EXTRACELLULAR MOLECULES STIMULATE THE FORMATION OF REPARATIVE DENTIN IN EXPERIMENTALLY EXPOSED ADULT DENTAL PULP OF RATS AND MICE.

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Pulp exposure may occur during the preparation of deep cavities or be the consequence of carious decay. For decades the only biological option have been to heal the pulp by forming a reparative dentinal bridge using calcium hydroxide. Over the past few years, bioactive molecules have been experimentally implanted, and variable results were obtained (Goldberg & Smith, *Crit Rev Oral Biol Med* 2004). We have developed an experimental model using the first maxillary molar in the rat, first, drilling a cavity after gingival electrosurgery, and then pushing the deeper part of the cavity with a steel probe to expose the pulp. We also used a surgical approach for the incisor, allowing implantation of bioactive molecules in the forming zone of the mandibular incisor in rodents. Agarose beads soaked in a culture medium supplemented with the bioactive molecule have been implanted for 8 to 90 days in the molar, and for 10-20 days in the incisor.

In separate experiments, we implanted in the rat molar structural extracellular molecules, which may be also matricellular proteins, including Bone Sialoprotein (BSP), or signaling molecules such as leucine-rich amelogenin peptides (LRAP) produced by ameloblasts, or the low molecular spliced amelogenins that are synthesized by odontoblasts (A+/-4). These procedures induced a slight inflammation at day 8, and stimulated the recruitment of cells involved in the reparative process. After 15 days, reparative dentin started to be formed in the exposure area, appearing as a dentinal bridge or a diffuse mineralization in the coronal pulp. In addition, with A-4, the root canals were totally occluded by reparative dentin. The recruitment of reparative pulp cells may involve 1- either dormant (or latent) adult committed odonto/osteoblast progenitors, or 2- cells issued from the transdifferentiation of pulp fibroblasts; or 3- selected lineage(s) derived from inflammatory cells that de-differentiate and re-differentiate into odontoblast-like progenitors. As shown by PCNA staining, at day 8 labeled cells were seen in the central part of the coronal pulp, near the agarose beads acting as carrier, and at the periphery of the root pulp beneath the Höehl's (sub-odontoblastic) layer. At day 15, proliferation was reduced in the coronal pulp and disappeared in the root. We used RP59, a marker for osteoblast progenitors, and positively labeled cells appeared near and around the carrier beads. Osteopontin (OPN)- positive cells increased in number between 1-3 and 8 days. At day 8, cells located around the beads were strongly OPN positive, whereas they were negative for dentin sialoprotein (DSP) staining, suggesting an early differentiation along the osteoblastic lineage. At day 15, a few cells located near the pulp exposure site were DSP positive, whereas the cells in close contact or around the carrier beads formed a ring immunostained for OPN. Mineralization, which started at day 15, was achieved at day 30, and did not vary much at day 90. The reaction created by Dentonin, a peptide from MEPE, differed from other biomolecules in that though cell recruitment was stimulated, with proliferation and commitments toward early stages of differentiation. The process was then apparently stopped at that point. Terminal mineralization was heterogeneous, some teeth forming reparative dentin whereas in other pulps, there was no evidence for such formation (Six et al. *J Dent Res*, 2007). In the rodent incisor, implantation of A+/-4 revealed at day 10 the formation of bone-like dendritic structures in the central part of the pulp, which increased in size and developed into a diffuse mineralization. The pulp exposure site where beads were implanted was filled firstly by osteodentin and later, with thickening in some areas by reactionary orthodentin.

Altogether, these experimental approaches allow a better understanding of the cascade of events occurring between cell commitment and the terminal differentiation. It also allows screening of the individual effects of bioactive molecules. The specificities of each molecule lead to their reclassification as matricellular, structural and bioactive molecules (growth factors), although most structural proteins appear to be involved as signaling and bioactive molecule.

