

Title : Identification and application of reaggregated tooth using bone marrow mesenchymal stem cells

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Abstract : Tooth bud requires specific and complex epithelial-mesenchymal interactions for development during organogenesis. The results of epithelial-mesenchymal recombination assay showed that molar tooth bud has the potential to develop tooth with reaggregated dental mesenchymal cells at E13.5. We could obtain the reaggregated tooth after recombination between the dissociated dental mesenchymal cells and oral epithelium. According to the number of the dissociated mesenchymal cells, teeth shapes were altered, such as Molar1-like (M1-like), slope-like and the crater-like shapes. The M1-like teeth were examined by H-E staining and in situ hybridization with signaling molecules. Moreover, We tried to produce reaggregated teeth by using bone marrow mesenchymal stem cells (BMMSCs) which have the ability to differentiate the source tissues of bone, cartilage and other mesoderm. BMMSCs committed to differentiate into teeth when BMMSCs were recombined with oral epithelium after reaggregation with the dental mesenchymal cells at certain ratio. These teeth were also examined by H-E staining and in situ hybridization with Shh and Bmp-4. In particular, the M1-like teeth were implanted into mouse alveolar bone to validate the possibility of bioengineered tooth. We could find that this bioengineered tooth, which was produced by using BMMSCs, might be a launching case for tooth development and tissue engineering.

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Title : IGFs Induce Enamel Formation by Increase Expression of Enamel Mineralizing Specific Genes

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Abstract : Insulin-like growth factors (IGF-I and IGF-II) have been shown to play an important role in growth and differentiation in a number of tissues including mineralizing bone. Little is known about their role in tooth mineralization. Previous work in our laboratory has shown the presence of IGFs ligands, their receptors, and their binding proteins during mouse tooth morphogenesis, and the expression of IGF I coincides with the expression of amelogenin, ameloblastin and enamelin at the late bell and secretory stage. Furthermore, the addition of IGF ligands to tooth organ cultures in vitro results in an increase in the thickness of the enamel layer while at the same time there is a decrease in the dentin layer. The objective of this study is to determine the mechanisms by which IGFs modulate enamel and dentin formation. Mouse first mandibular molars were dissected from E16, E17 and E18 mouse embryos and placed in organ culture in the presence of 100 ng of IGF-1 or IGF-2.!

The molars were harvested after 1, 3 and 6 days in culture and either RNA or nuclear proteins were extracted. RNA was subjected to real time RT-PCR and the nuclear extracts were used to determine the activity of transcription factors using a TF/DNA microarrays. Our results show an induction of enamelin, ameloblastin and amelogenin mRNA expression in E17 tooth organs treated with IGF-I for 24hrs. The microarray results showed changes in DNA binding activity for several transcription factors known to be related to tooth development. The DNA binding activity was increased for Pbx-1, NF-1, Yy-1, CREB(1) but decreased for Brn-3 and CBF as compared to the untreated control. These results suggest that IGFs increase enamel formation by the induction of enamelin, ameloblastin and amelogenin gene expression via the transcription factors Pbx-1, NF-1, Yy-1, CREB(1). Studies are underway to determine a possible mechanism for these factors. This study was supported by NIH-NIDCR grant DE!

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Title : The Nature and significant regeneration of primary enamel knot

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Abstract : The development of individual teeth is regulated by epithelial-mesenchymal interactions that are mediated by signals shared with other organs such as skin appendages. Three transient signaling centers in dental epithelium, such as early signaling center, primary enamel knot (PEK) and secondary enamel knots (SEKs), direct the differential growth and subsequent folding of the dental epithelium and also regulate the growth of the tooth shape. The present study shows the origin, some characteristics and inducing mechanism of enamel knots. The center part of dental lamina moved and formed the PEK region and SEKs were not from the PEK cells. After removal of signaling centers, the remained epithelium was recombined with dental mesenchyme. The regeneration possibility of enamel knots was investigated in these recombined tooth germs with two evidences. One is the epithelial cell cluster expressing Shh and Fgf4 in cap stage and the other is the sharp epithelial edge of inn!

er dental epithelium between SEKs in bell stage. The PEK could be regenerated at the tooth germ at stage E12 and E14. To find out necessary factors in PEK regeneration, some of gene expressions, such as Lef1, Bmp2 and Bmp4, were investigated in the E16 recombined tooth germ, which could not regenerate the PEK, and were compared with those gene expressions in E14 recombined tooth germ. Also, inter-stage recombination between epithelium and mesenchyme was applied to identify necessary tissue in PEK regeneration. The necessary tissue in regeneration of PEK might be dental epithelium holding important epithelial signals. Also, the position of regenerated PEK was determined by dental epithelium rather than dental mesenchyme. It is concluded that destiny of PEK including origin, regeneration, size and position is determined by dental epithelium rather than dental mesenchyme.

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Title : Dentition pattern, tooth development and amelogenin expression in the lizard, *Chalcides viridanus* (Scincidae, Squamata)  
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Abstract : We have studied the formation of the dentition pattern, tooth replacement, tooth morphology and tooth development, with particular attention paid on amelogenesis and amelogenin expression, in a series of the scincid lizard, *Chalcides viridanus*, from late embryos to 6 year-old specimens, using light, scanning and transmission electron microscopy, in situ hybridization on sections and immunogold labelling. Tooth shape changes during ontogeny. Through successive replacements, the simple, conical shape of the embryonic tooth modifies into bicuspid in the juveniles. The dental lamina of the first teeth branches off from the oral epithelium, while it spreads from the enamel organ of the previous tooth where a replacement tooth is initiated. The cells of the dental lamina proliferate and differentiate into the three layers of the enamel organ: the outer and inner dental epithelium (i.d.e) separated by the stellate reticulum. Next, the i.d.e cells differentiate into ameloblasts.

Facing them mesenchymal cells differentiate into odontoblasts and deposit predentin matrix. Opposite, the enamel matrix is synthesised by the ameloblasts. Mineralization of the dental matrices starts at the top of the tooth, where the ameloblasts stop to deposit the enamel matrix. Maturation of the enamel matrix progresses from top to base, while dentin mineralization proceeds centripetally. Tooth attachment is pleurodont. Replacement occurs from the lingual side, where the dentin cone is resorbed. Although tooth morphology in *C. viridanus* and in mammals is different, morphogenesis and differentiation are roughly similar. However, Tomes' processes and enamel prisms, which characterise forming enamel in mammals are absent in this lizard. The amelogenin gene was cloned and compared to available sequences in other tetrapods. The expression pattern of the gene and of its protein was described during tooth development, and compared to that currently known in mammals. This

sequence analysis confirms that the hydrophilic regions were well conserved during evolution, but it reveals that the hydrophobic region is more variable than in other tetrapods. However, more amelogenin sequences must be analysed to understand how this region is evolving in squamates. Indeed, the rapid evolution of this region seems to blur the phylogenetic information. In situ hybridization was performed on tooth sections at various stages of the amelogenesis, and immunogold labelling was obtained using an antibody directed against a synthetic peptide. This is the first report of an amelogenin gene in a lizard and the first demonstration, using specific probes, that reptilian ameloblasts express amelogenin during enamel development. In *Chalcides*, amelogenin expression during amelogenesis was similar to that described in mammals, i.e. from the moment at which the first enamel matrix was deposited to the post-secretory phase, when the ameloblasts faced mature enamel. In contrast to what has been described in mammals, amelogenin transcripts were

never found in the odontoblasts at any stage of their development.

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Title : The expression pattern of hyaluronan synthase during human tooth development  
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Abstract : In previous studies hyaluronan and its major cell surface receptor CD44 has been suggested to play an important role during tooth development. Therefore our aim was to describe for the first time the expression pattern of hyaluronan synthase (HAS) by immunohistochemistry in human tooth germs from different developmental stages. The distribution pattern of HAS in the various tissues of the "bell stage" tooth primordias corresponds to that of hyaluronan in most locations: positive HAS immunoreaction was observed on the dental lamina cells, inner - and outer enamel epithelium. On the stellate reticular cells moderate HAS signal was recorded, similar to the layers of the oral epithelium, where faint HAS immunolabeling was detected. At the early phase of dental hard tissues mineralisation strong HAS immunostaining was detected in the odontoblasts and their processes, as well as in the secretory ameloblast and their apical processes and the pulpal mesenchymal cells. Th!

e HAS signals observed in odontoblasts and ameloblasts gradually decreased with age. It is concluded that hyaluronan synthesised locally by different dental cells and these results provide additional indirect support to the idea that HA may also contribute both to the regulation of tooth morphogenesis and dental hard tissue formation.

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Title : Conservation of process for vertebrate dentitions but each with their own design

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Abstract : Recent palaeontological data has challenged long-standing assumptions that teeth in vertebrate dentitions are homologous, instead it has been proposed that basal taxa of stem group gnathostomes each have a unique pattern for tooth addition. We decided to investigate the genetic mechanisms that pattern and regulate odontogenesis in basal crown group gnathostomes, the rainbow trout (*Oncorhynchus mykiss*), to distinguish mechanisms common to those of tetrapods, from those determined as distinctive. In contrast to the mouse, with only two tooth types and one set, the trout has one type but multiple sets and many sites, marginal, palatal, lingual, and pharyngeal. Therefore, we can examine genes involved in patterning the first teeth of each set and also for their replacement, and can simultaneously compare expression patterns for a detailed range of developmental stages. A number of genes identified as homologous to the murine genetic cascade, responsible for tooth initiation,

and cytodifferentiation have been isolated from *O. mykiss* using RT-PCR. Key genes *Shh* and *Pitx2*, identified as early epithelial markers of odontogenic initiation, correlate with mesenchymal *Bmp4* expression at all first tooth loci. This confirms the conservation of developmental controls at one stage, between trout and mouse, at all sites both in initiation of the dentition and its replacement. We have also identified a reiterative sequential expression pattern through morphogenesis of the teeth, similar to that for the mouse. These data aim to test questions of homology and their evolutionary significance.

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Title : Laminin subchains and related integrins in the human tooth pulp: pulpal fibroblasts express laminin-2 (alpha2/beta1/gamma1) and laminin-8 (alpha4/beta1/gamma1), and these isoforms support trigeminal neurite growth

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Abstract : Laminins are involved in cell adhesion, migration and signalling, and play crucial roles in neuronal outgrowth, maturation and regeneration. Different isoforms of laminins, which are formed through the combination of various laminin alpha, beta and gamma chains, have different functional characteristics. Here, we report on the distribution of the different alpha, beta and gamma laminin chains and laminin-related integrins in the human tooth pulp. Immunohistochemistry demonstrated that pulpal nerve fibers express laminins alpha1, alpha2, alpha4, alpha5, beta1 and gamma1. Blood vessels showed immunoreactivity for laminin alpha3, alpha4, alpha5, beta1, beta2, gamma1 and gamma2. Among the laminin-related integrin receptors, alpha3, alpha6, beta1, and beta4 were all associated with nerve trunks, while alpha1, alpha3, alpha6, alpha3beta3 and beta1 were expressed by blood vessels. RT-PCR showed that the major laminin chain mRNAs expressed in cultured pulpal fibroblast were

alpha2, alpha4, beta1 and gamma1, and immunoprecipitation/Western blot revealed that the alpha2/beta1/gamma1 and alpha4/beta1/gamma1 subchain proteins in the fibroblasts assembled to form laminin-2 and laminin-8, respectively. Co-cultures of rat trigeminal primary sensory neurons and human pulpal fibroblasts showed that neurites sprouted extensively on the surface of the fibroblasts, where they formed elaborate networks. Laminin-2 and laminin-8 may be crucial in promoting and maintaining these contacts between pulpal cells and nerve fibers. This notion is strongly supported by the fact that trigeminal neurons grew extensively when cultured on laminin-2 and laminin-8 substrates. Laminin-2 and laminin-8 may be vital for sensory nerve terminal integrity and necessary for a functional nerve receptor in the tooth pulp.

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Title : Fragilitas ossium (fro/fro) and TGF-beta1 over-expressing mice: Is the lingual forming part of the incisor a structural entity?

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Abstract : The phenotypes of two mutant mice were examined histologically and by immunohistochemistry. In both cases dentin formation was especially altered in the labial part near the apex of the mandibular incisors. These mutations are adding evidences to the differences which have been already reported between the crown-analogue and root-analogue dentines (Beertsen et al., 1986). Fragilitas Ossium (fro/fro) is an autosomal recessive mutation induced in the mouse by tri (1-aziridinyl) phosphine sulfide (Thiotepa). The mice displayed severe forms of osteogenesis imperfecta (Type II and III OI) (Guenet et al., 1981; Sillence et al. 1993). No collagen abnormalities were found in bone, whereas osteonectin was decreased by 30-50% (Muriel et al. 1991). Our investigations demonstrated that in the adult mouse dentine formation was impaired in the labial zone near the apex of the mandibular incisor, which remained widely open during tooth formation. The large gap present at the margins of the cornet-like dentine was finally resolved by a groove and later by a simple line, which finally disappeared. The lingual part of the dentin was apparently normal. Small structural alterations were detected in enamel. In the molar, dentine filled a residual pulp chamber, a situation close to some forms of Dentinogenesis Imperfecta. Investigations carried out on newborn mouse revealed severe hypomineralization of alveolar bone and dentine, as shown by the von Kossa staining. This was correlated with an increased decorin and biglycan expression, and slight modifications in the SIBLINGs distribution (DSP, DMP-1, BSP and OPN). However, no significant changes were detected in dental tissues with respect to osteonectin. PCNA immunostaining established that in the wild type (WT) mouse proliferation is occurring exclusively in the lingual forming apical zone of the incisor, and at the tip of cusps in molar tooth germs. No labeling was detected in the fro/fro newborn mouse in molar tooth germs and in the incisors as well. In the fro/fro mouse, PCNA immunostaining was firmly reduced in the proliferative zone of the oral mucosa. The lack of cell proliferation explains why there is no backward growth of the mandibular incisor, which was about half of the length of the WT. The incisors of transgenic mice over-expressing TGF beta 1 (Thyagarajan et al., 2003) displayed a kidney-like appearance because the labial half of the tooth was formed alone, and the lingual half was totally missing. This suggests that the defects observed in the fro/fro mouse were intensified in the TGF beta1 mouse. In the labial dentine defects were aligned along the junction between between the mantle and circumpulpal dentines, at a 10-15 micrometers distance from the dentino-enamel junction. With its cellular inclusions, the circumpulpal dentine displayed an osteodentine phenotype. The same features were seen in the molar, together with a reduced pulp chamber and short roots, reminiscent of the DI phenotype. In conclusion, in the mouse mandib-

ular incisors, the lingual forming zone near the apex was affected by the two mutations. The fact that this area is the target for cell proliferation and consequently for an altered root-analogue dentinogenesis suggests that it is an anatomical entity bearing its own biological specificities.

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Title : Genetic and genomic approaches to understand Pax9 function in tooth development Oral Presentation Sunday 18th July "Initiation"

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Abstract : Pax9 encodes a transcription factor required for tooth formation in both mice and man but the molecular mechanisms that are regulated by Pax9 are largely unknown. To address this, we have started different projects using the mouse as a model system. Our experimental approaches include generation of novel hypomorphic and conditional Pax9 mutant alleles, genome-wide expression profiling of wild-type and Pax9 mutant tooth rudiments, and analyses of genetic interactions involving Pax9, Msx1, and Bmp4. Recent progress in these areas will be presented at the meeting.

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Title : Pax9 and Msx1 interact via Bmp4 in mammalian tooth development (Abstract for poster session)

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Abstract : Tooth formation is a tightly regulated process involving reciprocal tissue interactions between the dental epithelium and mesenchyme. At the bud stage, mesenchymal Bmp4 signalling is required for the expression of Shh, Bmp2, p21 and Msx2 in the overlying epithelium. Bmp4 expression in the dental mesenchyme is downregulated in Pax9 and Msx1 single homozygous mutants, suggesting the two transcription factors to act in the same genetic pathway. To test this, we have generated Pax9/Msx1 double mutant mice. In Pax9/Msx1 double heterozygous mice there is a complete absence of the lower incisors, while the remaining molars and upper incisors are normal. The absence of lower incisors is not observed in Pax9 and Msx1 single heterozygotes. In the double heterozygous mutants, the tooth rudiment of the lower incisors reached the early cap stage at E13.5, while at E14.5 the tooth rudiment was arrested. Interestingly, there was a complete absence of pre-odontoblasts between the ameloblasts and dental papilla in the double heterozygous mutants at E15.5. Analysis of targets of Bmp4 signalling, Shh, p21 and Bmp2, revealed their expression domains to be consistently smaller in Pax9/Msx1 double heterozygous mutants, indicating that Pax9 and Msx1 synergistically regulate signalling events required for maintaining the inductive potential of the odontogenic mesenchyme. Introduction of a transgene that directs Bmp4 expression to the dental mesenchyme was able to partially rescue the formation of the lower incisors in Pax9/Msx1 mutants. In addition to the dental defects we occasionally observed facial clefting affecting the lip and nose. The spectrum of defects is highly variable ranging from unilateral cleft lip only to bilateral cleft lip and cleft nose. Together, our results demonstrate a critical genetic interaction between Pax9 and Msx1 that is mediated via Bmp4 and suggest that different levels of Pax9 and Msx1 expression are required in different teeth.

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Title : Discontinuity of the early dental lamina in the human upper jaw in 3D reconstructions  
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Abstract : Objectives: Thickenings of the dental epithelium are already apparent on all the medial nasal and maxillary processes before their fusion. After the fusion of the facial processes, the dental epithelial thickenings also fuse, giving rise to a continuous horseshoe-shaped dental lamina. It is assumed that the lateral deciduous incisor originates at the fusion site and includes material from both the medial nasal and maxillary processes. Aim: Using serial frontal histological sections and computer aided three dimensional (3D) reconstructions, we searched for the fusion site on the human dental lamina at 6-7 weeks of prenatal development. Results: At embryonic day 40-42, reconstructions showed a mound of dental epithelium in the incisive region, separated from the dental epithelium in the maxilla by a narrow gap. At a later stage, these separated dental epithelia fused into a continuous horseshoe-shaped dental lamina. The fusion site was detectable on the dental lamina!

a until week 7 of prenatal development. At this specific position, the second deciduous incisor started to develop. Conclusion: The original insular pattern of the dental epithelium can also be traced after the fusion of the facial processes. The independent early development of the thickened dental epithelium of the not-yet fused medial nasal and maxillary processes can explain the dental anomalies frequently associated with cleft formation, such as the presence of incisors located on both sides of the cleft, and hypoplastic or missing incisors. This work was supported by the Grant Agency of the Czech Republic (304/02/0408) and Ministry of Education, Youth, and Sports of the Czech Republic (project COST B23.002).

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Title : Tooth engineering without scaffold from dissociated single cells in vitro  
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Abstract : Introduction: It has been reported that dental structures could be regenerated by implanting dissociated odontogenic cells seeded onto a pre-shaped scaffold in vivo. However, it is not clear how far the surrounding host tissues may participate in the regeneration and what is the exact role of the scaffold since the size of the tooth structures was much smaller than the scaffolds. Objectives: To address these questions, in vitro approaches were developed. Methods: Enamel organs and dental mesenchymes from the first lower molars from ED14 ICR mouse embryos were enzymatically separated. Each tissue was further dissociated into single cells. After centrifugation, the pellets of epithelial and mesenchymal cells were reassociated and co-cultured up to 14 days in vitro. Results: A rapid regeneration of tooth germs was observed. The bud stage was achieved after two days and the cap stage started after three days. The cap stage characteristic histogenesis was achieved after 4 days. At this stage, the IDE and ODE had differentiated, suggesting that a regionalization of the mesenchyme also took place in the meantime. BrdU incorporation and in situ hybridization for Shh confirmed the formation of primary enamel knot after 4 days hours. Bell stage was observed after 6 days. Multi-cusps teeth were observed in 46% of the recombinations. The IDE, ODE, SR and SI became distinct after 8 days in culture. Gradients of functional odontoblasts starting from the tip of the cusps were observed after 10 days. Ameloblast differentiated soon after. The tooth shape after 14 days culture was investigated by 3D reconstruction. Conclusion: Our results indicate that it is possible to bioengineer a tooth structure from dissociated cells in vitro. Tooth regeneration does not require external influences from non-dental surrounding tissues for crown formation. Furthermore, artificial tooth-shaped polymer scaffolds are not necessary.

