

# Odontogenesis - a Masterful Orchestration of Functional Redundancy or What Makes Tooth Bioengineering an Intrinsically Difficult Concept

## Abstract

Rapid increase of knowledge in stem cell research, bioengineering technology and molecular basis of odontogenesis has finally lead us to the point where it is possible to develop approaches for treatment of tooth loss with bioengineered teeth, which one day might completely replace conventional prosthodontics and dental implants. By holding onto the premise that in order to bioengineer teeth, full understanding of how teeth develop is required, it must be acknowledged that there are certain features of odontogenesis which create obstacles in gaining that understanding. One such feature is the functional redundancy in genetic networks responsible for molecular control of odontogenesis. Abundant data imply that having functional redundancy of various elements in regulatory genetic networks is more than just a failsafe built into the odontogenic sequence in order to secure unhindered development of teeth. This phenomenon plays important roles in determination of tooth numbers and positioning, and is increasingly recognized as important for enabling sufficient plasticity of regulatory genetic networks through which the appearance of tooth-type specific and species-specific diversity of mammalian tooth morphology can be explained. Unfortunately, most of what we know about molecular basis of odontogenesis, comes from studies of mouse molars. Novel insights from developmental biology, stem cell research, and bioengineering technology are still needed, if we want to make de novo tooth bioengineering a clinically viable method for treatment of tooth loss.

**Keywords:** Odontogenesis; Tooth bioengineering; Dental stem cells; Morphogenesis; Patterning; Homeobox genes; Growth factors; Primary enamel knot

## Mini Review

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**Darko Kero<sup>1\*</sup> and Mirna Saraga Babic<sup>2</sup>**

<sup>1</sup>Study Programme of Dental Medicine, University of Split, Croatia

<sup>2</sup>Department of Anatomy, Histology and Embryology, University of Split, Croatia

**\*Corresponding author:** Darko Kero, Study Programme of Dental Medicine, School of Medicine, University of Split, Soltanska 2, 21000 Split, Croatia, Tel: +385 21 557 846; Fax: +385 21 557 811; Email: dkero@mefst.hr

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**Abbreviations:** DPSCs: Dental Pulp Stem Cells; SHEDs: Stem cells from Human Exfoliated Deciduous Teeth; PDLSCs: Periodontal Ligament Stem Cells; SCAPs: Stem Cells of Apical Papilla; 1BA: First Branchial Arch; PEK: Primary Enamel Knot; SEK: Secondary Enamel Knot; FGFs: Fibroblast Growth Factors; BMPs: Bone Morphogenetic Proteins; FGFs: Fibroblast Growth Factors; BMPs: Bone Morphogenetic Protein; Lhx: LIM/homeobox Protein; Barx-1: BarH-like homeobox 1; Msx: Msh homeobox; Dlx: Homeobox Protein Dlx; Shh: Sonic Hedgehog; Wnt: Wingless-related Integration Site Homeobox; Lef-1: Lymphoid Enhancer-binding Factor 1; p21: Cyclin-dependent Kinase Inhibitor 1

## Introduction

Rapid increase of knowledge in stem cell research, bioengineering technology and molecular basis of odontogenesis has finally lead us to the point where it is possible to develop approaches for treatment of tooth loss with bioengineered teeth, which one day might supplement and replace conventional prosthodontics and dental implants [1,2]. What puts tooth bioengineering in the forefront of bio-regenerative medicine are the very features of tooth as an organ. Teeth are easily accessible, non-essential for life organs of relatively simple structure with their own complement of various stem cells niches [2]. There

are at least three possible ways to bioengineer tooth - to build and then to assemble individual tooth components crown, root; putting aggregates of dental stem cells to spontaneously re-organize within tooth-shape customized scaffolds; and to transplant artificially created tooth germs into adult jaws until they develop to fully functional teeth [3-5].