EVOLUTION OF VERTEBRATE HEAD DEVELOPMENT

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Vertebrate head development has been a major research topic in comparative zoology for two centuries, and questions around head segmentation were investigated even earlier (by Goethe and Oken around 1800). In my plenary talk i will present our research on the evolution of head development, which focuses on understanding the developmental origins of morphological innovations and involves asking questions like: How flexible (or conserved) are cell fates, patterns of cell migration or the timing of developmental events (heterochrony)? How do timing changes, or changes in life-history affect head development and growth? Our "model system" is a comparison between lungfishes and representatives from all three extant groups of amphibians. We use fate-mapping techniques, such as injection of fluorescent dyes or GFP mRNA, to investigate the migration and fate of important cell populations that contribute to the skeletal and muscular tissues in the head region, i.e. the cranial neural crest and the paraxial mesoderm. Changes in the timing of cranial muscle development are investigated using histology and whole-mount immunostaining. Within anuran amphibians, major changes in life-history such as the repeated evolution of larval specializations such as carnivory, or indeed the loss of a free-swimming larva, allows us to test for developmental constraints. Cell migration and cell fate are conserved in cranial neural crest cells in all vertebrates studied sofar. Patterning and developmental anatomy of cranial neural crest and head mesoderm cells are conserved within amphibians and even between birds, mammals and amphibians. However, radical changes in the timing of cranial neural crest stream emergence and migration occur, at least in anurans, even within a genus. The evolution of carnivorous larvae is correlated with changes in both pattern and timing of head skeletal and muscle development, and sequence-heterochronic changes are correlated with both feeding mode and phylogenetic relatedness.

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EVOLUTIONARY NOVELTIES: INTERACTIONS BETWEEN GENETICS, DEVELOPMENT AND SELECTION

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INTRODUCTION: Body plans are remarkably well conserved, but on (very) rare occasions important novelties evolve. Such novelties involve changes at the genotypic and phenotypic level affecting both developmental and adult traits. At all levels duplications play an important role in the evolution of novelties. Mutations for duplications, including mutations for duplications of body parts, as well as mutations for other body plan changes, in particular homeotic ones, occur surprisingly frequently. Hence mutation limitation is relatively unimportant for the conservation of body plans. However, mutations for duplications of body parts and homeotic changes rarely persist in populations.

RESULTS & DISCUSSION: We argue that the root cause of the conservation of body plans is the strong interactivity during the patterning of the embryonic axes, including the interactivity between patterning and proliferation processes. Due to this interactivity, mutations cause many negative pleiotropic effects (malformations and cancers) that dramatically lower fitness[1,2]. As an example we have shown in humans extreme selection against negative pleiotropic effects of the, surprisingly frequent, mutations affecting the number of cervical vertebrae (Fig. 1). Moreover, we argue for the relevance of relaxed selection, which temporarily allows just arisen novelties to persist, for the effective breaking of pleiotropic constraints. We illustrate this with two empirical examples, domesticated dogs and extinct Semionotus fishes that *Semionotus* fishes that invaded newly formed rift lakes in North Eastern America in the late Triassic and early Jurassic and that radiated into a species clade[3].