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Title : Expression of the even-skipped-related gene *eve1* suggests its requirement in initiation and morphogenesis of the first tooth, and in the differentiation of the ameloblasts in the zebrafish (*Danio rerio*)

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Abstract : The increasing knowledge of the genetic control of tooth development in mammals (in particular mice) stands in sharp contrast to the poor, and so far exclusively morphological characterisation of tooth development in zebrafish. Recently a strong *eve1* signal was identified in the pharyngeal region of zebrafish, potentially located in the developing teeth. Even though *evx* genes are well studied in mammals, their expression in developing teeth had never been reported, making the observation even more surprising. To precisely localize *eve1* expression in the teeth, we performed whole mount in situ hybridisation in zebrafish ranging from 44 hpf to 8 dpf (10 stages). For each stage, larvae of the same nest were fixed as controls. Both the hybridized and control specimens were embedded in epon, and serially sectioned for histological analysis. A comparison of the hybridized and control specimens allowed a detailed analysis of the cell populations expressing *eve1* during the four steps of tooth formation distinguished: initiation, morphogenesis, early and late differentiation. Clearly, *eve1* expression is restricted to the epithelium. The gene is activated first at 48 hpf in the placode area, a region of the pharyngeal epithelium that corresponds to the initiation site of the first tooth (4V1) (and possibly also includes the initiation sites of the adjacent teeth 3V1 and 5V1). Expression of *eve1* is maintained in the dental epithelium throughout 4V1 morphogenesis (72 hpf), but is then restricted to the inner dental epithelium during the early differentiation stage of the ameloblasts, until the first elements of the tooth matrix are deposited. The gene is downregulated as soon as the matrix of tooth 4V1 thickens (80 hpf). In contrast, there is no *eve1* expression during the initiation of the replacement teeth (4V2, 3V2 and 5V2). Except for 4V1, *eve1* expression in all tooth germs is restricted to the inner dental epithelium during the early differentiation stage of the ameloblasts and it disappears when the first matrix is deposited. Therefore, in the zebrafish *eve1* expression in the pharyngeal region is correlated with two key steps of tooth development: initiation and morphogenesis of the first tooth, and ameloblast differentiation of all developing teeth.

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Title : Cellular Differentiation of Dental Follicle in Mouse Embryo

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Abstract : In mice, dental mesenchymal cells consist of the dental papilla and dental follicle in developing mammalian tooth bud. It is thought that dental follicle cells have the ability to differentiate into fibroblasts, cementoblasts, and osteoblasts. However, cellular differentiation and the effects of environmental factors are not known exactly. To understand cellular differentiation and interaction, immunohistochemistry and in situ hybridization were used to study for osteoblastogenesis. At E14, the structure of dental follicle could be observed but there were no expression of BSP and OPN proteins, which are known as markers for differentiation into bone. At PN8, positive reactions of BSP and OPN in alveolar bone. We examined in vitro study to characterize dental follicle cells. At E14, dental follicle cells were dissected for in vitro culture. After 2 days, dental follicle cells were aggregated. To examine the level of differentiation to osteoblast and its potential to make bone, both Runx2 and Bsp expressed in aggregated cells. Furthermore, to evaluate effects of environmental factors, bony inducer such as developing calvaria and BMP4 were placed on to the cell, in which dental follicle cells were attached. After 2 days, Runx2 and Bsp were expressed in aggregated cell. These results suggest the dental follicle cells have the capacity to differentiate into osteoblast by the interaction with bony inducers

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Title : Involvement of maxillary process epithelium in rat maxillary incisor formation  
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Abstract : Teeth are formed through tissue interaction between dental epithelium and mesenchyme. In this study we characterized the rat maxillary incisor development. Histological observation shows that the maxillary incisor is composed by the fusion of medial and lateral primary dental laminae (M-PDL and L-PDL). This fusion is concomitant with fusion of facial process, medial nasal process (MNP) and maxillary process (MP), and the dental lamina forms at the boundary between MNP and MP. In order to investigate the contribution of MP to maxillary incisor we labelled the epithelium of MP with a fluorescent dye, DiI, before the fusion starts, and carried out whole embryo culture followed by maxillary organ culture. The result indicates that MP epithelium forms most lateral part of L-PDL. This is supported by observation of the homozygote of rat small eye mutant (rSey) that lacks lateral nasal prominence resulting in facial cleft between MNP and MP. The mutant develops PDL-structure at prospective fusion area of unfused MP. In addition, M-PDL and L-PDL in MNP do not fuse in the mutant, they stay at a distance. Eventually M-PDL proceeds incisor development; 25% of the homozygous L-PDL develop unilateral or bilateral incisor-like structure while the rest stop development at the bud stage. These results suggest that fusion of the facial processes contributes to maxillary incisor formation, to supply all components and possibly to assemble the components at one site. Defect in the fusion in the rSey mutant keeps MP epithelium from contributing to L-PDL formation and maintains M-PDL and incomplete L-PDL separated leading M-PDL and some of L-PDL to proceed further development.

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Title : Analysis of downstream targets of Runx2 during tooth development

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Abstract : Runx2 is a transcription factor which is expressed during and is essential for normal skeletal and tooth development. Loss of activity of one copy of the human Runx2 gene results in the disorder cleidocranial dysplasia. Loss of both copies of the gene in mouse leads to animals completely lacking bone, having arrested tooth development and supernumerary teeth. The general aim of this project is to elucidate the function of Runx2 and its target genes in bone and tooth development. In particular, to determine the role of Runx2 in signalling cascades regulating initiation of osteogenesis and key steps of tooth morphogenesis. In this project we have performed comparative gene expression analysis of Runx2 mutant and wild type embryonic mice molars using Affymetrix microarray chips. These experiments revealed a list of genes severely downregulated in Runx2 samples. In situ hybridization experiments have localised the expression of several signalling pathway genes to the osteogenic mesenchyme in wild type animals and revealed that in most cases, the expression of these genes is completely missing in the mutant tooth and/or developing bone. These genes include Ectonucleotide pyrophosphatase 1 (ENPP1), Bono1 (EST AI842353) and Wnt antagonist Dickkopf1 (Dkk1). EST AI842353 was localised to the developing bone and odontoblasts and renamed as Bono1 (in bone and odontoblasts). Tissue culture experiments revealed that Dkk1 and ENPP1 are induced by FGF in tooth mesenchyme.

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Title : Localization of Cholinesterase enzyme and its putative role in human tooth morphogenesis  
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Abstract : A number of studies available in the literature suggest non-neuronal actions of cholinesterase enzymes (ChE) in the processes of cell proliferation, differentiation, and apoptosis during embryonic development. However, the precise action of ChE in these processes remains unclear. The purpose of the present study was to demonstrate the ChE reactive sites in the developing human tooth germs to provide valuable insights into the role of ChE in embryonic tooth development. Human dead fetuses (around 22 week IUL), aborting spontaneously, were collected from the Teaching hospital Peradeniya, Sri Lanka after obtaining the consent from the hospital authorities. Immediately after the collection of fetuses, jaws were dissected and fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer followed by decalcification in 4 % neutral EDTA. 20 mm thick cryosections of the jaws were made and stained for ChE reactivity according to Karnovsky and Root (1964). The jaw sections we examined contained deciduous tooth germs at the “late bell” stage of development. The permanent tooth germs at the “bud” or “early cap” stages were also found close to the deciduous tooth germ in most of the sections. In the deciduous incisor tooth germ at the late bell stage of development, we detected moderate reactions for ChE activity in the epithelial cells of the cervical loop region, and the inner (IEE) and outer enamel epithelia (OEE). The staining intensity of ChE was consistent throughout the IEE, but became significantly more distinct in the preameloblasts, which lay on a thin layer of newly formed dentin matrix. However, secretory ameloblasts were devoid of any staining for ChE activity. Cells in the stratum intermedium and stellate reticulum, odontoblasts, and other dental papillary cells showed weak or no activity for ChE. Nerve fibers and the vascular endothelium in the dental papilla and the developing periodontal tissues were stained strongly for ChE. The distribution pattern of ChE activities in the deciduous molar tooth germ was similar to that of the incisor tooth germ at comparable stage of development. In the permanent tooth germ, distinct reactions for ChE were confined to the epithelial cells facing toward the lingual mucosa of the oral cavity. In contrast, cells of the tip and the center of the tooth bud, and those facing the deciduous tooth germ, were unstained for ChE reaction. Interestingly, the cells of the disintegrating dental lamina were strongly stained for ChE activity. In addition, the basal cells of the oral epithelium showed modest ChE reactions. The ectomesenchymal cells surrounding the permanent tooth bud were free from ChE reactions. Our current study has provided the first evidence for the presence of ChE activity in the dental epithelium of human tooth germs. Together with our previous observations of ChE activity in the continuously erupting guinea pig teeth (Jayawardena & Takano 2003), the present data further support the contribution of ChE enzyme in tooth morphogenesis through its putative roles in the regulation of cell proliferation, differentiation, and apoptosis of dental epithelia.

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Title : Amelogenin: lessons from evolution

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Abstract : Amelogenin, the major protein of forming enamel, plays a crucial role in enamel structure and mineralization as revealed by the numerous tooth defects resulting from anomalies occurring in different regions of the coding sequence (X-linked amelogenesis imperfecta, AI). However, the function of the different regions/amino acids of this protein is far to be understood. Among the various approaches aiming to better know the structure/function relationships of the amelogenin, the analysis of its origin and of its evolutionary pathways seem to be a promising way. The amelogenin story has begun 600 millions years ago, when it duplicated from SPARC (osteonectin) or from one of its relative (hevin), i.e. long before the presence of vertebrates in the fossil records. Comparisons with databank sequences have indeed revealed that the N-terminal region of the amelogenin, in particular the leader peptide, shares different motifs with the homologous region of SPARC. This suggests that the coding sequence of this region (the hydrophilic, so-called TRAP region: exon 3, 5 and beginning of exon 6) originated at this time, as probably do the C-terminal region (end of exon 6). Because they are involved in important functions, these regions of the molecule have slowly evolved. These constraints are still present in modern amelogenin as shown by the high number of conserved positions. The analysis of the central, hydrophobic region (most of the exon 6, i.e. 400 bp) of the amino acid sequence of 26 mammalian species has revealed that (i) it is rich in proline (P) and glutamine (Q), (ii) it possesses numerous repeats of three amino acids (PXX or PXQ), and (iii) it is highly variable (numerous insertions, deletions, substitutions). This region was created by several runs of triplet duplications, which have occurred long before mammal divergence, and at least in a stem tetrapod. Such mechanism of triplet insertions was used again during mammalian evolution, and occurred independently in various lineages. These insertions are always located in a particular region of the exon 6, which is considered a hot spot of mutation for mammalian amelogenin. This region appears therefore as a possible locus for amelogenin polymorphism in human. The study of amelogenin evolution has been extended to the reptilian lineage (6 crocodiles, 6 snakes and 20 lizards). The analysis largely confirmed the findings in mammals: highly conserved N- and C-terminal regions and presence of triplet repeats in the variable region. Interestingly, the amelogenin sequences are nearly identical in all crocodiles, revealing a high stability of their genome. Conversely, in lizards and snakes the amelogenins are very different, confirming the rapid evolution of squamate genome. Such a rapid evolution allowed to test in natura the importance of each amino acid position for amelogenin. Thirty-two amino acids, all located in the N- and C-terminal regions, were kept unchanged during 250 million years. This indicates the important role of these residues for amelogenin. These are predictable sites, which could lead to enamel defects when either deleted or substituted. The three amino acid positions known to lead to AI when substituted are validated by our evolutionary analysis. An evolutionary-based dataset of sequences is proposed. It will allow a rapid validation of the amelogenin mutations in human.

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Title : Tooth development and morphological variations through ontogeny in the amphibian, *Pleurodeles waltl*

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Abstract : Non-mammalian vertebrates replace their teeth several times during life (polyphyodonty). An appropriate polyphyodont species would permit to study the mechanisms controlling tooth development and replacement, and to monitor the changes occurring in tooth morphology (size, shape, orientation) and structure during ontogeny. In the amphibian *Pleurodeles waltl* teeth develop early, and through successive replacements (generations) their morphology and structure are modified. A growth series was fixed, from two days before hatching to the adult stage, for light, scanning and transmission electron microscopy, to know whether the tooth modifications either occur progressively during ontogeny or appear during one step, for instance at metamorphosis. To this goal we have followed a single tooth family, i.e., the first-generation tooth and all replacement teeth that succeeded until adult stages. Either three or four tooth generations occur before the metamorphosis. In larval stages teeth differ from those in metamorphosed juveniles by: a conical and monocuspidate shape versus bicuspidate; an homogeneous basal region of the dentin cone versus a pedicellate organisation; the presence of enameloid (both mesenchymal and epithelial contribution) and a thin enamel layer versus only a thick enamel layer; and a minimal structure with reduced pulp cavity and absence of dentinal tubules in the dentin versus large pulp cavity containing blood vessels, nerves endings and presence of dentinal tubules within the dentin matrix. The monocuspid-bicuspid transition occurs suddenly during the metamorphosis, while the other modifications occur progressively during the larval period. (Poster)

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Title : Ectodysplasin signalling and formation of dental placodes

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Abstract : Ectodysplasin (Eda) is a Tumour Necrosis Factor (TNF) family member protein. It is a secreted cell signalling protein which binds to its receptor Edar in surface of its target cells. This binding initiates a signal transduction cascade leading to changes in gene expression. In mice Edar is expressed in the molar tooth germs from very early stages onwards; in fact, it is one of the earliest markers of the molar placode. Dental placodes resemble morphologically and molecularly the placodes of other ectodermal organs such as hairs and some glands. All placodes are epithelial thickenings and act as signalling centers regulating the early steps of organogenesis. The molar placode later buds into the underlying mesenchyme, setting forth a cascade which leads to the formation of all molar teeth. Since Ectodysplasin is expressed only in the epithelium outside molar placodes the Ectodysplasin – Edar signalling represents a pathway for non-placodal – placodal interactions within epithelium. Lack of functional Ectodysplasin – Edar signalling pathway leads to ectodermal dysplasia, characterized by missing teeth and simplified molar shape, in both mice and men. Moreover, when Ectodysplasin is overexpressed in mice, molar shape becomes more complex and a supernumerary tooth appears in the molar area. We analysed the initiation of molar development in three cases: 1. Lack of functional Ectodysplasin, 2. Normal amount of Ectodysplasin and 3. Overexpression of Ectodysplasin. Our results show that placode formation and function is different in all groups. The significance of these changes, i.e. whether it is these early changes in tooth organogenesis which lead to changes in the final shape of the teeth, is yet to be evaluated. However this work together with other work in our laboratory on different ectodermal organs, such as hairs and skin glands, contributes to our understanding of the dynamics of placode formation and indicates that Ectodysplasin– Edar signalling stimulates the formation and function of ectodermal placodes.

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Title : Pattern formation and molecular studies in fungiform papillae development

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Abstract : Gap junctions are specialized areas of the cell membranes that allow direct exchange of signaling molecules and metabolites between the cytoplasm of neighboring cells. Gap junctions are observed in many developing tissues including cardiac muscle and nerve, and have been suggested in a variety of roles including the regulation of signal transmission, cell proliferation, differentiation, apoptosis, and tissue homeostasis. We show here that connexin43 protein expression significantly correlates with fungiform papillae development. The development of the fungiform papillae has similar pattern of formation with the other epithelial appendages. The fungiform papillae form a repeating unit with a highly regular spatial pattern through the epithelial-mesenchymal interaction and signaling pathway over the dorsal surface of the tongue during embryonic development. Antisense oligonucleotide (AS-ODN) Connexin 43 was treated to analyze the developmental functions of Connexin !

43. We investigated the function of the BMP during tongue development by ectopically expressing NOGGIN, a potent antagonist of the BMPs. The expression patterns of signaling molecules, Shh and Bmp-2 were altered by inhibition of gap junctions. Shh, which is known as a key molecule to determine spacing pattern, expression patterns were disturbed after the inhibition of Bmp-2 as well. These results revealed that pathway of the signaling molecules during fungiform papillae development.

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Title : Generation of an allelic series of Pax9 mutant mice: a model for oligodontia caused by mutations in the human PAX9 gene

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Abstract : Pax9 encodes a transcription factor that is essential for tooth formation in both mice and man. In homozygous mouse mutants carrying a null allele of Pax9 (Pax9-lacZ), tooth development is arrested at the bud stage whereas heterozygous Pax9-lacZ mutant mice do not show dental defects. In contrast, heterozygous mutations in the human PAX9 gene cause oligodontia suggesting different sensitivities to reduced Pax9 levels during tooth formation in mice and humans. To better understand the aetiology of oligodontia, we have generated a novel, mutant Pax9 allele (Pax9-neoflox) in mice resulting in reduced expression of the wildtype Pax9 mRNA. Preliminary analysis shows that homozygous Pax9-neoflox and Pax9-neoflox/Pax9lacZ compound mutant mice exhibit tooth agenesis with variable severity and thus mimic the predominant phenotype observed in PAX9 oligodontia patients. Interestingly, similar to some oligodontia patients, some of the remaining teeth in homozygous Pax9-neoflox and Pax9-neoflox/Pax9lacZ compound mutants are hypoplastic and show marked attrition, suggesting later functions of Pax9 in odontoblast differentiation. These data show that the allelic series of Pax9 mutant mice is a suitable model to investigate gene dosage-dependent functions of Pax9 and indicates that Pax9 is required for multiple processes during tooth formation.

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Title : Involvement of maxillary process epithelium in rat maxillary incisor formation

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Abstract : Teeth are formed through tissue interaction between dental epithelium and mesenchyme. In this study we characterized the rat maxillary incisor development. Histological observation shows that the maxillary incisor is composed by the fusion of medial and lateral primary dental laminae (M-PDL and L-PDL). This fusion is concomitant with fusion of facial process, medial nasal process (MNP) and maxillary process (MP), and the dental lamina forms at the boundary between MNP and MP. In order to investigate the contribution of MP to maxillary incisor we labelled the epithelium of MP with a fluorescent dye, DiI, before the fusion starts, and carried out whole embryo culture followed by maxillary organ culture. The result indicates that MP epithelium forms most lateral part of L-PDL. This is supported by observation of the homozygote of rat small eye mutant (rSey) that lacks lateral nasal prominence resulting in facial cleft between MNP and MP. The mutant develops PDL-structure at prospective fusion area of unfused MP. In addition, M-PDL and L-PDL in MNP do not fuse in the mutant, they stay at a distance. Eventually M-PDL proceeds incisor development; 25% of the homozygous L-PDL develop unilateral or bilateral incisor-like structure while the rest stop development at the bud stage. These results suggest that fusion of the facial processes contributes to maxillary incisor formation, to supply all components and possibly to assemble the components at one site. Defect in the fusion in the rSey mutant keeps MP epithelium from contributing to L-PDL formation and maintains M-PDL and incomplete L-PDL separated leading M-PDL and some of L-PDL to proceed further development.

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Title : Immunolocalization and signalling pathway of BMPs 2, 3, 4 and 7 during the mouse tooth root development.

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Abstract : The mechanisms that take place during periodontal regeneration and tooth root development are very similar and justify the studies concerning root embryogenesis. Using the mouse's first lower molar as a model for the study of tooth root development, we tried to explain the role played by certain factors, such as BMPs 2, 3, 4 and 7 during the development of the periodontium. Materials and methods : Twenty five mice ( breed ICR CD-1) were sacrificed one by one everyday from day 4 to day 28 ex-utero. Their cephalic extremity was immediately fixed in 4% paraformaldehyde, dissected to separate the right and left sectors of the mandible, then demineralized in 4% nitric acid for an average of 4 days. Each sample was then included in paraffin and a succession of 5µm para sagittal slides were colored by the Masson trichrom. Immunohistochemistry : Goat polyclonal primary antibodies against Bmps 2, 3, 4, 7, BMPRIa, BMPRIb and BMPRII (SantaCruz Biotechnologies), BMP 3 and BMPRIa (R & D Systems) were used at a dilution of 1/50 and 1/20 (Antibodies from R & D Systems) for stages 5, 8, 10, 12, 16, 20, 24, 25, 26 and 28 post-partum. Mouse monoclonal primary antibodies against Bmps 2, 4, 7, BMPRIb, BMPRII (R & D Systems) and ACTRIa (Santa Cruz Biotechnologies) were used at a dilution of 1/20 and 1/50 (ACTRIa). Rabbit polyclonal primary antibodies against BSP, OPN, ON (Fisher) and pSmad1 (TenDjike) were used at a dilution of 1/100. Rabbit anti-goat and Goat anti-rabbit secondary antibodies were then applied and revealed with the ABC complex (VectorLab), after endogenous peroxidase blocking. The slides were then observed under light microscope. Results : The 4 proteins were localized in the different cell compartments of the tooth germ, constantly and importantly in the odontoblasts, more irregularly in the future periodontal tissue. BMPs 2, 3, 4 and 7 had a similar profile of expression during the entire period even though the BMP3 staining was much less intense and more irregular. The young stages (D5) showed a concomitant intracellular, granular, staining of the 4 proteins on Hertwig's epithelial root sheath, while the dental follicle was negative in the future root territories. D8, as the first third of the root is under edification, and the cementogenesis just starting, the dental follicle becomes faintly positive and the tooth root external lining seems discreetly stained. Later (D10-16) a granular type staining can be seen in tooth root lining cells (cementoblasts of the intraradicular cellular and acellular cementum), bone cells and dental follicle cells. However, all cells belonging to the same phenotype were not stained. From D16 to D24, the staining decreases and becomes mostly matricial. On D25 we can notice an important come back of the intracellular granular type staining in the periodontal ligament and the cementoblasts, especially in the apical part of the cellular cementum (cementocytary cemenoblasts) Concerning the BMP signalling pathway, on D21 we can notice a concomitant expression and similar staining pattern of BMPRIa, Ib, II, pSmad1, BSP, OPN, ON. The intracellular staining is important in the coronal part of the dental follicle and decreases towards the apical part of the root. It is also present in the dental pulp cells especially in the apical part of the pulp. Conclusion: These results show that BMPs 2, 3, 4 and 7 are expressed during the mouse's first lower molar tooth root development, particularly around the tooth root morphogenetic structure represented by Hertwig's epithelial root sheath and in the dental follicle, holder of the periodontal stem cells. Later while the root is growing, the proteins seem to be expressed by differentiated cells of the periodontium, the cementoblasts. This secretion could invite the progenitor cells to differentiate themselves, thus implying that BMPs could play a key role in periodontal regeneration.

