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FUNDAMENTALS OF ORAL BIOMEDICINE

· EDITOR WANTAO CHEN ·

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CONTRIBUTORS

Chief Editor

Wantao Chen Shanghai Jiao Tong University College of Stomatology

Deputy Editors in Chief

Jerry Jian Q. Feng Texas A&M Health Science Center Baylor College of Dentistry

Li Mao University of Maryland School of Dentistry

Authors

Anika S. Voisey Rodgers Texas A&M Health Science Center Baylor College of Dentistry

Hui Huang Shanghai Jiao Tong University College of Stomatology

Jianjun Zhang Shanghai Jiao Tong University College of Stomatology

Lizhen Wang Shanghai Jiao Tong University College of Stomatology

Ming Yan Shanghai Jiao Tong University College of Stomatology

Ping Zhang Shanghai Jiao Tong University College of Stomatology

Qin Xu Shanghai Jiao Tong University College of Stomatology

Xinquan Jiang Shanghai Jiao Tong University College of Stomatology

Zhengwei Huang Shanghai Jiao Tong University College of Stomatology

Zhen Tian Shanghai Jiao Tong University College of Stomatology

Zhiyuan Zhang Shanghai Jiao Tong University College of Stomatology

Zhongjing Lv Shanghai Jiao Tong University College of Stomatology

PREFACE

Since the 16th century, the world has experienced five technology revolutions, which promote the rapid development and continuous improvement of science and technology. Since the first scientific revolution, piloted by innovative new physics, represented by Copernican and Newtonian mechanics, we are now in the fifth technology revolution symbolized by electronics and computer, information and the internet. Supposedly, 2020 will be the end of the fifth technology revolution and the beginning of the sixth technology revolution, whose central concept is "new biology" era and main contents are bionic, regeneration and creation, and also called as "life biology" century.

In this context, basic scientific research of stomatology, an important component of life to be continued, is facing wonderful and broad development prospects. How to grasp the opportunity to become a leader in the field of research is an important issue and task in front of every stomatology researcher. The full English textbook *FUNDAMENTALS OF ORAL BIOMEDICINE* edited by Professor Wantao Chen from Shanghai Jiao Tong University School of Stomatology, breaking the professional boundaries and barriers of stomatology basic research, using an integrated knowledge system instead of simply imparting knowledge, shows the latest research achievements and progress in the basic scientific research of stomatology. The textbook involves: the development and regulation of oral tissue, characterization of oral genetic disease related genes, microflora and oral diseases, new dental materials and tissue engineering principles and applications, progresses of molecular genetics and epigenetic of oral cancer and the related cell signaling pathways, research on oral epithelial stem cells and cancer stem cells, the transgenic and gene knockout animal models, oral cancer and immune, molecular diagnosis and classification of oral cancer, microRNA regulation mechanisms of oral cancer, and other latest research hotspots and progresses in oral basic scientific research. The editorial board includes both national and international young experts in the field of oral basic scientific research. Professor Jerry Jian Q. Feng from Texas A & M University, Baylor College of Dental and Professor Li Mao from University of Maryland School of Dentistry are invited as the deputy editors in chief.

This book is an important component of "Oral Medicine Graduate English Course System", which is belonging to the graduate course system construction project sponsored by National project "985" Stage Ⅲ and project "085" of Shanghai Municipal Education Commission Stage I, and is responsible by Professor Zhiyuan Zhang. Undoubtedly, building a full English graduate curriculum system as the starting point will contribute to a comprehensive graduate training of professional English skills and promote the internationalization ability of postgraduate training.

We warmly congratulate the officially publication of this book, and write these as the preface.

June 20th, 2013

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CHAPTER I

MOLECULAR REGULATION OF TOOTH DEVELOPMENT AND DENTAL STEM CELLS

Zhen Tian

PART I TOOTH DEVELOPMENT

There are many things that make teeth special: from an evolutionary genetic standpoint, they contain the hardest biological substance; much of our understanding of animal evolution is based on teeth; forensic science relies on dental records for identification. From a developmental perspective, the model of tooth development offers a useful paradigm for studying patterning and morphogenesis or the determination of position, size, shape and number during organogenesis (1).

