Mo ular Mechanisms Underlying Tooth Mo hogenesis and Cell Differentiation

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CONTENTS

ABBREVIATIONS

aa	amino acid
BMP	bone morphogenetic protein
BrdU	5'-bromo-2'-deoxyuridine
BSA	bovine serum albumin
Bsp	bone sialoprotein
Cbfa1	core binding factor alpha 1
cDNA	complementary deoxyribonucleic acid
CCD	cleidocranial dysplasia
DAB	3'-3' diaminobenzidine
DEPC	diethyl pyrocarbonate
Dlx	vertebrate homologue of *Drosophila distal-less* gene
DNA	deoxyribonucleic acid
DMEM	Dulbesso's Modified Eagle's Medium
E	embryonic day
EDTA	ethylenediamine tetra-cetic acid
Fgf	fibroblast growth factor
Fgf	fibroblast growth factor receptor
Gli	vertebrate homologue of *Drosophila cubitus interruptus* gene
GDF	growth and differentiation factor
Hh	hedgehog
Hox	vertebrate homeodomain box gene
Igf	insulin-like growth factor
K14-follistatin	overexpression of follistatin under keratin 14 promoter
Lef1	lymphoid enhancer factor 1
M1	first molar
M2	second molar
M3	third molar
mRNA	messenger ribonucleic acid
Msx	vertebrate homologue of *Drosophila muscle segment (Msh)* gene
MMP	matrix metalloproteinase
Osf2	osteoblast specific factor 2
p21	21 kD cyclin dependent kinase interacting protein
P	postnatal day
PCR	polymerase chain reaction
RT-PCR	reverse transcription – polymerase chain reaction
PFA	paraformaldehyde

Pitx2	pituitary homeobox 2
PTHrP	parathyroid hormone-related protein
Runx	vertebrate homologue of the *Drosophila runt* gene
Shh	sonic hedgehog
Smad	vertebrate homologue of *Drosophila mothers against decapentaplegic (Mad)*
TGFβ	transforming growth factor beta
Tnf	tumor necrosis factor
UTP	uridine triphosphate
Wnt	vertebrate homologue of the *Drosophila* segment polarity gene *wingless*

Genes are given in italics. Proteins are given in Roman using upper case letters.

LIST OF ORIGINAL PUBLICATIONS

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

I Åberg, T., Cavender, A., Gaikwad, J.S., Bronckers, A.L., Wang, X.P., Waltimo-Siren, J., Thesleff, I., and D'Souza, R.N. (2004). Phenotypic changes in dentition of Runx2 homozygote-null mutant mice. *J. Histochem. Cytochem*. 52: 131-9.

II Åberg, T.*, Wang, X.P.*, Kim, J.H., Yamashiro, T., Bei, M., Rice, R., Ryoo, H.M., and Thesleff, I. (2004). Runx2 mediated FGF signalling from epithelium to mesenchyme during tooth morphogenesis. *Dev. Biol.* 270: 76-93
* Equal contribution

III Wang, X.P., Suomalainen, M., Jorgez, C.J., Matzuk, M.M., Wankell, M., Werner, S., and Thesleff, I. (2004). Modulation of activin/BMP signalling by follistatin is required for the morphogenesis of mouse molar teeth. *Dev. Dyn.* 231: 98-108.

IV Wang, X.P., Suomalainen, M., Jorgez, C.J., Matzuk, M.M., Wankell, M., Werner, S., and Thesleff, I. (2004). Follistatin inhibits ameloblast differentiation by antagonizing BMP4 signaling and is responsible for the enamel-free area formation in the mouse incisors. Submitted.

 In addition, some unpublished data are presented.

SUMMARY

Mammalian organs comprise epithelial and mesenchymal tissues. During development, sequential and reciprocal interactions between these tissues regulate initiation, morphogenesis, as well as organ-specific cell differentiation. At the molecular level, these interactions are mediated by signaling molecules, their receptors, transcription factors, and cell adhesion molecules. It is now well known that during embryogenesis, many signals and signaling pathways have been remarkably conserved among different organs and species, and even between invertebrates and vertebrates. Studying the molecular mechanisms underlying one system can often provide clues to the studies of other models.

Teeth are typical examples of epithelial appendages and their early development resembles morphologically as well as molecularly other epithelial derived organs, such as hairs, feathers, and glands. Since developing mouse tooth germs are easily accessed and manipulated *in vitro*, they have been used for a long time as good models for studying the nature of epithelial-mesenchymal interactions and the molecular regulation of organogenesis. Teeth start to form from a narrow stripe of thickened epithelium on the oral surface of maxillary and mandibular primordia. Tooth germs pass through bud, cap, and bell stages with dental epithelium growing and folding into the specific shape of the tooth crown. Eventually, dental epithelial cells give rise to ameloblasts and mesenchymal cells into odontoblasts, which then secrete enamel and dentin matrices, respectively. Since the 1990s, there have been dramatic advances in our understanding of the genetic control of tooth development, and the molecular basis and signaling networks regulating tooth development is starting to be elucidated.

Runx2 (Cbfa1) is a runt domain transcription factor that plays pivotal roles in the formation of bones and teeth. Mutations of one allele of the *Runx2* gene in humans are responsible for cleidocranial dysplasia, a syndrome characterised by general bone dysplasia as well as supernumerary and unerupted teeth in permanent dentition. *Runx2* knockout mice completely lack bone formation and their teeth arrest at the late bud stage. Earlier work has shown that *Runx2* is expressed in the dental mesenchyme and regulated by epithelial FGF signals. In this study, we analyzed in detail the tooth phenotype in *Runx2* mutant mice. We showed that the *Runx2* mutant lower molars were affected more severely than the upper ones. Moreover, there was extra budding on the lingual aspects of *Runx2* mutant upper molars, which may represent the extension of dental lamina to form a secondary dentition in other animals. The differences between mutant upper and lower molars could also be detected molecularly with most of the enamel knot marker genes expressed normally in the mutant upper molars, whereas reduced or absent in the lower ones. More significantly, the expression of *Runx3*, another runt domain transcription factor, was dramatically

upregulated in *Runx2* mutant upper molars, which may substitute some of Runx2 function and contribute to the differences between the mutant upper and lower molars. Tissue recombination experiments indicated that the main defective tissue in *Runx2* mutant teeth is the dental mesenchyme, which is consistent with the expression pattern of *Runx2* in the mesenchymal cells. In *Runx2* mutant molars, *Fgf3* expression was downregulated and FGF4 protein releasing beads failed to induce *Fgf3* expression in the mutant dental mesenchyme as in the wild types. Based on these results, and also the finding that *Runx2* expression was downregulated in *Msx1* mutant tooth germs, we proposed a model where Runx2 functions in the dental mesenchyme between Msx1 and Fgf3 and mediates FGF signals and epithelial-mesenchymal interactions during tooth development.

The development of mammalian organs follows a rigid temporal and spatial schedule which is regulated by antagonistic interactions between activators and their inhibitors. The final outcome of these interactions determines the cell fate. Follistatin is an extracellular modulator of TGFβ superfamily signals, including activins, BMPs, and GDFs. Earlier work has shown that follistatin is transiently expressed in the primary and secondary enamel knots of the developing mouse molars with concominant expression of activin βA in the underlying mesenchyme, suggesting an important role of these molecules in tooth development. Here we studied the role of follistatin during tooth development by analyzing the tooth phenotypes in follistatin knockout mice and in transgenic mice overexpressing follistatin under keratin 14 promoter. Both mouse lines exhibited misshapen molars. In follistatin knockout mouse molars, the primary enamel knot has formed. However, its signaling function was apparently disturbed resulting in the defects in secondary enamel knot formation and aberrant tooth shape. These data suggested that finely tuned antagonistic interactions between follistatin and activin /BMP signals are critical for the precise size and shape of mouse molars. Interestingly, these antagonistic interactions are also involved in the regulation of cell differentiation in the tooth. Over-expression of follistatin in the dental epithelium inhibited ameloblast differentiation. Conversely, in follistatin knockout mice, functional ameloblasts differentiated on the lingual surface of mouse incisors, which is normally the root-analogue area without any enamel formation in wild type mice. We showed that BMP4, which is expressed in odontoblasts, is able to trigger the differentiation of inner dental epithelium into ameloblasts. Activin βA expressed in the dental follicle can induce follistatin in the dental epithelium. Follistatin acts locally on the dental epithelium antagonising the ameloblast-inducing activity of BMP4 from odontoblasts and thereby prevents enamel formation. Our results implicate a novel role for the dental follicle as a regulator of enamel formation and indicate that the differentiation of dental epithelium into ameloblasts is regulated by antagonistic actions between activin and BMP signals from two dental

mesenchymal cell lineages. Follistatin integrates these effects and spatially and temporarily regulates enamel formation. These results have helped us in understanding the molecular control of cell differentiation in the tooth, and furthermore emphasized the importance of negative regulation during development.

1. REVIEW OF THE LITERATURE

Tooth development is a multi-step and complex process involving coordinated interactions between different tissue layers. The early stages of tooth development resemble morphologically and molecularly other ectodermal derived organs, such as hairs, feathers, and mammary glands (Pispa and Thesleff, 2003). They all develop via sequential and reciprocal interactions between epithelial and mesenchymal tissues. At the initiation stage, local thickenings of the ectoderm form ectodermal placodes, which direct the condensation of underlying mesenchymal cells. Subsequently the ectodermal placodes invaginates into (tooth, hair) or grows out of (feather) the mesenchyme generating an ectodermal bud, which then grows and folds, or branches outlining the final shape of the organ. Eventually, differentiation of specialized cell types contributes to a functional organ in the body.

It is now well known that during embryogenesis, many signals and signaling pathways have been conserved between different organs, or even between invertebrates and vertebrates. There are also remarkable similarities in the developmental regulatory processes used in different systems, such as lateral inhibition in the early induction of primary axes and later in the induction of hair follicles and feather buds, as well as signaling centers in the limb bud, tooth, and hair follicle development (Patel et al., 1999; Nakamura et al., 2003; Smith, 1999). Studying the mechanisms underlying one model system can often shed light on the studies of other models. Since the developing mouse tooth germs are easily assessed and can be experimentally manipulated *in vitro*, they have been used for a long time as a powerful model for analyzing the molecular mechanisms of organogenesis (Thesleff and Nieminen, 1996). Over the last 15 years, rapid progress in molecular biology, genetics, transgenic mouse techniques, together with classical embryological approaches have led significant insight into the genetic regulation of tooth development. More than 300 genes have been demonstrated to be expressed in the developing tooth (see tooth database http://bite-it.helsinki.fi). The genetic pathways and signaling networks involved in tooth development have been studied in great detail particularly in several protein families, including fibroblast growth factor (FGF), transforming growth factor β (TGF-β), hedgehog (Hh), Wnt, and tumour necrosis factor (TNF) (Jernvall and Thesleff, 2000; Thesleff and Mikkola, 2002). In this review I will discuss the current knowledge of molecular and tissue interactions regulating tooth development from initiation and morphogenesis, to the final differentiation of ameloblasts and odontoblasts. A special focus will be given to the transcription factor *Runx2* and the soluble protein follistatin in embryogenesis and specifically during tooth development.

1.1. Developmental anatomy of the tooth

Mammalian dentition is usually heterodont with teeth of different shapes, and diphyodont consisting of two sets of

teeth (Berkovitz et al., 2002). In the oral cavity, from anterior to posterior region, there are basically three tooth forms: incisiform, caniniform, and molariform. Incisiform (incisors) teeth have thin blade-like crowns for cutting the food. Caniniform teeth (canines) are used for piercing or tearing food with a single pointed cone-shaped crown. Molariform teeth (premolars and molars) possess a number of cusps used for grinding and mastication (Berkovitz et al., 2002). Species-specific variations exist in the teeth. For example, humans have two generations of teeth: deciduous (primary) dentition and permanent (secondary) dentition, and the permanent dentition contains two incisors, one canine, two premolars, and three molars in each quadrant of the jaws. Mice only exhibit one dentition with one incisor in the front and three molars in the back of each half of the jaw. Between the incisor and molar teeth is a toothless diastema region containing rudimentary tooth germs arrested at the bud stage and eventually degenerated by apoptosis (Fig. 1; Keränen et al., 1999; Tureckova et al., 1995). Mouse teeth are also unique in that their incisors grow continuously throughout life and the enamel is solely formed on the labial surface of the incisors, whereas the lingual aspect is enamel-free and only covered by dentin.

Mammalian teeth develop on the oral surface of the frontonasal process, maxillary process, and mandibular process. The first evidence of tooth development in mice is seen around embryonic (E) day 11.5 with the formation of a horseshoe-shaped epithelial ridge, i.e. dental lamina, from the basal layer of the primitive oral epithelium into the mesenchyme. The dental lamina follows the line of the vestibular fold and marks the position of the future dental arch (Fig. 1). Further development of the dental lamina gives

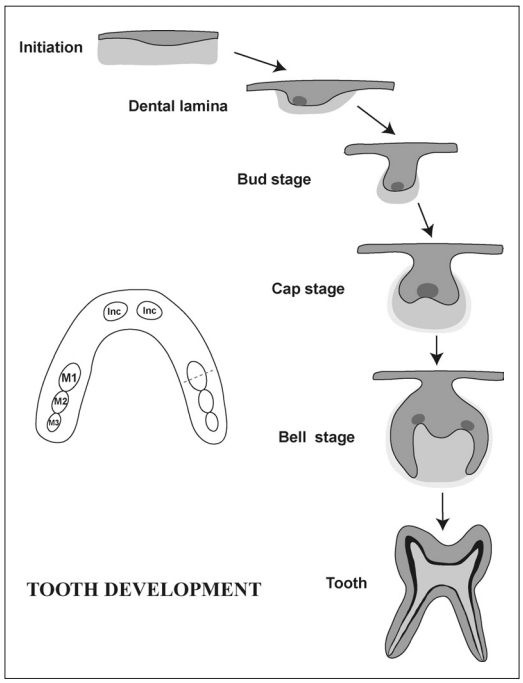

Fig.1. Schematic view of molar tooth development. Mouse has one incisor (Inc) and three molars (M) in each quadrant of the jaw. Teeth develop through dental lamina, bud, cap, and cell stages with signaling centers form at the tips of the tooth buds. Inc, incisor; M1, first molar; M2, second molar; M3, third molar.

rise to individual globular swellings which bud into the jaw mesenchyme (E12-E13, bud stage). The mesenchymal cells proliferate and condense around the bud. Meanwhile, the dental lamina grows backward giving rise to the second and third molar germs. The tooth bud grows and folds becoming progressively cap-shaped and enveloping the underlying dental mesenchyme, which is now termed dental papilla (E14-E15, cap stage). The surrounding mesenchymal cells form the dental follicle. At this stage, a cluster of condensed cells can be obviously seen at the tip of the tooth bud constituting the primary enamel knot, a transient signaling center immediately above the dental papilla mesenchyme. After the cap stage, the primary enamel knot degenerates soon by apoptotic removal. During the following bell stage (E16 onward), the tooth germ undergoes further morphodifferentiation and histodifferentiation forming distinct cell types and cell layers of the enamel organ. In the center of the enlarging enamel organ reside large and star-shaped cells containing conspicuous nuclei and many branching processes forming the stellate reticulum. The extracellular matrix of the stellate reticulum is fluid filled and rich in glycosaminoglycans, which have been suggested to be involved in the maintenance and protection of the enamel organ by balancing pressure from the dental follicle. The external epithelial cells remain cuboidal and are separated from the surrounding mesenchymal dental follicle by a basement membrane. Between the stellate reticulum and inner dental epithelium are two or three layers of flattened cells forming the stratum intermedium. The secondary enamel knots start to form at the tips of future cusps governing the folding of the dental epithelium and determining the shape of the tooth crown (Jernvall and Thesleff, 2000). At late bell stage, the inner dental epithelial cells become columnar, elongated, and polarized, and differentiate into enamel-secreting ameloblasts. The dental papilla mesenchymal cells lying adjacent to the inner dental epithelium differentiate into dentin-secreting odontoblasts, and the remaining dental papilla cells give rise to the dental pulp. The differentiation of ameloblasts and odontoblasts both start at the tips of future cusps, gradually sweeping down to the base of the tooth crown and they are coordinated with each other. When the odontoblasts start to secrete dentin matrix, the basement membrane between the dental papilla and pre-ameloblasts become degraded, and later ameloblasts secrete enamel matrix which then mineralizes forming the hardest tissue in the body (Kjoelby et al., 1994). The dental lamina connecting the enamel organ to the oral mucosa breaks down and degenerates. Once the formation of tooth crown is completed, roots start to develop. Dental follicle cells surrounding the enamel organ generate cementoblasts lining the root, and fibroblasts and osteoblasts forming the periodontal ligament and alveolar bone supporting the tooth. The dental follicle also plays a significant role during the eruption of teeth into the oral cavity (Berkovitz et al., 2002).