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Title : Direct evidence of FGF signaling in palatogenesis

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Abstract : During the secondary palatal development, two shelves elevated to horizontal position above tongue, then they completely fused in developing mouse embryo. Development of the secondary palate was related with developing forebrain, nasal cavity and oral cavity, as a boundary between nasal and oral cavity. Etiology of clefts is the failure of the secondary palatal fusion by genetic and environmental factors. We examined the sequential fusion point from at E13.5 to E15.5 and analyzed the pathway of FGF signaling integrating with FGFR, Slug and Tbx3. Palatal fusion starts the middle region, anterior region, and posterior region. At E13.5, Fgf8 and Fgf10 were expressed in the mesenchyme of medial edge. At E14.5, posterior region was strongly expressed than anterior and middle regions. At E15.5, Fgf8 and Fgf10 expression were restricted in ruge and medial edge, similar as Fgfr1b and Fgfr2b. Interestingly, Fgfr2b expression was disappeared in the middle region at E14.25, beginning to fuse palatal shelf. To investigate the inhibitory action of FGF signaling, SU5402 soaked beads implanted in the middle of medial edge at E13.5 in vitro culture. After 72 hours, Slug expression in anterior region was not effected, whereas in posterior region was inhibited. Also, Tbx3 expression was detected in the medial edge at E13 and E14. However, at E15.5, Tbx3 expression was restricted in ruge and medial edge. From these results, fusion of palate shelves begins to middle region of palate. Then, development of secondary palate closely associated with FGF signaling with Slug and Tbx3.

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Title : Dental epithelial histogenesis in vitro : Positional information does not require the memorization of cell history.

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Abstract : Reciprocal epithelial-mesenchymal interactions control odontogenesis. Two epithelial cell types are present at the bud stage but the epithelial histogenesis really starts during the bud to cap transition. Local changes in the basement membrane composition also take place during this transition. Using the first lower molar from ICR mouse embryos at ED13 (bud stage before the PEK forms) and ED14 (cap stage when the PEK was present), our objectives were 1) to evaluate the importance of positional information in the epithelial cells at the two stages 2) to test the role of the mesenchyme in specifying this information and 3) to test the potential role of the EK in modifying the potentialities of the dental mesenchyme. For this purpose, dissociation/reassociation experiments were performed. Reassociations between the dental mesenchyme and dissociated epithelial single cells were cultured in vitro. Using material from either ED13 or ED14, the epithelial-mesenchymal junction was rapidly restored and the epithelial cells in contact with the newly deposited basement membrane then elongated while the internal cells remained round. After 3 days in vitro, a transitory EK formed, characterized by the specific arrangement of its cells which were also BrdU-negative and expressed Shh. At this stage, the immunostaining for WNT-5a, -10b, Frizzled, FGF-4 and Flg gave similar patterns when compared to tooth germs ex vivo at the cap stage. The PEK was functional since cusps formed. Despite a complete loss of positional information in epithelial cells, the dental mesenchyme from ED14 was able to control a characteristic dental epithelial histogenesis. However, the initial steps were slightly accelerated when using tissue and cells from ED13 instead of ED14. In both cases however, the fast progression of epithelial histogenesis suggested a cell reprogramming instead of migration in the epithelial compartment. The cells initially behave according to their new position and independently from their own history. After 4 days in the reassociations, the inner and outer dental epithelia were specified. This is known to be controlled by the mesenchyme in the tooth, suggesting that regional specificities (dental/peridental) were maintained in this tissue. In conclusion, these observations showed a high plasticity of epithelial cells. Positional information, which is necessary to co-ordinate the histo-morphogenesis of the developing tooth, is a very dynamic process and does not require the memorization of the cell history.

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Title : Apoptosis related factors (Fas receptor, Fas ligand, FADD, caspase 3, DNA breaks) in early tooth development of the field vole (*Microtus agrestis*)

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Abstract : Apoptotic cells can be detected throughout the whole embryonic development of molar teeth. Restricted temporospatial distribution, particularly in enamel knots, dental lamina, stalk and rudimental diastemal primordia, suggests several important roles of apoptosis in tooth morphogenesis. However, functional studies as well as knowledge about mechanisms employed in dental apoptosis are necessary to confirm the hypothesis. Field vole (*Microtus agrestis*) as a valuable model of odontogenesis, due to taxonomical relationship to the mouse, the same tooth formula but different final molar shape and patterning, was exploited to investigate candidate factors related to dental apoptosis using immunohistochemistry. First molars were investigated at the stage 13.5 – 15.5 of embryonic development when primary enamel knots appear and are gradually terminated by apoptosis. Caspase activation and specific DNA fragmentation are two hallmarks of apoptosis. TUNEL test and anti-activecaspase 3 antibody were used to collocate TUNEL positive cells and activation of caspase 3. As further criteria to confirm apoptosis in these cells, morphological features were evaluated after hematoxylin – eosin staining (H&E). Fas (CD95/APO-1) belongs to the TNF receptor (TNFR) family. Fas ligand binding followed by Fas receptor oligomerization leads to formation of the death inducing signal complex starting with recruitment of the Fas-adaptor protein (FADD). Both, internal and external, termini of Fas receptor molecules were detected to avoid potential cross-reaction of Fas antibody and Fas ligand. Fas ligand and FADD antibodies were employed to detect other molecules engaged in Fas mediated apoptosis. Apoptotic cells were demonstrated in the most superficial layer of dental lamina. The number of TUNEL positive cells expanded in the middle part of the axis from later bud to cap stages. Restricted areas of apoptotic cells were found also in stalk and primary enamel knots. Caspase 3 activity corresponded with temporospatial distribution of TUNEL positive cells. Internal and external termini of Fas molecules were demonstrated in the same areas. Fas and FADD were collocated at the bud stage and in primary enamel knots, with strong expression of FADD molecules, probably due to sharing this domain among other TNF receptors. Fas ligand was also detected in proliferating cells where it could have a protective function as in immune privileged organs. Fas and Fas ligand were found in some areas corresponding with apoptotic cells at the bud stage and at the bell stage in the enamel knots. According to these immunohistochemical data, Fas mediated signalling may be involved in dental apoptosis. However, its role in triggering and enhancing dental apoptosis, respectively, remains to be confirmed. Supported by the Grant Agency of the Czech Republic (304/04/0101 and 204/02/P112).

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Title : Facial and tooth defects in mice lacking the Notch ligand Jagged2  
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Abstract : The Notch signalling pathway is an evolutionary conserved intercellular signalling mechanism that is essential for cell fate specification and proper embryonic development. We have analysed the expression and regulation of the Jagged2 gene, which encodes a ligand for the Notch family of receptors, in developing teeth of mouse embryos. Jagged2 is expressed from the earliest stages of tooth development in a layer of epithelial cells that will give rise to the enamel-producing ameloblasts. Tissue recombination experiments showed that Jagged2 expression is regulated by epithelial-mesenchymal interactions. Fgf-releasing beads up-regulate Jagged2 expression in explants of dental epithelium, while expression is downregulated by Bmp4. We have then examined the in vivo role of Jagged2 in the developing teeth of mutant mice that lack the domain of the Jagged2 protein required for interaction with the Notch receptors. Mice homozygous for this deletion die after birth and exhibit fusions between the tongue, palatal and mandibular shelves. Furthermore, tooth development is abnormal: additional cusps are formed in molars, while dentine and enamel deposition has not occurred in incisors. Notch1, Notch2 and Jagged1 expression is downregulated in the developing teeth of the mutant homozygotes. Similarly, in the enamel knot, an epithelial signalling structure involved in tooth morphogenesis, both Bmp4 expression and apoptosis are affected. These results demonstrate that Notch signalling mediated by Jagged2 plays an essential role during facial and tooth development in mice.

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Title : Effects of BMP2 on Gene Expression in Dental Epithelial Cell Line  
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Abstract : [Introduction] Recombination experiments demonstrated that epithelial-mesenchyme interaction plays a crucial role during tooth development. Molecules and signaling pathways responsible for this interaction have not been fully understood, but some fundamental principals have been reported. Bone Morphogenetic Proteins 2 (BMP2) is one of the key signaling molecules through tooth development and mainly expressed in dental epithelia. BMP2 is also provided from odontoblasts during ameloblast differentiation. However, the involvements of BMP2 signaling pathway in ameloblast differentiation is not clear. To identify the candidate genes involved in BMP2-induced differentiation of dental epithelia, we performed cDNA microarray analysis. [Methods] Rat dental epithelial cells (HAT-7) were cultured in DMEM/HamF12 medium with 10 % FBS until 70% confluency. The medium was then changed to serum-free medium with or without BMP2. After 24 hours, total RNA was isolated from them and microarray analysis was performed with rat cDNA array (Agilent) which contains 14,815 genes. Expression patterns of several selected genes were also analyzed in other dental epithelial cell lines. [Results] Seventy-five genes were up-regulated and 28 genes were down-regulated in BMP2-treated dental epithelial cells (more than 2-fold, less than 0.5-fold). This is the first time to analyze gene expression pattern induced by BMP2 in dental epithelial cells and may find the new candidates related with dental epithelial development.

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Title : Characterisation of the cytoskeleton of the fibroblasts in the periodontal ligament of the rat mandibular incisor during unimpeded eruption.  
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Abstract : Many studies suggest that eruptive forces are generated in the connective tissues of the periodontal ligament (or in its precursor, the dental follicle). However, the role of the fibroblasts in this process remains controversial, particularly since there is considerable evidence that eruption does not result from the generation of a tractional force mediated through the tissue's fibrous network. At the very least, the fibroblasts must be involved in the remodelling of the periodontal connective tissues as the tooth erupts. Nevertheless, quantitative electronmicroscopic studies have not provided an association between fibroblast morphology and eruptive behaviour. Our previous immunocytochemical studies however indicate that, at times of significant eruptive movements of the teeth in the rat dentition (i.e. during tooth emergence and in the aged animal), the periodontal fibroblasts co express vimentin and cytokeratin intermediate microfilaments. To assess this relationship further, the present investigation involves the immunocytochemical labelling of intermediate microfilaments within the fibroblasts of the rat incisor periodontal ligament at a time when eruption rates are significantly increased experimentally. Experiments were conducted on the right mandibular incisors of 12, 8-week-old Wistar rats where, by maintaining the teeth out of the bite (the inimpeded state), eruption was increased from about 0.3mm/day to approximately 1.1mm/day. Using standard immunocytochemical techniques and antibodies to label vimentin and cytokeratin 19 respectively, vimentin was always expressed within the fibroblasts but never cytokeratin. The results suggest that, contrary to the interpretation derived from our previous studies, there is no clear association between eruptive behaviour and the co expression of vimentin and cytokeratin intermediate microfilaments. Alternatively, unimpeded eruption differs sufficiently from non-experimental eruptive behaviour to raise some doubts as to the validity of its use in research on tooth eruption. For this reason, it remains important to study eruption using both impeded and unimpeded teeth.

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Title : Identification and Characterization of the Krüppel-like Factor Epiprofin Expressed in Epithelium of Developing Tooth, Hair Follicle, and Limb Bud

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Abstract : Introduction and Aims: The developing tooth is a good model for studying the mechanism of organogenesis. Tooth development initiates by the thickening and invagination of the oral ectoderm into the dental mesenchyme. The following reciprocal interactions between these two cell types give rise to differentiation into enamel-secreting ameloblasts and dentin-secreting odontoblasts and form unique structures specialized for tooth function. Our aims are to search for and identify genes involved in tooth development, characterize their functions, and search for human genetic disorders caused by mutations of these genes. Major Findings: We prepared a cDNA library using mRNA from tooth germ molars of embryonic day (E) 19.5 mice. To obtain cDNA clones preferentially expressed in tooth, we differentially screened DNA microarrays containing about 12,000 clones from this library with fluorescently labeled probes from E19.5 molar and E13.5 body mRNA. We identified 197 cDNA clones that were preferentially hybridized to E19.5 mRNA. The majority of these clones encode enamel matrix proteins, such as ameloblastin, amelogenin, and enamelin, indicating feasibility of the microarray analysis. We found that 7 out of the 197 clones encode either unknown proteins or correspond to ESTs previously deposited in GenBank. One of the clones, which we named epiprofin, was further characterized. Full-length cloning and sequencing revealed that epiprofin encodes a member of the Krüppel-Like Factor (KLF) family containing three characteristic C2H2-type zinc-finger motifs. Except for its 5' terminal sequence, the epiprofin mRNA sequence is almost identical to the predicted sequence of KLF14/SP6, which was previously identified in EST databases and GenBank through a sequence homology search for an Sp1 zinc-finger DNA-binding domain. We determined the 5' terminus and determined the exon 1 location of the gene. Transfection with a reporter gene construct containing the -200 to +30 bp promoter and SV40 enhancer confirmed that this region has basal promoter activity. In situ hybridization revealed that epiprofin mRNA is expressed by proliferating dental epithelium and also hair follicle matrix epithelium. In addition, whole-mount in situ hybridization showed transient expression of epiprofin mRNA in cells of the apical ectodermal ridge (AER) in developing limb. Transfection of an epiprofin expression vector showed that epiprofin is localized in the nucleus and promotes cell proliferation. Thus, epiprofin is a highly cell- and tissue-specific nuclear protein expressed primarily by proliferating epithelial cells of tooth, hair follicle, and limb that may function in the development of these tissues by regulating cell growth.

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## Regulation of Gene Expression in Dental Epithelial Cells

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[Introduction] Tooth enamel is the hardest tissue in our body. Biom mineralization in enamel organ is quite unique compared to those in bone, cartilage and dentin. In order to study for roles of amelogenin, which is a major component of enamel matrix proteins, we established the in vitro system for amelogenin gene induction by various nucleotide analogues including N<sup>6</sup>-Benzyladenine (6BAP), in dental epithelial cells. [Methods] We first isolated clones of dental epithelial cells derived from apical bud. Each clone was cultured in the presence or absence of nucleotide analogues. Total RNA was isolated from them and examined the expression levels of amelogenin mRNA by RT-PCR. In addition, we measured the levels of mRNA encoding several tissue specific markers. Cell growth was also determined by counting cell numbers. [Results] Amelogenin mRNA, a marker for ameloblast differentiation, was significantly increased in some clones. At the same time, the cell growth was inhibited. The levels of mRNA encoding several signaling molecules and differentiation markers were varied among the clones. [Conclusion] Our findings suggest that growth inhibition by 6BAP initiates ameloblast differentiation, resulting in induction of amelogenin gene expression. The precise mechanism of the amelogenin gene expression is under studying.

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Title : The „supernumerary” cheek tooth in the tabby/EDA mice – a reminiscence of the premolar in mouse ancestors  
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Abstract : Primitive rodents, as well as some of their actual descendants, displayed two maxillary and one mandibular premolars in the dentition. In mice, these premolars disappeared during the evolution. The fossil record documents that the disappearance of the mandibular premolar coincided with a lengthening of the first lower molar (M1) towards the mesial direction. However, a wide vestigial tooth bud transiently exists in front of the lower M1 in the mouse embryo and has been hypothetically related to the lost premolar. This vestige is incorporated into the M1 and involved during the morphogenesis of the most mesial cusps. The large vestigial bud may be absent in Ta/EDA mouse embryos at the appropriate developmental stage. It is not incorporated into the M1 but may develop autonomously and participate in the origin of the so-called “supernumerary molar” in Ta/EDA mice. Ontogenetic and phylogenetic data support the hypothesis that this “supernumerary molar” might be relat!

ed to the premolar lost during mouse evolution. Acknowledgements: This work was supported by the Grant Agency of the Czech Republic (grant 304/02/0408), the Ministry of Education, Youth, and Sports of the Czech Republic (project COST B23.002), the French Network on rare genetic diseases (grant APS02005MSA) and the COST action B23 (Oral facial development and regeneration), Brussels, EU.

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Title : Tracking the odontoblast phenotype

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Abstract : It is admitted that the dental pulp contains progenitor cells that can be recruited towards the odontogenic program. Recent studies have further shown that the post-natal human dental pulp contains multipotent stem cells capable of differentiating in neural cells, adipocytes, osteoblasts and odontoblasts. The phenotypic frontier between the odontoblasts and osteoblasts is narrow. At the present time, the characterization of the odontoblast phenotype essentially relies on the expression of extracellular matrix proteins such as the dentin matrix protein 1 (DMP1) or the dentin matrix protein 1 (DMP1). However, these molecules are also expressed by osteoblasts and therefore cannot, by themselves, be sufficient to accurately distinguish an odontoblast from an osteoblast. In the present study, we have derived a series of immortalized clonal cell lines from the tooth germ of day 18 embryos of mice transgenic for SV40 T antigen. We have made use of these clones to get a better understanding of the "essence" of the odontoblast phenotype. RT-PCR, western blot and immunocytochemical analyses were undertaken to characterize the pattern of expression of DSP and DMP1 but also of a series of transcription factors potentially involved in odontogenesis such as Pax 9, Msx1, Msx2, Dlx2, Dlx5 and Cbfa1. Some of the clones express DSP, DMP1 and osteopontin under appropriate culture conditions. Among those, four clones, form mineralized nodules with or without induction by  $\beta$  glycerophosphate. All the clones tested express Pax9, Msx1, Dlx2 and Cbfa1 transcripts. In addition, they also express Sox9, a transcription factor related to mesenchymal condensation and chondrogenic fate, as well as RP 59, a recently described marker that labels mesenchymal progenitors during recruitment towards the hematopoietic, osteoblastic or odontoblastic program. A differential pattern is observed for Msx2 and Dlx5. This suggests that we are dealing with different population of dental pulp cells. In parallel, we performed similar experiments with a tripotential mesenchymal progenitor cell line, C1, that can be induced to alternatively differentiate into chondrocytes, osteocytes or adipocytes. Surprisingly, we found that the C1 cells also express DSP, and DMP1 as well as Pax9, Msx1, Dlx2 and Dlx5. Therefore, in our experimental conditions, the combination of markers tested in this study allows to identify skeletal progenitor cells but does not permit an accurate distinction between the odontoblast and osteoblast phenotypes. Further studies involving a subcellular characterization of the expression pattern of the odontogenic transcription factors here analyzed or other gene marker potentially involved in odontogenesis will be performed. In summary, the dental pulp cell lines isolated in our laboratory appear as a useful tool to identify which gene or series of genes determines a cell to become an odontoblast. In addition, these cell lines may be of use to pave the way for the development of dental tissue engineering.

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Title : Explainable and critical periods during human dental morphogenesis and their control  
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Abstract : Rapid progress has been made in understanding dental morphogenesis. From perturbation experiments and from studies of knock out models we know about the signalling agents essential during tooth formation. There are, however, some phases during dental morphogenesis, which are critical but essential, and nonetheless well understood. Whereas, for the thickening of the epithelium in the oral cavity it seems to be understood that it is strongly correlated with the necessary signals. The establishment of the number of 5 teeth per quadrant seems not well understood for human development. Furthermore, the determination of the exact location makes a certain regularity obvious, but the underlying signalling pattern remains unknown so far. In addition, it is not clear how it is regulated that there are replacement primordia for the second dentition and additional primordia in the molar region. The transition from the bud stage to the cap stage seems to be explainable by accepting that the enamel knot cells retard (apoptosis) in growth while ongoing proliferation leads to a swelling and a bulging of the cap's brim. Here, a direct interdependency between growth and form becomes obvious. However, it is not understood, how the detailed, tooth specific proliferation in the cap is regulated, since we know that for each primordium its tooth type can be clearly characterized and identified. From murine studies we know which factors are essential to establish anterior and posterior teeth, but tooth identity is more specific in regard to the characteristic shape of each single molar or other tooth types in humans. It has been argued that the enamel knot, or the secondary enamel knots, are responsible to control growth of the inner enamel epithelium in order to form the specific tooth form. A comprehensive explanation could not be given so far according to which signals the ameloblasts would arrange their movement in order to create the shape of the finished crown. In enamel, the interdependency between outer form and inner structure - and how this is related to the movement of the ameloblasts - we are far from understanding the process. Finally, we do not know, in controlling traffic of the ameloblasts, how the stop signal is given to create the occlusal surface (cusps, ridges, tubercles, fissures), which is typical and serves functional needs for each single tooth. With our 3D-reconstructions of human dental primordia, and with scanning electron microscopical findings on human enamel structure, fetal enamel and ameloblasts, we want to visualize the critical phases during dental morphogenesis and point out ways for possible explanations.