Teeth are highly mineralized and the bulk of a tooth is composed of a mineralized tissue called the dentin, which is a bone-like matrix characterized by closely packed dentinal tubules that contain the cytoplasmic extensions of odontoblasts (2). Dentin is the major component of the tooth and is completely surrounded by enamel in the crown and by cementum in the root. It is a mineralized tissue, formed of a network of type I collagen fibrils and carbonated apatite crystals organized in a tubular pattern. Dentin surrounds the pulp, which is the soft connective tissue that occupies the central portion of the tooth. The dental pulp is rich in fibroblast-like cells, blood vessels and nerves. Odontoblasts continue to deposit dentin throughout life. The primary dentin forms till the completion of root development, secondary dentin forms at the roof and floor of the pulp chamber after root formation and, reactionary dentin (tertiary dentin) is formed by the original odontoblasts in response to stimuli, such as dental decay or restorative treatment. Dentin and pulp are related embryologically, histologically and functionally. They are usually described together as "dentin-pulp complex".

The dentition is derived from the first branchial arch, or from two major cell types: stomodeal ectoderm and cranial, neural crest derived ectomesenchyme cells (3). The tooth development begins as a thickening of the oral epithelium. Then the epithelium at specific sites invaginates into the underlying mesenchyme form the dental placode. The dental placode then further invaginates into the surrounding dental mesenchyme to form a tooth bud. At this stage, the mesenchyme proliferates and condensates around the tooth bud. After that, the epithelium of the tooth bud folds to form a cap-shaped structure. The folding divides the epithelium into the inner and outer enamel epithelium. The mesenchymal cells adjacent to the inner enamel epithelium will form the dental papilla and those near the outside will form the dental follicle. Morphogenesis from the bud to the cap stage involves the formation of enamel knot, which is an important signaling center

for tooth morphogenesis. The enamel knot is transient structure and will disappear at the end of the cap stage, but molars will continue to form secondary enamel knot, which determines the locations and shapes of the molar cusps (4).

Subsequently, the dental epithelium further folds and extends into the mesenchyme to form bell-shaped tooth germs and then the enamel knot disappears. At the late bell stage, cell differentiation starts: the cells of the internal enamel epithelium differentiating into ameloblasts (enamel-producing cells) while adjacent cells in the dental papilla differentiating into odontoblasts (dentine-producing cells). The ameloblasts and odontoblasts deposit enamel and dentin back-to-back and mineralization of the sematrices forms the two principal hard tissues of the tooth.

After the tooth crown formed, epithelial cells of the inner and outer enamel epithelium proliferate from the cervical loop of the enamel organ and form a double layer of cells known as *Hertwig's epithelial root sheath*. This sheath of epithelium extends and encloses all but the basal portion of the dental pulp. The inner enamel epithelial cells of the sheath initiate the differentiation of odontoblasts from ectomesenchymal cells at the periphery of the pulp and form the dentin of root. Cementum formation occurs later and cementoblasts are the cells responsible for cementogenesis. During the roots formation, the supporting tissues also developed. After tooth eruption, with the formation of root apex, the development of tooth is completed (5,6).

PART Ⅱ MOLECULAR REGULATION OF TOOTH DEVELOPMENT

The molecular regulation of tooth development shares many similarities with development of a number of other organs. Thus, the tooth provides an excellent model for studying how an organ develops. During tooth development, the initiation of tooth, the formation of enamel knot, the determination of tooth type and the formation of hard tissue are key stages for developing of a normal tooth. And all these stages involve the epithelial-mesenchymal interactions, which regulate both morphogenesis and cell differentiation. The epithelial mesenchymal interactions were described in detail by experimental embryologists in the 1950's. Progresses in the field were rather slow during the following three decades. Due to the great advances in molecular biology and technology, now the epithelial mesenchymal interactions can be studied at the molecular level. Recently, a reasonable amount of data on tooth developmental regulation have been accumulated. More than 300 genes have been associated with tooth development, such as transcription factors, growth factors, components of the cell surface and extracellular matrix (ECM), and matrix degrading enzymes. The majority of them are associated with conserved signaling pathways mediating cellular communication, in particular between epithelial mesenchymal tissues. In this chapter, we review some of the current literature concerning molecular regulation in the processes of tooth development (7).

Otherwise, most molecular experiments about mammalian tooth development have been done in mice because they are readily amenable to genetic analysis and manipulation, such as gene knockout mice, transgenic mice, etc. Mice develop two types of teeth: one is the incisors development in the distal part of the jaw, while molars develop in the proximal part of the jaw in each quadrant. Incisors continuously grow throughout the life. In mouse, tooth formation is initiated at embryonic day 10 (E10), and the dental epithelium proliferates and continues to develop through the bud (E12-13), cap (E14-15) and bell (E16-17) stages. Because of the stages of tooth development are well conserved throughout toothed vertebrates, the data from the mouse provide us the molecular signaling regulation of human odontogenesis (8).

