1* (IV Fig. 3D-L). However, *Sprouty2* and -4 were downregulated in the epithelium of *Runx2* *-/-* teeth (IV Fig. 3M-R). In conclusion, *Fgf3* is a possible target gene of *Runx2*. The specific reduction in the expression of *Sprouty2* and *Sprouty4* in *Runx2* *-/-* dental epithelium suggest that Fgf signaling was inhibited there and that *Fgf3* is a reciprocal signal affecting dental epithelium. In line with this, we showed that overexpression of *Runx2* can induce *Fgf3* expression in *Runx2* *-/-* calvarial cells. This result further indicates that *Fgf3* may be a target of *Runx2* in dental mesenchyme (IV Fig. 4A-B).

Previous work has shown that the expression of *Fgf3* in the dental mesenchyme is controlled by dental epithelium and that the epithelial effect can be mimicked by epithelially expressed *Fgfs* (Kettunen et al., 2000); Bei and Maas, 1998). In line with this we showed that *Fgf4* can induce *Fgf3* in the dental mesenchyme at E13 and E14 but failed to induce *Fgf3* in the *Runx2* *-/-* dental mesenchyme (IV Fig. 7A-F). In addition, *Fgf4* could induce *Tgfb1* and *Activin*  $\beta$ A in *Runx2* *-/-* molars (IV Fig. 7G-O). This result showed that *Runx2* mutant mesenchyme was competent to respond to Fgf signals and therefore the lack of Fgf receptors or other mediators of Fgf signals do not explain the lack of *Fgf3* induction by *Fgf4*. Thus, *Runx2* may directly regulate *Fgf3* expression in the dental mesenchyme. Furthermore, we showed that *Runx2* is expressed in the dental mesenchyme, regulated by epithelial Fgf signaling, and regulating *Fgf3* expression in the dental mesenchyme indicating that *Fgf3* may be a direct target gene of *Runx2*. It has also been shown that *Fgf3* is missing in *Msx1* *-/-* teeth and that its induction by Fgfs requires *Msx1* (Bei and Maas, 1998). These findings together with the downregulation of *Runx2* in *Msx1* *-/-*

teeth suggest that *Runx2* can be placed between *Msx1* and *Fgf3* and that it is responsible for mediating *Fgf* signaling from the dental epithelium to the mesenchyme (Fig. 11).

Although the enamel knot is morphologically discernible only at cap stage, its induction takes place already at the late bud stage when many enamel knot marker genes are upregulated (Jernvall and Thesleff, 2000). *Shh* is one of the best characterized enamel knot marker genes and is required for normal tooth morphogenesis. *Shh* expression was absent from the *Runx*  $-/-$  lower molar tooth buds, which suggested that the enamel knots may not have formed in the *Runx2*  $-/-$  teeth (IV Fig. 5C). However, the analysis of the expression of other enamel knot marker genes indicated that a partially functional enamel knot actually starts to form in the mutants. The genes analyzed included *Wnt10b*, *Lef1*, *Fgf4*, *Bmp2*, *Edar*, *Msx2* and *p21* (IV Fig. 5D-U). Surprisingly, most genes were expressed in the tips of mutant upper molar buds with similar intensities to those in the fully developed enamel knots of cap stage wild-types molars. *Shh* was weakly expressed in the upper molars of *Runx2*  $-/-$ . However, in the lower molars of *Runx2* mutant embryos, the enamel knot markers were either absent or reduced with the exception of *Wnt10b*, *Lef1*, and *Msx2*, which were expressed with similar intensity as in wild-type molars (IV Fig. 5D-I, data not shown). These observations indicated that the enamel knots start to form in *Runx2*  $-/-$  tooth buds and that there are differences between the upper and lower molar. Enamel knot signaling is believed to regulate the growth of the cervical loops either directly or indirectly via mesenchyme. Despite the expression of many enamel knot marker genes, the cervical loops did not form in either lower or upper *Runx*  $-/-$  molars which could be due to lack of *Shh* signaling in both upper and lower mutant molar. However, when *Shh* function was conditionally deleted in developing teeth (Dassule et al., 2000), cervical loop formation was only partly inhibited, which suggest that lack off *Shh* signaling can not be fully responsible for the arrest of *Runx2* mutant teeth at bud stage.