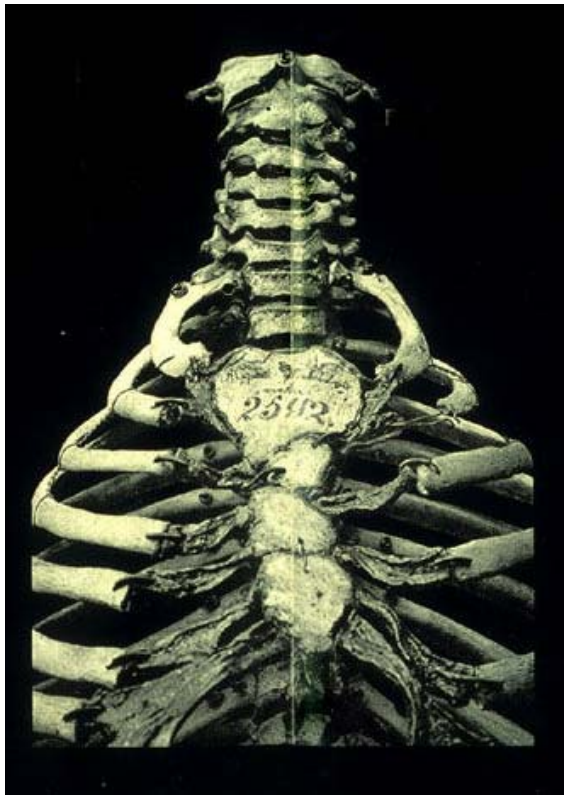

Figure 1. Adult human skeleton with a complete cervical rib, i.e. a rib on the seventh cervical vertebra. This change represents both the duplication of a structure, *i.e*. a rib, and a homeotic change, the change of identity of the seventh vertebra into that of a thoracic vertebra. Reproduced from ref. 3.

REFERENCES: [1] F. Galis, T.J.M. van Dooren, H. Feuth, S. Ruinard, A. Witkam, M.J. Steigenga, J.A.J. Metz and L.C.D. Wijnaendts (2006) Evolution **60**: 2643-2654. [2] F. Galis and JAJ Metz (2001 *J exp Zool B (Mol Dev Evol)* **291**: 195-204. [3] A. Fishel (1906) *Anatomische Hefte* **31**:462-588. [4] A.R. McCune (1990) *Evolution* **44**:71-85.

BUILDING DENTITIONS THROUGH REGULATED REPEATED TOOTH INITIATION

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INTRODUCTION: The dentition for each clade of gnathostomes is unique and all show great diversity in patterns for tooth addition and replacement. The formation of a sub-epithelial dental lamina promotes regulated tooth development with distinctive addition patterns in both chondrichthyans and osteicthyans. The evolution of such a structure, judged essential to produce iteratively patterned teeth at the margins of the jaws, may have occurred independently in more than one gnathostome group and is not a plesiomorphic character of jawed vertebrates [1]. Thus the pattern of tooth addition acquired a unique signature, for each clade of jawed vertebrates (Placodermi, Acanthodii, Chondrichthyes, Osteichthyes). This character 'teeth produced from a dental lamina' can occur more than once on a gnathostome phylogeny and suggests non-homologous developmental mechanisms. This site-specific, sequential, timed tooth addition as a mechanism for tooth renewal also ensures a sequestered environment for potential odontogenic epithelial cells. This may be a permanent dental lamina as in chondricthyans and tetrapods, or a transient and discontinuous one as in many osteichthyan fish.

A universal developmental model has been suggested [2] where all toothed fields start from a pioneer tooth. This autonomously regulates the pattern of tooth addition in the row and all successive replacement teeth in each family, envisaged as modular development. The aim is to obtain gene expression data for regulation of tooth sites for sequential succession of teeth both within a dental lamina, but also without, in osteichthyan and chondrichthyan fish.

RESULTS: The early pattern of tooth loci in the position of the dentary in the Australian lungfish (*Neoceratodus forsteri)* is observed from timed stages with gene expression data. There is a single pioneer tooth in position two of each side; the first in a triad of teeth with sequential teeth added in adjacent tooth positions, first three and then one. This is also the sequence identified in rainbow trout (*O. mykiss)* for the dentary bone, preceded at tooth initiation stage by activation of *Omshh*, *Ompitx2* and *Ombmp4* in a different spatial-temporal expression pattern for each dental field. In *Neoceratodus forsteri* different levels of *Nfshh* expression reveal cryptic sequential timing of dentary tooth initiation at the triad stages. Sequential tooth buds also form from the dental epithelium of the previous tooth germ, as in the trout, rather than from a dental lamina [3]. Preliminary data on expression of *Scshh* shows an intense broad band laterally and medially restricted to the odontogenic region of the lower jaw.