2.1 Initiation of the Tooth

The first source of initiating signals must be either the oral epithelium or the underlying neural-crest-derived mesenchyme. The early dental epithelium is shown to possess the potential to induce non-dental, neural crest-derived mesenchyme to form a tooth. Tooth forms from the combination of neural crest expanded from the trunk level and mandibular epithelium. This indicates that molecules located in the oral epithelium initiate the tooth formation. However, after 12 days of development, oral epithelium loses this odontogenic potentiality, and mesenchyme gains the ability to instruct non-dental epithelium to form tooth-specific structures. For instance, recombination of the late first arch ectomesenchyme with the embryonic foot epithelium elicits an enamel organ. Conversely, if the oral epithelium is recombined with the skin mesenchyme, the enamel organ assumes as those of epidermis. These results suggested that with time passed the odontogenic potentiality is transferred to the factors resident in the ectomesenchyme. Epithelial-mesenchymal interactions are now considered to constitute the most important mechanism regulating organogenesis, and some of transcription and growth factors are detected in these early and late tissues.

Tooth developments in mice start as localized thickenings of epithelium which can first be detected histologically at E11. The earliest mesenchymal marker of the appearance of tooth primordia is starting to be elucidated with the cloning of two Lim-homeobox domain genes, *Lhx6* and *Lhx7*. Unlike other homeobox genes, such as *Msx-1*, *Dlx-2* and *Barx-1*, which are expressed in all of the

branchial arches, *Lhx6* and *Lhx7* are expressed and restricted to the neural crest ectomesenchyme of the oral half of the first branchial arch as early as E9. The expression of both of these genes may due to a signaling molecule originated from the oral epithelium of first branchial arch. If recombined with epithelium from the oral surface of the first branchial arch, second arch ectomesenchyme, which normally does not express these genes, can be induced to express *Lhx6* and *Lhx7*. If first arch mesenchyme is recombined with second branchial arch epitheliums, the expression of these genes will be quickly down-regulated. These results indicate that the local epithelium-mesenchyme interactions induce the expression of *Lhx6* and *Lhx7*. Secreted fibroblast growth factor (*Fgf-8*) is the most likely candidate molecule for the induction of *Lhx* genes. Because *Fgf-8* is expressed in the oral epithelium overlying the *Lhx6* and *Lhx7* expression domains, but it is not expressed in the equivalent epithelium of second branchial arch, which demonstrates that *Fgf-8* may play a role in determining the positions where the tooth germs will form.

One of the most important questions in tooth development is what controls the position and the number of tooth germs along the oral surface. *Fgf-8* has been shown to relate with the position determination. Besides *Fgf-8*, *Sonic hedgehog* (*Shh*), *Bmp-4*, *Pax-9*, etc. may also play key roles in defining the location of tooth germs. The *Pax-9* gene is one of the earliest mesenchymal genes that its expression is restricted within the areas where tooth germs appear. *Pax-9* can be induced by *Fgf-8* and repressed by bone morphogenetic proteins (*Bmps*), such as *Bmp-2* and *Bmp-4* at E10.5. *Fgf-8*, *Bmp-2* and *Bmp-4* are expressed in nonoverlapping areas, with *Pax-9* being expressed at sites where *Fgf-8* but not *Bmps* exists. *Shh* is first found in incisor regions and localized to the presumptive dental ectoderm at E11. *Shh* has been shown to be involved in cell proliferation to produce tooth bud. *Shh*-soaked beads to oral ectoderm can induce local epithelial cells proliferation to produce invaginations into the underlying mesenchyme. *Shh* knockout mice have less developed tooth buds. So *Shh* is regarded as another signaling molecule in tooth initiation. *Bmp-4* expression is present in the thickened presumptive dental epithelium at early tooth morphogenesis, and then they shift to the underlying mesenchyme at about E12 during normal mouse tooth initiation. Results from *in vitro* studies indicate that *Bmp-4* acts as epithelial signaling to regulate gene expression in mesenchyme. When *Bmp-4* is expressed in dental epithelium, it can induce the expression of homeobox-containing genes *Msx-1* and *Msx-2*, but repress the expression of *Pax-9*. Interestingly, *Bmp-4* expression in the bud mesenchyme is required to maintain *Bmp-2* and *Shh* expression. A loss of mesenchymal *Bmp-4* and an arrest of tooth development at early stage are also observed after the loss of Wnt signaling, placing the Wnt pathway upstream of the mesenchymal *Bmp-4* (9).