In the treatment of tooth loss, tooth crown is of less importance than tooth root because the crown itself if there is a viable root can be easily replaced by the artificial one with adequate morphology following conventional prosthodontic treatment protocols. In deciduous and permanent teeth, researchers have already identified, cultivated and manipulated cellular elements needed to synthesize hard and soft tissue elements of tooth crown and root - DPSCs and SHEDs, for dentine PDLSCs, and SCAPs for cementum and alveolar bone anchoring periodontal ligament fibres [6-8]. Even though these cells show huge potential in regenerative treatment of dental pulp injuries and periodontal ligament defects, their odontogenic potential for tooth bioengineering has been less apparent [6]. In numerous attempts to bioengineer whole-tooth or tooth components by seeding dental stem cells onto bio-degradable scaffolds moulded in the shape of teeth, dentin-like or cementum-like structures with incorporated cellular elements were indeed produced, however, those chips

and globules of mineralized tissue were still far cry from what resembles to normal crown, root, or especially the whole tooth [3,9]. One of the main reasons why such an approach in tooth bioengineering does not yield as expected, is that scaffolds represent an over-simplification of micro-environment in which the tooth development normally takes place where stem cells only represent a niche, but not a full cellular complement. Another reason is that dental stem cells might be too lineage committed and thus have more limited proliferation, differentiation and self-organization potential in comparison to embryonic tooth germ cells [10,11]. Research of properties of various dental stem cell populations is still under way and it will probably take several more years before the viability of DPSCs, SHEDs, PDLSCs and SCAPs for prolonged preservation in dental stem cell banking can be properly evaluated [10,12]. It should also be noted that, intrinsically, these stem cell populations differ greatly. For example, DPSCs and SHEDs differentially express more than 4000 genes, and the latter population seems to be more resilient in retaining pluripotency after multiple passages in *in vitro* culture [2]. The third, and the most serious obstacle is that DPSCs, SHEDs, PDLSCs and SCAPs are all of mesenchymal origin, whereas no adequate source of adult odontogenic epithelial stem cells has been identified so far.

This stems directly from the way teeth develop, which is that of ectodermal derivatives of mixed epithelial and mesenchymal origin [13]. By the time teeth erupt into oral cavity, epithelial components of tooth germs are completely disintegrated, thus leaving us without the source of epithelial odontogenic stem cells. Furthermore, one of the basic features of odontogenesis is constant reciprocal signalling between epithelial and mesenchymal parts of the developing tooth germ in which either epithelial or mesenchymal tooth germ parts depending on the stage of development assume and relegate inductive odontogenic potential from/to the opposing tissue compartment [14]. Tissue recombination experiments have shown that inductive potential needed for initiation of tooth development sequence resides exclusively in parts of epithelium of primitive oral cavity, whereas underlying jaw mesenchyme takes the lead at later stages [15]. Modest successes in reconstruction of fully developed teeth by using dental stem cells and biodegradable scaffolds do imply that, in order to bioengineer teeth de novo, we have to learn how to faithfully emulate original setting in which odontogenesis takes place. Furthermore, human permanent teeth take on average several years to fully develop 15-20 years for human third molars being the most extreme example. So, what is even more daunting in making teeth bioengineering a therapeutically viable approach, is that we also have to learn how to artificially speed up the natural process of tooth development without detrimental effects.

By holding onto the premise that in order to bioengineer teeth, one needs to fully understand how teeth develop, in this mini-review we discuss why there are lots of obstacles before we achieve both of these goals. We do this primarily from the aspect of functional redundancy of genetic networks involved in molecular control of odontogenesis. Abundant data imply that having functional redundancy of various elements in regulatory genetic networks is more than just a failsafe built into the odontogenic sequence in order to secure unhindered development of teeth [16,17]. This phenomenon plays important roles in determination

of tooth numbers and positioning, and is increasingly recognized as important number for enabling plasticity of regulatory genetic networks through which the appearance of specific types of teeth, and species-specific morphological diversity of mammalian teeth can be explained [18,19]. Most of the findings discussed here come from studies performed on mouse molars, which are most commonly used as model teeth for research of molecular basis of odontogenesis.