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Title : Identification of Pax9 target genes in tooth development using microarray analysis  
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Abstract : Pax9 encodes a transcription factor with important functions during embryogenesis and organogenesis. Pax9-deficient mice lack all teeth due to an arrest of tooth development at the bud stage (E13.5) and heterozygous mutations in the human PAX9 gene result in oligodontia. Little is known about the molecular processes causing the morphological defects of tooth development in Pax9-deficient embryos, thus genes regulated by Pax9 during early phases of murine odontogenesis need to be identified. For this purpose, normal and Pax9-deficient tooth rudiments were dissected at E12.5, which is about 24 hours before a dental phenotype becomes apparent in Pax9<sup>-/-</sup> embryos. Total RNA was isolated from these rudiments, followed by generation and comparison of genome-wide expression profiles using Affymetrix microarray technology. From a total of 45,265 transcripts represented on the microarrays expression in Pax9-deficient tooth rudiments was significantly altered in 136 cases. Differential expression of two genes important for tooth development, Msx1 and Bmp4 was previously described in Pax9-deficient tooth rudiments and could be confirmed by the present analysis. Interestingly, the most upregulated genes included several factors important for cartilage formation. This result indicates that Pax9 influences the differentiation of the neural crest cell derived dental mesenchyme already at a very early stage and suggests that Pax9 is required for inhibition of chondrogenesis in this tissue. Furthermore, several factors with roles in modulating Tgf-beta/Bmp signalling were amongst the most differentially expressed genes. Since Bmp4 is implicated as an important mediator of Pax9 function during tooth development this finding might be significant. Downregulated expression of one of these factors, Sip1 (Smad interacting protein 1), could be confirmed by RT-PCR and in situ hybridisation. Analyses of further candidate genes are currently under way. The results from this study demonstrates that microarray analysis is a powerful system to determine genome-wide expression profiles during tooth development and to identify early changes of gene expression levels before morphological manifestation have occurred in mutant tooth rudiments.

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Title : Amelogenin: lessons from evolution

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Abstract : Amelogenin, the major protein of forming enamel, plays a crucial role in enamel structure and mineralization as revealed by the numerous tooth defects resulting from anomalies occurring in different regions of the coding sequence (X-linked amelogenesis imperfecta, AI). However, the function of the different regions/amino acids of this protein is far to be understood. Among the various approaches aiming to better know the structure/function relationships of the amelogenin, the analysis of its origin and of its evolutionary pathways seem to be a promising way. The amelogenin story has begun 600 millions years ago, when it duplicated from SPARC (osteonectin) or from one of its relative (hevin), i.e. long before the presence of vertebrates in the fossil records. Comparisons with databank sequences have indeed revealed that the N-terminal region of the amelogenin, in particular the leader peptide, shares different motifs with the homologous region of SPARC. This suggests that the coding sequence of this region (the hydrophilic, so-called TRAP region: exon 3, 5 and beginning of exon 6) originated at this time, as probably do the C-terminal region (end of exon 6). Because they are involved in important functions, these regions of the molecule have slowly evolved. These constraints are still present in modern amelogenin as shown by the high number of conserved positions. The analysis of the central, hydrophobic region (most of the exon 6, i.e. 400 bp) of the amino acid sequence of 26 mammalian species has revealed that (i) it is rich in proline (P) and glutamine (Q), (ii) it possesses numerous repeats of three amino acids (PXX or PXQ), and (iii) it is highly variable (numerous insertions, deletions, substitutions). This region was created by several runs of triplet duplications, which have occurred long before mammal divergence, and at least in a stem tetrapod. Such mechanism of triplet insertions was used again during mammalian evolution, and occurred independently in various lineages. These insertions are always located in a particular region of the exon 6, which is considered a hot spot of mutation for mammalian amelogenin. This region appears therefore as a possible locus for amelogenin polymorphism in human. The study of amelogenin evolution has been extended to the reptilian lineage (6 crocodiles, 6 snakes and 20 lizards). The analysis largely confirmed the findings in mammals: highly conserved N- and C-terminal regions and presence of triplet repeats in the variable region. Interestingly, the amelogenin sequences are nearly identical in all crocodiles, revealing a high stability of their genome. Conversely, in lizards and snakes the amelogenins are very different, confirming the rapid evolution of squamate genome. Such a rapid evolution allowed to test in natura the importance of each amino acid position for amelogenin. Thirty-two amino acids, all located in the N- and C-terminal regions, were kept unchanged during 250 million years. This indicates the important role of these residues for amelogenin. These are predictable sites, which could lead to enamel defects when either deleted or substituted. The three amino acid positions known to lead to AI when substituted are validated by our evolutionary analysis. An evolutionary-based dataset of sequences is proposed. It will allow a rapid validation of the amelogenin mutations in human.

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Title : Tooth development and morphological variations through ontogeny in the amphibian, *Pleurodeles waltl*

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Abstract : Non-mammalian vertebrates replace their teeth several times during life (polyphyodonty). An appropriate polyphyodont species would permit to study the mechanisms controlling tooth development and replacement, and to monitor the changes occurring in tooth morphology (size, shape, orientation) and structure during ontogeny. In the amphibian *Pleurodeles waltl* teeth develop early, and through successive replacements (generations) their morphology and structure are modified. A growth series was fixed, from two days before hatching to the adult stage, for light, scanning and transmission electron microscopy, to know whether the tooth modifications either occur progressively during ontogeny or appear during one step, for instance at metamorphosis. To this goal we have followed a single tooth family, i.e., the first-generation tooth and all replacement teeth that succeeded until adult stages. Either three or four tooth generations occur before the metamorphosis. In larva!

1 stages teeth differ from those in metamorphosed juveniles by: a conical and monocuspidate shape versus bicuspidate; an homogeneous basal region of the dentin cone versus a pedicellate organisation; the presence of enameloid (both mesenchymal and epithelial contribution) and a thin enamel layer versus only a thick enamel layer; and a minimal structure with reduced pulp cavity and absence of dentinal tubules in the dentin versus large pulp cavity containing blood vessels, nerves endings and presence of dentinal tubules within the dentin matrix. The monocuspид-bicuspid transition occurs suddenly during the metamorphosis, while the other modifications occur progressively during the larval period. (Poster)

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Title : Semi-quantitative Bioassay For Investigation of TGFB / Hydroxyapatite Interactions  
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Abstract : TGF-B isoforms in dentine matrix potentially provide a reservoir of bioactive growth factors that may be able to influence cells behaviour in the dentine-pulp complex. Studies indicate a number of interactions between extracellular matrix components and TGFs in the dentine matrix, however, little is understood about the nature of TGF interaction with the mineral component of the matrix or the effects of such interactions on TGF signalling. This study investigated the effects of hydroxyapatite bound TGF $\beta$ 1 on gene expression using a PCR bioassay with focus on the expression of TGF $\beta$ 1 due to its possible autocrine signalling effects. 100ul of human recombinant TGF $\beta$ 1 was incubated with 15mg of crystalline hydroxyapatite (HAp) for 30 minutes after which the insoluble hydroxyapatite was removed. Near confluent Swiss albino mouse 3T3 cells were stimulated by the addition of HAp-TGF $\beta$ 1 or non-mineral treated TGF $\beta$ 1 at concentrations of 0.1ng – 10ng/ml. After 24 hours of incubation with HAp-TGF $\beta$ 1 or free TGF $\beta$ 1, RNA was extracted; reverse transcribed and PCR performed using specific primers for TGF $\beta$ 1. Cells stimulated with non-mineral treated TGF $\beta$ 1 showed a general increase in TGF $\beta$ 1 expression from unstimulated assays to 10ng/ml TGF $\beta$ 1, however a decrease in expression was seen between 0.1ng/ml to 0.5ng/ml. A similar pattern of expression was observed with cells stimulated by HAp-TGF $\beta$ 1, although at a concentration of 10ng/ml a significantly lower expression level of TGF $\beta$ 1 was observed in the HAp-TGF $\beta$ 1 assays. We conclude that interactions between TGF $\beta$ 1 and hydroxyapatite may occur leading to a possible decrease in available TGF $\beta$ 1 in HAp-TGF $\beta$ 1 samples. Such interactions may be present in dentine and be important when considering tissue repair responses following injury and the nature of TGF-B / ECM interactions within dentine.

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Title : Analysis of Osteopontin in Bone Remodelling in Organ Cultures of Murine Neonatal Calvaria.

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Abstract : Remodelling of bone is important for tooth formation, tooth eruption as well as during tooth function. Osteopontin (OPN), a major protein in the bone matrix, is expressed in different forms by osteogenic cells and osteoclasts during bone remodelling. Although defects in the formation and activity of osteoclasts have been observed together with alterations in mineral crystal structure in OPN-null mice, these defects have minimal effects on the quality and quantity of bone. However, deficits in osteoclastic resorption have been reported in response to excessive functional demands. To determine the precise functions of OPN in bone remodelling we have initiated studies in organ cultures in which the effects of different forms of OPN can be analyzed in more detail. Neonatal calvariae (E-7) representing membranous bone were cultured for 3-7 days in 10% serum. Bone formation was studied using immuno- and histochemical staining and pulse-chase radiolabelling with <sup>35</sup>S-methionine. Bone resorption was determined by measuring calcium release and by resorption pits after Alizarin Red staining. Immunostaining of WT bone showed that OPN is expressed in osteogenic cells and osteoclasts and is enriched in remodelling sites in distinct layers representing early and late (Bone sialoprotein staining) stages of bone formation. Bones from OPN-null mice were structurally similar but showed no staining for OPN. Radiolabelling of normal calvarial bone revealed OPN in the culture medium and in demineralizing tissue extracts, but not in 4MGuHCl extracts which release proteins, including BSP, from the collagen matrix before and after demineralization. Thus, the radiolabelled OPN on secretion appears to rapidly associate with the bone mineral, whereas some of the BSP is present in 4MGuHCl extracts. Stimulation of bone resorption in OPN<sup>-/-</sup> calvaria by Vit D3, PTH or LPS revealed decreased formation of osteoclasts and decreased resorption activity, as indicated by fewer resorption pits and reduced calcium release. Confocal microscopy of resorbing WT osteoclasts showed that OPN is concentrated in the cell periphery where it co-localizes with CD44, F-actin and TRAP. In OPN<sup>-/-</sup> osteoclasts F-actin was reduced and disorganized, indicating that OPN expression is required for normal cytoskeleton formation. Conclusions: These studies show that organ culture systems can be used to evaluate the effects of OPN gene deletion on bone remodelling. Although no marked differences in development and growth have been observed in the bones from OPN-null mice, studies of cultured calvariae demonstrate defects in osteoclast development and activity that relate to OPN effects on cytoskeleton formation. These studies were supported by a Canadian Institutes of Health (CIHR) Grant, MOP-36333.

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Title : Proteolipid Protein and Nestin Expression in the Late Bell Stage of Developing Rodent Teeth

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**Abstract :** Introduction: Proteolipid protein (PLP), encoded by the proteolipid protein gene, is a major structural component of the central nervous system (CNS) myelin. In man, mutations of this protein can cause Pelizaeus-Merzbacher disease, a X-linked dysmyelinating neuropathy. To date, no data are present on the expression of PLP in odontoblasts. Nestin is the product of a gene that encodes a protein belonging to a distinct sixth class of intermediate filaments predominantly expressed at early stages of central nervous system and muscle development. Nestin plays an important role in induction, morphogenesis and differentiation of organs. Previous studies have showed that nestin is expressed in developing and adult human teeth under normal and pathological conditions. Aim of the study: The aim of this study was to investigate the expression of PLP and nestin in the late bell stage of developing rodent teeth in neonatal rats. Materials and Methods: Jaw bones with developingteeth were cut into 2 mm slices, fixed with formalin 4% (Unifix, Klinipath) for 1 hour, embedded in paraffin and sectioned at 4  $\mu$ m. Sections were deparaffinized and immunostained using an indirect visualization system, based on a peroxidase-labelled polymer (Dako EnVision System). Endogeneous peroxidase was quenched with 0.5% H<sub>2</sub>O<sub>2</sub> in methanol. Sections were incubated for 1 hour with either mouse anti-nestin monoclonal antibody (Chemicon) at a dilution of 1/2000 in PBS or mouse anti-PLP monoclonal antibody (Sigma) at a dilution of 1/20 in PBS. Nuclei were counterstained using Mayers' hematoxylin. Results: PLP: Strong immunoreactivity was observed in the odontoblast layer, the ameloblast layer and the stratum intermedium. In the odontoblasts and ameloblasts, both the cell bodies and the processes exhibited immunoreactivity. This was only seen in regions of active deposition of dentine and enamel. The cell-rich zone underlying the odontoblasts also stained positive in these regions. At the cervical loop, and in absence of dentine and enamel, the odontoblasts and preameloblasts did not present PLP immunolabelling. Further, immunoreactivity was seen in the external enamel epithelium but not in the stellate reticulum. Nestin: Strong immunoreactivity was seen in both the odontoblast cell bodies and processes. Immunoreactivity was observed up to the dentin-enamel junction from the cervical loop to the crown region. Strong nestin immunoreactivity was also observed in the cell-rich zone at the periphery of the pulp and in the stratum intermedium. Discussion: PLP and Nestin expression is present in odontoblasts, ameloblasts and stratum intermedium in developing teeth of neonatal rat. PLP functions as a mechanic anchor between two cell membranes and in this way PLP is a very important structural protein in the formation of myelin. PLP, present in odontoblasts and ameloblasts, could give a structural support for these cell layers when they deposite their secretions: dentin and enamel respectively. In a study concerning dental characteristics of patients with Duchenne Muscular Dystrophy, disturbances in tooth form, number and eruption of the second premolars were observed in 39% of the patients. One of the key mutations found in the Duchenne syndrome is a duplication of the PLP gene resulting in a failed transcription. In some other neurodegenerative diseases such as Pelizaeus-Merzbacher disease, where a duplication of the PLP gene is one of the possible causing mutations, it then might be possible to detect malformation of the dentin and enamel due to the loss of PLP. This suggests a crucial role for PLP in the developing tooth. PLP and Nestin expression taken together could point to a possible similarity in function between the oligodendrocyte and the odontoblast, both derived from the neural crest. Like the oligodendrocyte, the function of the odontoblast might be supporting the sensory nerves entering the dentinal tubules. Further investigation is necessary to confirm these hypotheses.

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Title : Modulation of activin/BMP signaling by follistatin is required for the morphogenesis of mouse molar teeth

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Abstract : The morphogenesis of teeth is regulated by sequential and reciprocal interactions between epithelium and mesenchyme. First signaling center form in the ectodermal placodes and afterwards new signaling centers, the enamel knots, appear reiteratively in the epithelium. The enamel knots control growth and folding of the inner dental epithelium and cusp patterning. Signaling molecules of several conserved families, such as transforming growth factor  $\beta$  (TGF $\beta$ ) superfamily, mediate cell communication during tooth development. The fine tuning of TGF $\beta$  superfamily members is critical for their functions. Follistatin inhibits extracellularly several members of TGF $\beta$  superfamily including activin A, BMP2, BMP4 and BMP7. We analysed molar phenotypes of follistatin knockout mice and transgenic mice over-expressing follistatin under the keratin 14 (K14) promoter. In both of the mice lines the folding of the crown was malformed. The cusps of the molars of follistatin knockout mice were shallow with reduced cell proliferation and lacked anterior-posterior polarization. The functions of both primary and secondary enamel knots seemed to be disturbed. In K14-follistatin transgenic mice, the occlusal surfaces of the molars were whorled and the enamel was prematurely worn. Furthermore, all the third molars were missing. The roles of follistatin during tooth development were also analysed from the expression patterns of follistatin, activin bA, Bmp2, Bmp4 and Bmp7. Our results indicate that follistatin regulates morphogenesis and shaping of the tooth crown. The antagonistic effects between follistatin and TGF $\beta$  superfamily signals were critical for the formation and function of the enamel knots, as well as for patterning of the tooth cusps.

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Abstract : The continuously growing tooth is the extreme form of increased tooth height, and exists in two forms, continuously growing 'crowns' and 'roots'. The latter are restricted to the Edentata, which includes the sloth. The design of the sloth tooth is reproduced in transgenic k14-Eda mice, in which the continuously growing incisor is changed from the crown into the root type. The epithelial dental stem cell niche is neither affected on a structural nor on a regulatory level in the k14-Eda incisor. Instead a defect in the differentiation prevents production of enamel and differentiation of the ameloblast cell lineage, and the epithelium acquires the root fate. Here we show that the maintenance and differentiation of dental stem cells are regulated independently, and that differences in their control determine the proximal-distal patterning of teeth. We propose that the independence of the mechanisms has allowed developmental flexibility and evolutionary variation in tooth character.

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Title : Teeth with continuously growing roots - from a rodent incisor to a sloth molar

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Title : Follistatin inhibits ameloblast differentiation by antagonizing BMP4 signaling and is responsible for the enamel-free area formation in mouse incisors  
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Abstract : Tooth specific cells, ameloblasts and odontoblasts, differentiate according to coordinated temporal and spatial patterns and secrete the enamel and dentin matrices, respectively. The molecular mechanisms regulating ameloblast differentiation are not known yet. Here we provide direct evidence that follistatin, an extracellular inhibitor of TGF $\beta$  superfamily signals, is the major negative regulator of ameloblast differentiation. In transgenic mice over-expressing follistatin in dental epithelium, ameloblast differentiation was inhibited in the incisors. Conversely, in follistatin knockout mice, functional ameloblasts occurred on the lingual surface of incisors, which is normally the enamel-free "root-analogue" area. Gene expression patterns and experiments on cultured tooth explants indicated that activin from the dental follicle induces follistatin expression in the dental epithelium, and follistatin inhibits the ameloblast inducing activity of BMP4 expressed by odontoblasts. 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 adjacent dental mesenchymal cell lineages. Follistatin integrates these effects and spatially and temporarily regulates enamel formation.

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Title : Diversity in eretmodine dentition & heterochrony: a tale of ontogenetic changes in tooth shape and pattern.

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Abstract : The Eretmodini, a tribe of cichlids endemic to Lake Tanganyika, currently comprise four nominal species mainly defined by their distinctive tooth shapes. Yet, during the past five years an extensive body of evidence has been collected, indicating high levels of variability with respect to dental traits within this tribe, emphasizing the plastic nature of the eretmodine dentition. However, little is known about the evolutionary and developmental mechanisms yielding this diversity in eretmodine dentitions. In order to better understand these phenomena, we have performed an ontogenetic analysis of the acquisition of tooth shape and tooth pattern in one genetic lineage of *Eretmodus cyanostictus* (lineage A) (Rüber et al., 1999; PNAS 96: 10230-10235). We have found that the adult taxon-specific dentition is acquired via a series of transitory tooth shapes and patterns. The dentition changes from a single row of conical, widely spaced teeth with extramedullary development, to a multi-rowed dentition of closely packed, spatulate teeth with intramedullary replacement. The change in tooth pattern is hypothesized to be the result of a gradual size increase of the teeth (via successive replacements), producing a more closely packed dentition, with the retention of functional teeth, thus yielding simultaneous functionality of predecessor and replacer. The change in tooth shape occurs in new tooth positions at the symphyseal end of the jaw, and appears to be subjected to a positional control. Intriguingly, the juvenile dentition of *Eretmodus cf. cyanostictus* (lineage A) reflects the adult *Spathodus* dentition, both in terms of tooth pattern and tooth shape. This raises the question whether the different dentitions of the Eretmodini (both in terms of tooth shape and pattern) could result from a process of heterochrony. Assumptions on heterochrony of course require a comparison with the ancestral situation. Yet, so far this is not possible since the ancestral tooth shape of the Eretmodini remains elusive. Nevertheless, if this hypothesis on heterochrony (paedomorphosis or peramorphosis, depending on the nature of the primitive condition) is corroborated, this could also potentially shed light on the aberrant position of some *Spathodus* and *Tanganicodus* individuals (based on tooth shape) within the otherwise *Eretmodus*-dominated lineages A and C (based on mtDNA).

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Title : Are breeding teeth in Atlantic salmon a component of the drastic alterations of the orofacial skeleton?

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Abstract : The upriver spawning migration of Atlantic salmon (*Salmo salar*) involves drastic skeletal alterations, most prominently, elongation of the lower jaw in males through a fast, "tumour-like" formation of chondroid bone (as shown by our recent studies). The literature also describes a toothless stage, followed by the appearance of a new set of teeth (breeding teeth) in association with the migration to spawn. To investigate the assumed complete tooth replacement we used serially sectioned and X-rayed jaws to elucidate the pattern of tooth initiation and replacement in different life stages of wild Atlantic salmon. The first teeth develop directly from the buccal epithelium. In all subsequent stages, a replacement tooth is connected to the lingual and caudal side of the enamel organ of its predecessor. In juveniles, every position in the tooth row holds both a functional and a replacement tooth. Odontogenic waves link teeth in similar developmental stages every three positions; replacement waves link teeth in alternate positions and slope cephalad. This pattern is maintained in pre-spawning animals, but through wider spacing of odontogenic waves, every position now holds either a functional or a replacement tooth. Our studies provide no indication for a complete change of dentition prior to spawning. At no time in the life cycle have we found edentulous stages; the pattern of tooth replacement observed in juveniles continues in pre-spawning individuals. We assume that earlier reports regarding complete tooth loss/replacement relate to the proliferation of the oral mucosa that covers and so disguises the teeth during the breeding period. Also, maceration techniques used in earlier studies may have removed all teeth with a base that is not fully mineralised.