1.2. Molecular regulation of tooth morphogenesis

1.2.1. Tooth initiation

The initiation of organogenesis involves both instructive and permissive factors, i.e. an inducer from one tissue (e.g. the epithelium) and the competence of the other tissue (e.g. the mesenchyme). In most epithelial derived organs, such as hairs, mammary gland, and kidney, the first inducer comes from the mesenchyme (Pispa and Thesleff, 2003). In the context of tooth development, the initial signals reside in the stomodeal epithelium. The mesenchymal component of the tooth derives from cranial neural crest cells, which migrate from caudal regions of midbrain and populate into the facial primordia (Imai et al., 1996; Chai et al., 2000). Classical tissue recombination experiments have demonstrated that the early stage (E9-E11) mandibular arch oral epithelium can induce tooth formation when recombined with neural crest-derived mesenchyme in the second branchial arch, or even with neural crest cells from the trunk level, but not with non-neural crest derived mesenchyme such as limb mesenchyme. Reversed recombinations between mandibular mesenchyme and nondental epithelium do not form teeth, indicating that the early stage oral epithelium possesses the odontogenic potential (Mina and Kollar, 1987; Lumsden, 1988). Moreover, the early stage oral epithelium can also determine the tooth identity, since recombination of incisor epithelium with induced molar mesenchyme forms an incisiform tooth (Kollar and Mina, 1991). After the initiation stage, around E12, the odontogenic potential shifts from the dental epithelium to the mesenchyme, which subsequently guides the tooth formation and also determines the tooth shape (Mina and Kollar, 1987; Lumsden 1988; Kollar and Baird, 1969).

The development of the teeth is confined in a U-shaped area in the maxilla and mandible. At the early stage, the oral ectoderm and mesenchyme appear homogenous in the facial primodia and all the neural crest derived mesenchymal cells possess odontogenic capacity (Mina and Kollar, 1987; Lumsden, 1988). It has been shown that at E10, mandibular arch mesenchymal cells are equally competent to respond to epithelial FGF8 signaling for the induction of the homeobox transcription factor *Lhx7* and form teeth. However, *Lhx6* and *Lhx7* expression are only restricted to the oral side mesenchyme of maxillary and mandibular processes, whereas the expression of *Goosecoid* is confined to the aboral side mesenchyme and is prevented by *Lhx7* expressing cells. The oral and aboral polarity of the mandible has been suggested to be specified by regionally localized signals from the oral ectoderm, such as FGF8, which in conjunction with another ectodermal signal endothelium-1 act by maintaining and gradually fixing the spatial expressions of oral (*Lhx7*-expressing) and aboral (*Goosecoid*-expressing) homeobox genes. By E10.5-E11.0, the fate of aboral side mesenchymal cells become determined and they gradually lose their competence to respond to FGF8 signal for oral-side genes expression (Grigoriou et al., 1998). It is thus appears that although the identity of the brachial arch is determined by neural crest cells, the

polarity of the first branchial arch is controlled by the ectoderm.

Tooth buds are generated from specific regions within the dental lamina. The correct position of individual tooth buds within the dental lamina was considered to be related to the antagonistic interactions between FGF and BMP signals, which regulate the mesenchymal expression of *Pax9* gene (Neubuser et al., 1997). *Pax9* is a paired box transcription factor specifically expressed in the prospective tooth mesenchyme prior to any morphological signs of tooth development (E10 in molar mesenchyme; E10.5 in molar and incisor mesenchyme). FGF8 induces *Pax9* expression, whereas BMP4 and BMP2 prevent this induction. However, in *Pax9* deficient mice, the tooth buds do form in the normal locations, indicating that some other genes are also required for the initial determination of individual tooth sites (Peters et al., 1998).

The definition of the boundaries of developing tooth germs has been demonstrated to involve antagonistic interactions between Shh and Wnt signals. *Shh* expression is highly restricted to the dental lamina of future incisor and molar regions at the early stage and later to the tips of the tooth buds (E11.5–E14.5). SHH protein acts as a long-range signal and application of SHH protein *in vitro* can induce oral epithelial cell proliferation. In sharp contrast, *Wnt-7b* is expressed reversely to *Shh*, throughout oral epithelium but remarkably absent in *Shh* expressing tooth-forming regions (Bitgood and McMahon, 1995; Hardcastle et al., 1998). Since ectopic- and over-expression of Wnt-7b in the dental epithelium represses Shh expression and prevents tooth bud formation, it has been proposed that Wnt7b acts by restricting Shh expression at specific tooth-forming regions within the dental lamina so that Shh can only locally stimulate cell proliferation for the tooth bud formation (Gritli-Linde et al., 2001; Hardcastle et al., 1998; Sarkar et al., 2000).

The localisation of tooth bud epithelial thickening may also involve other molecules which show restricted expression patterns in the developing tooth germs. For example, *Pitx2* (*Otlx2*) is a bicoid-related transcription factor which is expressed continuously in the dental lamina epithelium at E11 stage but subsequently (E12) limited to the budding tooth germs (Mucchielli et al., 1997; Keränen et al., 1999). Targeted mutation of the *Pitx2* gene in transgenic mice results in the development of maxillary teeth arrested at placodal stage and mandibular teeth arrested at bud stage. Similarly haploinsufficiency of *PITX2* gene in humans has been shown to be associated with Rieger syndrome comprising missing teeth (Oligodontia) (Semina et al., 1996; Flomen et al., 1998; Lin et al., 1999).

Mammalian dentitions are highly patterned with specific shapes of teeth for each locations of the jaw, i.e. mono-cuspid teeth located in distal (anterior) region and multi-cuspid teeth in proximal (posterior) region. The basic dental pattern has been suggested to be established early during embryogenesis, such that cranial neural crest cells may be specified first as odontogenic lineage and later further regionally specified as maxilla/mandible/molar/incisor (Weiss et al., 1998; Teaford et al., 2000).

In insects and vertebrates, the anterior-posterior axis of the body is

determined by homeotic genes or homeobox-containing Hox genes. In vertebrates, Hox genes are expressed in ectoderm and mesoderm during gastrulation and during organogenesis expression is found in the central nervous system, somites, and limb buds. The neural crest cells emanating from the neural tube maintain their Hox-gene expression during their migration (Duboule and Morata, 1994; Ramirez-Solis et al., 1993). However, no Hox gene is expressed in the first branchial arch mesenchyme. Instead, there are a number of non-Hox 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, Lhx6, -7,* and *Gsc*. They are expressed prior to the morphological sign of tooth development and also during tooth development (Peters et al., 1998; Cobourne and Sharpe, 2003). These homeobox-containing transcription factors do not exhibit genomic colinearity as in the Hox genes, but the combinational activities of these genes may specify the tooth shape via an "odontogenic homeobox code" (Sharpe, 1995). Evidence for the potential role of these homeobox genes in determining the identity of the teeth has come from *in vivo* expression pattern analysis and targeted gene mutagenesis, as well as *in vitro* experiments manipulating gene expressions in the tooth germ. It has been shown that *Barx1* is induced by FGF8 from overlying oral ectoderm and restricted to the proximal molar-forming region by antagonistic signaling from Bmp4 in the distal incisor-forming epithelium. Conversely, *Msx1* expression in the incisor-forming region mesenchyme is induced and maintained by BMP4 (Tucker et al., 1998). Inhibition of BMP4 in early mandibular arch (E9-E10) by applying Noggin beads extends *Barx1* expression domain to the distal incisor regions and downregulates endogenous expression of *Msx1* leading to the transformation of incisor to molar teeth (Tucker et al., 1998). Another example is *Dlx1/Dlx2* double knockout mice. *Dlx1* and *Dlx2* are also expressed in the proximal molar-forming ectomesenchyme prior to the initial manifestation of tooth development (E10). *Dlx1* and *Dlx2* double knockout mice show developmental defects in the maxillary molars. However, the mandibular molars and incisors developed normally, suggesting that *Dlx1* and *Dlx2* are only specifically required for mesenchymal cells in the maxillary molars, but not for mandibular molars (Qiu et al., 1997; Thomas et al., 1997). Most notably the double mutant maxillary ectomesenchyme is re-programmed to a chondrogenic fate, but not to an incisor fate. This feature implicates that the loss of "molar" patterning genes and the gain of "incisor" patterning genes may both be required for an incisor formation. Detailed analysis showed that only *Dlx1* and *Dlx2* are expressed in the upper jaws, whilst the other Dlx genes, *Dlx1-6*, are all expressed in overlapping domains in the mandibular primordial. The redundancy with other *Dlx* genes in mandibular molar regions may cause the regional defects in the maxillary molars. It thus appears that the tooth identify is not determined by only one specific gene but by many different genes, whose overlapping and combinational activities (presence and also absence) determines

the tooth shape (Cobourne and Sharpe, 2003).

Similar to the initiation of odontogenesis, the establishment of different domains of homeobox genes in the mesenchyme is also determined by spatially distributed ectodermal signals, which induce and maintain the expression patterns of homeobox-containing genes in the dental mesenchyme. It has been shown that before E10.5, removal of epithelium from the mandibular arch downregulates almost all the mesenchymal homeobox genes. FGF8 in the proximal region of oral ectoderm is able to induce a number of homeobox genes, including *Barx1, Dlx1, -2, Lhx6, -7, Pax9*, and *Msx1*. Conditional mutation of the *Fgf8* gene in the first branchial arch epithelium results in the absence of all the molars, whilst the distal region lower incisors develop normally, suggesting that Fgf8 controls the large proximal region of the facial primordia but not distal part (Trumpp et al., 1999). BMPs upregulate *Msx1, -2, Dlx2*, but inhibit *Barx1* and *Pax9* expression. SHH signal induces *Gli1, -2, -3* expressions (Vainio et al., 1993; Bei and Mass, 1998; Hardcastle et al., 1998; Tucker et al., 1999). After E11.5, the mesenchymal homeobox gene expression is no longer dependent on the overlying epithelium. Hence, before E11.5, the oral epithelium possesses the odontogenic ability and determines the tooth type (Kollar and Baird, 1969; Mina and Kollar 1987; Lumsden 1988; Kollar and Mina, 1991). After E11.5, when the homeobox code domains has been established and fixed, the dental mesenchyme acquires the odontogenic ability and signals back to the dental epithelium regulating the tooth identity and their morphogenesis (Ferguson et al., 2000). The molecular mechanisms regulating of the patterning the early epithelial signals remains unknown.

It is noticeable that although oral ectoderm is the source of initial signals instructing tooth development, the underlying neural crest derived mesenchymal cells seem to respond differently to the epithelial signals in certain genetic pathways. For example, FGF8 can induce *Dlx2* in both upper and lower jaw mesenchyme, but it can induce *Dlx5* only in the mandibular mesenchyme, not in the maxillary mesenchyme. On the other hand, maxillary oral epithelium is still able to induce *Dlx5* in mandibular ectomesenchyme, suggesting that the upper and lower jaw mesenchyme behave fundamentally different from each other (Ferguson et al., 2000). Targeted mutation of the activin βA gene in mice generates reversed tooth phenotype to that o*f Dlx1*/*Dlx2* double mutants, where the development of activin βA mutant mouse incisors and lower molars is arrested at the bud stage whilst upper molars developed normally. This phenotype cannot be explained by the redundancy of other TGFβ family signals since activin βA/βB double knockout mice show similar tooth phenotype with activin βA mutants. Moreover, activin's downstream target gene, follistatin, is downregulated in both upper and lower jaws, indicating that the maxillary molars may use some other signaling pathways for its development (Ferguson et al., 1998). As neural crest cells in the maxillary and mandibular primordia are actually derived from distinct regions of the neural tube (although very close with

each other), it is reasonable to hypothesize that these ectomesenchymal cells may have been slightly pre-patterned. Therefore even small changes in their competence to the epithelial signals may lead to the different tooth shapes between upper and lower jaws.

1.2.2. Early epithelial signaling center

Between the dental lamina and early bud stage (E11.5-E12), a transient early epithelial signaling center forms at the tip of the budding cells expressing locally a number of molecules including *Bmp2*, *Shh, Wnt10a*, as well as p21*, Msx2,* and *Lef1* (Jernvall and Thesleff, 2000). Signaling centers, such as the apical ectodermal ridge (AER) in the limb bud and the isthmus in the central nervous system, are defined as a group of cells that regulate the behaviour of surrounding cells by providing positive and negative intercellular signals (Hogan, 1999). It has been proposed that at the early developmental stage, the oral epithelium induces mesenchymal signals (Mina and Kollar, 1987; Lumsden, 1988). The mesenchymal signals then reciprocally act on the dental epithelium forming the early epithelial signaling center, now called the dental placode, which shares morphological and molecular similarities with placodes in other ectodermal organs (Pispa and Thesleff, 2003). Signals from the epithelial signaling center may function in maintenance and restriction of the previously induced mesenchymal genes, but may also induce new genes in the mesenchyme. It has been suggested that the dental mesenchymal cells may acquire the full competence to induce tooth development only after receiving signals sent back from this early epithelial signaling center (Jernvall and Thesleff, 2000). Two signaling molecules, Bmp4 and activin βA, have been suggested to act as the reciprocal mesenchymal signals for the induction of early epithelial signaling center and initiation of tooth bud formation (Jernvall and Thesleff, 2000). Bmp4 is initially expressed in the oral ectoderm and is able to induce *Msx1* expression in the underlying mesenchyme (Vainio et al., 1993). At E11.5, corresponding to the odontogenic shift from dental epithelium to the mesenchyme, Bmp4 expression also shifts to the underlying dental mesenchyme and forms a positive regulatory loop with Msx1 (Vainio et al., 1993; Mina and Kollar, 1987). Meanwhile, *Msx1*, which was widely expressed in the facial mesenchyme at the initiation stage, becomes restricted to the tooth bud regions (Cobourne and Sharpe, 2003). Bmp4 is also able to induce in the expression of p21 in the dental epithelium. P21 is a cycline dependent kinase inhibitor associated with stop of cell proliferation and has been shown to be expressed in a number of signaling centers (Jernvall et al., 1998). Activin βA, which is induced by epithelial FGF signals, is expressed in the dental mesenchyme at E11.5. Targeted mutation of the activin βA gene in mice results in the development of tooth germs arrested at the bud stage (incisors and lower molars). However, exogenous Activin A protein can only rescue the mutant tooth phenotype at E11.5, but not at a later stage such as E13.5, implicating the early requirement

of this signal during tooth development (Ferguson et al., 1998).

1.2.3. Primary enamel knot and bud to cap stage transition

When the tooth bud has reached its full size it folds and invaginates forming a cap-shaped structure surrounding the mesenchymal dental papilla. Meanwhile a new signaling center, the primary enamel knot, forms at the tip of the enamel organ. The transition from bud to cap stage is a critical step in tooth development since many knockout mice have the tooth germs arrested at this stage, such as *Msx1, Lef1, Pax9, Runx2*, and *activin βA* mutants (Satokata and Maas, 1994; Kratochwil et al., 1996; Peters et al., 1998; D'Souza et al., 1999; Ferguson et al., 1998). In all of them, the formation of the enamel knot is affected. Hence, the primary enamel knot has been suggested to be a prerequisite for the tooth bud to develop into cap stage. The primary enamel knot is a non-proliferating transient structure (Jernvall et al., 1998; Jernvall and Thesleff, 2000), but it may regulate the growth of the flanking epithelial cervical loops and may also provide a fixed point for the epithelial folding. The primary enamel knot starts to form at the late bud stage and is marked by the centralized expression of a number of molecules, including *Fgf9, Bmp2, Bmp7, Shh, Wnt10b*, *Msx2*, *Edar*, p21, and follistatin (Vaahtokari et al., 1996; Kettunen and Thesleff, 1998; Dassule and McMahon, 1998; Laurikkala et al., 2001; Jernvall et al., 1998; Heikinheimo et al., 1997). By the cap stage, when the primary enamel knot is histologically recognizable as a cluster of condensed cells at the tip of the tooth germ, some other molecules such as *Fgf4, Bmp4, Wnt3,* and *Wnt10a* are also upregulated in this region (Kettunen and Thesleff, 1998; Åberg et al., 1997; Sarkar and Sharpe, 1999).