## Discussion

### Cap stage - starting point for bioengineering of tooth germ

Embryonic tissues have extreme self-organizing ability. That has been firstly demonstrated by mixing different types of dissociated amphibian germ layer cells together [20]. Within the medium, cells of similar type were able to spontaneously rearrange themselves into structures corresponding to the original germ layers in developmentally correct positions. This type of embryonic cell behaviour has also been exploited for tooth germ bioengineering in the series of experiments on cap-staged mouse incisor and molar tooth germs [5,21-23] (Figure 1). Namely, after complete cellular dissociation of epithelial and mesenchymal parts of the cap stage tooth germ, cells were able to spontaneously reorganize *in vitro* into a viable tooth germ only when seeded in close contact layers simulating epithelial-mesenchymal interface. Following the transplantation into jaw of adult animal, bioengineered tooth germs developed into teeth, which displayed normal alignment of hard and soft tissues in both crown and roots, and had properly developed root-periodontal ligament complex. They were also able to erupt normally forming occlusal contacts with antagonist teeth, and responded normally to various noxious stimuli. However, the teeth were somewhat smaller premolar size and had slightly altered crown morphology, but it was subsequently demonstrated in another study, that the crown size can actually be increased or reduced depending on the size of contact area between epithelial and mesenchymal tooth germ cells during the seeding.

We should note that when the same tooth bioengineering method was applied to bell-staged tooth germs, results were not equally good. So, what is it that makes up for the amazing plasticity of cap-staged tooth germs and significantly less so of the bell-staged germs?

### Odontogenic sequence

Teeth are organs of mixed epithelial and mesenchymal origin. They develop due to reciprocal signalling between the embryonic oral ectoderm which gives rise to enamel organ and underlying cranial neural crest cells-derived mesenchyme of 1BA which gives rise to dental papilla and dental follicle [24-26]. Tooth germs undergo several histologically distinctive stages before they are fully prepared for synthesis of hard dental tissues of tooth crown and subsequently those of the root (Figure 1). Following the determination of tooth forming sites, epithelial thickenings placodes begin to appear in designated areas of the embryonic oral ectoderm. In bud stage, epithelial thickenings continue to invaginate into the underlying mesenchyme, thus forming tooth buds. The mesenchyme around the tip of the buds starts to condense, whereas the opposing epithelial cells

gradually stop proliferating as they get to pack themselves into a transient structure called primary enamel knot. Around PEK epithelium begins to fold designating the onset of cap stage or morphodifferentiation of enamel organ. During the cap stage, parts of epithelium closer to PEK grow more intensely, which leads to formation of cervical loops at buccal and lingual ends of enamel organ. Cervical loops are highly proliferative and they will continue to ingress through the underlying mesenchyme now dental papilla in the subsequent bell stage of development. Once the transition from cap to early bell stage is complete, PEK is eliminated by programmed cell death apoptosis. However, in tooth germs of multicuspid premolars and molars novel SEKs form at the sites of additional epithelial folding designating future cusp tips. Thus, at the early bell stage the layout of interface between enamel organ and dental papilla acquires the shape of future tooth crown. Progressively, growth becomes even more confined to cervical loops while the epithelium around SEK, and directly underlying cells of dental papilla, begin to differentiate into hard dental tissue secreting cells ameloblasts for enamel and odontoblasts for dentine.

### **Functional redundancy in tooth germ initiation and jaw patterning**

Streams of data about molecular basis of odontogenesis, provided us with a framework of more than 300 different genes involved in molecular regulation of tooth development [27]. Several genes encoding for growth factors such as FGFs, BMPs, following the reports about genetic activity during the early stages of organogenesis in general, for a particular subset of homeobox genes called the non-hox homeobox genes, became of special interest [14,16,28-30]. While growth factors act as signals to target cells in order to change cellular behaviour proliferation, differentiation, apoptosis, etc., members of the latter group are transcription factors, which direct joint actions of many other genes by binding to their DNA via evolutionarily highly conserved homeodomain sequence [16,31]. Homeobox genes in general are deemed as master organizers of organogenesis responsible for arrangement of total body plan and that of individual organs. Non-hox homeobox genes comprise the subset of homeobox genes which are expressed very early in the oral ectoderm and underlying 1BA mesenchyme from which teeth, jaws and most of other structures in oral cavity develop [31,32].