DISCUSSION & CONCLUSIONS: Primary rudimentary teeth and all successional teeth are formed in a permanent and continuous deep epithelial dental lamina in the catshark *S. canicula.* Each module starts from one tooth to provide both alternate families. Conversely, neither initial teeth nor successional teeth of the rainbow trout *O. mykiss* are formed in a classical dental lamina but are sited in the dental epithelium of the predecessor tooth [4], as are those of the lungfish. It is proposed that a transient site in the dental epithelium of the preceding tooth germ is one stage in evolution of the permanent successional lamina of tetrapod osteichthyans. A mechanism for retaining a localised epithelial progenitor cell population for continuous tooth renewal deep to the oral surface is considered essential to regulate tooth addition to each specific pattern.

REFERENCES: [1]Smith M. M. and Johanson, Z. 2003. Separate evolutionary origins of teeth from evidence in fossil jawed vertebrates. *Science*: 299: 1235-1236.

[2]Smith, M. M. 2003. Vertebrate dentitions at the origin of jaws: when and how pattern evolved. *Evol. & Dev.* 5: 394-413.

[3]Fraser, G., Berkovitz, B. K., Graham, A and Smith, M. M. 2006. Gene deployment for tooth replacement in the rainbow trout (*Oncorhynchus mykiss*): a developmental model for evolution of the osteichthyan dentition. *Evol. & Dev*. 5: 446-457.

[4]Fraser, G., Graham, A. and Smith, M. M. 2006. Developmental and evolutionary origins of the vertebrate dentition: molecular controls for spatio-temporal organisation of tooth sites. *J. Exp. Zool. (Mol. Dev. Evol.)* 306B: 183-203.

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ORAL MORPHOGENESIS IN AXOLOTL AND THE FIRST EVIDENCE OF ORAL ENDODERMAL TEETH FOR GNATHOSTOMES

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INTRODUCTION: The textbook schemes explain that the oral cavity of vertebrates is formed via an ectodermal invagination that forms a stomodeum. Oral tooth buds are thought to arise exclusively in the ectodermal areas where the ectoderm contributes to tooth enamel epithelium and the neural crest mesenchyme to dentin.

METHODS: Aside from histology and molecular markers (like e.g., a-fibronectine, a-calbindin), we have used orthotopical transplantations of oral ectoderm plus transverse neural fold from GFP+ transgenic to wild-type embryos of the Mexican axolotl (*Ambystoma mexicanum*) in order to fate-map the contribution of the oral ectoderm to mouth formation and to tooth germs. Next, this approach was combined with focal DiI injections into the exposed foregut endoderm in order to directly prove contributions of cells of endoderm origin to tooth formation and morphogenesis.

RESULTS: We found that oral and tooth development in the Mexican axolotl is very different when compared to classical textbook predictions. The stomodeum is not shaped by an ectodermal invagination; instead, it is a solid tube of the foregut endoderm that loads the oral area. The ectodermal layer only later populates superficially the anterior part of this endodermal tube as a stomodeal (ectodermal) collar.

Our double fate-mapping approach demonstrates conclusively that in the Mexican axolotl the enamel epithelia of oral tooth germs are derived either from ectoderm (maxillary, dentary and palatal tooth fields), or from endoderm (splenial and vomeral tooth fields). Moreover, at the contact zone between the ecto- and endodermal oral epithelium we have noted tooth germs that consistently show mixed contribution of both the ecto- and endodermal cells to their enamel epithelia (splenial and vomeral tooth fields).

DISCUSSION & CONCLUSIONS: Despite vivid discussions on the subject, to our knowledge this is the first reliable evidence of endodermal origin of oral teeth. Without any doubts the key to the understanding of the development of teeth is a correct appreciation of the formation and relation of the stomodeum. In Urodeles, stomodeum develops via ectodermal collar and thus the endodermal layer protrudes much more anteriorly than would be expected. In fact, we can clearly conclude, that in the Mexican axolotl about a half of mouth area is lined by the endoderm, moreover, that the endodermal lining can be found even on the outer mouth surface. It seems plausible to expect teeth of endodermal origin to be present in all animals where mouth develops throughout structures similar to ectodermal collar. We thus speculate that oral teeth of endodermal origin might be present in Urodele and lungfish species, at least, maybe even in some of Anurans (e.g., Ascaphus). However, it would be beyond scope of this study to decide what germ-layer origin of teeth might be considered as a plesiomorphic feature. Nevertheless, our results suggest that the major organizing agent of tooth initiation is not the epithelium but neural crest mesenchyme cells that are apparently capable to interact with any epithelial cells surrounding them not essential whether of ecto- or of endodermal origin.