The formation and function of the enamel knot is tightly regulated by the reciprocal epithelial-mesenchymal interactions. The induction of the primary enamel knot may involve signals from the dental mesenchyme, in particular BMP4. *In vitro* bead experiments have shown that BMP4 can induce the expression of two enamel knot marker genes, p21 and *Msx2* in the dental epithelium (Jernvall et al., 1998). *Bmp4* expression is downregulated in *Pax9* and *Msx1* knockout mouse dental mesenchyme. Moreover, exogenous BMP4 protein can almost completely rescue *Msx1* mutant tooth phenotype, suggesting that mesenchymal BMP4 signaling may reciprocally act on the dental epithelium for further tooth development (Chen et al., 1996; Bei and Maas, 1998; Bei et al., 2000; Peters et al., 1998).

Lef1 is a member of the high mobility group (HMG) family DNA-binding proteins. *Lef1* is expressed in the dental mesenchyme and in the enamel knot region at bud and cap stages. In *Lef1* mutant mice tooth development is also arrested at the bud stage, which is similar to *Msx1* mutants (Kratochwil et al., 1996). However, detailed tissue recombination experiments between *Lef1* mutant and wild type mouse dental tissues demonstrated that Lef1 is only transiently required in the dental epithelium for inducing *Fgf4* expression in the enamel knot. Lef1 is dispensable in the dental mesenchyme, which may be

due to the redundancy with other TCF/ LEF transcription factors. Since the *Lef1* mutant tooth phenotype can be rescued by either epithelial or mesenchymal FGF proteins, a signaling pathway has been proposed where Lef1 upregulates *Fgf4* in the primary enamel knot region. FGF4 signaling is transduced to the dental mesenchyme where it induces the expression of mesenchymal *Fgfs*, which then reciprocally acts on the dental epithelium and stimulates cell proliferation in the cervical loops (Kratochwil et al., 1996, 2002). Lef1 may also integrate Wnt and BMP signaling, as well as cell adhesion via E-cadherin (Teaford et al., 2000). Both Wnt and BMP can induce *Lef1* expression in the dental mesenchyme and Lef1 interacts intracellularly with β-catenin, which also regulates cell adhesion with E-cadherin (Dassule and McMahon, 1998). Shh is an early enamel knot marker gene and has been shown to repress *Wnt10b* expression in the dental epithelium (Dassule and McMahon, 1998). Activin βA is a signaling molecule expressed in the dental mesenchyme (Heikinheimo et al., 1997; Ferguson et al., 1998). It can reciprocally act on the dental epithelium and stimulate the expression of ectodysplasin receptor *Edar* in the enamel knot (Laurikkala et al., 2001). Thus, it is apparent that various signaling pathways are linked during bud to cap stage transition and that the enamel knot plays a critical role in integrating these pathways (Thesleff and Mikkola, 2002a).

1.2.4. Secondary enamel knots and cusp formation

Between E14.5 and E15, when the primary enamel knot has fulfilled its task, it rapidly undergoes apoptosis and progressively disappears except for its anterior portion, in which area forms the first secondary enamel knot (Jernvall and Thesleff, 2000). Subsequent secondary enamel knots form sequentially at the tips of future cusps within the tooth crown base. Similar to the primary enamel knot, secondary enamel knots are also composed of packed and non-proliferative cells showing centralized expression of *Fgf4*, and are removed by apoptosis (Vaahtokari et al., 1996; Coin et al., 1999a). However, the secondary enamel knots do not show as much morphological difference from the adjacent tissues as the primary enamel knot. In addition, p21, *Shh*, and *Fgf9* are expressed more broadly than in the primary enamel knot.

It has been proposed that the cap stage dental mesenchyme regulates the primary enamel knot formation, which then determines the tooth crown base and subsequent secondary enamel knot formation. *In vitro* heterotopic epithelial and mesenchymal recombination experiments demonstrate that E14 cap stage dental mesenchyme controls individual molar cusp patterning, even the mirror symmetry of right and left handed teeth (Schmitt et al., 1999). The distance between adjacent secondary enamel knots may be regulated by the antagonistic interactions between cusp activators (e.g. FGFs) and cusp inhibitors (e.g. BMPs). Position, time and order of appearance of these secondary enamel knots define the

location and relative height and size of cusps for a species-specific tooth shape (Jernvall and Thesleff, 2000). This has been well illustrated by comparison of enamel knots and tooth crowns in two closely related rodents, mice and voles (Keränen et al., 1999). More evidence comes from the study of spontaneous *Tabby* mutant mice, in which the molar crowns are small and flattened with fused and fewer cusps (Gruneberg, 1971). Correspondingly, the mutant primary enamel knot is small and most secondary enamel knots are fused. Although the primary enamel knot expresses all the signal molecules analyzed, the expression levels are greatly reduced. Hence, the cusp defects in *Tabby* mutant mice can be traced back to the early stage small tooth germs, which results in a small sized primary enamel knot and thus limited tooth crown base leading to the fusion of secondary enamel knots and the following cusps. It thus appears that early disturbance in the primary enamel knot can affect the later cusp formation (Pispa et al., 1999). Mutations in the human homologues of *Tabby* gene, as well as its receptor *downless* and intracellular adaptor *crinkled*, can cause similar ectodermal congenital defects named anhidrotic (or hypodrotic) ectodermal dysplasia syndrome (HED) comprising hypodontia of the teeth (Headon et al., 2001; Monreal et al., 1999; Thesleff and Mikkola, 2002b; Yan et al., 2002).

FGF signals have been suggested to be involved in the regulation of growth and folding of the enamel organ during advancing tooth morphogenesis. FGF proteins can stimulate cell proliferation in both dental epithelium and dental mesenchyme (Jernvall et al., 1994; Kettunen and Thesleff, 1998). There are abundant FGF receptors expressed in the epithelial cervical loop regions and in the dental papilla mesenchyme, but none in the enamel knot cells. In contrast, *Fgf3, Fgf4*, and *Fgf9* transcripts are restricted in the enamel knot non-proliferating cells. *Fgf3* is also expressed in the dental mesenchyme and *Fgf10* expression is confined to the dental mesenchyme (Kettunen et al., 2000). In transgenic mice over-expressing a dominant negative FGF receptor and in mutant mice lacking a functional FGFR2 receptor IIIb, tooth germs failed to develop beyond the bud stage (Celli et al., 1998; de Moerlooze et al., 2000). *In vitro* experiments have shown that epithelial FGFs (FGF4 and FGF8) can induce mesenchymal *Fgf3* expression, whereas mesenchymal FGF10 can only stimulate cell proliferation in the dental epithelium but not in the dental mesenchyme (Kettunen et al., 2000). Therefore, FGF signals may mediate the interactions between dental epithelium and mesenchyme, and stimulate the proliferation of epithelial cells forming cervical loops, as well as the underlying mesenchymal cells forming the dental papilla.

Shh signaling was shown to be required for the asymmetrical growth of the enamel organ. Conditional deletion of *Shh* under keratin 14 promoter in mice results in small and abnormally shaped tooth with defects in lingual cervical loop growth and missing dental cord (Dassule et al., 2000). *Shh* is expressed in the epithelial enamel knot region. However, the action of Shh on the dental epithelium is not direct, since ablation of Smoothened, the receptor of Shh, in the

epithelium, does not inhibit cervical loop formation, suggesting that Shh functions on the dental mesenchyme which then reciprocally regulates the growth of the epithelial cervical loops (Gritli-Linde et al., 2002).

1.3. Cell differentiation of the tooth

As in early tooth morphogenesis, cell differentiation of the tooth is also governed by sequential and reciprocal interactions between epithelial and mesenchymal tissues. Terminal differentiation of the cells requires both temporally and spatially regulated epigenetic signals and the cells to be competent to the signals. The signaling is mediated by soluble growth factors, their receptors, transcription factors, extracellular matrix, as well as cell adhesion molecules. Cell-cell junctions may also play a role in these processes. Compared to the active studies on the early stage of tooth development, information concerning the molecular regulation of odontoblast and ameloblast cell differentiation is quite limited. This is mainly due to the reiterative use of the same signals or signaling pathways during development resulting in knockout mice with either early embryonic lethality or arrested tooth development before cap stage (Jernvall and Thesleff, 2000; Thesleff, 2003; Thesleff and Mikkola, 2002a). Direct investigation of ameloblast and odontoblast differentiation has also been hindered by the difficulty to isolate pure populations of a limited number of cells in the tooth. Since dentin is quite similar to bone, studies on the odontoblasts have extrapolated a lot from the information of bone cell biology.

1.3.1. Odontoblast differentiation

Odontoblasts are tall columnar postmitotic cells that differentiate according to a specific temporal and spatial pattern. Dental papilla mesenchymal cells are seemingly uniform in appearance with large nuclei, sparse cytoplasm, and few organelles. Only the mesenchymal cells adjacent to the inner dental epithelium and in contact with the basement membrane differentiate into odontoblasts. Pre-odontoblasts are initially cylindrical organizing in a single layer at the peripheral of the dental papilla. As differentiation proceeds, these cells elongate and polarize with an obvious increase in the number of organelles and movement of the nucleus away from the basement membrane. When synthesizing and secreting dentin matrix, the odontoblasts form long cell processes that become embedded in the dentin matrix (Ruch, 1987). The dentin matrix proteins consist of type I collagen (approximately 86%) and some non-collagenous proteins, including proteoglycans, glycoproteins, and dentin sialophosphoprotein (DSPP) (MacDougall et al., 1998). Several odontoblast cell lines have been established from dental mesenchyme or dental pulp cells for studying the function and regulation of odontoblasts (MacDougall et al., 1995; Couble et al., 2000).

Classical tissue recombination experiments have confirmed that

differentiation of odontoblasts is controlled by the inner dental epithelium (Thesleff and Hurmerinta, 1981; Ruch et al., 1995). Preodontoblasts never give rise to a functional odontoblast layer when the dental papillae are isolated and cultured alone (Kollar and Baird, 1969). Since the differentiation of odontoblasts starts from the tips of the cusps, directly underneath the secondary enamel knot, it has been proposed that signals from the secondary enamel knot may regulate the terminal differentiation of odontoblasts (Thesleff, 2000).

The growth factors that have been shown to stimulate the odontoblasts differentiation *in vitro* include TGFβ superfamily signals, FGFs, nerve growth factor (NGF) and insulin-like growth factor (IGF) (Vaahtokari et al., 1991; Cam et al., 1992; Mitsiadis and Luukko, 1995; Joseph et al., 1993). *In vitro* culture of dental papilla in agar solidified medium with different growth factors demonstrated that TGFβ superfamily proteins, including TGFβ1, -3, and BMP2, -4, -6, are all able to induce polarization of pre-odontoblasts and stimulate matrix secretion when combined with heparin or fibronectin (Begue-Kirn et al., 1992, 1994; Martin et al., 1998; Ruch, 1998; Lesot et al., 2001). Heparin or fibronectin alone had no effect, but they may alter the interaction of growth factors with the extracellular matrix and potentiate the activities of the signals, or restrict the diffusion of growth factors to the cultured tissues. Follistatin, which is expressed in preameloblasts, is also able to promote odontoblast differentiation *in vitro*. The combination of follistatin protein with heparin produces a more pronounced effect. It has been proposed that follistatin may block the mitogenic effect of activin and facilitate the terminal differentiation of odontoblasts (Heikinheimo et al., 1997, 1998). FGF1 or FGF2 protein alone does not stimulate odontoblast differentiation. A combination of FGF1 and FGF2 treatment only causes pre-odontoblast cell polarization, but no matrix secretion. However, the combination of FGF1 and TGFβ1 protein appears to work in a synergistic manner and can induce functional odontoblast differentiation *in vitro*, including polarization of the cells and secretion of extracellular matrix similar to predentin. Treatment of dental papilla with FGF2 and TGFβ1 only stimulated cytological but not functional differentiation of pre-odontoblasts (Unda et al., 2000). IGF1 is not expressed in the tooth germs, but exogenous IGF1, combined with heparin, stimulates extended polarization of pre-odontoblasts without apical matrix deposition (Begue-Kirn et al., 1994). Shh is not necessary for the differentiation of odontoblasts and ameloblasts, but for the normal organization of these cell layers. In conditional knockout mice with deletion of Shh activity under keratin 14 promoter, the polarity and organization of the odontoblasts and ameloblasts were disrupted, although the differentiation of odontoblasts and ameloblasts was not affected (Dassule et al., 2000).

During cell differentiation of the tooth, basement membrane at the tissue interface may act as a substrate and as a reservoir of paracrine signaling molecules (Meyer et al., 1983; Lesot et al., 2002). The basement membrane is a dynamic remodelling and asymmetric structure consisting of basal lamina and

the lamina fibroreticularis, including laminin1, collagen type IV, nidogen, and heparin sulfate (Merker, 1994; Lesot, 2000). During odontoblast differentiation, fibronectin, which surrounds pre-odontoblasts, is redistributed and accumulates at the apical pole of the polarizing odontoblasts (Lesot et al., 1981; Thesleff and Hurmerinta, 1981). A non-integrin 165kDa fibronectin binding protein is also transiently expressed at the apical pole of odontoblasts. The interactions between this protein and fibronectin may reorganize microfilaments inside the cells for the polarization of odontoblasts (Lesot et al., 1990, 1992). Latent TGFβ binding protein (LTBP) is also present at the epithelial-mesenchymal interface and may target and activate TGFβ proteins (Flaumenhaft, 1993; Ruch, 1998). Functional odontoblasts express and secrete gelatinase A, which may contribute to the degradation of basement membrane at the tissue interface (Sahlberg et al., 1999).

Besides epigenetic inducing signals, the expression of competence of preodontoblasts is also necessary for the terminal differentiation of these cells. Heterochronal recombination between tissues from different developmental stages cannot give rise to anticipated differentiation of odontoblasts (Ruch et al., 1995). The expression of specific receptors or matrix molecules with affinity for growth factors is required for responding to the induction signals. It has been hypothesized that in order to become the competent responding cells, preodontoblasts have to count their cell divisions and reach a minimal number of cell cycles (Ruch et al., 1995; Lesot, 2000). Based on that, the following model has been proposed: TGFβ superfamily signals or other growth factors secreted by preameloblasts may be trapped and activated by the basement membrane and stimulate terminal differentiation of preodontoblasts. The gradient of functional odontoblast in the tooth cusp is generated by the gradual emergence of competent preodontoblasts, which is related to their sequential withdrawal from the asynchronic cell (Schmitt and Ruch, 2000).

1.3.2. Ameloblast differentiation

Preameloblasts are derived from precursor cells in the inner dental epithelium of the enamel organ. Upon differentiation, preameloblasts reverse their polarity and the originally basal basement membrane contacting end becomes structurally and functionally the apical end. Their sizes increase dramatically with extensive development and redistribution of cytoplasmic organelles (Ten Cate, 1998). Secretory ameloblasts are highly columnar and polarized with oval-shaped nuclei elongated along the apical-basal axis. Meanwhile, the overlying stratum intermedium cells also increase in size and become cuboidal. Functional ameloblasts synthesize and secrete a number of enamel matrix proteins, including amelogenin, ameloblastin, enamelin, tuftelin, dentin sialophosphoprotin (DSPP), laminin 5, as well as proteolytic enzymes belonging to the metalloprotease and serine protease families for degradation of matrix proteins during maturation stage of enamel formation (Robinson et al.,

1995, 1998). After deposition of the full thickness of enamel matrix, post-secretory ameloblasts shrink in size and stop secretion of enamel matrix. During this transitional stage, about one quarter of ameloblasts undergo apoptosis. During the following maturation stage, another one-quarter of the cells die by apoptosis. The remaining ameloblasts become short and small and together with outer dental epithelium form protective layers on the enamel until the eruption of the tooth into the oral cavity (Smith, 1998).

Reciprocal epithelial-mesenchymal interactions also regulate ameloblast differentiation. Preameloblasts never differentiate into ameloblasts when the isolated enamel organ is cultured *in vitro* alone. Differentiation of ameloblasts requires the presence of functional odontoblasts and/or predentin-dentin matrix. It is remarkable that even cell-free predentin-dentin matrix is able to stimulate functional ameloblast differentiation *in vitro* (Karcher-Djuricic et al., 1985). When preodontoblasts differentiate into functional odontoblasts and start to secrete dentin matrix, the basement membrane breaks and degrades allowing direct interactions between preameloblasts and predentin-dentin (Ruch, 1987). Secretion of enamel matrix is only initiated when the dentin matrix starts to mineralize (Boukari and Ruch, 1981; Coin et al., 1999a).