From the aspects of both odontogenic potential and jaw patterning or how particular types of teeth get to develop at specific sites in jaws, non-hox homeobox genes expression domains in 1BA mesenchyme during the initiation stage of odontogenesis, are more instructive than their expression domains & remove those observed in the overlying oral ectoderm. Here we list several examples. Prior to the initiation of tooth development, the oral half of 1BA mesenchyme starts to express two LIM-domain homeobox genes, Lhx6 and Lhx7 [33]. It should be noted that similar phenomenon cannot be seen in the neighbouring 2BA mesenchyme unless it is artificially recombined with embryonic oral ectoderm. Within Lhx6 and Lhx7 expression domains, other non-hox homeobox genes display more localised expression patterns; Barx-1 in the area of future molars, Msx-1 in the area of future incisors, whereas several Dlx genes tend to be differentially expressed in the area of upper and lower future molars, having Dlx1 and Dlx2 the only Dlx genes being the only group members

expressed in both future upper and lower molars area [34-36]. Expressions of other non-hox homeobox genes such as Pax-9 appear throughout the odontogenic mesenchyme of the 1BA, but in response to inductive signals from the overlying oral ectoderm initiating tooth development sequence [37]. At this stage, a group of non-hox homeobox genes which are exclusively expressed in the oral ectoderm such as Shh, Wnt and Msx-2, display completely overlapping expression domains approximating the sites of all future tooth germs in both jaws equally [16]. As the tooth development progresses, some of their expression domains gradually extend into the underlying 1BA mesenchyme Msx-2, but the overall layout of non-hox homeobox gene expression patterns in epithelial and mesenchymal compartment at initiation stage of odontogenesis strongly implies that oral ectoderm is the tissue with odontogenic potential, while the blueprint for tooth type determination resides in the 1BA mesenchyme [31]. That notion was also corroborated by tissue recombination experiments of which those performed on avian embryonic oral ectoderm provided the most striking results most striking. Although birds have no teeth, avian embryonic oral ectoderm retains odontogenic potential, and when recombined with rodent 1BA mesenchyme is able to give rise to the teeth [38]. Furthermore, the odontogenic potential of embryonic oral ectoderm can also be amplified by knocking out specific inhibitors of non-hox homeobox genes expressed. This either promotes over expression of those genes in embryonic oral ectoderm e.g. Wnt, or speeds up the epithelial-to-mesenchymal shift of their activity e.g. Shh that normally occurs at later stages of development [39-41]. The outcome of such genetic manipulation is formation of different variants of supernumerary teeth with poor or properly shaped crowns in both upper and lower jaw.

In contrast, tinkering with mesenchymally expressed non-hox homeobox genes such as Barx-1, Msx-1, Dlx1 and Dlx2 creates more specific dental phenotypes. That is partly in agreement with layout of those genes' expression domains and jaw patterning properties of 1BA mesenchyme, but has also a lot to do with functional redundancy that Barx-1, Msx-1, Dlx1 and Dlx2 seem to display. Namely, shift of Barx-1 expression domain in future incisor area, results with ectopic development of molars [34]. On the other hand, single null mutations of either Dlx1 or Dlx2 have no apparent effect on tooth development, but if both Dlx1 and Dlx2 are knocked out, only upper molars fail to develop. This effect is clearly due to functional redundancy of Dlx1 and Dlx2 because 1BA mesenchyme in lower molar region expresses additional complement of Dlx genes. Further analyses have also revealed that the timing of the arrest of tooth development caused by the knockout of various non-hox homeobox genes expressed in 1BA mesenchyme is variable, and can also occur in advanced stages of development. Therefore, to some extent these genes seem to be functionally redundant for initiation of odontogenesis. Accordingly, signalling networks of other non-hox homeobox genes and growth factors respond in highly unpredictable ways. They either continue to operate normally, or jointly switch to other developmental programs [36].