REFERENCES:

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CRUCIAL ROLES OF RETINOIC ACID SIGNALLING DURING TEETH INDUCTION IN TELEOST FISH

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INTRODUCTION: In contrast with other teleosts, the zebrafish has teeth only localized in the pharynx. FGF signalling has been shown to be crucial during pharyngeal and oral teeth induction in teleosts [1,2]. In absence of this signal, several genes including *dlx2a* and *dlx2b* required for teeth development are no longer expressed and teeth are absent. To date, no other signalling pathways have been investigated during fish teeth induction. Since retinoic acid (RA) has pleiotropic functions during vertebrate development we investigate its precise roles during teleosts teeth induction. We used several fish models with teeth only in the pharynx or teeth located both in the pharyngeal and oral cavity. RA is synthesized by *aldh1a* enzymes (four in mammals) and binds RA receptors that are ligand-dependant transcription factors.

RESULTS & DISCUSSION: We used the zebrafish *neckless* (*nls*) mutant, which is devoid of full retinoic acid production from the RA-synthesizing enzyme *aldh1a2* to show that teeth induction does not occur in absence of RA signalling.

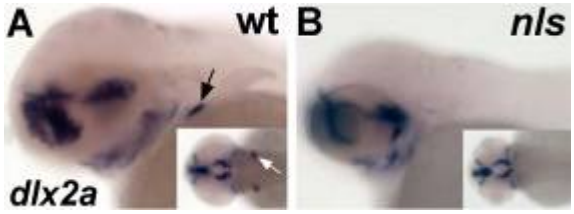

Fig. 1: Teeth induction does not occur in nls *mutant. (A) wild type embryo with* dlx2a *expression in the first pair of teeth (arrows). (B)* nls *mutant with lack of dlx2a expression in the teeth.*

By abolishing RA signal using pharmacological treatments at different times during embryonic development, we showed that RA is necessary during three different phases for the induction of the first pair of teeth in zebrafish: 1- for the proper migration of the neural crest cells (NCCs) at around 10 hpf, 2- for the maintenance of the NCCs in the ventral posterior pharynx at 26 hpf and 3- for the first pair of teeth induction *per se* at 43 hpf.

We further demonstrate that the third phase of RA (the teeth induction phase) is solely dependant on *aldh1a2* and not of any other *aldh1a* enzymes. By abolishing other cellular domains of *aldh1a2* expression, we show that the origin of RA signalling is located in the ventral posterior pharynx close to the fifth ceratobranchial arch.

We next demonstrate that RA and FGF signalling are not required at the same developmental time for the induction of the first pair of teeth and that FGF signalling acts downstream of RA signaling during teeth induction.

Finally, we used two other teleost fish, the medaka *Oryzias latipes* and the mexican tetra *Astyanax mexicanus* that possess teeth in both the oral and pharyngeal cavity to determine the roles played by RA in oral teeth induction. We showed that the NCCs phases only affect the induction of the pharyngeal teeth and not the oral teeth induction. Furthermore, we demonstrate that oral teeth induction is RA independent.

CONCLUSIONS: We uncover new roles for RA signalling in the induction of the first pair of teeth. We show that RA signaling is generated via the RA–producing enzyme aldh1a2 in the ventral posterior pharynx. In an evolutionary perspective, we show that similar tissue like teeth are dependant on different signaling pathway for their induction depending in their localization.