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Title : Gene Expression and Protein Distribution of Matrix Metalloproteinases and Their Inhibitors During Mouse Molar Tooth Development

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Abstract : Remodeling of the extracellular matrix is an important regulator of tooth histomorphogenesis and cytodifferentiation. Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that are capable of degrading different substrates of extracellular matrices, and their activity is suppressed by tissue inhibitors of metalloproteinases (TIMPs). It is now widely appreciated that TIMPs have pluripotential effects on cell growth, apoptosis, and differentiation, in addition to their MMP inhibitory activity. In this study we have analyzed the gene expression and protein distribution of MMP-2, -9, MT1-MMP, as well as of TIMP-1, -2 and -3 during mouse molar development. Immunohistochemical data demonstrated that all the MMPs investigated were expressed in the dental epithelium and mesenchyme. In contrast, gene and protein expression analysis for TIMPs showed that they were differentially expressed. TIMP-1 was expressed in the dental epithelium and mesenchyme between E13-16, and RNA transcripts were transiently upregulated at E14, the cap stage. TIMP-1 transcripts were also detected in differentiating odontoblasts. TIMP-2 RNA transcripts were found in the peridental and dental mesenchyme, odontoblasts and ameloblasts. Protein analysis revealed high expression in the jaw mesenchyme at the lingual side of the tooth and in the oral and dental basement membrane facing the TIMP-2- positive jaw mesenchyme during the bud, cap, and early bell stages, together with transient expression in the enamel knot at E14. TIMP-2 staining was detected in the gingival tissue and enamel matrix post-natally. TIMP-3 RNA transcripts were found in discrete regions of the dental epithelium including at high levels in the cervical loop at E16. Expression was also detected in dental papilla mesenchyme during the cap to bell transition. Analysis of the protein distribution revealed a lower level of TIMP-3 on the lingual side of the dental epithelium at E14 and the protein was no longer detectable on the dental basement membrane at E16. MT1-MMP RNA transcripts were found in the dental mesenchyme between E13-16, at relatively high levels in the cervical loop at E14, and in the odontoblasts and ameloblasts. The distinct temporo-spatial distribution patterns of the TIMPs suggest that these inhibitors should be involved in regulating extracellular matrix remodeling and cytodifferentiation during tooth development.

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Gene targeting in embryonic stem (ES) cells has been traditionally used to dissect gene function in vivo. RNA interference (RNAi) technology has provided an alternative loss-of-function method for assessing gene function in the mouse, as well as in a number of different organisms. The introduction of small interfering RNA (siRNA) molecules homologous to target genes mediates specific gene knockdown. Using RNA polymerase III (RNA pol III) promoters, plasmid-based systems have been devised to drive short hairpin RNA (shRNA) molecules to stably mediate RNAi. Transgenic shRNA was used to generate knockdown ES cell lines and embryonic phenotypes were directly assessed by the tetraploid complementation method. The merging of RNAi and ES cell technology will increase the efficiency of investigating gene function in vivo.



Abstract : The bulk structure and the properties of hydroxyapatite (OHAP) have been extensively studied but much less is known about the OHAP surface. The interaction between the OHAP surface and molecules or cells relates to various surface properties: e. g. surface functional groups, acidity, basicity, surface charge, hydrophilicity, porosity, reactivity to molecules or cells, catalytic activity... Surfaces charges create dipoles at the surface of ionic solids due to the different relaxation and polarization of anions and cations. Adsorbed species can accept to donate electrons to the substrate to generate further dipoles and hence possibly modify the substrates themselves. Surfaces do not need to be electrically neutral except at a macroscopic scale. Dipoles fields are due to space charges. Typical space-charge layers extend for 10-100 nm from the surface, and lead to energy difference of perhaps 0.2 eV and fields of  $10^6$ - $10^7$  V/m (Stoneham and Harding, 1998). Several models have been proposed for the surface structure of OHAP. Three kinds of P-OH groups acting as the adsorption sites for CO<sub>2</sub>, CH<sub>3</sub>OH, CH<sub>3</sub> and H<sub>2</sub>O on the surface of OHAP have been proposed (Ishikawa, 1996). The regulation of surface P-OH groups by for example alkyl phosphates in acetone and in water acetone solutions maybe expected to give novel functions to OHAP. (Tanaka et al., 2004). Furthermore, the significance of positively charged adsorption sites on the exposed ac or bc crystal faces influences the amount of bovine serum albumin adsorbed (Kandori et al., 1995) but the adsorption of lysozyme is independent from both cation/P molar ratio nor the number of ac or bc sites (Kandori et al., 1997) which indicates that adsorption phenomena is a complex process and is influenced not only by substrate and adsorbed materials but also by the interaction between them. Clearly, the adsorbed materials interact with the substrates and in some cases modify it. Observations of the surface structure of human tooth enamel crystals (Brès and Hutchison, 2002) have shown structural instabilities leading to a subsurface reconstruction. These finding and the possible implications to the understanding of the OHAP surface structure will be discussed in the light of ionic crystal instabilities (Tasker et al., 1979).  
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Abstract : Patients with the syndrome cleidocranial dysplasia (OMIM#119600) have defects in both bone and tooth formation. Genetically this is caused by haploinsufficiency of the transcription factor gene Runx2 (Cbfa1). Mice homozygous for null mutations in Runx2 completely lack bone and have arrested tooth development prior to the cap stage. As this arrest in tooth development takes place during morphogenesis, downstream targets of Runx2 may offer insights into the molecular events controlling tooth morphogenesis. We therefore attempted a transcriptional profile of molar tooth germs isolated from wild type and Runx2 mutants using Affymetrix microarrays. Using stringent selection criteria we have identified 49 downregulated and 15 upregulated genes in Runx2 mutant tissue. So far 11 downregulated genes have been validated using either RT-PCR or in situ hybridization techniques. The expression patterns of these genes reveal that 6 of these targets are expressed in tooth, while 5 of the genes are expressed only in the underlying osteogenic mesenchyme and apparently associated with the development of mandibular bone. This result demonstrates that the downstream targets of Runx2 are different in the developing bones and teeth.

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Title : Transcriptional profiling of arrested tooth development in Runx2 mutant mice

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Abstract : Heat shock protein (HSP)-25, the family of low molecular weight HSPs, is induced under stressful conditions such as heat shock and other deleterious environmental and pathophysiologic stresses. HSP-25 has been also reported to possess diverse functions including molecular chaperones, a modulator of actin dynamics, and a specific inhibitor of apoptosis under normal conditions and during development in addition to stressful conditions. Recently, we demonstrated the transient and/or continuous expression of HSP-25 in the dental pulp and enamel organ during odontogenesis. To clarify the functional significance of HSP-25 during tooth development, we analyzed the relationship between the expression of HSP-25 in the dental epithelial and mesenchymal cells and their proliferation and differentiation during odontogenesis at the postnatal stages (P0 - P100) of rat molars by immunohistochemistry using antibodies to HSP-25 and 5'-bromo-2'-deoxyuridine (BrdU) that determined cell proliferation. Throughout postnatal stages, differentiated odontoblasts showed the intense HSP-25 immunoreactivity (IR) except for the root and floor pulp, where they were initially weak or negative but increased in IR in the later stages. BrdU-immunoreactive cells in the dental pulp were located beneath cervical loop or Hertwig's epithelial root sheath (HERS). Interestingly, this proliferative zone did not show HSP-25-IR. From postnatal 60 to 100 days, HSP-25-IR was exclusively located in the odontoblast layer, and BrdU-immunoreactive cells were hardly recognizable in the dental pulp. In the enamel organ, on the other hand, BrdU-immunoreactive cells were detected in the stellate reticulum, inner and outer enamel epithelium, and cervical loop, whereas HSP-25-immunopositive preameloblasts and ameloblasts did not show BrdU-IR. These findings indicate that dental epithelial and mesenchymal cells become to acquire HSP-25-IR after they complete the cell division. Thus, HSP-25-IR is suggestive to act as a switch between cell proliferation and differentiation during tooth development.

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Title : Relationship between the Expression of Heat Shock Protein-25 in the Dental Epithelial and Mesenchymal Cells and Their Proliferation and Differentiation during Odontogenesis in Rat Molars

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## **Genetics of craniosynostosis: clinical applications, suture biogenesis, and selfish testes**

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Craniosynostosis, the premature fusion of the cranial sutures, occurs in 1 in 2500 children and often presents challenging clinical problems that are best managed in a multidisciplinary setting. Until a decade ago, little was known about the causes of craniosynostosis but the discovery of mutations in the *FGFR1*, *FGFR2*, *FGFR3*, *TWIST* and *MSX2* genes in both syndromic and nonsyndromic cases, has led to considerable insights into the aetiology, classification and developmental pathology of these disorders. Key findings have included the recognition of entirely new syndromes, most notably Muenke syndrome caused by heterozygous P250R substitution in *FGFR3*, the use of molecular data to provide prognostic information, and the partial description of the molecular pathways required for the maturation of established cranial sutures. An exciting new line of research is opened up by the recent discovery that mutations of *EFNB1*, encoding ephrin-B1, cause the X-linked disorder craniofrontonasal syndrome: this focuses attention on the previously unknown mechanisms by which position of the coronal suture is established. Finally, the study of Apert syndrome provides a paradigm for understanding the forces shaping mutation. This has given new insight into the relationship between developmental malformations and cancer, and highlights a novel form of evolutionary conflict.

**Regeneration of the Periodontium**  
**Bruce Rutherford, DDS, PhD**

A major goal of periodontal reconstructive therapy is to establish treatment modalities that predictably restore periodontal tissues, i.e., bone, cementum and a functional periodontal ligament (PDL), lost as a consequence of disease. An attractive approach to determining the required factors and cells for regenerating these tissues is to ascertain the factors necessary for their development. Using such an approach, our laboratory, in collaborative efforts with other researchers, has identified several candidate factors and is exploring the mechanism of action of these candidate genes/proteins using both *in vitro* and *in vivo* models. This presentation will provide information on some of these putative factors including: a) genes/proteins that may be involved in regulating epithelial-mesenchymal interactions during root development; b) bone morphogenetic proteins; c) genes/proteins that modulate phosphate transport, as regulators of cementogenesis; and d) proteins controlling osteoblast/osteoclast activity, as protectors for use during periodontal regenerative therapy.

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Abstract : LM04 is a novel member of the LIM-only (LMO) subfamily of LIM domain-containing transcription factors. LIM-only transcription factors have known to be involved in development and patterning in several systems by interacting with DNA-binding proteins. To further explore the role of the broader LMO genes in tooth development, we examined the expression pattern of LM04 in the developing tooth. Wild-type embryos used in this study were generated by mating Kun-Ming mice. Pregnant mice were scarified by cervical dislocation on E10.5;«E14.5. DIG labeling RNA probes of LM04 were generated by in vitro transcription from LM04 cDNA. The expression pattern of LM04 mRNA were analysed by in situ hybridization for whole-mount and paraffin sections. The results show that LM04 expression defined in epithelium including those of the epidermis, limb, mandibular reagon, brain and somites during mouse organ development. On E11.5, LM04 transcripts are most prominently expressed in the ! dental lamina. LM04 was detected in the tooth bud on E13.5 and in the enamel organ on E14.5. Conclusion: The expression pattern of LM04 indicate this transcription factor might play a role in the specific tooth crown shape formation. Supported by NSFC grant 30171011. \* Corresponding authorf°zhi\_chen@163.com

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Title : Spatiotemporal Expression of LM04 in tooth morphogenesis

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**Abstract :** Primates demonstrate considerable variation in the timing of major developmental events, including development of the dentition. The age at completion of dental development in the higher primates shows clear grade differences which separate monkeys, apes and modern humans. The duration of dental development within these grades is correlated to the secretion rates of ameloblasts, irrespective of tooth identity or the amount of enamel being formed. The relationship between coronal odontoblast secretory rates and the time available during development is unknown but it could be hypothesised that odontoblast secretion rates, like ameloblasts, reflect the length of the developmental period available. This hypothesis was tested using polarising light microscopy of ground tooth sections from primate species representing old world monkeys, great apes and humans. A wide range of tooth types were sampled for each species. Secretion rates for mid-axial coronal dentine were ca!

culated for each tooth from incremental markings in the dentine (Andresen lines). Dentine incremental markings were calibrated from daily incremental lines in the corresponding enamel. Results suggest that, unlike ameloblasts, differences in odontoblast secretion rates occur between tooth types within each species. Odontoblast secretion rates are unrelated to any grade differences in the length of the period available for dental development but instead appear to be correlated to tooth morphology, specifically that of the enamel dentine junction. This finding highlights the fundamental differences in the apparent control of enamel and dentine secretion rates occurring at the same time.

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**Title :** Odontoblast secretory activity: constrained by tooth identity or time?

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**Abstract :** We have proposed that geometrical property of the artificial extracellular matrices (ECM) must be taken into consideration for successful development of tissue engineering (JBJS, 83-A: S1-105-115, 2001). Geometry is defined in this case as the three-dimensional (3D) structure of artificial ECM at the order of micrometer, which can direct growth of tissues and organs in vivo and in vitro. Typical in vitro example is the remarkable difference between the cell cultures on flat dishes and 3-D artificial ECM (Nature, 424: 870, 2003). Previously we have shown that honeycomb-shaped hydroxyapatite (HC-HAP) with 100-micron pores induced endochondral ossification, while one with 350-micron pores induced direct bone formation. Inspired by the fact that a single large blood vessel was always noticed in the center of the 350-pores of HC-HAP, we attempted to direct the haversian-type bone formation by an artificial ECM with similar geometry to HC-HAP but with biodegradable property. We chose a honeycomb-shaped collagen product (HC-COL) (Honeycomb sponge, Koken, Japan). It is a reconstituted pepsin-treated collagen, which equips numerous straight tunnels with semi-hexagonal walls, size of which is controllable from 0.1 to 1.0 mm. HC-COL was cut into block-form, combined with the purified BMP cocktail (0.5 mg) or rhBMP-2 (0.005 mg) and implanted into rat skin. HC-COL was calcified with a series of the solutions, which contained calcium and phosphate (multi-step calcifying method, submitted) to obtain the hydroxyapatite-coated honeycomb collagen (HAP-HC-COL), which was also implanted into rat skin. Two weeks after implantation, it was found that the concentric layered osteogenesis occurred within the each tunnels of HC-COL/BMP, the centers of which, there was always a single large blood vessel. The structure was reminded us the haversian system. More interestingly, HAP-HC-COL itself (without BMP) induced the concentric fibrous tissue formation with a blood vessel in the center of the tunnel, although bone formation was not observed. It was concluded that the characteristic 3D-structure (vasculature-inducing structure) of HC-COL or HAP-HC-COL induced vasculature first, irrespective of the presence of BMP, then bone formation will occur if the mesenchymal cells were differentiated into osteoblasts by BMP.

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Title : Mechanism of Haversian-type Bone Formation as Evidenced by Artificial ECM of Vasculature-Inducing Geometry

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## Therapeutic Applications of Human Embryonic Stem Cells

>

> There has been intense interest in the potential therapeutic application of human embryonic stem (ES) cells. However, only a limited number of human ES cell lines throughout the world have been derived and characterised, and many of these are difficult to propagate. Under license from the Human Fertilisation and Embryology Authority and with ethical approval from King's College London, we have been endeavouring to generate new human ES cell lines. We have recently derived a novel human ES cell line, WT-3, that expresses a number of characteristic genes and proteins indicative of human ES cells, including Oct-4, Nanog, SSEA-3 and SSEA-4. These cells also differentiate into cells of all three primitive germ layers, including cells that express characteristic markers of neurons, liver cells, pancreatic beta cells, muscle and heart tissue. We have also established two additional new human ES cell lines, one of which was derived from an embryo screened by preimplantation genetic diagnosis and shown to harbour Cystic Fibrosis. This line represents the first known human ES cell line containing a known genetic lesion, which should provide a valuable resource for biomedical research.

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**Abstract :** Rodent incisors are known to be continuously growing teeth that are maintained by both cell-proliferation at the apical end of the tooth and the attrition of incisal edges. In tooth developmental biology, discovery of dental epithelial stem cells is recent breakthrough. The dental epithelium of continuously growing tooth has a special structure for maintenance of the stem cells at the apical end including the cell-proliferative region. In three-dimensional views by scanning electron microscopy, the specific bulbous protrusion of the incisor epithelium, which is obtained from the lower mandibles of 2 or 3-day-old mice by the treatment with 2% collagenase, is observed as a human head-like structure at the apical edge of the labial epithelium. Furthermore, morphological transition of the epithelial-mesenchymal compartment by the serial transverse section from the apical end to the incisal edge is likely to reflect the development of tooth germ in the prenatal stage. Especially, the morphological feature of the epithelium and mesenchyme was identical to the histological section of bud-cap stages in developing molar tooth germ. These structures are composed of the cells of the inner and outer enamel epithelium, and stellate reticulum, and these distinct epithelial compartments of the enamel organ are also confirmed by transmission electron microscopy. Based on these histological and molecular biological studies, the special structure at the apical end is obviously different from the cervical loop of the developing molar germ of human, mouse and rat. Continuously growing teeth are represented not only by rodent incisors but also molars in certain other species including guinea-pigs. Hence, we compared the morphological features of apical end in rodent incisors with those in the guinea-pig molars. Interestingly, the guinea-pig molars also possessed the specific proliferative region at the apical end. We propose a new concept that the eternal tooth bud producing various dental progeny is formed at the apical end of continuously growing teeth, and a new term "apical bud" for indicating this specialized epithelial structure.

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**Title :** The eternal tooth germ is formed at the apical end of continuously growing teeth

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**Abstract :** We describe the expression patterns of Runx1 and Runx2/Osf2 during the development of teeth and other craniofacial tissues and compare them to Runx2 expression reported earlier. Runx1 was expressed in several cartilage primordia and Runx2 was intense in all mesenchymal condensations of teeth. Runx1 and Runx2/Osf2 were coexpressed in future osteogenic regions. However, Runx1 was markedly downregulated upon ossification of the tissues. Only Runx1 was expressed in epithelia, and in tooth germs transcripts were detected in outer dental epithelium. Runx1 was also intensely expressed in the midline epithelium of palatal shelves. In addition, we examined Runx2/Osf2 expression in the developing face of Runx1 knockout mice at E12.5 and Runx1 expression in Runx2 knockout mice at E14.5. Runx2 expression was not affected by gene disruption of Runx1, whereas the expression domains of Runx1 were extended in Runx2<sup>-/-</sup> mice compared with wild type mice. Our results suggest that Runx1 may play a role in tooth development, palate formation and ossification of bones.

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Reichert theory states that the skeletal components of the mammalian middle ear are homologous to the bones and cartilages of the primary jaw joint in non-mammalian jawed vertebrates.

The middle ear consists of three ossicles the malleus, incus and the stapes and two structural components the gonium and the tympanic ring.

Bapx1 is expressed in both systems at the sites of articulation i.e. between the malleus and incus in mammals and the quadrate and articular in other gnathostomes.

In zebrafish and chick knock down of Bapx1, directly or indirectly leads to fusion of the quadrate and articular, and loss of the joint marker Gdf5. A similar fusion of the malleus and incus is, however, not seen in Bapx1 knockout mice, and in keeping with this the expression of Gdf5 is unaffected. This indicates a possible difference in the formation of these two articulations.

In the chick the quadrate and articular can be observed initially as one condensation expressing type II collagen, which later becomes defined as two separate elements. How the malleus and incus develop in the mouse however has not been clearly studied. I have used Chlorantine fast red and Alcian blue staining of sagittal sections E14-Nb to visualise the middle ear at different ages in mouse embryos. It was seen that at E14.5 the malleus and incus are already separate. By E16.5 Bone has started to form as well as the cartilaginous components.

Collagen type 11 immunohistochemistry was used to determine how the ossicles develop at E12-13.5, prior to overt cartilage differentiation. Do they start off as one condensation that splits in two as in the chick, or are they always separate?

**Preliminary results so far show that they follow the same pattern as the chick.**

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## **Distribution and Structure of Dental Enamel in Incisors of Tabby Mice**

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In Tabby mice the *Ta* (*Eda* in humans) gene is not expressed, resulting in a syndrome that is homologous to hypohidrotic ectodermal dysplasia in humans. Among the characteristics of the Tabby phenotype are aberrations in number and morphology of the teeth, both molars and incisors. The effect on the structure of enamel, however, has not been investigated. It was the aim of the present study to describe the enamel phenotype in Tabby incisors, with emphasis on its distribution, thickness and structure.

All four incisors were obtained from five female Tabby mice and from three female wild type mice. The incisors were sectioned and ground transversely at a level just apical to the abraded incisal part, etched for 45 seconds with 0.1% nitric acid, and sputter-coated with gold-palladium. The transverse surface was observed in SEM.

All measured dimensions were more variable, often considerably more, in Tabby mice than in wild type mice. Also the outline of the enamel-dentin junction was more variable in Tabby mice, the mesial concavity was often reduced or lacking. Maxillary incisors were wider in Tabby mice, while mandibular incisors were wider in wild type mice. No significant difference in enamel thickness between Tabby and wild type mice was observed. The enamel on the mesial aspect tended to extend further lingually in Tabby mice than in wild type mice both in maxillary and in mandibular incisors. On the lateral aspect this tendency was only significant in mandibular incisors. In one Tabby mouse the enamel on the mandibular incisors extended as a thin layer far beyond the bends in the dentin contour that normally coincide with enamel termination, both mesially and laterally on the right incisor and mesially on the left incisor. In this most affected mouse the right maxillary incisor was abortive with no enamel, while the left maxillary incisor was relatively small. Instances of hypoplastic enamel were observed. The complex mouse enamel structure was generally well preserved in the Tabby mice, only few instances of aberrant structure were observed. Based on observation of the enamel crystals in the SEM, slight hypomineralization of the enamel was present in about 60% of the Tabby incisors.

A major effect on incisor morphology in Tabby mutants seems to be a variation in mesial and, to a lesser extent, lateral enamel termination. Since the *Ta* gene during tooth development is only expressed in the outer enamel epithelium, it may be speculated that in Tabby mice the transition from inner to outer enamel epithelium is less well defined, resulting in a less well defined termination of enamel.