Tinkering with activity of after with non-hox homeobox genes and/or elements of their signalling networks has been important for understanding of molecular basis of tooth development initiation and jaw patterning. However, the fact that changes in tooth number and positioning are the only effects caused by



null mutations, conditional knockout, or transgene-driven over-expression of non-hox homeobox genes, does not make non-hox homeobox genes likely candidates involved in determination of more sophisticated morphological features of teeth such as crown shape, or cusp patterning, etc. [17]. How that is done can partly be explained by interpretation of certain factors' expression patterns in PEK and by understanding of mechanisms involved in maintenance of stem cell niches in cervical loops.

### **Primary enamel knot - the keeper of cusp pattern blueprint**

Since the discovery that the expression domain of non-homeobox gene *Msx-2* is closely confined within the emerging PEK, this structure has been receiving substantial attention in the research of molecular basis of odontogenesis [42]. Among many factors, the expression domains of key members from four major signalling networks involved in odontogenesis *Shh*, *Wnt*, *FGF*, *BMP* have been observed in PEK. Similar expression patterns of *Shh*, *Wnt*, *FGF* and *BMP* have also been observed in dental placode and at the tip of the tooth bud, so in that way, PEK represents cellular and molecular link between the initiation and advanced stages of tooth development. Function of PEK resembles to that of signalling centres in other developing organs such as Zone of Polarizing Activity and Apical Ectodermal Ridge in limbs. Namely, PEK relays signals which orchestrate direction and rate of growth of surrounding odontogenic tissues from both epithelial and mesenchymal compartment of the tooth germ [43,44].

The effects of impairment of PEK function were described in null mutants for *Edar*, which is one of the main receptors in TNF signalling pathway [45]. TNF signalling pathway plays important role in epithelial morphogenesis of developing tooth germ. *Edar* null mutants fail to properly form PEK, since PEK cells end up aligned in sheet forming a structure called the enamel rope. As a consequence, tooth germs develop into teeth which exhibit extremely flattened crowns [46]. Furthermore, early removal of PEK by excessive activation of apoptotic machinery is also detrimental for tooth crown development. Gaps in the inner enamel epithelium appear and its folding fails. Thus, small teeth develop displaying severely deformed crowns with hypoplastic defects in both enamel and dentine [47]. In contrast, delayed removal of PEK has rather confusing outcome; apart from transient morphological changes in enamel organ, teeth develop normally. In this case, alternative apoptotic pathways are probably activated [48]. It is intriguing, though, that neither the failure to form PEK, nor its premature removal can actually cause the arrest of tooth germ development. Functional redundancy of regulatory networks of factors involved in apoptotic removal of PEK, can be deemed as a necessary failsafe, but there might be even more to it. Namely, even though the expression profiles of members of key signalling networks in PEK and dental placode closely resemble (which makes PEK a cellular and functional successor of dental placode) it should be noted that PEK operates in significantly different circumstances of which the most important is shift of odontogenic potential from epithelial part of tooth germ to the underlying mesenchyme.

How does pattern of molecular activity within PEK reflect on crown morphology is also interesting question. In previous

section we described connection between regionalization of non-hox homeobox genes' expression domains in 1BA mesenchyme and tooth type determination. Does something similar occur in PEK? Computational analysis and comparison of expression domains of 4 PEK-marker genes *Fgf4*, *Lef1*, *p21* and *Shh* from horizontal sections of mouse and vole tooth germs aged between cap and late bell stage showed that certain correlation between expression patterns and basic crown shape is actually present [17]. Namely, layout of peak intensity of these genes' expression domains within PEK corresponds to arrangement of cusps cusp patterning in species-specific manner. Furthermore, it seems that such an arrangement of PEK-marker genes' expression domains precedes even the first histological signs of cusp formation, i.e. the additional folding of inner enamel epithelium. From the aspect of basic crown shape determination and cusp patterning, it seems that not only if, but also where within PEK particular genes express. This type of gene expression domain localisation is much more sophisticated than that described in 1BA mesenchyme during determination of tooth type at initiation stage because it occurs within much smaller area. How this is regulated is not exactly known, but the activation of diverse groups of factors with mutually opposing or similar activity functional redundancy could be the underlying regulatory mechanism. In that sense, fine tuning of molecular activity in PEK is of paramount importance.