TGFβs and Bmp2 are expressed in odontoblasts and these proteins have also been reported to be trapped in predentin-dentin matrix (Begue-Kirn et al., 1994; Smith, 1998). Exogenously added TGFβ1 and BMP2 proteins can induce cytodifferention of ameloblasts. In addition, BMP2 coated apatite is able to induce functional differentiation of ameloblasts as indicated by secretion of amelogenin, whereas TGFβ1 coated apatite does not have this effect. The special role of apatite in this process is still unknown. Cytokine interleukin 7 (IL-7) has also been suggested to be involved in maintaining the polarization state of ameloblasts (Coin et al., 1999). Laminin5 is expressed in the inner dental epithelium and functional ameloblasts, and may be related to the ameloblast differentiation (Yoshiba, 2000). Bmp2, Bmp4, Bmp5, and Bmp7 have also been reported to be expressed in the differentiated ameloblats (Åberg et al., 1997; Heikinheimo et al., 1998).

Although the dental papilla mesenchyme is necessary for functional differentiation of dental epithelial cells into ameloblasts, recent studies have shown that signaling within the dental epithelium, such as Shh, is also needed for the proper cytodifferentiation of ameloblasts *in vivo* (Gritli-Linde et al., 2002). Shh is expressed strongly in both proliferating preameloblasts and differentiated ameloblasts. There are also intense Shh signals in the stratum intermedium cells. Smoothened is a multi-pass membrane receptor for transduction of Shh signals into the cell. Conditional removal of smoothened activity from the dental epithelium results in the deletion of Shh signaling in the enamel organ without affecting the signaling in the dental mesenchyme. Conditional smoothened mutant mice exhibit cuboidal shaped ameloblasts with centrally located round nuclei. The mutant preameloblasts withdraw from the cell cycle prior to dentin secretion and undergo premature differentiation. Although the mutant ameloblasts express

several molecular markers of differentiated cells, no Tomes's processes form at the apical end and the enamel matrix is absent. Thus, Shh is necessary for regulating cell proliferation within the dental epithelium and controlling proper cytodifferentiation of preameloblasts. It is noticeable that *Patched 2* and *Gli1*, which are the downstream target genes of Shh, exhibit polarized localization in the secretory ameloblasts with enriched expression at the basal and perinuclear compartment. Conversely, *Patched 1, Msx2, and Dlx3* transcripts are enriched apically. It is therefore proposed that signaling from the stratum intermedium may play a role in the asymmetric distribution of these RNAs (Gritli-Linde et al., 2002). It appears that pre-odontoblasts and preameloblasts in association with stratum intermedium, basement membrane, and extracellular matrix constitute a dynamic developmental unit leading to coordinated cell differentiation of the tooth.

To date, several knockout or transgenic mouse models have been established exhibiting enamel defects. For example, in the transgenic mice with over-expression of ectodysplasin under keratin 14 promoter, enamel is absent in the incisors (Mustonen et al., 2003). Wnt3 is normally expressed in stratum intermedium, stellate reticulum, and outer dental epithelial cells, but not in the ameloblasts. Over- and ectopic-expression of *Wnt3* in the whole dental epithelium under keratin 14 promoter causes progressive loss of ameloblasts in postnatal adult mouse incisors, which has been explained by defects in the proliferation of preameloblasts or stem cells (Millar et al., 2003).

A characteristic feature of rodents is that their incisors erupt continuously throughout life by virtue of stem cells in the cervical loops at the base of the tooth (Smith and Warshawsky, 1975, 1976; Harada et al., 1999, 2002). In addition, enamel is solely formed on the labial side of the incisors, whereas the lingual surface is enamel-free and only covered by dentin. It has therefore been thought to be the root-analogue area (Fig. 2). Odontoblasts and dentin matrix are distributed similarly on both labial and lingual surfaces of incisors. Classical tissue recombination experiments have shown that the lingual side dental epithelium has lost the ability to differentiate into ameloblasts, whilst lingual side dental mesenchyme is still able to trigger ameloblast differentiation when recombined with labial side dental epithelium of incisors or with the inner dental epithelium of molars (Amar et al., 1986, 1989). There are also some enamel-free areas on the occlusal surface of the molar crowns. Studies on the matrix in the enamel-free area have revealed that it is actually composed of a mixture of enamel and cementum related proteins. There also exist secretory cells capable of enamel-like matrix secretion, including amelogenin, ameloblastin, and bone sialophosphoproteins (BSP) (Sakakura et al., 1989; Bosshardt and Nanci, 1997; Bosshardt et al., 1998). However, these cells are not polarized and do not have Tome's process as the normal ameloblasts. In addition, the enamel matrix in the enamel free area is irregular with some of the enamel matrix even between the epithelial cells (Nakamura et al., 1991). The molecular mechanism of the formation of the enamel-free areas remains unknown yet.

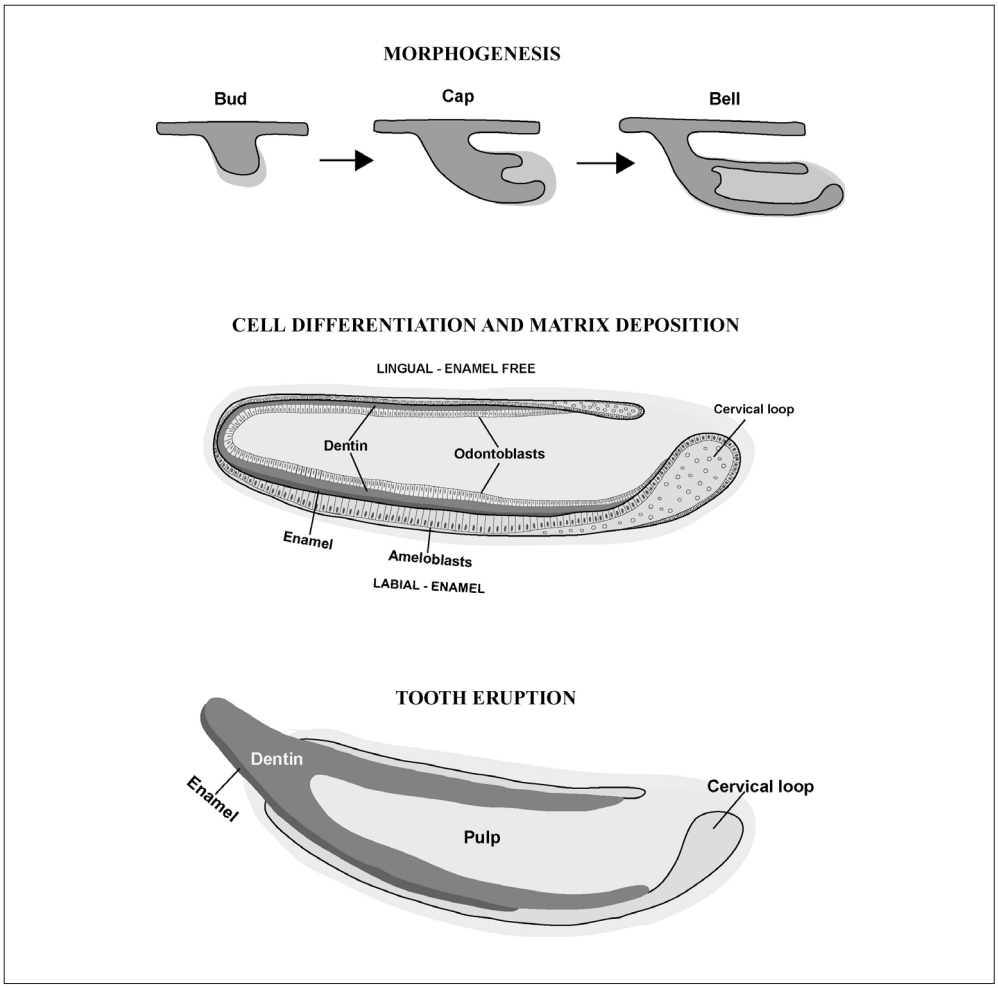

Fig.2. Schematic view of mouse incisor development. After initiation, the incisor bud rotates anteroposteriorly parallel to the long axis of the incisor. At the late bell stage, only the labial side dental epithelial cells differentiate into ameloblasts giving rise to enamel. There is no ameloblast differentiation and enamel formation on the lingual surface. Odontoblasts and dentin are distributed similarly on the labial and lingual aspects. The stem cells in the cervical loop region support the continuous growth of mouse incisors.

1.4. Runx2 in embryogenesis

In 1997, four papers were published in the same issue of Cell confirming that *Cbfa1*, now named as *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). The knockout mice die at birth and have no bones and teeth. Heterozygous mutant mice are viable but show a number of skeletal changes that are phenotypically similar to those observed in the human skeletal

syndrome, cleidocranial dysplasia (CCD). Since then, extensive studies have been performed on identifying the structure, function, and regulation of *Runx2* gene.

1.4.1. Structure and biological function of Runx2

Runx2 is a runt domain transcription factor, which is the mammalian homologue of the fly Drosophila genes *runt* and *lozenge*. In mammals, there are three Runx genes, Runx1 (Cbfa2/ Pebp2αB /Aml1), Runx2 (Cbfa1/ Pebp2αA /Aml3), and Runx3 (Cbfa3/ Pebp2αC /Aml2). They encode the α subunit protein which, together with another β subunit protein, form heterodimeric complexes. The β subunit is encoded by the CBFβ gene. It does not bind or contact DNA itself but can increase the binding affinity of Runx protein to DNA and perhaps also stabilize Runx from degradation (Coffman, 2003; Ito and Miyazono, 2003). Inactivation of CBFβ dramatically eliminates the function of Runx protein (Huang et al., 2001).

Runt domain proteins bind to DNA through the runt domain, which is a 128 amino acid motif highly conserved among distinctly related species (Fig. 3). The runt domain can direct DNA binding of Runx proteins, and also contributes to protein-protein interactions, ATP binding, and nuclear localization (Crute et al., 1996; Kanno et al., 1998). The N-terminal region is rich in glutamine and alanine repeats (Q/A). Toward the C-terminus is a proline/serine/threonine (P/S/T) rich region, which is necessary for nuclear matrix targeting, transcriptional activation or repression of target genes, and also contains phosphorylation sites for MAP kinases (Thirunavukkarasu et al., 1998; Xiao et al., 2000, 2002; Coffman, 2003). Most Runx proteins terminate with a common pentapeptide, valine-tryptophan-arginine-proline-tyrosine (VWRPY; Fig. 3) which serves to recruit the Groucho/TLE family of co-repressors (Levanon et al., 1998; Javed et al., 2001).

Runt domain transcription factors bind to the core site of 5-PYGPYGGT-3' on a number of enhancers and promoters, including murine leukemia virus, polyomavirus enhancer (Ducy et al., 1997; Karsenty and Wangner, 2002).

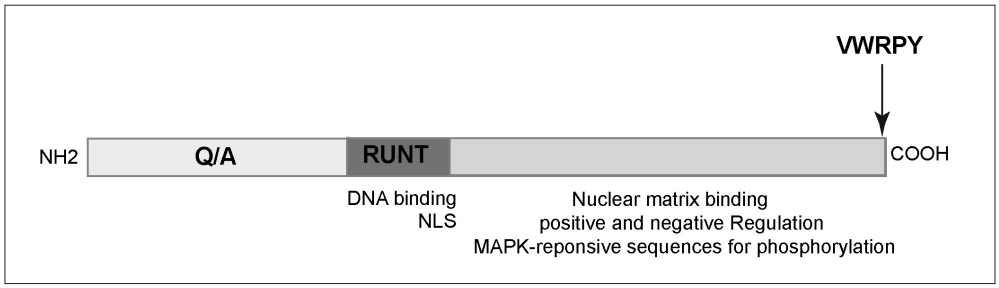

Fig. 3. Schematic representation of the Runx2 protein. Runx2 protein is composed of a glutamine/alanine-rich region (Q/A) in the N-terminal region, a centrally located DNA binding domain and nuclear localization signal (NLS), and the C-terminal proline/serine/threonine-rich (P/S/T) region.

The three Runx proteins can bind to the same DNA motif and interact with a common transcriptional modulator (Ito, 1999). However, they are reported to mediate distinct biological functions *in vivo* (Okuda et al., 1996; Speck and Gilliland, 2002; Ducy et al., 1997; Levanon et al., 2002; Li et al., 2002).

Runx2 knockout mice die soon after birth. The most remarkable finding in the mutant mice was that the bone was completely missing. Skeletal staining revealed only some calcified cartilage left in the mutants (Komori et al., 1997; Otto et al., 1997). Molecular analysis using osteoblast cell lines confirmed that Runx2 is the earliest specific marker of osteoblast lineage and that its expression is both necessary and sufficient for osteoblast differentiation (Ducy et al., 1997). Abrogation of Runx2 activity disturbs osteoblast differentiation in both intramembranous and endochondral ossification. Runx2 can directly stimulate transcription of a number of osteoblast-related genes, including osteocalcin (OCN), type I collagen, and collagenase (Ducy et al., 1997; Kern et al., 2001; Selvamurugan et al., 1998). In addition, Runx2 also has a positive role in the differentiation of hypertrophic chondrocytes during endochondral ossification. In *Runx2* mutant mice, conversion of proliferating chondrocytes into hypertrophic chondrocytes is very slow (Unda et al., 2000).

Runx2 heterozygous mice are viable but show a number of skeletal changes which are phenotypically similar to those observed in the human CCD syndrome (Mundlos et al., 1997). Genetic studies revealed that CCD is an autosomal dominant congenital disorder caused by the lack of function of one allele of Runx2 gene (haploinsufficiency) (Mundlos et al., 1997). CCD patients are characterized by general bone dysplasia with short stature, hypoplasia or aplasia of clavicles, patent fontanelles, and wormian bones (additional cranial plates caused by abnormal ossification of the calvaria), as well as supernumerary teeth and unerupted teeth in the permenant dentition. The phenotype of CCD patients varies among individuals, and some patients only exhibit dental anomalies and are thus first identified in clinic by the dentists (Jensen, 1990; Kreiborg et al., 1999; Quack et al., 1999). CCD syndrome shows variable clinical severity. A number of mutations have been reported in the *RUNX2* gene, such as deletion, insertion, missense, nonsense, or frameshift mutation. Most of the mutations affect the runt DNA binding domain and this further emphasizes the importance of amino acid conservation in this region (Lee et al., 1997; Mundlos, 1999; Quack et al., 1999).

Runx1 is essential for hematopoiesis and angiogenesis. In humans, about 30% of leukemia cases are caused by loss of function mutations of *Runx*, and most of them are chromosomal translocations with fusion of the N-terminal region of Runx1 (containing the entire runt domain) to the C-terminus of another gene. *Runx1* knockout mice exhibit early embryonic lethality with massive hemorrhage, which is caused by the lack of definitive hematopoietic stem cells and defective angiogenesis (Okuda et al., 1996; Wang et al., 1996; Speck and Gilliland, 2002). Runx3 controls neurogenesis in the dorsal root ganglia and is also shown to be the major tumour

suppressor of gastric cancer (Levanon et al., 2002; Li et al., 2002).

1.4.2. Regulation of Runx2 activity

All three mammalian Runx genes exhibit two alternative promoters, a distal promoter P1 and a proximal promoter P2. Different Runx products transcribed from the different promoters may have distinct functions (Bangsow et al., 2001; Coffman, 2003). In addition, mammalian Runx genes can generate alternatively spliced transcripts (Levanon et al., 1996; Bangsow et al., 2001). Three alternative products of Runx2 have been identified in humans and nine in mice. These differently spliced Runx proteins may play different roles during embryogenesis.