The *Wnt* signaling pathway also plays essential role in early tooth development (10-13). The canonical *Wnt* signaling pathway involves nuclear accumulation of β-catenin. Activation of *Wnt* signaling inhibits β-catenin phosphorylation and leads to its accumulation in the cellular nuclei where it interacts with and converts the *TCF/Lef* family DNA-binding proteins from transcriptional repressors to activators. An inhibitor of canonical Wnt signaling, *Dkk1*, causes tooth developmental arrest at the early bud stage. Several *Wnt* genes, including *Wnt4*, *Wnt6*, *Wnt10a*, *Wnt10b*, and *Wnt* receptor gene *Fz6* are strongly expressed in the presumptive dental epithelium in mice. *Wnt7b* is expressed in the oral epithelium, but is absent in presumptive dental epithelium. Ectopic expression of *Wnt7b* in dental epithelium down-regulates *Shh* expression in the dental ectoderm and the tooth development is subsequently arrested. This indicates that the expression of *Wnt7b* in oral ectoderm may relate to the location of tooth germs.

Lef-1 is a member of the high-mobility group nuclear protein family that includes the T-cell factor proteins, known to be nuclear mediators of *Wnt* signaling pathway. Mouse lacking *Lef-1* exhibits tooth developmental arrest at the bud stage. *Lef-1* is firstly expressed in thickening dental epithelium and then shifts to the underlying mesenchyme. Tissue recombination experiments suggested that *Lef-1* function is required in the dental epithelium and ectopic expression of *Lef-1* in the oral epithelium results in ectopic tooth formation (14).

Obviously, a lot of other genes are also expressed (e.g., *Msx-1*, *Msx-2*) in dental epithelia at the same time. To date, more than 90 genes have been identified from the oral epithelia, dental epithelia and dental mesenchymes at the initiation of tooth development. Much more up-to-date information can be searched on the website "*Gene Expression in Tooth*" (http://bite-it.helsinki.fi).

2.2 Bud-to-cap Transition

At around E13.5, the tooth bud is clearly formed accompanied by the formation of a condensate of dental mesenchyme, which expresses a host of signaling molecules such as *Bmp-4*, *Msx-1*, *Activin-βA* and *Pax-9*. *Pax-9* plays an important role in the bud-to-cap transition. All teeth *of Pax-9* mutant embryos are arrested at the bud stage. *Pax-9* is expressed in domains similar to *Activin-βA* in bud stage mesenchyme. Despite being co-expressed and essential for tooth development to progresses beyond the bud stage, *Pax-9* and *Activin-βA* appear to act independently. However, changes occur for other genes such as *Bmp-4* and *Msx-1*. Not only in mouse but also in human, the loss of *Msx-1* and

Pax-9 may lead to down-regulation of *Bmp4* and an arrest of tooth development at the bud stage, suggesting that *Msx-1* is required for *Bmp-4* expression. *Bmp-4* induces its own expression via *Msx-1*. Tooth development can be rescued in *Msx-1*-/- embryos by addition of exogenous *Bmp-4* (15,16).

Bmp-4 expression in the bud mesenchyme is needed to maintain *Bmp-2* and *Shh* expression in dental ectoderm. Blocking *Shh* with neutralizing antibodies leads to the loss of *Bmp-2*, implying that *Shh* and *Bmp-2* may be within the same signaling pathway. *Shh* is an epithelial signaling necessary for epithelial proliferation. Blocking *Shh* signaling at different stages of tooth development may show different effects. If blocking *Shh* at E11-E12, the tooth development will be arrested at bud stage, but tooth buds will still be developed to form teeth if blocking occurs at E13 (17).

2.3 Condense of Mesenchyme

The first molecules that are found to be specifically localized in mesenchymal condensates are extracellular matrix glycoprotein tenascin and the cell membrane proteoglycantsyndecan-1. Tenascin interacts with cells and other matrix molecules and is initially localized in the mesenchyme of developing teeth, and is regulated by epithelial signals. Syndecan-1 acts as a receptor for several matrix molecules and is shown to interact with several components of the extracellular matrix. It may also be required for *Fgfs* binding to its receptors. Syndecan-1 may bind to tenascin, which suggests that the two molecules may interact with each other. These two molecules constitute a mechanism of homotypic interaction between dental mesenchymal cells and contribute to their condensation around the epithelial tooth bud.