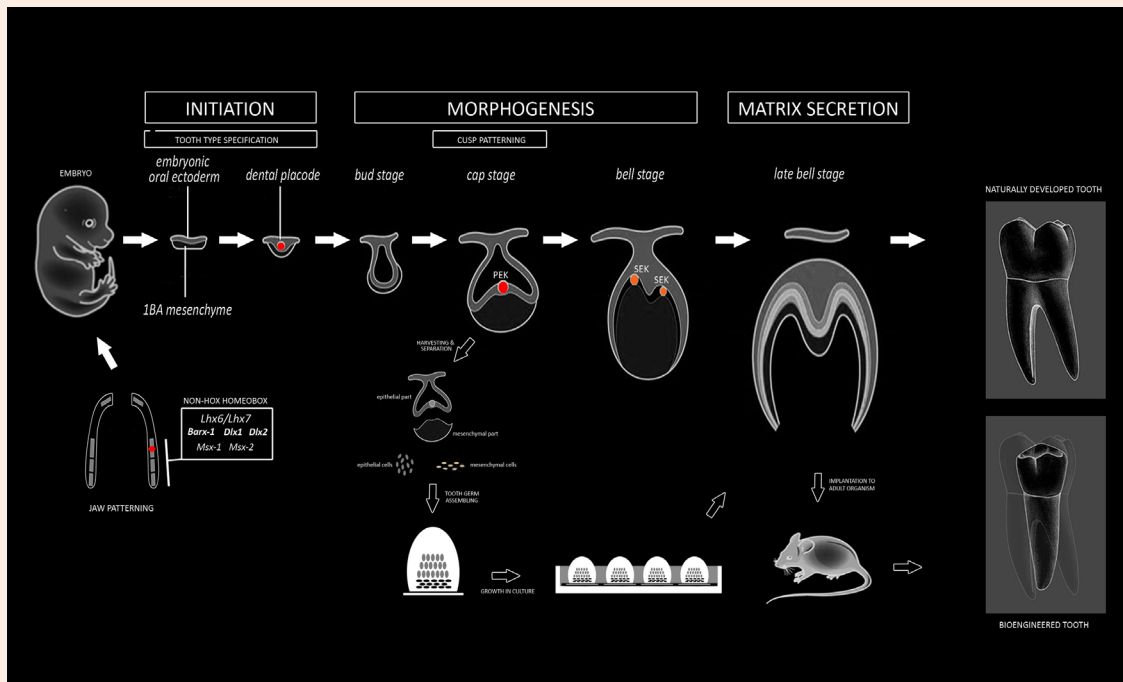
### **How to make a difference between squat crown and continuous growth**

There are two major theories about development of variable tooth shapes in different mammalian species. According to one theory, this occurs due to introduction of new genetic networks, in odontogenic sequence, whereas the other theory suggests that variability of tooth shapes is not the consequence of introduction of new genetic networks, but rather the outcome of modulating the existing ones [27,49]. Continuously growing rodent incisors are perfect model for investigating the mechanisms which regulate maintenance and function of stem cell niches [50]. Namely, roots of those teeth harbour permanently active cervical loops driven by non-depleting complement of stem cells. Rodent incisor stem cell niche resides within the stellate reticulum, a vacuolated tissue compartment enclosed by a single layer basal epithelium of cervical loops [51]. Mesenchymal cells surround the epithelium and provide regulatory signals, which determine proliferation and self-renewal or differentiation of stem cells [52,53]. Balance is established by co-expression of various growth factors *FGF-3*, *FGF-10*, *BMP-4*, their inhibitors *Activin*, *Sprouty* genes, *Noggin* and by all-round inhibitors *Follistatin* which seem to be capable to inhibit both growth factor and its immediate inhibitor [51,54-56]. This type of functional redundancy is quite an exemplary display of plasticity of odontogenic regulatory networks, which (in case of rodent incisors) yields with quite an astonishing phenotype - cervical loops of rodent incisors are asymmetric (labial one being wider than the lingual one) and they possess differential activity with regard to hard dental tissue synthesis, which is subsequently reflected by deposition of enamel on the labial side only. Interestingly, knocking out any of these inhibitors involved in cervical stem cell niche regulatory networks (or simultaneously a group of inhibitors) always leads to substantial enlargement of crown [57,58]. However, in some cases crown ends up with