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**Abstract :** Amelogenesis can be influenced by hormonal, nutritional or environmental changes. Previous studies have demonstrated that a hypocalcemic state observed in thyro-parathyroidectomy (TPTX) rats was associated with the development of enamel hypoplasias. It has been established that a severe hypocalcemic state is installed one week after the thyro-parathyroidectomy, but there are no additional data regarding when the promoted alterations are morphologically detected. The aim of this study was to evaluate the morphology of the amelogenesis of rat mandibular incisors after different periods of TPTX, and to immunolocalize the enamel protein amelogenin. Fifty Wistar rats were TPTX under ether anaesthesia. The control groups (C) were sham operated. After 14, 30 and 57 days, TPTX and C animals were intraperitoneally anaesthetized and fixed by intracardiac perfusion. The incisors were dissected out, decalcified and routinely processed for transmission electron microscopy. Another group was fixed and processed for immunocytochemistry. Semi-thin sections from the secretion and maturation stage were stained with toluidine blue and studied under light microscopy. Ultra-thin sections of TPTX and C rats were processed for post-embedding colloidal gold immunolabeling using an antibody against 24 kDa rat amelogenin. After 14 days, the amelogenesis of TPTX rats was morphologically similar to the control. After 30 and 57 days, however, the TPTX rats presented alterations in the late secretion and early maturation stages. The immunocytochemical study showed differences in the immunolabeling for amelogenin between C and TPTX animals. The absence of enamel defects after 14 days may suggest an insufficient period in the hypocalcemic state to induce morphological alterations. The difference in amelogenin immunolabeling may be due to an altered protein secretion, and it can indicate its association with the induction of enamel hypoplasia.

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**Title :** A Morphological And Immunocytochemical Study Of The Amelogenesis Of Rat Mandibular Incisors After Thyro-Parathyroidectomy.

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**From:** S.J.BROOKES@LEEDS.AC.UK  
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**Abstract :** Introduction: The protein matrix of developing enamel is dominated by amelogenins with smaller amounts of ameloblastin and enamelin. The ultra structural arrangement of these proteins results in the formation of "nanospheres" which presumably control enamel crystal growth, habit and organisation. The aim of the present study was to examine close neighbour relationships between amelogenins prior to their secretion from ameloblasts - in other words to examine whether nanosphere assembly is initiated intracellularly before amelogenin is secreted into the matrix. Methods: Rat incisors were dissected free of the jaws and the enamel organ carefully removed. The tissues was incubated in 1mL of PBS containing 1mM of the reversible protein cross linker dithiobis[succinimidyl propionate] for 5 minutes at room temperature. The cross linking reaction was quenched by adding lysine to a concentration of 10mM. The tissue was removed and sonicated in non-reducing SDS PAGE sample loading buffer and subjected to SDS PAGE. The resulting gel lane was excised and incubated for 15 minutes in reducing SDS PAGE sample loading buffer (to break any cross links formed). The gel lane was then placed on a second SDS gel and the reduced proteins separated by SDS PAGE (essentially 2D-electrophoresis where both dimensions comprise SDS gels). The second gel was electroblotted on to nitrocellulose and probed with anti-amelogenin antibodies. Results: When subjected to 2D electrophoresis using SDS PAGE in both dimensions proteins run in a diagonal line across the gel. However, proteins that were cross linked prior to reduction will run in the second dimension at their monomeric molecular weight below the diagonal line. Monomeric proteins at 27KDa (corresponding to full length rat amelogenin) were identified that would have had molecular weights of 55, 110 and 135KDa prior to reduction. These cross linked complexes appear to represent dimers, tetramers and pentamers of the 27KDa amelogenin. Other cross immunoreactive species appeared on the diagonal (i.e. proteins not involved in the cross linking reaction) including a prominent band at about 6KDa. These proteins are presumably alternatively spliced amelogenins variants with the prominent band at about 6KDa probably representing LRAP on a molecular weight basis. Conclusion: In vivo molecular cross linking indicates that parent 27KDa rat amelogenins appears to be closely associated with each other prior to secretion from ameloblasts suggesting that nanospheres may be pre-assembled intracellularly prior to secretion. Other immunoreactive species present in the enamel organ (one tentatively identified as LRAP) were not cross linked suggesting that they may not be closely associated intracellularly and play no part in intracellular nanosphere assembly.

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Title : Evidence for intracellular amelogenin nanosphere assembly in rat ameloblasts

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Abstract : Introduction : During cranio-facial development and osteoblast differentiation, the fine tuning of Msx1 expression was suggested to involve an "unusual" mechanism : a non-coding antisense (AS) RNA transcribed from the same locus than the coding sense (S) RNA (PNAS 98 : 7336, 2001). Several bioinformatic studies have established that, in fact, bidirectional transcription of gene loci is not exceptional. Aims : The present study aimed to explore Msx1 antisense RNA as a model system for the study of antisense RNA role in development and ponderate these data with an opening window on potential antisense RNAs within the field of tooth morphogenesis and differentiation. Results : LargeT-SV40 immortalized cells allowed the investigation of 3 distinct differentiation stages of odontoblasts. Interestingly, in contrast to previous findings in neo-natal mandibular bone, antisense RNAs were not restricted to differentiated cells. However, identified relationships between S/AS ratios and protein levels with/without transfection of S/AS vectors supported previous concept of an inhibitory role on protein expression pathway for AS-RNA. Based on such a possibility, pre- and post-natal cartilage growth plate was studied by in situ hybridization and histoenzymology of a chimeric b-galactosidase/Msx1 protein in knock-in heterozygous mice. The data showed extremely high fluctuations of Msx1 protein, sense and antisense RNAs perinatally. Apparent different locations of sense and antisense transcripts in the cytoplasm and nucleus were observed but difficult to definitively establish by using conventional microscopy. After sense cDNA transfection in largeT-SV40 immortalized odontoblasts, S-RNA accumulation was observed in the nucleus by RNA-FISH and confocal microscopy. This distinct distribution of sense and antisense transcripts is critical as it orientates hypothesis on antisense RNA function(s) in the inhibition of gene expression. Indeed, in the cytoplasm, antisense RNAs are able to trigger Dicer enzyme responsible for RNA interference (RNA degradation through 21-24 nt duplexes), and interferon pathway. In the nucleus, antisense RNAs trigger ADAR enzymes responsible for post-transcriptional RNA edition, induce sense RNA retention and degradation and finally hide splicing sites, resulting in splicing modification. There are also involved in epigenetic gene silencing. As more than 8 % of the murine EST are overlapping bidirectionally, the tooth development database was predicted to contain approximately 20 bidirectional loci among the 248 different indexed genes. A systematic search showed cis-natural endogenous antisense RNA examples already isolated in other tissues for 11 gene loci. Various types of genes were involved, encoding transcription factors, growth factors and matrix proteins. Conclusion: The physiological impact of various mechanisms of gene expression inhibition by antisense RNAs is established in some circumstances (for instance early failure of Dicer -/- mouse development). The study of these unusual transcripts suggests the existence of another level of complexity in developmental pathways, as suggested here for Msx1 and craniofacial morphogenesis and differentiation. Grants: INSERM, and GIS maladies rares.

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Title : Msx1, endogenous antisense transcripts, Tooth Morphogenesis and Differentiation

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Abstract : Enamel synthesis is dependent on mesenchymal/epithelial interactions between odontoblasts (mesenchymal) and ameloblasts (epithelial) cells in the tooth organ. Ameloblasts progress through several stages of differentiation to form the enamel matrix. Previously we reported the culture of epithelial cells, with ameloblast-like characteristics, by selection with a serum free media. In this study, we seek to further characterize these cells and determine whether micromolar levels of fluoride can alter gene expression and cell function. Tooth organs were dissected from approximately 20 week old human fetal cadaver tissue. The tooth organs were first digested in collagenase/dispase (1mg/ml in PBS) for 1hour at 37°C. The tissue/cell digest was then collected, centrifuged at 2500 rpm for 5 minutes, washed with PBS and further digested with STV (0.05% trypsin, 0.025% versene, in saline), for 5 minutes at 37°C. The cells were washed and 5 X10<sup>4</sup> cells were plated on 100 mm Primaria (Becton Dickinson) tissue culture plates. Cells were cultured in supplemented keratinocyte media (KGM-2) with and without serum, as well with different calcium concentrations (0.05, 0.075, 0.15, 0.25, 1.0mM) added to the media. Further separation of the cells was attempted by FACS. The cells were characterized for amelogenin and ameloblastin by RT-PCR and western blot. Cells with a cobblestone-like epithelial phenotype were the screened for as osteogenesis/odontogenesis-related genes using a commercially available osteogenesis DNA array. This array was further used to screen for differences in mRNA expression of cells grow in media containing 10 µM fluoride as compared to a chloride control. Cells isolated from the tooth germs grown in KGM-2 media in serum concentrations from 2 to 10 % contained a mixture of cells with both a cobblestone-like epithelial phenotype, and a fibroblast-like spindle shape. FACS revealed that the mixed cell population could not be separated based on cell sorting by shape. Cells grown in serum free KGM-2 + 0.05 mM calcium proved to have the most selective advantage for cobblestone-like cells. These cells showed no intercellular bridging when observed under light microscopy. TEM revealed tonofibrils concentrated in the perinuclear region of the cytoplasm, and no typical attachment apparatus. Cells grown at calcium concentrations above 0.05 mM showed intercellular bridge formation, this intercellular bridge formation, occurring within 1 hour after the addition of increased calcium. Amelogenin and ameloblastin expression was detected by RT-PCR, and at the proteins identified by Western blot. Osteogenesis SuperArray showed relatively strong expression of integrins, fibronectin, EGFR and TNF-?. Triplicate SuperArray analyses showed that 10 µM fluoride down-regulated expression of TNF-? in the cobblestone-shaped ameloblasts-like cells. These studies showed that enamel organ epithelial cells with ameloblast-like characteristics can be selectively grown in serum free media with 0.5 mM calcium. When the cell were exposed to 10 µM F, TNF? was down-regulated. Further studies are needed to determine the role of TNF? in enamel formation, and how fluoride may affect enamel formation by a direct effect on cell function.

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Title : Characterization of Primary Enamel Organ Epithelial Cells in vitro.

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Abstract : In development of mouse- and rat-molars, root formation starts after dental epithelium mold shape of crown. However, the molecular mechanism for the transition from crown to root in the development has not been elucidated. Fibroblast growth factor (Fgf) -10, which is expressed in the dental mesenchyme, plays important role in cell-proliferation and -differentiation of dental epithelium in the stage of crown morphogenesis. But, the expression of Fgf-10 disappears in the stage of root formation. On the other hand, in continuously growing teeth such as mouse- and rat-incisor, vole- incisor and molar, continuous expression of Fgf-10 results in eternally forming crown analog (labial side in case of incisors) consisting of enamel and dentin. To clarify the significance of disappearance of Fgf-10 in root formation, we observed the postnatal growth of incisors of Fgf-10 deficient mice (lethal at postnatal 0 day) by implantation underneath the kidney capsule. In the labial side of the mutant type, the formation of enamel stopped halfway and continuous dentin was observed to the apical end. The histological feature was similar to be that of mouse-molar development exactly. The dental epithelium like Hertwig' epithelial sheath was formed at both labial and lingual sides of apical end and the sheath was fragmented concomitantly with the formation of root dentin and periodontal ligament. These results suggest that the disappearance of Fgf-10 is key for the transition from the crown to root formation in development of mouse molars.

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Title : Fgf10 is a regulatory factor for transition from crown to root formation in tooth development

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Abstract : The tribosphenic molar is a synapomorphy of mammals and it is retained with certain clade-specific modifications even in many clades of extant mammals such as in all of about 800 species of microbats. Our contribution is intended to discuss why just such a type of dental arrangements is so constant and why possible variations on it are largely constrained. We reexamined state of 22 fine dental characters in 60 taxa of vespertilionid bats and in some of them also investigated micromorphology of the molar enamel coat with particular respect to the patterns of enamel maturation. The results suggest that the major goal of tribosphenic organisation is in its modular structure and in that the respective structural modules interface at broad suture zones. In combination with a delayed enamel maturation this allows extensive spatial rearrangements during late odontogenesis and the perieruptional enlargements of the tooth which produce a maximisation of adult occlusion space. Moreover, the heterochrony of the enamel maturation and delayed final setting of the tooth design also allows a fine response of adult tooth design to the most derived rearrangements of the dental and oro-facial context (i.e. the rearrangements appearing late in ontogeny) and, hence, a very rapid and effective adaptive microevolution (within the functional limits of tribosphenic design).

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Title : Structural constraints on tribosphenic molar

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Abstract : The existence of stem cells for repair and renewal of epithelial tissues has been demonstrated for many years in different epithelia, or in organs with an epithelial component, such as lungs, mammary glands and hairs. Like many other organs in the vertebrate body, teeth develop from an epithelial and a mesenchymal component. Teeth are usually replaced during the lifetime of the animal (repeatedly in most non-mammals, but only once in most mammals, including part of the human dentition). Although epithelial stem cells have been reported in continuously growing teeth in rodents, they have never been associated with the process of repeated tooth renewal. Recently, we have hypothesized that epithelial stem cells are required for continuous tooth replacement in non-mammals (Huyseune & Thesleff, 2004). This hypothesis relies on several arguments, largely based on observations on the development of teeth in the zebrafish (*Danio rerio*), a widely used animal model: (1) the morphological resemblance between the epithelial crypts surrounding the erupted, functional teeth (and from which the replacement teeth bud off), and intestinal crypts (in vertebrates known to harbour epithelial stem cells); (2) the observation that first-generation teeth form *in vitro* whereas replacement teeth fail to do so; this coincides with failure of crypt formation and possible absence of stem cells; (3) the fact that initiation of replacement teeth appears to be under a different genetic control than that of first-generation teeth (Laurenti et al., 2004), (4) proliferation data in the enamel organ of developing replacement teeth in Cichlidae (a family of advanced bony fish) that are in accordance with what can be expected from stem cell progeny behaviour. Given the resemblance, at different levels, between hair and tooth formation, and given the importance of Wnt signaling for the cyclical replacement of hairs from stem cells, we furthermore hypothesize that putative epithelial stem cells in the pharyngeal epithelium in the zebrafish are under control of the Wnt signaling pathway. As an experimental model, the zebrafish undoubtedly will prove its value in future studies aiming to test the hypothesis of epithelial stem cell involvement and Wnt signaling in the process of continuous tooth replacement in non-mammalian vertebrates. Huyseune A, Thesleff I (2004). *BioEssays* 26: 665-671. Laurenti P, Thaeon C, Allizard F, Huyseune A, Sire JY (2004). *Dev Dyn* (in press).

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Title : Are epithelial stem cells involved in continuous tooth replacement in non-mammalian vertebrates? Lessons from the zebrafish.

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Abstract : The prevailing dogma of “one protein – one function” has been questioned in recent years and the concept of “Moonlighting” proteins - proteins which have multiple, seemingly unrelated functions which are not due to gene fusions or alternative splicing – is becoming increasingly accepted (Jeffrey, CJ. Trends Genet 19: 415-417 (2003)). The results of the human genome project, which suggested fewer genes in the human genome than was previously predicted, further supported this view. Examples of moonlighting proteins include key catabolic enzymes such as phosphoglucose isomerase and fumarate hydratase which also function as a cytokine/nerve growth factor and tumour suppressor protein respectively. Structural proteins are also known to moonlight. Crystallins in the lens of the eye, for example, have a wide range of moonlighting functions, including acting as heat shock proteins (Piatigorsky, J. Ann New York Acad Sci 842: 7-15 (1998)). Recent work has suggested that matrix proteins of dental tissues previously thought to play an exclusively extracellular role in odontogenesis may also have moonlighting functions, including acting as signalling molecules [eg. DSPP: MacDougall et al Connec. Tiss Res 39: 329-341 (1998); SIBLING matrix proteins: Jain et al J Biol Chem 277: 13700-13708 (2002)]. Amelogenin, long held to be a structural protein of the developing enamel matrix, has now been shown to have a possible signalling function in epithelial-mesenchymal conversations and to induce cytokine production by fibroblasts in vitro (Lyngstadaas et al J Clin Perio 28: 181-188 (2001)). The amelogenin splice product (LRAP) has also been shown to be osteoinductive (Veis et al J Biol Chem 275: 41263-41272 (2000)). However, methods capable of quantifying any interaction of amelogenin with target cells are limited. The aim of the present study was to develop methodologies based upon chemical force spectroscopy (CFM) to directly measure the actual forces of interaction between amelogenin molecules and target cells. Primary cultures of human periodontal fibroblasts were obtained by scraping residual ligament tissue from the roots of freshly extracted teeth. Full length amelogenin (M179, kindly supplied by Dr O Ryu, University of Texas, USA) was covalently attached to chemically functionalised silicon-nitride AFM tips (silanized using N'-(3-(trimethoxysilyl)-propyl)-diethylentriamine) via NHS activated carboxyamiloze spacers. Amelogenin-fibroblast force measurements were on the living cells after replacing the culture fluid with PBS in the Bioscope AFM (Digital Instruments). Preliminary results indicated that the softest AFM tips (up to 10 pN/nm) were necessary for carrying out the measurements. Analysis of the resulting force-distance curves indicated a Gaussian-like distribution of amelogenin-cell forces in the 200-800 pico-Newton range. Unmodified tips gave a much narrower distribution of forces at around 100 pN. CFM can provide a direct and quantitative measure of the forces of interaction between protein ligands and their target cells, potentially addressing the questions posed by multifunctional proteins and assisting in identification of receptor-mediated processes. The forces measured here for amelogenin-cell interactions approach characteristics of ligand-receptor binding in other systems though a great deal of further work, using different protein ligands and a range of target cells is still required. These preliminary data suggest that amelogenin may well be another example of a moonlighting matrix protein, perhaps explaining its apparent efficacy in the treatment of periodontal lesions with extracts of developing enamel matrix (EMDOGAIN) and the observed osteoinductive capabilities of its splice products. Supported by BBSRC.

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Title : Moonlighting and the Matrix: Amelogenin Multifunctionality?

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Abstract : Dioxins are widespread environmental pollutants that are developmentally toxic. The most toxic congener, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), can not only arrest early morphogenesis of rat and mouse embryonic molars but also interfere with mineralization of the dental matrices. Dentin sialophosphoprotein (Dspp), which is unique to dentin and a major component of dentin matrix, plays a key role in dentin mineralization. The aim of this study was to find out whether the retardation of dentin mineralization by TCDD could involve altered expression of the Dspp gene. E18 mandibular molars from NMRI mice were cultured in a Trowell-type organ culture for 5 days. After 2 days of culture the medium was changed and the explants were cultured for 3 more days in the presence of 1  $\mu$ M TCDD. The explants were examined stereomicroscopically, fixed with 4% paraformaldehyde, demineralized with EDTA and processed to serial paraffin sections. Some sections were stained with HE. Dspp mRNA was detected by non-radioactive in situ hybridization. After 5 days of culture, deposition of predentin was in progress in the first molar crowns and was slightly more advanced in control teeth than in TCDD-exposed teeth. Morphogenesis of the second molars had been completed. Dspp mRNA expression in odontoblasts coincided with predentin secretion and was also visible in ameloblasts but only in those facing the newly differentiated odontoblasts. In control explants, expression was intense throughout the zone of secretory odontoblasts but was gradually faded toward the cusp tips after TCDD exposure. TCDD decreased the overall intensity of Dspp gene expression, more in odontoblasts than ameloblasts. The results show that TCDD reduces Dspp transcripts in secretory odontoblasts and that the reduction is accelerated with the progression of dentinogenesis. This could contribute to the retardation of dentin mineralization observed earlier after TCDD exposure.

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Title : TCDD decreases dentin sialophosphoprotein expression in cultured mouse embryonic molars

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Abstract : The ultimate aim of odontogenetic research is to enhance the therapy of human dental diseases. Unfortunately, in most studies, mainly in such aimed on molecular regulation of tooth development, the mouse is used as a model species. Extrapolation of results obtained in the mouse to the human dentition is not easy due to the severe reduction of the tooth number and the highly specialised tooth shape in rodents. The domestic pig could represent a more appropriate model species for odontogenic research. Its dentition is not reduced (tooth formula 3.1.4.3/3.1.4.3), it contains two tooth generations, and all teeth are brachyodont as in the man. Studies on tooth development in the pig could particularly elucidate the spatial relationship between the dental laminae of the incisor and the cheek tooth region. This question cannot be answered based on studies on mouse odontogenesis, since in the mouse (in contrast to the human and the pig) a large diastema is present. The aim of our work was to assess the spatial relationship of the dental laminae in incisor and cheek tooth region in the pig. From the dental and the adjacent oral epithelium of both upper and lower jaw of pig embryos (ED 20 - 40), three-dimensional computer-aided reconstructions have been made. The first morphological signs of odontogenesis in the pig have been observed at ED 20. In the youngest embryos under study, two distinct epithelial thickenings could be detected in each half of the upper jaw - one in the prospective incisor region, one in the prospective cheek tooth region. During further development, these thickenings increased and connected to each other, so that a horseshoe shaped dental lamina developed. At least one tooth primordium originated from the incisor part of the dental lamina. In the lower jaw of the youngest embryos under study was detected a rather complex epithelial thickening consisting of an anterior (incisor) part and a posterior (cheek tooth) part, which were interconnected by a narrow epithelial lamina. From this thickening, both dental and vestibular lamina developed. In younger specimens, the incisor dental lamina was separated from the canine primordium, which represented the anterior limit of the cheek part of the dental lamina. However, incisor and cheek part joined secondarily. Studies on spatial distribution of molecules regulating segregation of incisor and cheek tooth part of the swine dentition could enhance our knowledge on determination of different tooth classes in other mammals than the mouse. This work was supported by the Grant Agency of the Czech Republic (grant No 206/04/P197) and the Ministry of Education, Youth and Sports of the Czech Republic (COST Programme B23, grant OC B23.001).