REFERENCES: [1] W.R. Jackman, B.W. Draper, and D.W. Stock (2004) *Dev. Biol.* **274**:139-157. [2] D.W. Stock, W.R. Jackman, and D. Trapani (2006) *Development* 133:3127-37. [3]

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SIMPLE COMPLEXITY OF TEETH

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INTRODUCTION: Dentitions appear to be more complex in vertebrates that eat plants rather than animals. Increased complexity is especially apparent in herbivorous taxa that eat fibrous plants. For example, specializations to eat bamboo have evolved several times in mammals. At least primates, bears, and muroid rodents have living species that rely largely on a bamboo diet. Whereas phylogeny, size, and life history are highly divergent among these bamboo specialists, their cheek tooth morphology show high overall complexity irrespective of the taxon-specific morphological details. The high complexity values can be related to the high number of tooth crown features, or 'tools', required to process fibrous bamboo. Yet this kind of high dental complexity may require simple developmental changes. For example, the lack of 'developmental individualization' of specific cusps is indicated by experiments in which increasing levels of the same signaling molecules can increase the number of several cusps. Thus, pandas teeth, for example, may be morphologically complex but developmentally simple. This would, of course, also indicate that tooth cusps are not characters as such, but rather character states of different tooth shapes.

CUSP DEVELOPMENT IN THE MOUSE FIRST LOWER MOLAR

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INTRODUCTION: Within the mouse dentition, the first lower molar (M_1) is considered as a model for odontogenetic studies. Recently, investigations combining the use of very precise embryo staging [1] and high resolution 3D reconstructions made it possible to follow step by step the morphogenesis and to go into the minutest anatomical details of a tooth germ [2]. From the utilisation of these new techniques, numerous questions have been raised about the development of the dentition in mice. Peterková *et al.* [3] and Viriot *et al.* [2] discovered that many rudimentary dental elements appeared and regressed during early dental development in the diastemae of both upper and lower jaws, whereas diastemae are by definition areas where teeth are absent. At the transition between bud and cap stages, one of the rudimental tooth buds located just in front of the M_1 incorporated into the mesial extremity of the M_1 cap. From these latter observations, a scenario of heterogeneous origin for the M_1 germ has been proposed [4,5]. The aim of the present work is to evaluate the morphological consequences of this bud concrescence on the shape and inter-relationships of the seven cusps of the M_1 crown in the mouse.

MATERIALS & METHODS: The ontogeny of the M_1 was investigated in mouse embryos whose age was determined in embryonic days (ED) specified by the wet body weight. Females were mated overnight and the midnight before the morning detection of the vaginal plug was taken as ED 0. Harvested embryos were fixed in Bouin-Hollande fluid. Five micrometers thick frontal serial sections from paraffin embedded heads were stained with alcian blue-hematoxylin-eosin. The M_1 dental epithelium was reconstructed in 3D from ED 14 to 20. Complementary reconstructions of the dental papilla mesenchyme were made to visualize the occlusal shape of the crown. We adopted the Cope-Osborn cusp nomenclature [6].

RESULTS: The mouse M_1 is composed of three mesio-distal opposite pair of cusps which are forming transversal cusp rows. Presumptive cusps of the second row (Fig. 1: protoconid and metaconid) rose first at about ED 14. Short after ED 15, presumptive regions for cusps of the first (anteroconids) and the third (hypoconid and entoconid) rows appeared quite conjointly. From ED 16 to ED 17, two mesial elements appeared and became integrated into the mesial extremity of the M_1. Finally, the posteroconid appeared at about ED 17.5 (Fig. 1).

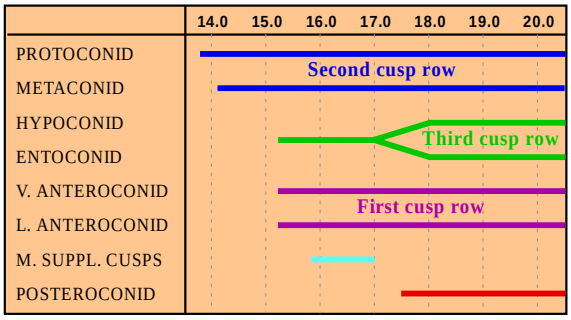

Fig. 1: Chronology of cusp development in the mouse first lower molar. V: vestibular, L: lingual, M: mesial, Suppl.: supplementary.