In the condensing mesenchyme, many other molecules, including *Tgf-β1, Fgf-3, Egr-1* and *Bmp-4*, are up-regulated. Msx-1 expression also becomes up-regulated and markedly restricted to the condensed dental mesenchyme at the bud stage. Tissue recombination studies indicate that the expression of some of molecules that are up-regulated in the condensing mesenchyme is controlled by the dental epithelia. When cultured alone, the expression of *Egr-1*, *Msx-1* and *Bmp-4* was undetectable in mesenchyme, whereas when cultured with dental epithelia, the expression was induced.

2.4 Enamel Knot And Tooth Type Determination

The enamel knot is a population of cells in the center of the invaginating dental epithelia. The enamel knot can be histologically visible as a bulge in the centre of the inner enamel epithelium at the cap stage. It was first visualized both in incisors and in molars by Ahrens in 1913, but its significance as an important structure and signaling center in tooth development was only recently discovered. It expresses genes for many signaling molecules including *Shh*, *Bmp-2*, *Bmp-4*, *Bmp-7*, *Fgf-4*, *Fgf-9*, *Shh*, *Lef-1, Wnt3*, *Wnt6*, *Wnt10b*, *MFz6* and *Slit-1*, etc. To be an important signaling center, the enamel knot is required for the transition of the epithelial bud to the cap stage. The cells of the enamel knot do not proliferate and thus act as an anchor to constrict the movement of cells in the tooth. High proliferation outside the enamel knot and low proliferation within the knot therefore act to fold the epithelium of the tooth germ, forming a cap-shaped structure at E14.5.

The enamel knot is a transient structure. It will disappear at the end of the cap stage. At E13, *Shh*, *Bmp-2* and *Bmp-7* are all expressed in the enamel knot. *Shh* and *Bmp-2* share same domains, whereas the *Bmp-7* domain is slightly larger. By E14, the expression of *Bmp-4* and *Fgf-4* can be detected in this structure. *Bmp-4* is expressed only in the distal half of enamel knot, and the expression of *Bmp-4* in oral epithelia leads to an up-regulation of enamel knot markers, such as *p21*. *P21* gene, a cyclin-dependent kinase inhibitor, may be the first gene to be turned on in the enamel knot and is expressed in the enamel knot precursor cells at the tip of tooth buds. *Ectodin* or *wise* known as the *Bmps* antagonist can restrict the *Bmps* action. *Bmp-4* induces *ectodin* expression, which then acts back on *Bmp-4*, leading to the down-expression of *Bmp-4* and restricted induction of *p21*. The *ectodin* knockout mouse is characterized by the over-expression of p21 with enlarged enamel knots and cuspal defects. These genes will be turned off when the enamel knot disappears, but *Fgf-4* is still expressed in the secondary enamel knots, which may associate with cuspal morphogenesis.

Transcription factors expressed in the mesenchyme, such as *Msx-1*, may play an important role in the formation of enamel knot. *Msx-1* is strongly expressed in mesenchyme surrounding bud. Another transcription factor, *Lef-1,* is also highly expressed in ectomesenchyme at the bud stage. *Bmp-4* expressed in the mesenchyme may also induce the expression of *Msx-2,* leading to the development of enamel knot.

Wnt3, *Wnt6*, *Wnt10b* and *Mfz6* are expressed in the primary enamel knot, while *Wnt5a* and *MFrzb1* are strongly expressed in the dental papilla mesenchyme. *Wnt* inhibitors, such as *Dkk* family, are up-regulated in oral mesenchyme, but excluded from enamel knots. Therefore, Wnt/β-catenin signaling is active in the enamel knot. In mutant embryos carrying an activating, stabilizing mutation of β-catenin, super-numerary teeth are developed due to new enamel knot buds grow off from the existing dental epithelium. This suggests that β-catenin signaling is an upstream activator for the enamel knot formation.

The genetic pathways that control morpho-

genesis are established in cells of tooth buds at the cap stage, which will differentiate into enamel knot. Thus, at this time, future development of the tooth germ must already be determined to be mono- or multi-cuspid, incisor tooth buds will develop into incisors, while molar tooth buds will develop into molars. The determination of specific tooth type is a remarkably consistent process. Most mammals have three-tooth shapes: incisor form, canini form and molar form. Incisor tooth germs only have a single enamel knot whereas molars go on to form secondary enamel knot. The secondary enamel knots again lead to the folding of the inner enamel epithelium at the bell stage, resulting in the formation of a complex multi-cuspid tooth. Thus the shape of the tooth crown is driven by the number of enamel knots formed. In the process of forming secondary enamel knots, *Wnt/β-catenin* signaling is active. If *Wnt* signaling is blocked at the early bell stage by a *Wnt* inhibitor, MFRZB1, teeth will be reduced in size with blunter cusps. Otherwise, the size and shape of primary enamel knot is also the key to produce exact degree of curvature of the oral epithelium. Small enamel knot in molars affects the folding of the tooth and the positioning of the secondary enamel knots, leading to a molar with few flattened cusps.