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Title : 3D reconstruction of the developing dentition in the incisor region of the pig

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Abstract : Teeth are formed through tissue interaction between dental epithelium and mesenchyme. In this study we characterized the rat maxillary incisor development. Histological observation shows that the maxillary incisor is composed by the fusion of medial and lateral primary dental laminae (M-PDL and L-PDL). This fusion is concomitant with fusion of facial process, medial nasal process (MNP) and maxillary process (MP), and the dental lamina forms at the boundary between MNP and MP. In order to investigate the contribution of MP to maxillary incisor we labelled the epithelium of MP with a fluorescent dye, DiI, before the fusion starts, and carried out whole embryo culture followed by maxillary organ culture. The result indicates that MP epithelium forms most lateral part of L-PDL. This is supported by observation of the homozygote of rat small eye mutant (rSey) that lacks lateral nasal prominence resulting in facial cleft between MNP and MP. The mutant develops PDL-structure at prospective fusion area of unfused MP. In addition, M-PDL and L-PDL in MNP do not fuse in the mutant, they stay at a distance. Eventually M-PDL proceeds incisor development; 25% of the homozygous L-PDL develop unilateral or bilateral incisor-like structure while the rest stop development at the bud stage. These results suggest that fusion of the facial processes contributes to maxillary incisor formation, to supply all components and possibly to assemble the components at one site. Defect in the fusion in the rSey mutant keeps MP epithelium from contributing to L-PDL formation and maintains M-PDL and incomplete L-PDL separated leading M-PDL and some of L-PDL to proceed further development.

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Abstract : The aim of this study was to explore root growth and eruption of M1 and M2. Data were collected from panoramic radiographs of healthy children aged between 3 and 15 years (N=447 boys, 377 girls). Developing molars were staged into root fractions (root length relative to crown length) and eruption stages (cusp tips relative to alveolar crest and occlusal level) and tooth length was also measured. More accurate measurements were collected for 67 boys and 75 girls (age range 7.2 to 13 years) using a digitiser; this included longitudinal data for 13 children. The distribution of root stages for each eruption level was calculated. The timing of root stages and eruption stages was calculated using probit analysis. The average rate of increasing root length for M2 was calculated as well as individual rate where longitudinal radiographs were available. Results suggest that variation in the timing of eruption stage is similar to root growth stage. For both molars the most frequent root stage at alveolar eruption was R1/4; when cusp tips were between alveolar and occlusal levels the most frequent root stage was R1/2. A small proportion of molars at R1/2 had reached the occlusal level. Girls reached all growth stages earlier than boys. The average ages of attainment of R1/4 and alveolar eruption were similar (between 4.2 and 4.7 years for M1, 8.9 and 9.4 years for M2). The average time for R1/2 and eruption level halfway to occlusal level, ranged from 5.4 and 5.7 for M1 and 9.9 and 11.3 for M2. Root stage R1/2 preceded the eruption stage cusp tips halfway to the occlusal levels by a few months for M1. For M2 this difference was 6 months for boys and 1 year for girls. The average increase in tooth length between 7.2 and 13 years was 0.80 mm per year for M2. Further analysis of longitudinal radiographs suggest the average rate of root growth in individuals for M1 was 2.4 (SD 0.48) mm per year and 2.2 (SD 0.62) for M2. These results highlight the large variation in timing of eruption and root growth in molars and the difficulty comparing growth data from cross-sectional and longitudinal studies.

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Title : Eruption and root growth of first and second permanent mandibular molars.

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Abstract : Previous studies have implicated noncollagenous proteins in the regulation of extracellular matrix mineralization in skeletal and dental tissues, and also in pathologic calcification. While much of this work was initially obtained from in vitro investigations and from immunolocalization data on tissue sections, recent genetic manipulations in transgenic mice have unambiguously identified several potent inhibitors of biomineralization in vivo. More specifically, we have shown that constitutive expression and secretion of matrix Gla protein (MGP) inhibits calcification of arteries and cartilage - genetic ablation of the Mgp gene in mice results in massive calcification of the extracellular matrices found in arteries and cartilage (Luo et al., 1997, Nature 386:78-81). To determine its mineral-inhibiting potential in physiologically mineralizing tissues, MGP was ectopically expressed in bones and teeth of mice using an osteoblast/odontoblast-specific 2.3kb proximal promoter for the alpha 1 chain of type 1 collagen. Mandibles and long bones of these Coll1a1-Mgp mice and wild-type littermates (1-4 weeks of age), were analyzed by Faxitron<sup>TM</sup> radiography (FR), Piximus<sup>TM</sup> dual-energy x-ray absorptiometry (DEXA) and micro-computed tomography ( $\mu$ CT). In addition, light microscopy and transmission electron microscopy were performed. While bone and tooth extracellular matrices appeared normally established in Coll1a1-Mgp mice in terms of deposition of an otherwise normally structured extracellular matrix, examination of mineralization by FR, DEXA and  $\mu$ CT, together with histological mineral localization by light microscopy after von Kossa staining of undecalcified tissue sections, and by electron microscopy, revealed extensive hypomineralization of bone and tooth extracellular matrices. In the skeleton of Coll1a1-Mgp mice, alveolar bone of the mandible was most heavily affected (compared to long bones), showing a 50% increase in the unmineralized osteoid volume compared to wild-type littermates. For teeth, mineralization was virtually absent in root dentin of both incisors and molars, and absent in molar cellular cementum, whereas crown dentin showed sporadic, localized "breakthrough" areas of mineralization. Acellular cementum formation and mineralization was absent in the Coll1a1-Mgp mice. Immunohistochemical staining of bone and tooth extracellular matrix proteins in Coll1a1-Mgp mice showed variations in staining relative to wild-type tissues, with immunostaining generally restricted to areas of mineralization. In conclusion, these results confirm in vivo that secreted proteins (in this case MGP), when released into the extracellular matrix, can act as inhibitors of bone and tooth mineralization. [Supported by the Canadian Institutes of Health Research, the Canadian Arthritis Network of Centres of Excellence and the National Institutes of Health]

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Title : Ectopic expression of matrix Gla protein in transgenic mice inhibits mineralization of bone and tooth extracellular matrices

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Abstract : In mammalian tooth development, tooth root continues to expand after completion of tooth crown formation until it reaches proper length in postnatal stage. It is believed that Hertwig's root sheath elongates itself to initiate root development and induce dental papilla cells to differentiate into odontoblast, however, the detailed molecular mechanism of tooth root formation is still unknown. The tooth root formation process mainly contains cell proliferation and cell differentiation. First, we examined cell proliferation during tooth root formation by BrdU labeling experiment and anti-PCNA immunostaining. The BrdU (+) and PCNA (+) cells were observed at the tip of Hertwig's epithelial root sheath and surrounding mesenchyme. Next, we examined the expression of tumor-related genes, Patched1 (Ptc1) and Twist1, which are previously reported in developing dental mesenchyme and also known to be expressed in many types of undifferentiated cells during developmental process. The transcripts of both Ptc1 and Twist1 were identified in the epithelial diaphragm at the tip of developing epithelial root sheath and pulp proliferation zone, and their signals decreased in differentiated odontoblast. This is the first description of Ptc1 and Twist1 expression in postnatal tooth development, which suggests that they possibly support tooth root formation to maintain the cell viability and undifferentiated-state in this region. It is recently reported that Ptc1 regulates Shh-pathway and Twist1 is involved in Wnt-pathway in vitro, and further analysis will focus on these pathways.

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Title : The expression pattern of Patched1 and Twist1 in postnatal tooth development

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Abstract : Understanding of the molecular basis for tooth agenesis in humans gives insights into the developmental mechanisms of teeth and dental patterning. PAX9 and MSX1, mutations in which cause isolated oligodontia, as well as several genes mutated in syndromic tooth agenesis, participate in the signaling pathways that regulate development. However, gene mutations identified so far explain only a minority of cases of severe types of tooth agenesis (oligodontia), and the molecular basis for hypodontia, one of the most common developmental malformations in man, is virtually unknown. We have recently described genetic mapping of oligodontia in a Finnish multigeneration family and subsequent identification of mutations in AXIN2, an intracellular regulator of the Wnt signaling pathway. The inactivating mutations were identified in patients with a tooth agenesis pattern distinct from earlier findings. In most severe cases only three permanent teeth had developed while deciduous teeth were seldom affected. In subsequent colorectal examination, neoplasia was found in most of the family members with the AXIN2 mutation. The finding underlines the commonalities of developmental pathways and regulation of tissue homeostasis and suggests that in some cases tooth agenesis may be an indication of cancer predisposition. It also connects Wnt signaling to tooth development in humans. The failure of permanent tooth development caused by inactivating mutations in AXIN2 presumably follows from stimulated Wnt signaling whereas experimental inactivation of Wnt signaling in mouse cause an early arrest in tooth development. We suggest that intracellular attenuation of Wnt signaling may be especially necessary for the development of secondary dentition.

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Title : Failure of regulation of Wnt signaling causes tooth agenesis and colorectal cancer

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Abstract : The primary enamel knot is considered as a signaling center during tooth morphogenesis (Jernvall et al. 1994). At the cap stage, it can be distinguished from other parts of the enamel organ by showing a specific morphological appearance (densely packed cells), very few cell divisions and the presence of apoptotic cells (Jernvall et al. 1994; Lesot et al., 1996, 1999). In molar tooth germs, the cells of the primary enamel knot in contact with the basement membrane are later redistributed to be localized at the tip of the forming cusps. These segregating groups of cells were suspected to play a role in cusp formation and described as Organizers of Morphogenetic Units (OMUs) (Coin et al., 1999). However, the process and mechanisms of the redistribution of the OMUs are not well understood yet. To investigate it more in detail, we analyzed the distribution of non-dividing cells and apoptotic cells in the mouse first molar tooth germs from the cap to the early bell stag!

e. The histological organization of the inner dental epithelium and the enamel knot was visualized after immunostaining for P-cadherin, a cell surface marker for these cells, on the same sections. In the tooth germs at ED14, the cells lining the enamel knot at its lateral sides and also at the posterior end transiently showed strong incorporation of BrdU. At ED15, the non-dividing area laterally bifurcated at its posterior end. At this position, the mitotic activity in the lingual half of the enamel organ was higher than in the buccal part. In the P-cadherin-positive domain around the lingual non-dividing area, a significant number of cells were BrdU-positive. However, the buccal area was entirely negative for BrdU-incorporation. At ED15, most of the inner dental epithelium visualized by P-cadherin-immunostaining was pseudo-stratified except in the posterior portion. At ED16, the non-dividing area laterally extended in the anterior part of the tooth germ and the state of stratification differentially changed within the inner dental epithelium. As a result, the inner dental epithelium at ED 16 remained pseudo-stratified in some areas and organized as a monolayer in others. Both the alteration of the histological structure and the differential cell proliferation might account for the extension of the inner dental epithelium and the segregation of the OMUs.

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Title : Cell proliferation and structural change of the inner dental epithelium and the enamel knot of the molar tooth germs during the cap to bell transition.

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Abstract : Amelogenesis Imperfecta (AI) is a clinically and genetically diverse group of hereditary conditions characterized by quantitative or qualitative abnormalities of dental enamel. Non syndromic forms of AI are the most prevalent however, syndromic forms have also been reported. A rare syndrome associating amelogenesis imperfecta with nephrocalcinosis, precipitation of calcium salts in the kidney has been reported in very few families. The purpose of the present study is to describe a consanguineous family with smooth hypoplastic enamel, delayed permanent teeth eruption and nephrocalcinosis. The histopathological features of a deciduous tooth and a biopsies enlarged dental follicles were also studied. For this purpose a complete dental and medical examination was performed, ground sections of two deciduous teeth and paraffin embedded cross sections of enlarged dental follicles biopsies were observed under light microscopy. A thirteen year old male patient was referred to the Dental Anomalies Clinic of University of Brasilia, Brazil with a presumptive diagnosis of autosomal recessive AI. The case index as well as his only sister and parents were examined. The parents were cousins in the first degree. Either parent or sister had enamel defect or any systemic disease. The dental examination of the case index showed shape alteration of the upper central incisors, yellow discoloration, retention of deciduous teeth and delay of the permanent teeth eruption. Panoramic radiographs revealed intrapulpal calcifications in the mandibular molars and the multiple pericoronal enlarged dental follicles in all impacted permanent teeth. Renal ultrasound showed the presence of nephrocalcinosis, serum levels of calcium and phosphate appeared normal but phosphatase alkaline was slightly decreased. Histologically the enamel appeared hypoplastic, and the biopsy of dental follicles suggested hyperplastic dental follicles. Some of the clinical manifestations of the AI/nephrocalcinosis previously reported were observed in the present case. However, the presence of delayed eruption due to presence of hyperplastic dental follicles has not been reported before. Further studies are necessary clarify the genetic defect behind the association of AI, nephrocalcinosis and impaired tooth eruption.

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Title : Amelogenesis Imperfecta, delayed tooth eruption and nephrocalcinosis in a consanguineous family. A morphological study.

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Abstract : In Tabby mice the Ta (Eda in humans) gene is not expressed, resulting in a syndrome that is homologous to hypohidrotic ectodermal dysplasia in humans. Among the characteristics of the Tabby phenotype are aberrations in number and morphology of the teeth, both molars and incisors. The effect on the structure of enamel, however, has not been investigated. It was the aim of the present study to describe the enamel phenotype in Tabby incisors, with emphasis on its distribution, thickness and structure. All four incisors were obtained from five female Tabby mice and from three female wild type mice. The incisors were sectioned and ground transversely at a level just apical to the abraded incisal part, etched for 45 seconds with 0.1% nitric acid, and sputter-coated with gold-palladium. The transverse surface was observed in SEM. All measured dimensions were more variable, often considerably more, in Tabby mice than in wild type mice. Also the outline of the enamel-dentin junction was more variable in Tabby mice, the mesial concavity was often reduced or lacking. Maxillary incisors were wider in Tabby mice, while mandibular incisors were wider in wild type mice. No significant difference in enamel thickness between Tabby and wild type mice was observed. The enamel on the mesial aspect tended to extend further lingually in Tabby mice than in wild type mice both in maxillary and in mandibular incisors. On the lateral aspect this tendency was only significant in mandibular incisors. In one Tabby mouse the enamel on the mandibular incisors extended as a thin layer far beyond the bends in the dentin contour that normally coincide with enamel termination, both mesially and laterally on the right incisor and mesially on the left incisor. In this most affected mouse the right maxillary incisor was abortive with no enamel, while the left maxillary incisor was relatively small. Instances of hypoplastic enamel were observed. The complex mouse enamel structure was generally well preserved in the Tabby mice, only few instances of aberrant structure were observed. Based on observation of the enamel crystals in the SEM, slight hypomineralization of the enamel was present in about 60% of the Tabby incisors. A major effect on incisor morphology in Tabby mutants seems to be a variation in mesial and, to a lesser extent, lateral enamel termination. Since the Ta gene during tooth development is only expressed in the outer enamel epithelium, it may be speculated that in Tabby mice the transition from inner to outer enamel epithelium is less well defined, resulting in a less well defined termination of enamel.

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Title : Distribution and Structure of Dental Enamel in Incisors of Tabby Mice

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**Abstract :** Introduction: Interaction between extracellular matrix proteins and apatite crystals is considered to be the main mechanism by which crystal growth rate and morphology are modulated in the skeletal tissues. In enamel it has been shown that amelogenins, (90% of the matrix) are able to interact with regular positively charged bands on crystal surfaces (1). While removal of such matrix proteins is necessary for crystal growth (2), the mechanism of protein removal is still unclear. The chemistry of the mineral surface and its interaction with matrix proteins is therefore of high significance. During enamel maturation, after the full thickness of tissue has been laid down, matrix proteins are almost completely removed from the tissue presumably after their removal from crystal surfaces. During this stage, tissue pH apparently oscillates between ~7.2 and 6.8 (3). Approach: To investigate possible implications of local pH on matrix removal, a study of apatite surface chemistry in terms of local pH was undertaken. Using Atomic Force Microscopy with cantilever tips functionalised with hydroxyl or carboxyl groups, adhesion force measurements between hydroxylated or carboxylated cantilever tips and enamel apatite surfaces were made over the range pH 2 to pH 10. The tips were brought into contact with isolated enamel crystals and the adhesion force required to withdraw the tips from the surface was measured. Results: Cantilever tip adhesion increased from pH 10 to a maximum at pH 6.6 and decreased thereafter down to pH 2. These effects were most likely due to increasing hydrogen bonds due to progressive replacement of surface cations (Na, Ca) by protons and protonation of surface phosphates. Below pH 6.6 very variable results were obtained indicating that the surface had become unstable, probably due to removal of loosely held dihydrogen phosphate from the surface by adherence to the cantilever tip. Frictional force studies indicated that below pH 6.6, protonation and accompanying instability had occurred selectively

on the bands previously identified as protein binding sites, each band revealing 2- 3 positively charged spherical domains each ~30nm dia. The location of these spherical domains mirrored the binding pattern of similarly sized amelogenin aggregates seen in vivo (ibid) and in vitro (4) suggesting that hydrogen bonding may play a role in protein attachment. The lowest enamel tissue pH, 5.8 - 6 occurs in juxtaposition with the ruffle ended ameloblasts which are believed to remove protein. While local conditions such as ionic strength cannot be known precisely, the data suggests that protonation of the enamel crystal surfaces produces a surface instability which may play a role in releasing protein which is then removed from the tissue by ruffle ended ameloblasts. Both removal of protein and reduction of surface energy (instability) would predispose the crystals to grow as mineral is pumped into the tissue at this site and the pH oscillates to ~7.2. Conclusion: The fall in pH during enamel maturation and protein removal destabilizes crystal surfaces such that detachment of protein could occur facilitating protein degradation and removal and subsequent crystal growth. 1. Kirkham J, Zhang J, Brookes, SJ, Shore RC, Ryu OH, Wood SR, Smith DA, Wallwork ML, and Robinson C, J.Dent Res., 2000, 79,(12),1943. 2. Robinson,C.,Kirkham,J.,Stonehouse, NJ and Shore,RC.Connect Tiss Res 1989, 22,139. 3. Sasaki S, Takagi T, Suzuki MT, Arch Oral Biol. 1991,1 ,33:159. 4. Wallwork ML, Kirkham J, Zhang J, Smith DAM, Brookes SJ, Shore RC, Wood SR, Ryu O & RobinsonC.Langmuir,2001,17.pp2508.

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Title : Surface Chemistry of Apatite in Developing Enamel:Implications for Matrix Protein Binding and Crystal Growth

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Abstract : Evolutionary reduction of the vertebrate dentition is a frequent phenomenon, while regain of lost teeth is rare. We have used the loss of oral teeth in cypriniform fishes as a model to investigate whether this trend is the result of selection, ease of tooth loss relative to gain, or a combination of the two. Despite a diversity of species and feeding modes, as well as the retention of teeth in the pharyngeal region, no cypriniform lineage has re-acquired oral teeth in the 55 million year history of the clade. To identify whether the developmental genetic mechanism of cypriniform tooth loss has rendered it irreversible, we compared gene expression and function in a representative species, the zebrafish (*Danio rerio*) and one of its closest relatives retaining oral teeth, the Mexican tetra (*Astyanax mexicanus*: Characidae). We first examined Fibroblast growth factor (FGF) signaling from the odontogenic epithelium to the underlying mesenchyme, an important component of tooth initiation in the mouse. We found that elements of this signaling pathway, including the epithelial ligand *Fgf8* and the mesenchymal transcription factor targets *Pax9*, *Lhx6*, and *Lhx7* are expressed similarly in the oral regions of *A. mexicanus*, the zebrafish, and the mouse. Furthermore, application of the FGF Receptor inhibitor SU5402 revealed that these transcription factors are indeed targets of FGF signaling in the zebrafish. These data suggest that FGF signaling from the oral epithelium to the mesenchyme occurs in the zebrafish. We next examined the expression of orthologs of the mammalian dental lamina markers *Pitx2*, *Shh*, and *Dlx2*. *Pitx2* and *Shh* were expressed in the oral epithelium of both species. In contrast, expression of the two fish orthologs of mammalian *Dlx2* (*dlx2a* and *dlx2b*) was present in dental epithelium of *A. mexicanus*, but was completely absent from the oral epithelium of the zebrafish. As *dlx2a* and *dlx2b* are likely related by genome duplication, loss of their expression from oral epithelium in the zebrafish lineage is explained more parsimoniously by evolutionary change in a common upstream regulator than by changes in the regulatory regions of each. Application of SU5402 to *A. mexicanus* indicates that the expression of *dlx2a* and *dlx2b*, but not that of *pitx2*, is dependent on FGF signaling; moreover, treated individuals failed to form oral teeth. These data identify loss of FGF signaling to the dental epithelium as a candidate cause of absence of oral teeth in cypriniform fishes. Gain-of-function studies should provide a test of this hypothesis and may yield insight into the potential for re-acquisition of oral teeth in cypriniforms.