To date, most of the information concerning regulation and function of the Runx2 gene comes from studies on osteoblasts. BMP, the critical inducer of osteoblast differentiation and bone formation, has been shown to upregulate Runx2 expression in osteoblasts (Ducy et al., 1997). BMP-induced osteogenic signaling pathway is mediated by Runx2, and mutated Runx2 cannot transmit BMP signaling to the downstream target genes (Zhang et al., 2000). It has been noticed that the expression level of Runx2 does not always correlate with its activity *in vivo*. The activity of Runx2 is also modulated at the post-translational level. Runx2 can be phosphorylated and activated by the mitogen-activated protein kinase (MAPK) pathway, which may be stimulated either by extracellular matrix binding to integrin on the cell surface or by FGF2. The classic PKA pathway activated by parathyroid hormone/parathyroid hormone related peptide is also able to phosphorylate and activate Runx2 protein (Thirunavukkarasu et al., 1998; Nugent and Iozzo, 2000; D'Alonzo et al., 2002). The activity of Runx2 can be also altered by direct interactions with other partner transcription factors such as Smads, AP-1 factors, and Twist (Fainsod et al., 1997; Bialek et al., 2004). Runx can form a complex with Smads, the mediators of TGFβ/BMP signaling, and function synergistically to regulate the target genes (Lee et al., 2000; Hanai et al., 1999; Pardali et al., 2000; Ito and Miyazono, 2003). Recent studies have shown that Runx2 activity is inhibited by the helix-loop-helix containing transcription factor Twist. Twist can inhibit osteoblast differentiation without affecting the expression of *Runx2*. *In vitro* experiments demonstrate that twist protein can directly interact with the runt domain of Runx2 protein and decrease the DNA binding activity of Runx2 and thereby its transactivation function. Double heterozygous *Twist1+/-; Runx2 +/-* mice can rescue the calvarial phenotype in *Runx2* +/- mice, and double *Twist2+/-; Runx2 +/-* can rescue the clavicle phenotype of *Runx2* +/- mice. Thus, the initiation of osteoblast differentiation may actually be determined by the relief of inhibition of Runx2 protein by Twist proteins (Bialek et al., 2004).

Osterix, a zinc finger containing transcription factor, was shown to act downstream of Runx2 during osteoblast differentiation. In *Osterix* null mutant mice, no endochodral and no intramembranous bone formation occurs, but the expression of *Runx2* is normal.

Osterix mutant mice do not have tooth defects (Nakashima et al., 2002).

1.4.3. Runx2 in tooth development

Detailed analysis of Runx2 in the tooth region showed that Runx2 is required for advancing tooth morphogenesis and histodifferentiation (D'Souza et al., 1999). In mice, *Runx2* expression was first detectable in the tooth region at E12, with only weak signals in the dental mesenchyme. At E13 bud stage, *Runx2* expression is intensely upregulated with strong signals in the condensed dental mesenchymal cells contacting the tip of the tooth bud. By the E14 cap stage, *Runx2* remains intense and restricted to the dental mesenchyme. At E16 early bell stage, *Runx2* expression becomes downregulated in the dental papilla mesenchyme. By E18, *Runx2* expression is downregulated in the dental papilla mesenchyme, whereas the dental follicle region is still intensely positive (D'Souza et al., 1999). Analysis of *Runx2* knockout mouse teeth revealed that the mutant molar tooth germs are severely hypoplastic and misshapen. Mutant tooth germs are arrested at the late bud stage without obvious differentiation of odontoblasts and ameloblasts. Tissue recombination experiments demonstrated that the E13 and E14 stage dental epithelium is able to stimulate *Runx2* expression in the isolated dental mesenchyme, whilst early stage dental epithelium (E11 and E12) does not have this effect. FGF4-soaked beads can mimic the function of dental epithelium inducing *Runx2* expression in the dental mesenchyme whereas BMP-soaked beads cannot, which is different from the osteoblasts where BMP can strongly upregulate *Runx2* expression (D'Souza et al., 1999). These data suggest that Runx2 functions in the dental mesenchyme and regulates the expression of mesenchymal signals, which may act reciprocally on the dental epithelium regulating tooth morphogenesis.

In *Runx2* heterozygous mice, the teeth appear normal. However, deletion of one allele of *RUNX2* gene in humans (CCD) often causes tooth defects. In CCD patients, the primary dentition is almost normal with slightly delayed eruption, but the eruption of their permanent dentition is severely delayed. Moreover, many patients have multiple supernumerary teeth, which can be clearly detected in the X-ray pictures. Sometimes, the supernumerary teeth even form more or less a third dentition. Clinical studies have shown that these supernumerary teeth are related to the permanent dentition, but not to the primary dentition (Jensen, 1990). The mechanism of the development of supernumerary teeth in humans is still unknown. It has been suggested that these supernumerary teeth may develop from remnants of dental lamina which haven't been dissolved at the expected time. Since the delayed tooth eruption even occurs in regions without supernumerary teeth, and also based on the observation that immature osteoclasts exit in *Runx2* knockout mice at the periphery of the bone region, it has been proposed that insufficient Runx2 activity may affect the differentiation of both osteoblasts and osteoclasts, and thus in CCD patients, the bone remodelling is also disturbed leading to diminished resorption on bone surfaces

and delayed resorption of the primary teeth (Kreiborg et al., 1999).

There have been reports that in the roots of CCD patients' permanent teeth, cellular cementum is absent and acellular cementum is partially hyperplastic (Jensen, 1990; Seow and Hertzberg, 1995; Lukinmaa et al., 1995). However, analysis on the *Runx2* heterozygous mice did not find defects in the molar root regions (Zou et al., 2003). This result, as well as the absence of supernumerary teeth in *Runx2* heterozygous mice, has been explained by the fact that mice have only one dentition and it may just represent the primary dentition in humans (Kreiborg et al., 1999).

During early tooth development, *Runx1* mRNA is detected on the buccal side outer dental epithelium. *Runx3* is expressed very weakly in several thin layers of mesenchymal cells directly underlying dental epithelium. *Runx3* mutant mice do not show any obvious tooth phenotype (Yamashiro et al., 2002).

1.5. Follistatin as a modulator of TGFβ superfamily signaling during development

The transforming growth factor β (TGFβ) superfamily growth factors contain over 50 structurally related proteins, which play diverse roles during embryogenesis and in the regulation of homeostasis in adult tissues (Table 1). The biological activities of the TGFβ superfamily signals have to be tightly controlled. Dysregulation can result in congenital malformation or cancer (Massague and Wotton, 2000). Follistatin is an extracellular modulator of TGFβ superfamily signals, including activin, BMPs, and GDFs. Follistatin was initially identified in the reproductive system and has been shown to play significant roles in the reproductive system regulating the function of pituitary, ovary, and testis (Lin et al., 2003). Recent work has demonstrated that follistatin is also involved in embryonic development (Patel, 1998; Jhaveri et al., 1998; Maeshima et al., 2001; Nakamura et al., 2003).

1.5.1. TGFβ superfamily proteins and their modulators in embryogenesis

TGFβ superfamily proteins can be further divided into several subfamilies, including the TGFβ family, activin/inhibin family, and BMP family. They all contain the highly conserved seven cysteine residues in the carboxyl-terminal domain. After the proteins are secreted and processed, the ligands form hetero-or homodimers by disulfide bonds, and bind to the type II and type I serine/threonine kinase receptors forming an activated receptor complex. Upon ligand binding, the type II receptors phosphorylate the type I receptors, which then relay the signal by binding and phosphorylating the intracellular receptor-regulated Smad transcription factors (R-Smads). R-Smads recruit and associate with the common partner Smad (Co-Smad or Smad4), and subsequently these heteromeric complexes translocate into the nucleus and activate transcription of target genes (Massague, 2000; Balemans, 2002). Activin and TGFβ

signals are mediated by Smad2 and Smad3, whereas BMP signaling is mediated through Smad1, Smad5, and Smad8.

BMP signals have been shown to have a wide range of biological activities regulating cell growth, proliferation, differentiation, chemotaxis, and apoptosis in various cell types. They are active in many processes during embryogenesis and play important roles in most major organs, such as brain, lung, kidney, and liver. *Bmp2* and *Bmp4* knockout mice are early embryonic lethal (Winnier et al., 1995; Zhang and Bradley, 1996). *Bmp7* knockout mice are perinatal lethal with defects in kidney and skeleton, but have no tooth defects, which may be due to the redundancy with other Bmps coexpressed in the tooth (Luo et al., 1995; Dudley et al., 1995; Karsenty et al., 1996). *Bmp2, Bmp4,* and *Bmp7* are all expressed in the enamel knot region. During early tooth development, *Bmp4* expression shifts from dental epithelium to mesenchyme corresponding to the shift of odontogenic potential. Bmp4 can induce *Msx1* expression in the dental mesenchyme and upregulate its own expression (Vainio et al., 1993; Åberg et al., 1997). During bud to cap stage transition, it can induce p21 and *Msx2* in the dental epithelium and is associated with enamel knot formation (Jernvall et al., 1998).

Activins regulate growth and differentiation in many biological systems, including mesoderm induction, 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). There are different forms of activin proteins. Activin A is a homodimer protein containing two βA subunits. There are also activin B (βB-βB), activin AB (βA-βB), activin βC, activin βD, and activin βE. Activin βC and βD subunits are mainly found in the liver and activin βD exits only in frog *Xenopus* (Oda et al., 1995; Fang et al., 1996; Schmitt et al., 1996). Activin βA mutant mice develop to term, but die soon after birth with defects in whiskers, hard palate, and teeth (Matzuk et al., 1995a). Activin βA mutant mandibular molars and incisors are arrested at the bud stage, but the maxillary molars develop normally (Ferguson et al., 1998). Exogenous activin A protein can induce follistatin and Edar expression in the dental epithelium, although the induction of Edar requires the presence of other mesenchymal factors and may be indirect (Ferguson et al., 1998; Laurikkala et al., 2001). Activin βB knockout mice have obvious failure of eyelid fusion. Activin βA/βB double knockout mice do not have additional defects, suggesting that these two molecules are not redundant *in vivo* (Matzuk et al., 1995a).

The biological activities of TGFβ superfamily signals are delicately controlled at different steps of signal transduction. For example, the BMP signaling is modulated intracellularly by inhibitory Smads (I-Smads) and Smurfs; at the plasma membrane by pseudoreceptor BAMBI; and extracellularly by many binding proteins (Balemans and Van Hul, 2002). The extracellular modulators of BMP signaling include noggin, chordin, chordin-like, DAN/Cerberus protein family, sclerostin, ectodin, follistatin and

Table I.Major defects in knockout mice of TGFβ family members and their binding proteins

Molecule	Phenotype of knockout mice	Reference
Activin βA	Perinatal lethal, cleft palate, lack of whiskers, incisors and lower molars arrest at bud stage	Matzuk et al., 1995a Ferguson et al., 1998
Activin βB	Viable, failure of eyelid fusion, reproductive abnormality in females	Vassalli et al., 1994
Activin βA/βB	Combinational defects of activin βA and activin βB mutants, no additional defects	Matzuk et al, 1995a
Bmp2	Embryonic lethal (E7.5-10.5), failure of proamniotic canal close and cardiac defects	Zhang et al., 1996
Bmp3	Viable, increased bone density in adults	Daluiski et al., 2001
Bmp4	Embryonic lethal (E7.5-9.5), defects in mesoderm, allantois and posterior structures	Winnier et al., 1995
Bmp5	Viable, defects in skeleton and cartilage	Kingsley et al., 1992
Bmp7	Perinatal lethal, defects in skeletal patterning, eye, and kidney, no tooth phenotype	Dudley et al., 1995 Luo et al., 1995
Bmp5/Bmp7	Embryonic lethal (E10.5), defects in allantois, heart, branchial arches, somites and forebrain	Solloway and Robertson, 1999
TGFβ1	Embryonic lethal (E9.5-11.5, >50%), defects in yolk sac and haematopoiesis, survivors have inflammation and autoimmunity, die within one month	Dickson et al., 1995
TGFβ2	Perinatal lethal, defects in cardiac, skeletal, inner ear, eye, lung, and kidney	Sanford et al., 1997
TGFβ3	Perinatal lethal, cleft palate and lung defects	Kaartinen et al., 1995
Chordin	Defects in inner and outer ear, pharynx and heart (strong inhibitor of BMP2, -4, -7, similar to noggin)	Bachiller et al., 2000
Noggin	Perinatal lethal, opened neural tube, lack of caudal vertebrate and abnormality in limb	McMahon et al., 1998
Chordin/Noggin	Severe defects in forebrain formation, and defects in specification of three body axes	Bachiller et al., 2000
Follistatin	Perinatal lethal with taut and shiny skin, defects in skeletal, whisker, hard palate, and tooth (strong activin inhibitor, also binds BMPs and GDFs)	Matzuk et al., 1995b
DAN	No obvious phenotype (BMP inhibitor)	Dionne et al., 2001
Cerberus	No obvious phenotype (inhibit BMP, activin, Nodal, and Wnt signals)	Simpson et al., 1999

follistatin related proteins (Balemans and Van Hul, 2002; Laurikkala et al., 2003). Noggin is a high-affinity BMP binding protein that prevents BMP signal to bind to its receptors. Noggin knockout mice exhibit numerous defects including absence of caudal skeleton, opened neural tube, and malformed limbs (McMahon, 1998; Brunet, 1998). Mutations of noggin in humans affect joint morphogenesis and have been genetically related to multiple synostoses syndrome and autosomal dominant stapes ankylosis (Gong et al., 1999; Brown et al., 2002).

Follistatin is a secreted glycoprotein. It was originally isolated from ovarian fluid and characterized by its ability to inhibit follicle stimulation hormone (FSH) secretion from the pituitary gland (Yan et al., 2002; Ueno et al., 1987). Further studies in reproductive system linked follistatin directly with activin and showed that follistatin neutralizes the FSH stimulatory activity by activin protein (Nakamura et al., 1990). *In vitro* studies have demonstrated that follistatin binds activin with high affinity and prevents the binding of activin to its receptors (de Winter et al., 1996). The binding of follistatin triggers clearance of activins by endocytotic degradation (Hashimoto et al., 1997). Follistatin gained the interest from developmental biologists after the study by Hemmati-Brivanlou and coworkers showing that it was expressed in the Spemann Organiser in early *Xenopus* embryo and acts as a neural and dorsal inducer (Hemmati-Brivanlou et al., 1994). Since then, many studies on follistatin have demonstrated that this protein is broadly distributed in adult tissues, including brain, bone marrow, endochondral bone, pancreas, and liver, and also plays important roles during embryogenesis (Patel, 1998; Lin et al., 2003).

1.5.2. Structure and biological function of follistatin

Follistatin is highly conserved among vertebrate species. It has various isoforms that are actually encoded by one single gene. Follistatin gene contains six exons and the first exon encodes a putative signal peptide. Alternative mRNA splicing between exon 5 and exon 6 produce two protein forms, follistatin-288 and follistatin-315. Subsequent glycosylation and proteolytic cleavage modifications generate proteins with a variety of molecular masses (31-39 kDa) (Ueno et al., 1987; Shimasaki et al., 1988; Lin et al., 2003). The majority of follistatins isolated from pig ovary is follistatin-303, which may be derived from proteolytic cleavage of follistatin-315 (Sugino et al., 1993). Follistatin-288 binds with high affinity to cell surface heparin sulfate whereas follistatin-315 binds with low affinity. The ability to bind to the extracellular matrix may be an important property that allows follistatin to act as a local modulator of TGFβ superfamily proteins (Lin et al., 2003).

Genetic and molecular evidence has demonstrated that follistatin prevents activin signaling in various biological systems. During hair follicle morphogenesis, follistatin is expressed in the hair follicle epithelium whereas activin βA is mainly in the mesenchyme. Both follistatin knockout mice and activin βA overexpression transgenic mice exhibit a retardation of hair follicle

development. In addition, follistatin protein stimulates hair follicle development *in vitro*. It appears that follistatin accelerates hair follicle morphogenesis by antagonizing the activity of activin.

This activin-follistatin system also regulates the cycling of hair follicle at a later stage in adult mice with activin promoting antigen development whilst follistatin antagonises this effect (Nakamura et al., 2003). The activin-follistatin system is also involved in epithelial branching morphogenesis during mammalian organogenesis. In the developing pancreas, kidney, and salivary gland rudiments, exogenous activin severely disrupts the branching of the epithelium, whereas follistatin counteracts the effect of activin (Ritvos et al., 1995; Maeshima et al., 2001). In addition, activin and follistatin are both expressed in the migrating endothelial cells and may be involved in the regulation of angiogenesis (Kozian et al., 1997). In the developing pancreas, follistatin can mimic the effects of developing pancreatic mesenchyme for the induction of exocrine tissues and repression of endocrine tissues (Miralles et al., 1998).