DISCUSSION & CONCLUSIONS: Contrary to what is generally supposed [7], the sequence of cusp development in mouse M_1 differs from the order of cusp appearance through evolution. Only the appearance of the five central and distal cusps follows the evolutionary sequence. The mesial part of the M_1 seems to develop independently to the rest of the molar. The very early appearance of the anteroconids, as well as the occurrence of supplementary mesial cusps could probably be related to the heterogeneous composition of the M_1 germ [4,5].

REFERENCES: [1] M. Peterka, et al (2002) *Connect. Tissue Res.* **43**:186-190. [2] L. Viriot, et al (2000) *Int. J. Dev. Biol.* **44**:233-240. [3] R. Peterková, et al (1998) *Eur. J. Oral Scpi.* **106**:667-670. [4] R. Peterková, et al (2002) *Connect. Tissue Res.* **43**:120-128. [5] L. Viriot, et al (2002) *Connect. Tissue Res.* **43**:129-133. [6] R. H. Biggerstaff (1968) *J. Dent. Res.* **47**:508. [7] P. M. Butler (1956) *Biol. Rev.* **31**:30-70.

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UNIQUE AND CONSERVED CHARACTERS IN SALMON TOOTH DEVELOPMENT

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INTRODUCTION: As part of a large scale investigation on the development and renewal of the dentition in Atlantic salmon (*Salmo salar* L.), we have recently analysed patterns of early tooth initiation and of replacement tooth formation throughout nearly all life stages [1,2]. Characters peculiar to salmon teeth include structural features, such as the persistence of atubular dentine even in adult fish, and developmental features, such as the gradual establishment of multiple cell layers between inner and outer dental epithelium. We here term these cell layers middle dental epithelium. Placing the histogenesis of replacement teeth into an evolutionary context, suggests that the formation of replacement teeth is a result of heterochrony and we hypothesise that the middle dental epithelium plays an essential role in the replacement process [3].

METHODS: To explore the molecular basis of tooth replacement, we have embarked upon a gene expression study by means of *in situ* hybridisation on cryosections of juvenile salmon, using digoxigenin-labeled antisense riboprobes directed against a number of key regulatory genes such as *bmp2, bmp4*, and *sox9* and structural genes such as *col1a1* and osteocalcin (= *bgp*, Bone Gla Protein). We have compared expression patterns of these genes to those in other skeletogenic cells such as osteoblasts, chondroblasts, and chondrocytes at the animals' lower jaw.

RESULTS & DISCUSSION: Our studies reveal both, a localisation of transcripts that is in accordance to studies on mammalian tooth development and a localisation of transcripts that is specific to salmon (respectively specific to teleosts). The epithelial expression of *sox9* and a shift of the expression of *bmp2* from epithelium to mesenchyme have also been observed during mammalian tooth development. Different from previous reports, are the expression of *col1a1* and *bgp*. Apart from being expressed in odontoblasts, *col1a1* is strongly expressed in the inner dental epithelium, representing the first report of ameloblast involvement in collagen type I production. *col1a1* is also observed in the basal layer of the oral epithelium, in agreement with a previous study reporting collagen type I alpha 2 production in the basal epidermal layer of fish skin [4]. In agreement with studies of *Bgp* expression in mouse bone [5] but in contrast to what has been reported about *bgp* expression in zebrafish [6], *bgp* is not expressed in odontoblasts, nor in the osteoblasts involved in the attachment of the teeth. At the lower jaw, we find *bgp* expression in old and resting osteoblasts only. These unusual findings are discussed in the light of the features particular to salmon teeth.