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Title : Genetic changes associated with reduction of the dentition in cypriniform fishes

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**Abstract :** The regeneration process of the dental pulp after tooth replantation was investigated by use of microcomputed tomography (micro-CT), immunohistochemistry for heat shock protein (HSP)-25, and histochemistry for both alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP). Under deep anesthesia, the upper right first molar of 4-week-old Wistar rats was extracted and then immediately repositioned into the original socket. The first molar on the opposite side was used as a control. The animals were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at the intervals of 1, 3, 5, 7, 10, 12, 14, 28, 60 days after replantation. The maxillae including both the replanted and control teeth were removed en block and overall changes occurring in the dental pulp and periodontium following tooth replantation were evaluated by micro-CT before tissue preparation. Following decalcification in 10% ethylenediaminetetraacetic acid (EDTA) solution, the specimens were cut sagittally with a cryostat for both immunohistochemistry and histochemistry. Micro CT clearly demonstrated that at least two types of healing processes, i.e. tertiary dentin or bone-like tissue formation, occurred in the dental pulp, and that the root elongation of the replanted tooth stopped after operation. In control teeth at postnatal 4 weeks, the odontoblasts showed intense ALP- and HSP-25-positive reactions in the coronal dental pulp, whereas no TRAP-positive cells occurred there. Tooth replantation weakened or ceased ALP-positive reactions in the dental pulp during postoperative days 1-3, whereas HSP-25-positive reactions remained in the degenerated odontoblast layer. Three to seven days after operation, ALP-positive region recovered from the root apex to the coronal pulp according to the revascularization followed by HSP25-positive reactions in the successful cases. In the other cases, TRAP-positive cells appeared in the dental pulp of replanted tooth where frequently root resorption concomitantly occurred in the periodontal side, and subsequently ALP-positive cells appeared in the area including TRAP-positive cells. After 12-60 days, tertiary dentin and/or bone-like tissue formation were clearly recognized in the dental pulp of replanted teeth. Furthermore, comparison between the time needed for the operation and the susceptibility to root resorption demonstrated that the retardation of the operation time for tooth replantation easily induced root resorption. These data suggest that the pattern of ALP- and TRAP-positive reactions reflects the healing pattern of the dental pulp following tooth replantation and that the appearance of TRAP-positive cells in the dental pulp may be a trigger to induce bone-like tissue formation in the dental pulp. On the other hand, the alignment of ALP- and HSP-25-positive odontoblast-like cells along the pulp-dentin border is suggestive of the decisive factor to induce the tertiary dentin formation after tooth replantation.

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**Title :** Hard Tissue Formation in the Dental Pulp during the Regeneration Process after Tooth Replantation in Rat Molars

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Abstract : The specific small amelogenins splice isoforms composed of exons 2,3,4,5,6d,7, and exons 2,3,5,6d,7 (designated A+4 and A-4, respectively) have been shown to have cell signaling activity in both in vitro and in vivo assays. However, these quite similar proteins have distinct effects. In vitro, A+4 induced expression of Sox9, while A-4 unregulated Runx2. In vivo, A+4 induced dentinal bridge formation when implanted into rat molar pulpal perforations. A-4 implanted similarly induced a generalized mineralization of pulpal tissue. The purpose of this study was to determine the mechanisms of the observed signaling by A-4, beginning with cell binding. <sup>3</sup>H-labeled [A-4], prepared by N-terminal acetylation of r[A-4] with tritiated acetic anhydride, was used in cell binding assays of C2C12 mouse myoblast cells at both 40 and 37°C. At 40°C, a dose-dependent saturable binding was observed. The binding data were plotted via Scatchard analysis to determine both KD, and the approximate number of receptors per cell. Binding at 37°C indicated that radiolabeled A-4, in addition to surface binding, was also internalized. An [A-4] affinity column was prepared, and C2C12 mouse myoblast cell membrane proteins were isolated and passed over the affinity column. 2 separate affinity column chromatography methods indicated that a 95 KD membrane protein, was bound. Ligand-affinity labeling immunohistochemistry with biotin labeled [A-4] was performed on E18 mouse developing incisors and first molars. Binding was evident in the incisor labially in both developing odontoblasts and ameloblasts. The staining was extinguished at the point where mineralized dentin was evident. Staining was seen in the stratum intermedium up to the cusp tip. Interestingly, staining was also robust in ameloblasts at the tip of the incisor in the enamel-free area. In the molar, staining was seen in the polarizing odontoblasts and ameloblasts at the cusp tip, and decreasing apically. Staining was also in the SI, but not in the stellate reticulum. The cells of the dental follicle surrounding the tooth germ were also stained. We conclude that labeled [A-4] binds to the cell surfaces of C2C12 mouse myoblasts, and also to ameloblasts, odontoblasts, and follicle cells in the developing mouse tooth to initiate its signaling activity. (Supported by NIDCR Grant DE-08525)

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Title : The 59 Amino Acid amelogenin splice isoform [A-4] (LRAP) binds to mouse myoblast cells and to odontoblasts, ameloblasts and stratum intermedium cells of the developing mouse tooth.

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Abstract : The Eretmodini, a tribe of closely related cichlids originating from Lake Tanganyika, possess oral tooth shapes ranging from conical (in *Tanganicodus*) over cylindrical (in *Spathodus*) to spatulate (in *Eretmodus*). Furthermore, during ontogeny tooth shape in *E. cf. cyanostictus* (lineage A) changes from conical in first-generation teeth to spatulate in adult teeth. In order to better understand how these distinctly different tooth shapes can be acquired in such closely related taxa, we performed a detailed histological study on the shape changes occurring within the enamel organ in different taxa of eretmodines. In teleosts, the shape in which this epithelial structure folds during tooth development foreshadows the shape of the fully developed tooth. Tooth formation in adult *E. cf. cyanostictus* (lineage A) roughly corresponds with what is known for teleost tooth development in general. Remarkable features include the localization and shape of the epithelial downgrowth, the presence of a layer intermediate between IDE and ODE, the asymmetric shape of the enamel organ and the steady retraction of the pulp cavity, and finally, the pattern of matrix mineralisation and the presence of pigment. Starting from camera lucida drawings of serial sections of eretmodine jaws, 3D reconstructions were made of tooth germs in different ontogenetic stages of *E. cf. cyanostictus* (lineage A) and of germs in different developmental stages in adult *E. cf. cyanostictus* (lineage A) and *T. cf. irsacae* (lineage E). In juveniles, tooth shapes range from teeth whose enameloid cap extends into a finger-like process over teeth with an oblong enameloid cap, to teeth with a tip that foreshadows the adult, spatulate shape. In adult *E. cf. cyanostictus* (lineage A), there is no conical 'precursor' that becomes gradually modified into a spatulate-shaped tooth. Our observations indicate that the ingrowth of the enamel organ proceeds in an asymmetrical way, leading to a more advanced state of differentiation along one side, and resulting in an asymmetric matrix deposition starting in the "corner" of the future tooth crown. In contrast, in *T. cf. irsacae* (lineage E), which possesses conical-shaped oral teeth, the formation of the tooth crown begins at the tip of the tooth and continues symmetrically. Based on the observations in both juvenile and adult *E. cf. cyanostictus* (lineage A) we propose a model on how the ingrowth (folding) of the enamel organ changes between conical and spatulate shapes.

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## NEMO and Friends: The IKK Complex in Tooth Development

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The transcription factor NF $\kappa$ B plays an important role in many physiological processes and in recent years it has become apparent that this includes the development of ectodermal organs such as teeth, sweat glands and hair. In non-stimulated cells NF $\kappa$ B is sequestered in the cytoplasm by I $\kappa$ B. Activation in response to an external stimulus involves the I $\kappa$ B kinase (IKK) complex which phosphorylates I $\kappa$ B causing the release of NF $\kappa$ B into the nucleus. The IKK complex comprises the catalytic subunits IKK $\alpha$  and IKK $\beta$  along with the regulatory subunit IKK $\gamma$  (also known as NEMO). We have previously shown that mutations in IKK $\alpha$  result in molar teeth with abnormal cusps, further implicating NF $\kappa$ B in cusp morphogenesis, and also an early incisor phenotype which appears to be NF $\kappa$ B independent. We have now further investigated the IKK complex in tooth development by using both gain and loss of function approaches to analyse the roles on IKK $\beta$  and IKK $\gamma$ .

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In early 1990s, tissue engineering emerged as a concept to regenerate biological tissues by ultimately seeding the patient's own tissue-forming cells in biocompatible polymers. A decade-long intense effort in tissue engineering has convincingly provided the proof of principle for cell-based regeneration of a number of individual tissues such as the skin, bone, and skeletal muscles. Recent work in stem-cell based restoration of multiple tissue phenotypes by composite tissue constructs such as osteo-chondral, fibro-osseous, and musculo-osseous grafts has offered clues for biological replacement of complex anatomical entities consisting of multiple cell lineages such as the synovial joint condyle, musculo-tendon complex, bone-ligament junction, the teeth and the periodontium. Of greater significance is a tangible contribution by current attempts to restore the structure and function of complex, multi-tissue structures using stem cell-based composite tissue constructs to the understanding of bioengineered restoration of complex organs such as the kidney or liver. Two examples will be provided in this seminar to demonstrate the current status of our knowledge regarding stem cell-based regeneration of a human-shaped articular condyle, and a generic fibro-osseous construct that may serve as the proof of principle for cranial sutures, ligaments, tendons and the periodontal ligament. In each example, adult stem cell derived tissue forming cells are seeded or encapsulated in biocompatible and biodegradable polymer scaffolds tailored into given shape and dimensions to accommodate target natural tissues. *Ex vivo* and *in vivo* approaches are coupled to manipulate cells and polymers in a reverse engineering process with an end goal that the tissue-engineered osteochondral and fibro-osseous tissues gradually approach the structural and mechanical properties of the target natural tissues.

Abstract : The morphological events associated with root formation have been extensively studied; however, our understanding of the biological mechanisms involved in root formation remains unclear. Root formation anomalies are found in dentin dysplasia type I (OMIM 125400), which is an autosomal dominant condition. At present, no root formation anomalies have been reported as autosomal recessive conditions. Recently, it was reported that nuclear factor I-C transcription-replication factor (NFI-C) knock-out mice showed as prominent phenotype the absence of molars root formation and alterations in the shape and size of the incisors. The aim of the present study was to describe the clinical manifestation of two consanguineous families that were referred to the Dental Anomalies Clinic, University of Brasilia, Brazil. A complete dental and medical examination was done and in the both families the parents were cousins in first degree. The index case, parents and siblings were examined and no dental anomalies or systemic disease were observed. In family 1, the case index, a 12 year old boy has been followed up since the age of 5 because of the early exfoliation of the deciduous and permanent teeth. No systemic disease or syndrome were detected, the only clinical manifestation observed was the early loss of teeth. Panoramic X-ray showed small crowns of the deciduous and permanent teeth and complete absence of all roots. Histological observation of demineralized permanent molars sections did not show any dentin abnormality. At present the patient loss all his permanent teeth except for two lower very small third molars which remain intraosseous. The case index in the family 2 is a two and a half year old boy with no other alteration other than root formation defects. The incisors are more affected than the molars which have one third of the root formed. The pedigrees both families suggest a recessive mode of inheritance in this uncommon condition.

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Abstract : Cleidocranial Dysplasia (CCD) is an autosomal dominant disorder characterized by abnormal skeletal genesis and dental disorders. Mutations in the runt-related transcription factor 2 (RUNX2) are associated with this disorder. Alteration of gene expression in the CCD dental phenotype remains unknown. Objective: The purpose of this study was investigate CCD dental pulp cells versus age and sex matched controls related to: 1)cell morphology and proliferation; 2) altered gene expression profiles to identify downstream target genes of Runx2; and 3) rescue of the altered CCD gene expression profiles by over-expression of Runx2 or Osterix. Methods: Cell morphology and proliferation were observed and counted. To determine the effect of a Runx2 mutation on the production of down-stream target gene expression a DNA microarray analysis was performed. RNA was extracted from the both CCD and normal dental pulp cells, converted to 33P-cDNA labeled probes and hybridized to a human cytokine/receptor array. Results: The CCD dental pulp cells are flat, larger (4-fold) than that of the normal dental pulp cells. In contrast, the cell proliferation rates of the normal dental pulp cells were two- to three-fold greater than the CCD cells at the time points tested. Among the 266 genes analyzed in DNA microarray, 24 genes were up-regulated by 2-fold or greater, and 17 genes were down-regulated by 2-fold or greater. Expression of selected genes was further analyzed by quantitative reverse transcriptase polymerase chain reaction (RT-PCR). The RT-PCR data confirmed several of the significant differences. Several of genes such as bone morphogenetic protein 2 (BMP2), leukemia inhibitory factor are involved in cell cycle, proliferation as well as tooth development. Overexpression of Runx2 or Osterix in the CCD dental pulp cells was able to partially rescue the down-stream gene expression profiles. Conclusion: This study indicates that altered Runx 2 gene expression associated with CDD in dental pulp cells alters the expression levels of a broad number of down-stream genes. These results suggest that such alterations may combine to contribute to the dental phenotype associated with CCD. This work was supported by NIDCR grant DE P01 DE13221.

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## Dual role of Gas1 during mouse tooth development

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In the developing mouse mandible, localized expression of Sonic hedgehog (Shh) marks the sites of future tooth development. Evidence suggests that Shh mitogenic activity is responsible for localised epithelial proliferation in the early tooth thickenings and therefore, tight control of Shh expression/activity plays a key role in the positioning of future teeth. Taking advantage of the mouse diastema, a toothless area situated between molars and incisors, we have previously shown that at the epithelial thickening stage, Shh protein diffusing from the molar and incisor tooth buds is rendered inactive in this non-odontogenic area to prevent tooth formation. The diastema epithelium plays an important role in restricting Shh activity in diastema mesenchyme. Indeed, the Shh target genes *Patched1* (*Ptc1*) and *Gli1* are ectopically induced in the diastema mesenchyme in the absence of epithelium. There is strong evidence to suggest that Gas1, a GPI-linked membrane protein that binds Shh, antagonises Shh activity in the diastema mesenchyme, since *Gas1* expression in the diastema depends on the overlying epithelium, and restoration of *Gas1* expression in the diastema of mandibles deprived of their epithelium prevents *Ptc1* and *Gli1* ectopic expression. We have examined *Gas1* expression at later stages of tooth development and show that it is restricted to the mesenchyme. In order to investigate if *Gas1* also functions as a Shh antagonist at the bud stage of tooth development, we have ectopically expressed *Gas1* in tooth buds or overexpressed it in the mesenchyme. An unexpected upregulation of Shh target genes was observed, which suggests that Gas1 may have a dual role in odontogenic and non-odontogenic tissues of the mandibular process.

## **Patterning of the first pharyngeal arch is regulated by embryonic cell origin**

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The mesenchyme of the embryonic jaw primordia is patterned by spatially restricted signalling molecules such as FGF8 and BMP4. These molecules are secreted from the oral epithelium. These signals regulate the spatial expression of homeobox genes such as *Msx1, 2, Dlx's* and *Barx1* that provide cells with their positional cues. This positional information determines the morphogenic fates of cells to develop into teeth of different shapes. The initial pattern of expression of *Fgf8* and *Bmp4* in the oral ectoderm thus provides the basis for subsequent tooth cusp morphogenesis. An understanding of how the oral ectoderm is patterned is thus critical to understanding dental patterning. One hypothesis is that the epithelial cells in the distal oral mandible are fundamentally different from those in the proximal and that this might be the result of a different developmental history. We have fate thus mapped the proximal and distal oral epithelium in the developing chick embryo and shown that the cells that give rise to the proximal oral epithelium and distal epithelium are segregated prior to pharyngeal arch development. We also have shown that removal of the embryonic endoderm early in development disrupts *Fgf8* gene expression, indicating that the early expression of *Fgf8* in the pharyngeal arch is regulated by signals arising from the endoderm.

Abstract : Teeth are formed through tissue interaction between dental epithelium and mesenchyme. In this study we characterized the rat maxillary incisor development. Histological observation shows that the maxillary incisor is composed by the fusion of medial and lateral primary dental laminae (M-PDL and L-PDL). This fusion is concomitant with fusion of facial process, medial nasal process (MNP) and maxillary process (MP), and the dental lamina forms at the boundary between MNP and MP. In order to investigate the contribution of MP to maxillary incisor we labelled the epithelium of MP with a fluorescent dye, DiI, before the fusion starts, and carried out whole embryo culture followed by maxillary organ culture. The result indicates that MP epithelium forms most lateral part of L-PDL. This is supported by observation of the homozygote of rat small eye mutant (rSey) that lacks lateral nasal prominence resulting in facial cleft between MNP and MP. The mutant develops PDL-structure at prospective fusion area of unfused MP. In addition, M-PDL and L-PDL in MNP do not fuse in the mutant, they stay at a distance. Eventually M-PDL proceeds incisor development; 25% of the homozygous L-PDL develop unilateral or bilateral incisor-like structure while the rest stop development at the bud stage. These results suggest that fusion of the facial processes contributes to maxillary incisor formation, to supply all components and possibly to assemble the components at one site. Defect in the fusion in the rSey mutant keeps MP epithelium from contributing to L-PDL formation and maintains M-PDL and incomplete L-PDL separated leading M-PDL and some of L-PDL to proceed further development.

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## **How Protein-Protein Interactions Affect the Function of Key Transcription Factors That Control Normal and Abnormal Patterning of Dentition**

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The legacy of inheritable anomalies involving human dentition offers a powerful system for studying the genetic pathways that control patterning and cell differentiation during tooth development. Driven by a long-standing interest in understanding how the selective functions of transcription factors are precisely regulated in tooth morphogenesis and cell differentiation, we have analyzed two conditions with opposite defects in dentition. The first genetic disorder is non-syndromic tooth agenesis, a condition where mutations in genes like *PAX9*, *MSX1* and *AXIN2* result exclusively in the congenital absence of teeth. The second, cleidocranial dysplasia, is a disorder caused by mutations in *RUNX2*, and one that results in phenotypic changes in the craniofacial skeleton as well as supernumerary teeth. The fundamental goal of our research is to improve our understanding of how these patterning defects arise in dentition. This talk will summarize important data from our recent experiments that unify human and mouse genetic studies with biochemical and molecular approaches. Our genetic analyses of several kindred affected with similar patterns of tooth agenesis reveal new mutations in *PAX9* and *MSX1*. Intriguingly, in certain families/individuals combinatorial effects of coexisting alterations in *PAX9* and *MSX1* suggest the possibility that the two genes may interact on the transcriptional and/or post-transcriptional levels. Our more detailed structure-function studies and biochemical analysis confirms a partnership between *PAX9* and *MSX1* and offers an explanation of the relationship between genotype and phenotype in tooth agenesis. Our studies of *Runx2* also point to the importance of its interactions with another nuclear protein partner in mediating key events in tooth morphogenesis as well as in the control of the onset of odontoblast terminal differentiation. Collectively, our results provide new and compelling hypotheses about the importance of protein-protein interactions in controlling the patterning, determination and fate of forming dental tissues. Such insights should improve our understanding about the pathogenesis of human disorders involving dentition. Supported by grants from the National Institutes of Health.

## **Distribution and Structure of Dental Enamel in Incisors of Tabby Mice**

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In Tabby mice the *Ta (Eda)* gene is mutated, resulting in a syndrome that is homologous to hypohidrotic ectodermal dysplasia in humans. Among the characteristics of the Tabby phenotype are aberrations in number and morphology of the teeth, both molars and incisors. The effect on the structure of enamel, however, has not been investigated. It was the aim of the present study to describe the enamel phenotype in Tabby incisors, with emphasis on its distribution, thickness and structure.

All four incisors were obtained from five female Tabby mice and from three female wild type mice. The incisors were sectioned and ground transversely at a level just apical to the abraded incisal part, etched for 45 seconds with 0.1% nitric acid, and sputter-coated with gold-palladium. The transverse surface was observed in SEM.

All measured dimensions were more variable, often considerably more, in Tabby mice than in wild type mice. Also the outline of the enamel-dentin junction was more variable in Tabby mice; the mesial concavity was often reduced or lacking. Maxillary incisors were wider in Tabby mice, while mandibular incisors were wider in wild type mice. No significant difference in enamel thickness was observed between Tabby and wild type mice. The enamel on the mesial aspect tended to extend further lingually in Tabby mice than in wild type mice both in maxillary and in mandibular incisors. On the lateral aspect this tendency was only significant in mandibular incisors. In one Tabby mouse the enamel on the mandibular incisors extended as a thin layer far beyond the bends in the dentin contour that normally coincide with enamel termination, both mesially and laterally on the right incisor and mesially on the left incisor. In this most affected mouse the right maxillary incisor was abortive with no enamel, while the left maxillary incisor was relatively small. Instances of hypoplastic enamel were observed. The complex mouse enamel structure was generally well preserved in the Tabby mice, only few instances of aberrant structure were observed. Based on observation of the enamel crystals in the SEM, slight hypomineralization of the enamel was present in about 60% of the Tabby incisors.

A major effect on incisor morphology in Tabby mutants was a variation in mesial and, to a lesser extent, lateral termination of the enamel covering the labial aspect of the teeth. Since the *Ta* gene during tooth development is only expressed in the outer enamel epithelium, it may be speculated that in Tabby mice the transition from inner to outer enamel epithelium is less well defined, resulting in a less well defined termination of enamel.