Although follistatin has been shown to be a neural inducer in the early *Xenopus* embryos, follistatin knockout mice do not have any neural or mesoderm defects during early embryogenesis. Follistatin knockout mice develop to term but fail to breathe, and so die shortly after birth. The mutant mice have taut and shiny skin and exhibit generalized growth retardation and defects in respiratory muscle, whisker, as well as skeletal abnormalities including hard palate. The mandibular regions surrounding the lower molars are less prominent in the mutants (Matzuk et al., 1995b). The defects in follistatin mutant mice are more widespread than those seen in activin deficient mice, indicating that follistatin may also modulate the actions of other members of the TGFβ superfamily during embryogenesis. Indeed, recent work has shown that besides activin, follistatin is also able to bind certain other members of the TGFβ superfamily, including BMP2, BMP4, BMP7, BMP15/GDF-9B, GDF8/ myostatin, GDF9, and GDF11/BMP11, but not TGFβ1 (Yamashita et al., 1995; Iemura et al., 1998; Otsuka et al., 2001; Gamer et al., 2001). In early *Xenopus* embryos and mouse teratocarcinoma cells, follistatin interacts directly with BMP2 and BMP4 proteins (Fainsod et al., 1997; Iemura et al., 1998). Follistatin has also been shown to antagonize BMP-mediated lateral inhibition and stimulate feather bud formation in the chick (Patel et al., 1999). However, the binding of follistatin to BMPs is much weaker than to activin and the binding between follistatin and BMPs may be reversible. During the development of the chick limb, follistatin promotes the activity of BMP7 for induction of muscle growth, but meanwhile inhibits the apoptotic activity of BMP7. Follistatin may store and present BMPs in a subapoptotic concentration which promotes continuous muscle growth and thus released BMPs could regain biological activity (Amthor et al., 2002). It appears that follistatin can act either as antagonist or anagonist of BMP depending on the tissue.

Unlike other BMP inhibitors such as noggin and chordin, follistatin does not limit the availability of BMP to its

receptors. Instead, they form a trimeric complex with BMP and its receptors and cause conformational changes of BMP proteins thus preventing the receptor activation (Fig. 4). The affinity of follistatin binding to BMPs is also much weaker (24-fold weaker) compared to Chordin and Noggin (Iemura et al., 1998; Balemans and Van Hul, 2002).

1.5.3. Regulation of follistatin activity

The expression of follistatin is regulated by various factors, including its ligands activin and GDF11 (Ferguson et al., 1998; Gamer et al., 2001). During early tooth development, activin βA from the dental mesenchyme is able to induce follistatin expression in the dental epithelium (Ferguson et al., 1998). Exogenous FGF4 and BMP7 proteins have also been shown to induce follistatin expression during chick feather bud development (Patel et al., 1999). In addition, epidermal growth factor and TGFβ1 can induce follistatin expression in keratinocytes *in vitro* (Wankell et al., 2001). Follistatin promoter region contains several DNA binding motifs for a number of

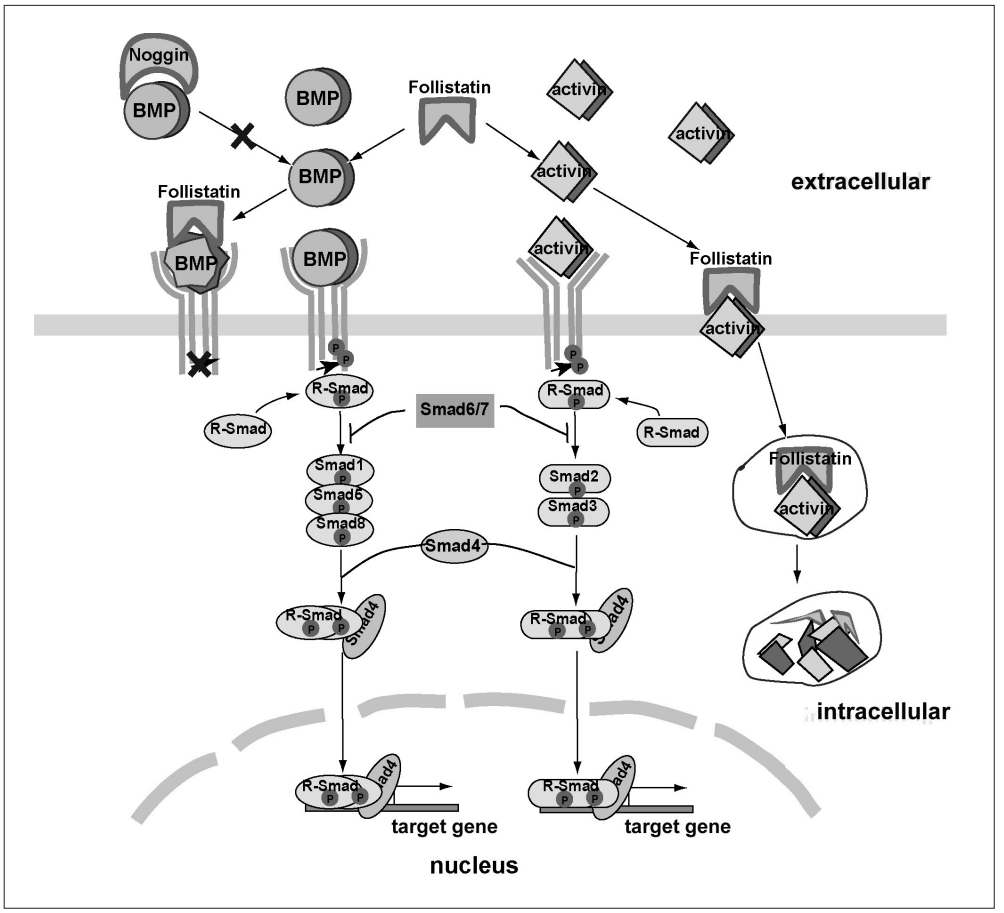

Fig. 4. Schematic presentation of the modulation of BMP and activin signaling by follistatin and noggin.

transcription factors, such as Sp1, Ap-1, Ap-2, Brachyury-T, CRE binding protein, and TCF (de Groot et al., 2000). Microarray analysis on Wnt protein stimulated human embryonic carcinoma cells demonstrates that follistatin may be a putative downstream target gene of Wnt signaling and the TGF binding site in the follistatin promoter region is required for the induction of follistatin (Willert et al., 2002).

There exits several proteins which share homology with follistatin, such as follistatin-related gene (FLRG), agrin, testican, SPARC, and Flik. They all have one or more follistatin domains. Only FLRG was shown to bind activin or related TGFβ family members, but the others cannot (Shibanuma et al., 1993; Zwijsen et al., 1994; Hayette et al., 1998; de Groot et al., 2000). FLRP binds to activin and BMP with similar affinity and sensitivity as that of follistatin, but the expression patterns and biological functions of these two genes are different (Tortoriello et al., 2001; Wankell et al., 2001; Shibanuma et al., 1993). Whether they are functionally redundant or fulfil unique biological roles is still unknown.

It has been suggested that members of the TGFβ superfamily may interact with each other *in vivo*. Different TGFβ family proteins can bind to the same receptor, for example both activin and BMP7 can bind to the ActRII and ActRIIB receptors, therefore they may compete at the cell membrane level for the common receptors (Balemans and Van Hul, 2002; Fig. 5). In addition, there is only a limited amount of Co-Smad (Smad4) in the cell and thus these proteins may also compete for Smad4 in mediating their signals (Candia et al., 1997; Lin et al., 2003). To date, all the

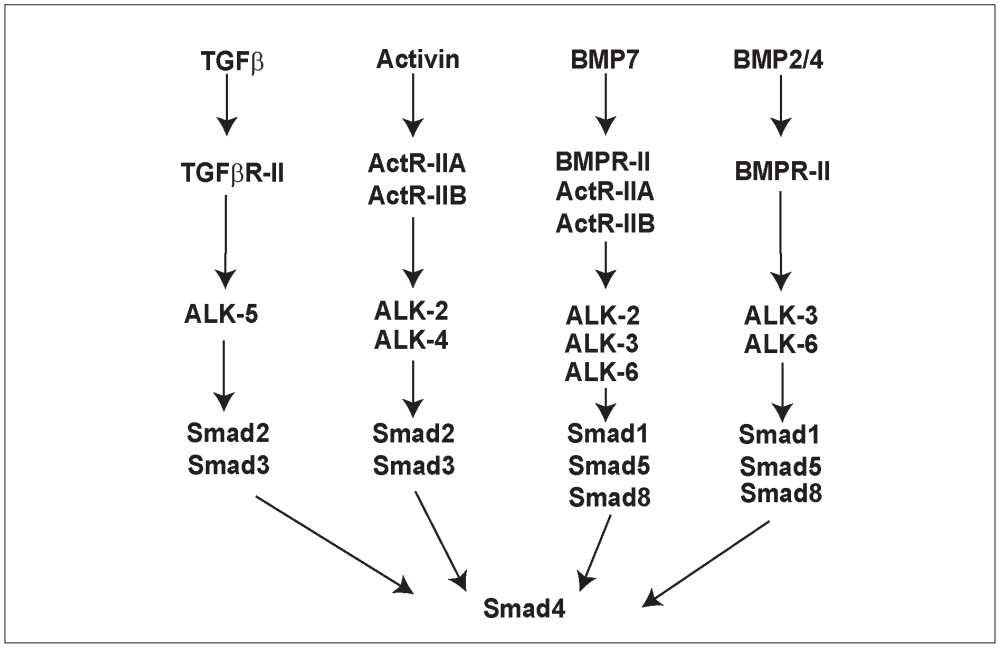

Fig. 5. TGFβ superfamily proteins can bind to the same receptors or use the same Smad proteins.

functions of follistatin are exclusively related to its binding and neutralizing various members of the TGFβ superfamily. There is no evidence for the existence of a specific follistatin receptor (Patel, 1998; Balemans and Van Hul, 2002). This, as well as the possible interactions between different TGFβ superfamily signals, leads us to speculate that the developmental defects in the follistatin mutant mice may be due to (1) increased activin signaling (follistatin binds and inhibits activins); (2) increased activin and BMP signaling (follistatin binds and inhibits both activin and BMPs); (3) increased activin signaling and reduced BMP signaling (activins compete with BMPs for activin receptors and Smad4); (4) reduced activin and increased BMP signaling (BMPs compete with activins for receptors and Smad4); (5) altered TGFβ signaling (both activin and BMPs compete with TGFβ for Smad4). The exact mechanism *in vivo* may depend on the specific developmental system and the final outcome of these interactions will determine the cell fate.

1.5.4. Follistatin in tooth development

Follistatin is expressed in the dental epithelium of the developing rat embryo between E16 and E20 stage (Roberts and Barth, 1994). During mouse embryogenesis, follistatin was reported to be expressed in the dental epithelium and can be induced by mesenchymally expressed activin βA signal (Ferguson et al., 1998). Studies by Heikinheimo and coworkers have demonstrated that follistatin transcripts are located in the mouse dental epithelium during bud stage. At later stages (E17 and E18), follistatin transcripts were restricted to the epithelial pre-ameloblasts, adjecent to the dental mesenchymal cells expressing activin βA at the tips of the forming cusps (Heikinheimo et al., 1997). The unique expression pattern of follistatin in the developing tooth and its close relationship with activin βA suggested that these molecules may function together during tooth development, especially for cusp formation and odontoblast differentiation (Heikinheimo et al., 1997, 1998). *In vitro* studies have shown that exogenously added follistatin recombinant protein can trigger odontoblast differentiation in isolated dental papilla mesenchyme, and this effect was enhanced by adding follistatin together with heparin. However, Activin A protein did not show any effect on odontoblast differentiation *in vitro* (Heikinheimo et al., 1997).

Follistatin knockout mice die at birth and exhibit abnormal tooth development. It has been reported that in 6/34 null mutants the lower incisors were absent, and in 23/34 mutants the development of incisors was delayed (Matzuk et al., 1995b). The precise role of follistatin in the developing tooth *in vivo* remains to be analyzed.

2. AIMS OF THE STUDY

The aim of this study was to investigate the molecular mechanisms underlying tooth morphogenesis and cell differentiation. The specific aims were:

1. To examine in detail the tooth phenotype of Runx2 mutant mice;
2. To identify the upstream regulators and the downstream targets of Runx2 in the developing mouse tooth;
3. To study the role of follistatin during tooth development by using follistatin knockout mice and transgenic mice over-expressing follistatin under keratin 14 promoter.

3. MATERIALS AND METHODS

3.1. Mouse strains

Strain	Used in article	Purpose
NMRI or C57BL/6 mice	I, II, III, IV	Analyze the expression pattern of mRNA and protein
Runx2 knockout mice	I, II	Analyze the role of Runx2 during tooth development
Follistatin knockout mice	III, IV	Analyze the tooth phenotype of follistatin mutant mice
K14-follistatin mice	III, IV	Analyze the tooth phenotype of over-expression of follistatin under the keratin 14 promoter
Msx1 knockout mice	II	Determine the upstream regulator of Runx2 in tooth development
Lef1 knockout mice	II	Determine the upstream regulator of Runx2 in tooth development
Tabby knockout mice	II	Determine the upstream regulator of Runx2 in tooth development

3.2. Probes

The following probes were used for *in situ* hybridisation:

Probe	Description or reference	Used in
Activin βA	Erämaa et al., 1992	II, III, IV
Ameloblastin	Lee et al., 1996	I, IV
Bmp2	Åberg et al., 1997	II, III, IV
Bmp4	Åberg et al., 1997	II, III, IV
Bmp7	Åberg et al., 1997	II, III, IV
Proα1(I) collagen	Metsaranta et al., 1991	I
Dan	Dionne et al., 2001	II
Dentin sialophosphoprotein	D'Souza et al., 1997	I, IV
Dentin matrix protein 1	D'Souza et al., 1997	I
Eda	Laurikkala et al., 2001	II
Edar	Laurikkala et al., 2001	II, III
Fgf3	Kettunen et al., 2000	II
Fgf4	Jernvall et al., 1994	II, III
Fgf10	Kettunen et al., 2000	II
FgfR1	Trokovic et al., 2003	II
Follistatin	full length, Wankell et al., 2001	III, IV
GDF11	Gift from Anne Calof (Univ. California, Irvine)	III
Lunatic fringe	Mustonen et al., 2002	II
Hes1 and Hes5	Mustonen et al., 2002	II
Lef1	Travis et al., 1991	II
MMP2	Sahlberg et al., 1999	II
MMP20	Gift from Dr. Jan Hu (Univ. of Texas School of Dentistry)	IV
Msx1 and Msx2	Jowett et al., 1993	II
Osteocalcin	Ducy et al., 1997	I
p21	Jernvall et al., 1998	II, III, IV
Pax9	Neubuser et al., 1997	II
Runx2	D'Souza et al., 1999	II
Runx1 and Runx3	Yamashiro et al., 2002	II
Sprouty1, Sprouty2, Sprouty4	Zhang et al., 2001	II
TGFβ1	Vaahtokari et al., 1991	II
Timp2 and Timp3	Sahlberg et al., 1999	II
Twist	Rice et al., 2000	II
Wnt 5a, 10a, 10b, and 11a	Dassule et al., 1998 ; Sarkar et al., 1999	II
Shh	Vaahtokari et al., 1996	II, III
Ectodin	Laurikkala et al., 2003	II
Hairless	Derived from mouse EST AI181388 by subcloning a SmaI-BamH1 fragment into EcRV-BamH1 cleaved PCRII-TOPO	II

3.3. Methods used and described in articles I-IV

Method	Article
Histology	I, II, III, IV
Skeletal preparation of adult tissues	III, IV
Tissue culture and bead implantation	I, II, III, IV
Genotyping by PCR	I, II, III, IV
Radioactive *in situ* hybridisation on sections	I, II, III, IV
Whole mount in situ hybridization	II, III, IV
Immunohistochemistry	II, III, IV
Cell proliferation assays (BrdU staining)	III
RT-PCR	I
Kidney transplantation	II
Cell transfection and analysis of gene induction	II

4. RESULTS AND DISCUSSION

4.1. Phenotypic changes in *Runx2* mutant mouse dentition (I)

Runx2 is a runt domain transcription factor that plays critical roles in bone development and tooth morphogenesis (Otto et al., 1997; Karsenty and Wagner, 2002; D'Souza et al., 1999). Earlier studies by Åberg in our laboratory have shown that *Runx2* is expressed in the dental mesenchyme and regulated by FGF signals from dental epithelium, and that in *Runx2* mutant mice tooth development is arrested at late bud stage (D'Souza et al., 1999). Our first aim was to study in detail the tooth phenotype of *Runx2* mutant mice. We performed histological analysis of *Runx2* mutant teeth at different developmental stages starting from E12 to E18. *Runx2* mutant molars had reached the bud stage with condensed mesenchymal cells underlying dental epithelium. However, at E14, when the wild type molar germs underwent folding morphogenesis and formed a cap shape structure, *Runx2* mutant molars were still arrested at the late bud stage without any morphologically visible enamel knot formation at the tip of the tooth bud. In the absence of Runx2 activity, the lower molars seemed to be affected more severely than the upper molars. Moreover, we observed epithelial budding on the lingual aspect of the mutant upper molar germs, indicating that Runx2 normally prevents lingual epithelial bud formation in the wild type mice. Mice exhibit only one dentition, which has been suggested to represent the primary dentition in humans (Kreiborg et al., 1999). Also based on the fact that CCD patients often possess supernumerary teeth, we propose that this lingual epithelial bud in *Runx2* mutant molars may represent the growth of dental lamina for successor dentition formation. It appears that Runx2 is required for tooth morphogenesis, but inhibits extra epithelial bud protrusion and successor dentition formation. These apparently contradictory effects at different developmental windows are not unusual and have also been demonstrated in other systems. For example, activin inhibits hair follicle development, but on the other hand promotes anlagen development and prevents catagen transition during hair follicle cycling (Nakamura et al., 2003). Further studies need to be performed to determine the nature of the lingual dental epithelial bud.