enamel being deposited on both labial and lingual side, while in other cases there is no enamel at all.

These examples show how striking lapses between dental phenotypes non-continuously growing teeth vs. continuously growing teeth can be caused by a slight modulation of existing genetic regulatory networks. Any kind of altering of activity or resilience of stem cell niches in cervical loops dramatically affects

the basic outlook of a tooth. Namely, continuously growing teeth have extremely tall crowns in contrast to relatively short roots hypsodontia. This also point out to a possible operational modes in genetic regulatory networks required for setting up the timing of transition from development of crown to development of root [59,60]. So far, that particular stage of odontogenesis has been relatively poorly investigated.



**Figure 1:** Odontogenic sequence and molar tooth germ bioengineering.

Tooth germ undergoes several histologically distinctive stages in preparation for enamel and dentine matrix secretion and mineralization- initiation dental placode, bud stage, cap stage and bell stage. Tooth type specification occurs prior to the initiation stage. Cranial neural crest cell-derived mesenchyme of the first branchial arch 1BA provides positional information through orchestrated activity of several non-hox homeobox genes, whereas the overlying embryonic oral ectoderm holds inductive odontogenic potential. During the transition from bud to cap stage, mesenchymal part of tooth germ takes over and acts inductively to drive morphogenesis of enamel organ. At the cap stage, a signalling centre called primary enamel knot PEK is formed, which is responsible for cusp patterning of future tooth crown. PEK is removed by the beginning of the bell stage when secondary enamel knots SEK assume its role. SEKs are located at the sites of future cusp tips from where the secretion of enamel and dentine matrix commences.

Bioengineered molar tooth germ split sequence - cap-staged molar tooth germs are harvested from embryo. Epithelial and mesenchymal parts are separated, their cells dissociated and then seeded in layers to emulate epithelial-mesenchymal interface. Re-assembled molar tooth germs are cultivated up to late bell stage and then transplanted into adult jaws. They develop into teeth with normal structure of hard dental tissues. They have viable roots and respond normally to noxious stimuli, however, they are smaller than natural teeth and have only molar-like crown morphology.

## Conclusion

Some features of odontogenesis, such as reciprocal epithelial-mesenchymal signalling, have already proven their value by helping to devise approaches in tooth bioengineering. Other features, such as functional redundancy of regulatory genetic networks, still await proper appraisal.

Unfortunately, knowledge of molecular basis of human odontogenesis does not nearly amount to that of rodent odontogenesis. Although similar genetic networks are involved in development of both human and rodent teeth, differences between human and rodent teeth with regard to tooth morphology, duration of odontogenic sequence and dental formulae, do imply different modes of regulation that need to be elucidated.

Investigating expression profiles can be informative, but due to functional redundancy of various factors, it precludes us from reaching unambiguous conclusions [61-64]. "Furthermore, research of some aspects of molecular basis of odontogenesis is extremely difficult and can only be performed on a narrow subset of animal models - immediate post-transcriptional regulation of odontogenesis by microRNAs is a good example" [65]. These methodological limitations may represent a serious gap which has to be jointly bridged by the novel insights from developmental biology, stem cell research, and bioengineering technology, if we ever want to make de novo tooth bioengineering a clinically viable method for treatment of tooth loss.

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