**Fig. 11.** Schematic representation of the genetic pathway involving *Runx2* during bud to cap transition in tooth development.

### **Attempts to rescue *Runx2* mutants tooth development (III, IV)**

We performed tissue recombinations where dental epithelium and mesenchyme from wild-type and *Runx2* *-/-* mandibular E13 molars were recombined in different combinations and cultured for 6-8 days. When *Runx2* *-/-* mesenchyme was recombined with wild-type epithelium, no obvious development occurred. When *Runx2* *-/-* epithelium was recombined with wild-type mesenchyme, normal and bell-staged molar morphology was seen. Hence, the primary defect in the *Runx2* *-/-* tooth organs resides in the dental mesenchyme and cannot be rescued by wild-type epithelium.

Furthermore, we cultured *Runx2* *-/-* molar organs under the kidney capsule of nude mice to examine if their development would be rescued under long term culture *in vivo*. However, only cyst-like structures formed and no teeth were observed. A wild-type tooth cultured for the same time formed a well-mineralized late bell stage tooth with surrounding alveolar bone.

We also tried to rescue the *Shh* expression in the enamel knot by adding exogenous Fgf but no rescue was observed. Hence, our results indicate that lack of Fgf3 in the *Runx2* mutant teeth is not the only reason for the downregulation of Shh expression in the enamel knot. There may be other mesenchymal signal molecules that regulate Shh expression in the enamel knot.

Since *Fgf3*, *Activin*  $\beta$ A, and *Shh* were all downregulated in *Runx2* mutant teeth, we attempted to rescue the morphology by culturing them in the presence of the recombinant proteins Fgf4, Fgf10, *Activin*  $\beta$ A and *Shh*. None of the signal molecules rescued the morphogenesis of *Runx2* mutant molars, either when introduced alone or in various combinations.

In conclusion, we observed that *Runx2* has a dual role in tooth development: it is necessary for the bud stage tooth to proceed to cap stage but it is also needed to prevent extra budding from the epithelium.

### **Runx2 and CCD**

The apparent difference between *Runx2* heterozygote mouse and CCD human syndrome is the lack of supernumerary teeth in mice which is likely due to the lack of secondary dentition in mice. A correlation of genotype and phenotype have been observed in CCD patients. If a mutation in the runt domain takes place, which is necessary for DNA binding, a classical CCD occurs with a wide variety of affected structures (Zhou et al., 1999). However if the mutation occurs in the WVRPY - 3'end, which is the binding site for the TLE coreceptor, a CCD phenotype with only supernumerary teeth has been reported (Quack et al., 1999). This could be a coincidence but it fits well with the idea that *Runx2* is needed to repress the budding of the dental lamina and to inhibit formation of the successional teeth. Other studies have concluded that there is no correlation between the phenotype and genotype but that could be due to small number of patients and perhaps that no mutations were observed in the TLE binding site (Otto et al., 2002). Another correlation that has been observed is the height of CCD patients (SD) and the number of supernumerary teeth which is more difficult to explain (Yoshida et al., 2002). De-

tailed study of mutations of *Runx2* in CCD patients would be needed to detect further genotype/phenotype correlation regarding supernumerary teeth. A recent study showed that activation of Wnt signaling leads to multiple extra teeth which suggests that the repressor function of Runx2 could be downstream of Wnt (Järvinen et al., 2006). It has also been shown that Wnt acts through  $\beta$ -catenin to interact with Runx2 and Smad which integrates Smad and Wnt pathways in interaction with Runx2 (Gaur et al., 2005). Furthermore, it is also evident that Bmp regulate *Runx2* in bone tissues and Fgf in tooth development (James et al., 2006). This could indicate that Bmp and Wnt regulate the repression function of Runx2 on the extra budding and successor tooth formation and that Fgf may regulate the activation of *Runx2* that promote tooth development.