Runx2 is a transcription factor specifically expressed in the dental mesenchyme (D'Souza et al., 1999). To assess the defective tissue in *Runx2* mutant molars, we performed tissue recombination experiments between *Runx2* mutant and wild type molars. Since *Runx2* mutant tooth germs are rather small, we combined E14 stage *Runx2* mutant molar tissues with E13 wild type molars. When *Runx2* mutant dental epithelium was recombined with wild type dental mesenchyme and cultured *in vitro* for 6-8 days, the explants could develop into bell stage morphology. However, when the *Runx2* mutant dental mesenchyme was recombined with wild type dental epithelium, no obvious development

occurred. These results indicate that the primary defective tissue in the *Runx2* mutant teeth resides in the dental mesenchyme. This is in line with the expression pattern of *Runx2* in the dental mesenchyme, suggesting that Runx2 regulates the expression of genes in the dental mesenchyme, which then reciprocally act on the dental epithelium for the tooth bud to cap stage transition.

Since *Runx2* mutant mice die at birth, it is unclear whether the development of mutant tooth bud is markedly delayed or is a true arrest at the late bud stage. Therefore, we transplanted the E13 and E14 Runx2 mutant tooth germs under the kidney capsule of nude mice, together with some wild type molars as positive controls. After 2 weeks *in vivo* culture, the mutant tooth explants degraded and only formed some cyst-like structure, whereas wild type molars had developed into bell stage with dentin matrix secretion. Hence, the tooth arrest in *Runx2* mutant mice cannot be rescued by long time culture *in vivo*.

4.2. The role of Runx2 during tooth development (II)

To clarify the role of Runx2 during tooth morphogenesis, we searched for the downstream target genes of Runx2 by extensive *in situ* hybridisation analysis. Since *Runx2* mutant mouse molars arrest at the bud to cap transitional stage, we analyzed and compared the expression of suspected genes between E14 *Runx2* mutant molars and both E13 and E14 wild type molars. For most genes involved in mesenchymal condensation, such as *Msx1, Bmp4, Pax9, Lef1,* and tenacin, the expression patterns were similar in *Runx2* mutant teeth to that of wild types. Only activin βA expression was reduced in the mutant dental mesenchyme. This was consistent with the morphological observation that condensation of dental mesenchyme around the tooth bud is not affected in *Runx2* mutants. However, in the ablation of Runx2 activity, *Fgf3* expression was completely downregulated in the dental mesenchyme of lower molars, and only few signals were detected in the mutant upper molars. Our *in vitro* bead experiments demonstrated that exogenous FGF4 beads could induce *Fgf3* expression in isolated E13 and E14 stage wild type dental mesenchyme, but failed to induce *Fgf3* expression in *Runx2* mutant dental mesenchyme. Meanwhile, FGF4-soaked beads were still able to induce *activin βA* and *TGFβ1* expression in the mutant dental mesenchyme indicating that the failure of Fgf3 induction by FGF protein is not due to the defects in FGF receptors or mediators. Further studies using Runx2 -/- calvarial cells demonstrated that *Fgf3* was also absent in Runx2 -/- calvarial cells and overexpression of Runx2 in these cells could rescue *Fgf3* expression. These results indicate that Runx2 acts in the dental mesenchyme and mediates FGF signals for the induction of Fgf3. Furthermore, by database searching, we found two putative Runx2 binding sites in the Fgf3 gene promoter region. Based on these results, we propose that Fgf3 may be a direct downstream target gene of Runx2 in the dental mesenchyme.

Previous work has shown that *Fgf3* is also downregulated in *Msx1* mutant molars and its induction by FGFs requires Msx1 (Bei and Maas, 1998). Therefore, we analyzed the relationship

between *Msx1* and *Runx2* genes during tooth development. We found that *Msx1* was expressed normally in *Runx2* mutant molars, whereas *Runx2* expression was down-regulated in *Msx1* mutant dental mesenchymal cells, although *Runx2* signals remained normal in the osteogenic regions. Therefore, we proposed that Runx2 functions between Msx1 and Fgf3 and mediates epithelial FGF signals to the dental mesenchyme.

In *Lef1* mutant mice, tooth development is also arrested at the bud stage. Earlier tissue recombination experiments have demonstrated that Lef1 is only transiently required in the dental epithelium for the bud to cap stage transition and Fgf4 is a direct downstream target gene of Lef1 in the dental epithelium. The tooth phenotype in *Lef1* mutant mice can be rescued by both epithelial and mesenchymal FGFs (Kratochwil et al., 1996, 2002). However, both *Msx1* and *Runx2* are expressed normally in *Lef1* mutant molars (Kratochwil et al., 1996 and our present data). It has been shown that epithelial BMP4 could induce Msx1 expression in the dental mesenchyme. However, BMP4 is not able to induce *Runx2* expression, suggesting that besides FGFs, Runx2 may be regulated by some other epithelial signals (Bei and Maas, 1998; D'Souza et al., 1999). Since *Fgf3* is also downregulated in *Lef1* mutant teeth, we propose that FGF4 signals from the enamel knot region may also use some other signaling pathway which convergent with Runx2 for the induction of *Fgf3* (Fig. 6).

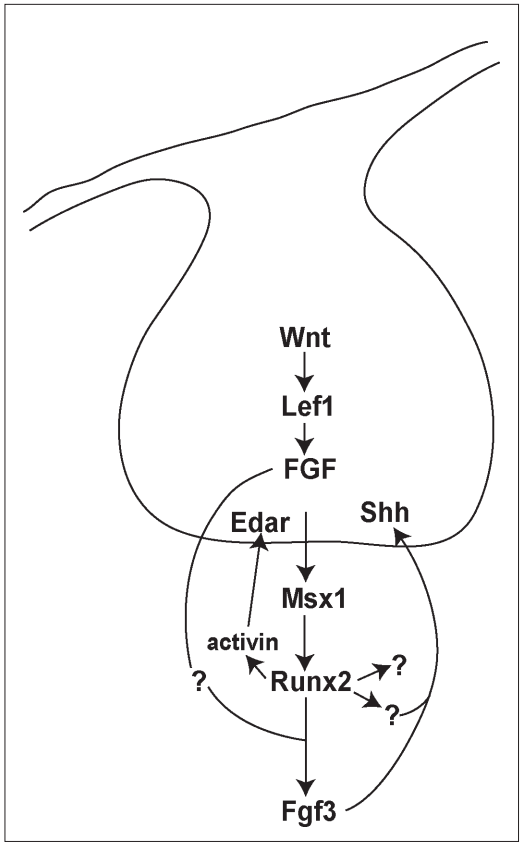

Fig. 6. Schematic representation of signaling pathways involving Runx2 during bud to cap stage transition in tooth development. Lef1 is required for the expression of Fgf in the enamel knot. Epithelial FGF signals induce Runx2 expression in the dental mesenchyme through Msx1. Runx2 regulates mesenchymal Fgf3 expression, which together with some other downstream targets of Runx2 induce Shh expression in the epithelial enamel knot. FGF may also use other signaling pathways which converge with Runx2 pathway for the induction of Fgf3 in the dental mesenhcyme. Runx2 also affects activin expression, which reciprocally acts on the dental epithelium and stimulates Edar expression in the enamel knot.

Although the primary enamel knot is morphologically discernible at the cap stage, its induction occurs already at the bud stage. The formation of enamel knot is induced by signals from the dental mesenchyme. In *Msx1, Pax9,* and *Lef1* mutant mice, tooth development is arrested at the bud stage and the enamel knot does not form. However, the tooth phenotype of *Msx1* mice can be rescued by exogenous BMP4 proteins and Lef1 mutants by FGF4 proteins. They all rescued the formation of the enamel knot so that the tooth development can proceed further (Bei et al., 2000; Kratochwil et al., 2002). Since *Runx2* mutant tooth development was also arrested at the late bud stage, we analyzed the enamel knot marker genes to see whether there were defects in enamel knot formation. We found that *Wnt10b, Lef1,* and *Msx2* were all localized at the tip of *Runx2* mutant tooth buds corresponding to the enamel knot region. However, *Edar,* p21*, Fgf4,* and *Bmp2* were expressed normally in the mutant upper molars, but down-regulated in the mutant lower molars. It appears that the enamel knot has started to form in *Runx2* mutant molars, but the formation is disturbed by the absence of Runx2 activity. The formation of enamel knot is disturbed more severely in the mutant lower molars than in the upper molars, which may be related to the morphological differences observed between mutant lower and upper molars.

Interestingly, we found that *Runx3*, which is another Runt domain protein, was dramatically upregulated in the *Runx2* mutant upper molars with a similar pattern with that of *Runx2*. In wild type mice, *Runx3* is only expressed weakly in several cell layers in the dental mesenchyme. The expression of the other mammalian Runx gene, *Runx1,* was not affected in *Runx2* mutant teeth. It has been shown that both Runx2 and Runx3 exhibit binding sites in their own promoter region and may autoregulate their own expressions. This has been suggested to explain the sudden upregulation of *Runx2* gene during development (Drissi et al., 2000; Bangsow et al., 2001). Our results indicate that Runx2 normally suppresses *Runx3* expression in the upper molars, and that upregulation of *Runx3* in *Runx2* mutant upper molars may compensate for some of the action of Runx2 there. This may account for the differences observed between mutant upper and lower molars.

It has been shown that exogenous FGF10 protein could rescue *Shh* expression in the dental epithelium of *Lef1* mutant molars (Kratochwil et al., 2002). We also tried to rescue the expression of *Shh* by adding FGF10-releasing beads on the E13 stage *Runx2* mutant molars. However, FGF10-releasing beads failed to rescue *Shh* expression, indicating that Runx2 may also regulate some other reciprocal signals from dental mesenchyme to the epithelium inducing *Shh* expression.

Our results indicate that Runx2 is involved in several signaling pathways during tooth morphogenesis. Therefore, we tried to rescue the mutant tooth phenotype by growth factors including FGF4, FGF10, SHH, and activin A. These growth factors were added either alone or in different combinations. However, none of them rescued the morphogenesis of *Runx2* mutant molars.

In conclusion, we demonstrated that Runx2 is an important transcription factor mediating epithelial FGF signals to the dental mesenchyme and regulating reciprocal mesenchymal signals during tooth bud to cap stage transition. We also showed that Runx2 acts downstream of Msx1 and directly regulates *Fgf3* expression in the dental mesenchyme. Runx2 may be involved in several different signaling pathways and the complex signaling networks determine the temporal schedule of tooth morphogenesis.

4.3. Antagonistic interactions between follistatin and activin/ BMP signals determine the shape of mouse molar teeth (III)

To further investigate the role of follistatin during tooth development, we analyzed the tooth phenotypes in two lines of mutant mice, follistatin knockout mice and the transgenic mouse line (K14-follistatin). Follistatin mutant mice die shortly after birth, whereas most of the K14-follistatin transgenic mice can live to adult.

We first performed comparative analysis on the expression patterns of follistatin and its ligands, activin βA, Bmp2, Bmp4, and Bmp7 in the normal mouse molars from E14 cap stage to the new born stage (NB). During cap and bell stages, the inner dental epithelium undergoes folding morphogenesis enveloping the underlying dental papilla mesenchyme and outlining the shape of the tooth crown. At E14 stage, follistatin was expressed in the primary enamel knot in the dental epithelium with some transcripts in the dental mesenchyme. Activin βA mRNA was localised in the dental mesenchyme with intense signals directly underlying dental epithelium. *Bmp2, Bmp4*, and *Bmp7* were all expressed in the primary enamel knot, and *Bmp4* was also expressed intensely in the dental mesenchyme.

By the E16 early bell stage, follistatin was expressed in the inner dental epithelium including secondary enamel knots, as well as in the dental follicle and the dental papilla mesenchymal cells near the cervical loop regions. Activin βA mRNA became restricted to the mesenchymal cells at the tips of the future cusps. *Bmp2, Bmp4,* and *Bmp7* were all expressed in the secondary enamel knots, and *Bmp4* was also detected in the mesenchymal cells near the cusp regions. At newborn stage, when the shape of the tooth crown has almost been determined, follistatin and activin βA were downregulated in the dental epithelium. Follistatin mRNA was restricted to the dental papilla mesenchymal cells near the cervical loop regions and activin βA was only expressed in the dental follicle. *Bmp2, Bmp4*, and *Bmp7* transcripts were all detected in the pre-odontoblasts and weakly in the preameloblasts. The unique expression patterns of these molecules during advancing tooth morphogenesis suggest that they may be involved in the regulation of cusp formation of the tooth. These results were consistent with previous studies (Åberg et al., 1997; Heikinheimo et al., 1998).

We first analyzed the molar tooth phenotypes of K14-follistatin mice. We found that when follistatin was over-expressed in the dental epithelium, the crowns of the molars were mostly

abnormal (two-month-old mouse skeletal preparation). In some severe cases, the occlusal surface of the tooth appeared whorled with no clear cusp formation and their enamel was prematurely worn. In addition, all the third molars in both upper and lower jaws were absent and this occurred in all the transgenic mice analyzed.

To determine the role of follistatin *in vivo,* we also examined the tooth phenotype in follistatin knockout mice. Follistatin mutant mice survive to term but die soon after birth due to the defects in respiratory muscles (Matzuk et al., 1995b). At E14 cap stage, the mutant molars are morphologically similar to the wild types. However, by the E16 early bell stage, the abnormalities in follistatin mutant molars started to be progressively more obvious. Compared to the clear, deep, and regular cusp patterns in wild type molars, follistatin mutant cusps were shallow and irregular. Their inner dental epithelium folded aberrantly and lacked antero-posterior polarization seen in the wild types. In freshly dissected E18 molar germs, the aberrant tooth morphology can be clearly visualized. Since follistatin mutant mice die at birth, we cultured E17 mutant molar germs *in vitro* to culturing for 7 days follistatin mutant molar germs generated multiple shallow and aberrant foldings. However, the differentiation of odontoblasts and ameloblasts was not affected, suggesting that follistatin only affects the morphogenesis of the molars but does not inhibit cell differentiation.

We further investigated the molecular mechanisms underlying follistatin mutant molar phenotype. It has been suggested that enamel knots play critical roles in tooth morphogenesis. We analyzed the expression of some enamel knot marker genes, including *Fgf4, Shh*, and *Edar* in E14 mutant molars and in wild type mice. It appeared that in the follistatin mutant molars, the primary enamel knot had formed, as indicated by the seemingly normal expression of Fgf4, Shh, and Edar. However, the secondary enamel knots were obviously abnormal. In E16 wild type mouse molars, p21, a cyclin dependent kinase inhibitor, was localised in three regions of the inner dental epithelium corresponding to the secondary enamel knots. In contrast, in follistatin mutant molars, p21 was only detected in two regions of the inner dental epithelium, and one of them was significantly larger than in the wild type. Cell proliferation analysis by BrdU incorporation revealed that the proliferating cells in the mutant inner dental epithelium were reduced and irregular without any antero-posterior polarity as in the wild types. Since the primary enamel knot determines the tooth crown base and the subsequent formation of secondary enamel knots (Jernvall and Thesleff, 2000), we proposed that in the ablation of follistatin activity, although the primary enamel knot had formed, its signaling function was disturbed resulting in the abnormal number and size of secondary enamel knots.

It has been reported that BMP4 from the dental mesenchyme can induce p21 expression in the primary enamel knot (Jernvall et al., 1998). We found that exogenous activin A-soaked beads could also induce p21 expression in the isolated dental epithelium, even though the induced signals were much weaker compared to that of BMP4 protein.