The loss of the primary enamel knot is due to apoptosis. Apoptosis may be used as a mechanism to remove the tooth signaling centers after they have finished patterning. Apoptosis is first seen in regions corresponding to the position of the inner cells of the future enamel knot. *P21* induces cells to leave the G1 phase of the cell cycle and play a role in apoptosis. *Bmps* and *Msx-2* have been involved and played positive roles in this apoptotic process, while *Fgf-4* may limit the spread of apoptosis to the enamel knot alone. *Fgfs* may be responsible for the observation that apoptosis increases in mesenchyme after the removal of the epithelia. *Fgfs* and *Bmps* have been shown to have antagonistic roles in apoptosis in the enamel knot. Cell death continues from the inner restricted epithelial populations till reaching the highest levels at E14.5. By E15, no apoptotic cells can be seen, and the primary enamel knot has largely disappeared. The secondary enamel knots of molars contain high numbers of apoptotic cells present at E18. The budding, folding and branching of epithelial tissues are associated with extensive remodeling of the extracellular matrix, in particular the basement membrane, at the epithelial-mesenchymal interface. The basement membrane is composed of type IV collagen, laminin, perlecan, nidogen (entactin) and various proteoglycans. These molecules have been shown to locate in the developing teeth and the integrity of the basement membrane is a prerequisite for tooth morphogenesis.

2.5 The Homeobox Code

Homeobox genes are characterized by a conserved 180bp DNA sequence coding for a 60-amino acid DNA-binding domain called "homeodomain". They function as transcription factors recognizing specific DNA sequences and regulating target genes. The homeobox genes are rapidly found in all animal species and act as control roles and are required in vertebrate development. Homeobox genes are divided into two classes. Class Ⅰ genes, called Hox genes, share a high degree of identity in their homeodomain; while Class Ⅱ genes share a lower degree of identity in their homeodomain. Class Ⅱ homeobox genes are grouped into subfamilies based on the presence of additional conserved functional domains besides the homeodomain. Homeobox genes play a crucial role in specifying cell identity and positioning during embryonic development, and the mutations in these genes can cause dramatic developmental defects, which are known as "homeotic transformations".

Hox genes are not expressed in the first branchial arch derivatives; thus, Hox genes are not involved in tooth development. But several genes belonging to the class Ⅱ homeobox families are expressed during odontogenesis. They are expressed and located in the anterior border between the first and second pharyngeal arch in nested patterns across the jaw. In 1998, Thomas and Sharpe proposed that the patterning of murine dentition was determined by the complex and specific distribution of homeobox genes prior to the initiation of tooth formation. The overlapping domains of homeogene expression in the jaw appear to confer a positional code that specifies them orphogenesis of teeth, termed as "*homeobox code*". And the teeth of mandibular and maxillary are involved in different developmental pathways, although the origin of them is histologically and morphologically identical. The different homeobox-containing genes are expressed in very distinct domains.

The mandible homeobox genes are divided into oral (*Lhx-6, Lhx-7*), aboral (*Gsc*), presumptive incisor (*Msx-1*, *Msx-2*) and presumptive molar (*Dlx-1*, *Dlx-2*, *Barx-1* and *Pitx-1*) domains (Fig. 1-1). For example, the early expression of *Msx-1* and *Msx-2* is restricted to distal, midline ectomesenchyme in regions where incisors, but not molars, will develop; if a presumptive molar gene, such as *Barx-1*, is abnormally expressed in the presumptive incisor region, the tooth germs will develop as molars, with the formation of multiple cusps. *Dlx-1* and *Dlx-2* are expressed in ectomesenchyme cells where multi-cuspid teeth, but not incisors (or canines) will develop. If *Dlx-1* and *Dlx-2* are knocked out, the molars fail to develop in the maxilla but development of these teeth in mandible will not be affected. A possible explanation for this phenomenon is that the loss of *Dlx-1* and *Dlx-2* in mandibular mesen-