## CONCLUDING REMARKS

The understanding of molecular mechanisms controlling tooth development has greatly advanced during the last years. The developing tooth has proven to be a powerful model for studying the sequential signaling events involved in organogenesis in general. Our expression analysis of six different *Bmps* indicated that Bmp signals are produced by both mesenchyme and epithelium and are important reciprocal regulators of tooth development. The present study also showed that *Runx2* is a transcription factor that is absolutely required for tooth development. In *Runx2* null mutant mice tooth development is arrested at bud stage, and our analysis of *Runx2* function gained us further information about signaling pathways involved in tooth development. We have demonstrated that *Runx2* is an essential transcription factor mediating Fgf signaling and epithelial-mesenchymal interactions in developing teeth during the transition from bud to cap stage. In addition, we show that *Runx2* is required for the upregulation of *Fgf3* gene expression. Our results also indicate that *Runx2* regulates additional mesenchymal signals which are required for continued morphogenesis of teeth.

The finding that mutations in *Runx2* cause in human and mice CCD, where bones as well as teeth are affected, provided an excellent opportunity to examine and compare the functions of one transcription factor in two different organs. Our studies suggested that *Runx2* is differentially regulated in dental and bone mesenchyme. Interestingly, recent work has shown that *Runx2* acts on different target genes and affects different signaling pathways in bones and teeth (James et al., 2006). In addition, *in vitro* experiments, in the same study, determined that *Runx2* is part of the Fgf and Bmp signaling pathways in tooth and bone development, respectively. Hence, the available evidence suggests that *Runx2* has a specific direct function on tooth formation, which is not secondary to its effect on bone.

In *Runx2* null mutants, tooth development is arrested at bud stage, similarly as in *Msx1*, *Pax9* and *Lef1* mutants. However, unlike the other mouse mutants, the lower molars in *Runx2* mutants were more severely affected than upper molars. In addition, in the upper molars of *Runx2* mutants extra buddings occurred at the palatal side of the tooth bud. Although mice do not develop a secondary dentition, these buds provided clues for the pathogenesis of CCD syndrome which is caused by *RUNX2* haploinsufficiency and characterized by multiple supernumerary teeth. We suggested that these extra buds occurred at sites where the formation of the secondary teeth is normally prevented in mice and that *Runx2* acts as an inhibitor of successional tooth formation by preventing advancing development of the buds.

We also showed that several genes expressed in the enamel knot were downregulated or missing in the *Runx2* *-/-* lower molar but normal in the upper molar. In particular, *Shh* was absent in the lower molars, and very faintly expressed in the upper molars. In a subsequent study in our laboratory on *Shh* involvement in *Runx2* signaling, it was shown that *Shh* as well as its mediators *Ptc1*, *Ptc2*, and *Gli1* were downregulated in the lower molars of *Runx2* null mutant whereas active *Shh* signaling was present in the extra buds of upper molars of *Runx2* null mutants as well as of *Runx2* mutant heterozygotes (Wang et al., 2005). This linked *Shh* expression in the enamel knot with the onset of successional tooth formation.

It was shown recently that continuous tooth generation in mouse could be induced by activated Wnt/beta-catenin signaling, and it was suggested that this unlocked the capacity for tooth renewal that was lost in rodents more than 60 millions of years ago (Järvinen et al., 2006). Interestingly, these supernumerary teeth formed successively from enamel knots expressing known markers including *Shh*. It was suggested that the mechanism of continuous tooth generation involved the iterative formation of ectopic enamel knot signaling centers where enamel knot activation and lateral inhibition is the underlying key mechanism. The results from my studies are in line with this suggestion and it is tempting to speculate that the formation of the supernumerary teeth in cleidocranial dysplasia as a result of *Runx2* haploinsufficiency results from modulation of the lateral inhibition mechanism that regulates enamel knot activation. The exact function of *Runx2* in this process remains to be demonstrated. *Runx2* was recently linked to Wnt signaling by the demonstration that *Dkk1*, a secreted Wnt antagonist, is a target of *Runx2* in dental mesenchyme (James et al., 2006), and the work in this thesis work linked *Runx2* to Fgf signaling. Since *Runx2* influences the expression of numerous genes in dental mesenchyme as shown by microarray analysis (James et al., 2006), it is possible that its role in the inhibition of successional tooth formation involves the modulation of several signaling pathways. Further studies are needed for more detailed dissection of the molecular basis for the pathogenesis of CCD.

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