Signals from the enamel knot, such as FGF and SHH, have been shown to regulate the growth and folding of adjacent cervical loop epithelium (Dassule et al., 2000; Jernvall and Thesleff, 2000). FGF signals from the dental mesenchyme are also able to stimulate cell proliferation in the cervical loops (Kettunen and Thesleff, 1998). Given the fact that BMPs can induce p21 expression in the dental epithelium to stop cell proliferation, it has been proposed that the antagonistic interactions and balance between activators (FGFs and SHH) and inhibitors (BMPs) regulate the position of secondary enamel knots and the growth of cervical loops (Jernvall and Thesleff, 2000; Salazar-Ciudad et al., 2002). Since follistatin can modulate the activity of activin/BMP signals and their expression patterns correlate well with their functions, we propose that follistatin may function by fine-tuning the balance between the activators and inhibitors of tooth morphogenesis and thus is important for normal tooth shape formation. Since both over-expression and ablation of follistatin activity affected tooth morphology, the amount of follistatin signal seems to be critical for tooth morphogenesis. The absence of third molars by over-expression of follistatin may be explained by the defects in the formation of molar field during the early stages of tooth development.

Another interesting feature observed in follistatin mutant molars was that most cells in the stellate reticulum were closely packed with round nuclei, which was in contrast with the star-shaped and sparsely distributed cells in the wild type mice. Since the stellate reticulum becomes highly vascularised during advancing tooth morphogenesis, and also follistatin and activin have been suggested to be involved in angiogenesis (Kozian et al., 1997), we visualized the blood vessels in follistatin mutant molars by using laminin antibodies to see whether there were links between angiogenesis and the abnormal tissue in the mutant molars. Our results showed that both wild type and follistatin mutant mouse molars contain blood vessels in the stellate reticulum, indicating that neither the reduction of extracellular matrix nor the low cell proliferation rate in the mutant molars was due to the failure of angiogenesis.

It has been demonstrated that during early tooth development, activin βA from the dental mesenchyme induces follistatin expression in the overlying dental epithelium (Ferguson et al., 1998). Based on this and our data, we propose that activin βA, which is expressed in the dental mesenchyme under the primary and secondary enamel knots, induces follistatin expression in the dental epithelium. Follistatin functions by finely tuning the activity of activin/BMPs signals and thus the balance between activators and inhibitors of tooth growth. The final outcome of these interactions determines the formation and function of the enamel knots and regulates the cusp patterning of the teeth. It is thus reasonable to hypothesize that variations in follistatin expression may have influenced the evolutionary divergence of mammalian teeth.

4.4. Follistatin spatially and temporally regulates ameloblast differentiation (IV)

In contrast to the multi-cuspid molars, incisors are mono-cuspid teeth. We further investigated the role of follistatin in the mouse incisors. We analyzed the incisor phenotype of K14-follistatin mice. In one-month-old wild type mice, incisors had fully erupted and exhibited yellow-brown color. Ground sections showed a thick layer of enamel exclusively on the labial surface of the incisors, whilst the lingual surface was enamel-free and covered only by dentin. This lingual surface of mouse incisors has been suggested to be the root-analogous surface (Amar et al., 1986, 1989). The incisors of heterozygous transgenic mice appeared grossly normal. However, in the homozygous transgenic mice, which express high levels of follistatin gene, incisors were chalky white and ground sections showed that the enamel layer was totally absent. Therefore, in this study, we focused on the analysis of homozygous transgenic mice.

To further understand the enamel defects in K14-follistatin mouse incisors, we examined the incisor phenotypes in new born mice. At the new born stage, wild type mouse incisors exhibited obvious asymmetry with columnar and polarized functional ameloblasts located only on the labial surface, whereas the lingual surface dental epithelial cells were small and flattened. Odontoblasts in the dental mesenchyme were similar on both labial and lingual sides. However, the K14-follistatin mouse incisor had lost the asymmetrical character with similar appearance of the labial and lingual surfaces. There were no obvious polarized ameloblasts on the labial surface, which correlates with the phenotype seen in the adult mice. Hence, over-expression of follistatin in the dental epithelium had inhibited ameloblast differentiation.

We further analyzed the incisor phenotypes in follistatin knockout mice. To our surprise, we found a layer of polarized ameloblasts on the lingual surface of mutant incisors. This was an unanticipated finding since classical tissue recombination experiments had suggested that the lingual side dental epithelium of mouse incisors has lost the competence to respond to epigenetic inducing signals, although the lingual side dental mesenchyme is still able to stimulate ameloblast differentiation when recombined with labial epithelium or the molar inner dental epithelium (Amar et al., 1986, 1989). Our results indicate that the lingual dental epithelium still possesses the competence to differentiate into ameloblasts in the absence of follistatin. Hence, follistatin is essential for the enamel-free area formation in mouse incisors.

We confirmed the identity of the dental epithelial cells in K14-follistatin and follistatin knockout mouse incisors by *in situ* hybridisation and immunohistochemistry using some molecular marker genes, including p21, amelogenin, ameloblastin, DSPP, and MMP20. p21 associates with the stop of cell cycle and thus is an early marker of differentiating cells. Amelogenin and ameloblastin are secreted by functional ameloblasts. DSPP is expressed in newly differentiated ameloblasts and down-regulated in secretory stage ameloblasts

(Zeichner-David et al., 1995). MMP20 is a proteolytic enzyme required for enamel matrix degeneration during enamel maturation (Caterina et al., 2002). In K14-follistatin mouse incisors, all these genes were down-regulated on the labial side dental epithelium. In sharp contrast, all these genes were up-regulated on the lingual surface of follistatin knockout mouse incisors. These results confirmed that ameloblast differentiation was prevented by over-expression of follistatin in the dental epithelium and in the ablation of follistatin activity, functional ameloblasts differentiated on the lingual surface of mouse incisors. The differentiation of odontoblasts was not affected in these two mouse lines.

Since the function of follistatin has been exclusively related to binding and neutralizing the activity of members of TGFβ superfamily, the effect of follistatin on the differentiation of ameloblasts should directly link to the TGFβ superfamily signals. Earlier experiments have demonstrated that terminal differentiation of ameloblasts is triggered by functional odontoblasts or predentin-dentin matrix (Coin et al., 1999a). To investigate the inducers of ameloblast differentiation, we compared the expression patterns of follistatin with its ligands, activin, Bmp2, Bmp4, and Bmp7 during mouse incisor development. At E16, follistatin transcripts were located in the dental epithelium on both labial and lingual sides. Some differentiating ameloblasts on the labial side were devoid of follistatin signals. By E18, follistatin was down-regulated in most part of the labial dental epithelium, whereas the lingual dental epithelium continued to express follistatin transcripts. Interestingly, in the molars of postnatal 4 days mice, there were also intense follistatin signals located in the dental epithelium lining the enamel-free areas. The unique expression patterns of follistatin in the mouse teeth correlates with its function as an inhibitor of ameloblast differentiation.

From E16 to new born stage, activin βA was mainly expressed in the dental follicle surrounding the tooth germs. *Bmp2, Bmp4,* and *Bmp7* were expressed in the dental papilla mesenchyme and later in the odontoblasts. However, only *Bmp4* was expressed in both labial and lingual dental mesenchyme under the epithelium. At new born stage, intense *Bmp4* and moderate *Bmp7* expression were also seen in the secretory ameloblasts on the labial surface. It has been shown that the lingual side dental mesenchyme is also able to stimulate ameloblast differentiation. In addition, only *Bmp4* was expressed on both labial and lingual side mesenchymal cells underlying inner dental epithelium, which led us to conclude that BMP4 is the major BMP signal regulating ameloblast differentiation *in vivo*.

To further identify the inducer of ameloblast differentiation, we performed *in vitro* bead induction experiments by placing growth factor releasing beads on isolated mouse incisor tooth germs. The induced genes were analyzed by whole mount *in situ* hybridisation. We first analyzed the induction of p21 to see which signals were able to trigger to cells to escape from the cell cycle. In E15 incisor germs, BMP2, BMP4, and BMP7-soaked beads all induced intense signals around the beads. Activin A-soaked beads could also stimulate p21 expression, but the expression was quite

weak and not around the beads, but confined to the anterior cells that expressed p21 endogenously. The induction of the other ameloblast marker gene, ameloblastin, showed similar results. In both E15 and E16 incisors, BMP proteins induced very intense ameloblastin signals around the beads, whilst activin A-releasing beads only stimulated endogenous expression of ameloblastin. To further confirm the major role of BMPs on the differentiation of ameloblasts, we introduced noggin-releasing beads on E15 and E16 incisors. Noggin is a strong extracellular inhibitor of BMPs. Noggin-releasing beads dramatically down-regulated endogenous ameloblastin expression. These results, together with the specific expression pattern of Bmp4 in the dental papilla mesenchyme, suggest that BMP4 is the major regulator of ameloblast differentiation and that follistatin functions locally in the dental epithelium by inhibiting the ameloblast-inducing activity of BMP4 from the underlying mesenchyme. However, noggin is not expressed in the developing teeth (our unpublished data). Since the lingual ameloblast differentiation is not completely rescued in follistatin mutant mice, we propose that there may be some other BMP inhibitors that act redundantly with follistatin on the lingual side dental epithelium and prevent ameloblast differentiation.

Previous work has shown that during early molar tooth development, activin βA from the dental mesenchyme can induce follistatin in the dental epithelium. In the chick ectoderm, exogenous BMP is also able to induce follistatin expression (Patel et al., 1999). To determine the upstream regulator of follistatin during incisor development, we performed *in vitro* bead induction experiments with activin A and BMP2, BMP4, and BMP7 beads. In both E15 and E16 incisor germs, activin A-releasing beads induced intense signals around the beads, whilst none of the BMPs had any inductive effects. Since activin βA is expressed in the dental follicle, our results unravelled a novel role of the dental follicle as a regulator of enamel formation. Dental follicle cells are derived from condensed dental mesenchymal cells at bud stage. During cap stage, condensed dental mesenchymal cells segregate into dental papilla cells enveloped by the enamel organ and dental follicle cells surrounding the whole tooth germ. The function of the dental follicle has been related to the formation of cementum and periodontal tissue attaching teeth to the alveolar bone (Ten Cate, 1998). The dental follicle can recruit osteoclasts for resorption of bone on the coronal tooth surface and help with tooth eruption into the oral cavity. This activity has been shown to be regulated by parathyroid hormone-related protein (PTHrP) secreted from the enamel organ epithelial cells formation. Our finding is the first indication that the dental follicle can also function on the dental epithelium and negatively regulate ameloblast differentiation. It appears that the integrating interactions between the dental follicle, dental epithelium, and odontoblasts spatially and temporarily regulate enamel formation.

Based on these results, we propose a model where BMP4 signals from odontoblasts trigger ameloblast differentiation in the overlying dental

epithelium; Activin A from the outside dental follicle induces follistatin expression, which in turn acts locally in the dental epithelium and inhibits the ameloblast-inducing activity of BMP4. The differentiation of ameloblasts only starts after follistatin expression is down-regulated in the labial side dental epithelium. The lingual dental epithelium continues to express follistatin and thus prevents enamel formation (Fig. 7). Since activin βA was expressed in both labial and lingual side dental follicle regions, the mechanism for the down-regulation of follistatin only on the labial aspect remains to be resolved. Taken together, our studies have shown that the differentiation of ameloblasts of mouse incisors is regulated by antagonistic effects of activin and BMPs from two adjacent mesenchymal tissue layers, and that follistatin integrates these activities and spatially and temporarily regulates enamel formation.

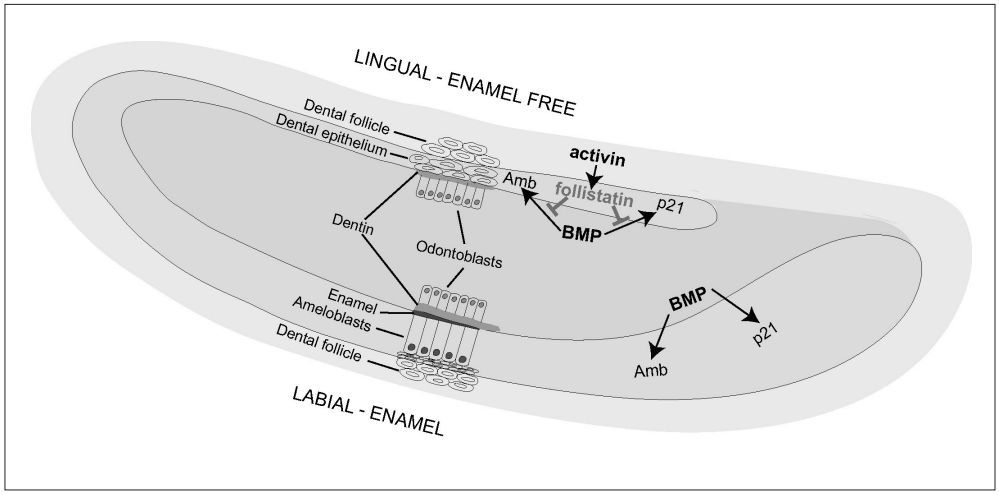

Fig. 7. Schematic representation of the molecular regulation of ameloblast differentiation in mouse incisors. Odontoblasts express BMP, which induce ameloblast differentiation. Activin from the dental follicle induces follistatin expression in the dental epithelium. Follistatin inhibits the activity of BMPs and thereby regulates enamel formation.

5. CONCLUDING REMARKS

During the last 15 years, we have seen remarkable advances in the understanding of the molecular mechanisms of tooth development. Signals and signaling pathways, as well as their interactions have started to be elucidated. One of the most important findings has been the identification of signaling centers, i.e. enamel knots, in the developing tooth. The importance of enamel knot has been well demonstrated previously in several knockout mouse models, including *Msx1, Pax9*, and *Lef1*. In all these mutant mice, development of the tooth was arrested at bud stage with no obvious enamel knot formation. Our studies have shown that in *Runx2* mutant mouse molars, enamel knot had started to form, but the formation was severely disturbed and thus the tooth bud was still not able to develop into the cap stage. In follistatin mutant mice, although the primary enamel knot had formed and expressed normal enamel knot marker genes, its function was apparently affected resulting in the defects in secondary enamel knot formation and aberrant tooth shapes. All these data emphasize the importance of the enamel knot as a signaling center during tooth development. The enamel knot itself does not proliferate but it regulates cell proliferation in adjacent cells. To date, more than 10 different signal molecules have been demonstrated to be expressed in this enamel knot region and they may play various roles in the teeth. The enamel knot may function by integrating these diverse actions and thus regulating the rigid temporal and spatial schedule of tooth development.

Another important finding in tooth development is the reiterative use of signals at different developmental stages. For example, BMP4 is involved in tooth identity determination and primary enamel knot formation. In this study, we provide further evidence showing that BMP4 also regulates the function of primary and secondary enamel knots as well as terminal differentiation of ameloblasts. Meanwhile, the activin/BMP signaling inhibitor, follistatin, also acts in different processes corresponding to the temporal and spatial activities of its ligands. The transcription factor Runx2 was also involved in several different signaling pathways during early tooth morphogenesis and later in odontoblast differentiation. The precise actions of these genes during different developmental windows may depend on the environment they work in and also the developmental competence of the target cells.

During our studies, an unanticipated finding was that the differentiation of ameloblasts is spatially and temporarily regulated by antagonistic interactions between BMPs and their inhibitor follistatin, and so terminal differentiation of ameloblasts is achieved by down-regulation of follistatin. A similar developmental regulatory process was recently demonstrated during osteogenesis as the differentiation of osteoblasts is actually determined by the relief of a Runx2-inhibitor twist (Bialek et al., 2004). With animals as complex as they are, it is not surprising that many inductive pathways have multiple additional levels of regulation. In fact, inhibiting signals may be as important as the inductive signals. The FGF signal inhibitor sprouty, Wnt signal inhibitor Dickkopf1, as well as Shh inhibitor

patched, are all expressed in the developing tooth, however, the precise roles of these inhibitors are still not fully understood. As many developmental regulatory processes are also conserved during embryogenesis, it will not be surprising to find more processes regulated by activator-inhibitor interactions in tooth development. In this study, we also provide evidence that the dental follicle negatively regulates cell differentiation in the dental epithelium and counteracts the inducing activity of odontoblasts. Sequential and reciprocal interactions between dental epithelium and dental papilla mesenchyme have been well characterised, but the interactions between dental follicle and dental epithelium are poorly understood. Our results have unravelled the reciprocal interactions between three tissue layers during tooth development and further emphasized the rigid and complex controls of organogenesis.

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