REFERENCES: [1]P.E. Witten, B.K. Hall, A. Huysseune (2005) Are breeding teeth in Atlantic salmon a component of the drastic alterations of the oral facial skeleton? Arch Oral Biol 50:213-217. [2]A. Huysseune, B.K. Hall, P.E. Witten (2007) Establishment, maintenance and modifications of the lower jaw dentition of wild Atlantic salmon (*Salmo salar* L.) throughout its life cycle. J Anat. (in press). [3]A. Huysseune, P.E. Witten (submitted) An evolutionary view on tooth development and replacement in wild Atlantic salmon (*Salmo salar* L.). Evol Dev. [4]D. Le Guellec ,G. Morvan-Dubois, J.Y. Sire (2004) Skin development in bony fish with particular emphasis on collagen deposition in the dermis of the zebrafish (*Danio rerio*). Int J Dev Biol 48(2-3):217-231. [5]T. Ikeda , S. Nomura, A. Yamaguchi, et al. (1992) In situ hybridization of bone matrix proteins in undecalcified adult rat bone sections. J Histochem Cytochem 40(8):1079-1088. [6]P.J. Gavaia, D.C. Simes, J.B. Ortiz-Delgado, et al. (2006) Osteocalcin and matrix Gla protein in zebrafish (*Danio rerio*) and Senegal sole (*Solea senegalensis*): Comparative gene and protein expression during larval development through adulthood. Gene Expr Patt 6:637-652.

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EVOLUTIONARY ANALYSIS OF DMP1

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INTRODUCTION: Dentin Matrix Protein 1 (DMP1) is expressed in both pulp cells and odontoblasts during dentinogenesis. The role of DMP1 during dentinmineralization is to facilitate initiation of mineral nucleation at specific sites and to prevent spontaneous calcium phosphate precipitation in areas in which mineralization is not desirable. DMP1 is a member of SIBLING (Small Integrin-Binding LIgand, N-linked Glycoprotein) family of secreted glyco-phosphoproteins. DMP1 is not specific of dental tissues; it is also highly expressed in osteocytes during bone mineralization, like other members of SIBLING family, and genetic defects in DMP1 encoding gene results in an hypomineralized bone phenotype. *In vivo* DMP1 depletion leads to a decrease of bone and dentin mineralization in mice. In humans, some rare mutations in *DMP1* are described to cause defective mineralization in bone and dentin. Since a few years, we are investigating the role of dental proteins using evolutionary analysis approaches. In the present study, we have compiled DMP1 sequences in amniotes (mammals and reptiles), with the aim to highlight residues and/or regions that appear to be important for a correct function of the protein.

MATERIALS: Five mammalian sequences of DMP1 were found in NCBI and 3 reptilian sequences were obtained from the literature[2,3]. We have completed our dataset in sequencing DMP1 in some species and in blasting sequenced genomes.

METHODS:
DNA and RNA Extraction: Genomic DNA was extracted (DNeasy tissue kit: Qiagen-GmBH,Ilden, Germany) from soft tissues conserved in ethanol. mRNAs were obtained (RNeasy kit: Qiagen) from the lower jaw of an iguanid lizard *Anolis carolinensis* and converted into cDNAs (ReverAid kit: MBI Fermentas, USA). Primers were defined from the alignment of known DMP1 sequences. *PCR Amplification:* Genomic DNA or cDNA (1 µL) was amplified in a mixture composed of 5 µL Taq buffer (10x), and 1 µL dNTP 10 mM, in the presence of sense and antisense primers, and 2.5 µL Red taq polymerase (Sigma-Aldrich). Amplification was performed in a thermocycler (G-Storm, GRI France) for 38 cycles, each cycle consisting of 1 min of denaturation at 94°C, 1 min of annealing at 59°C, and 1 min of extension at 72°C. The final extension was for 20 min at 72°C. *Cloning:* One microgram of PCR product was isolated, ligated to pCR 2.1-TOPO plasmid vector (Invitrogen, USA) by the TA-cloning method, then used to transform competent E. coli TOP10F bacteria. Sequencing was done by GATC Biotech SARL. *Molecular Analyses*: DMP1 sequences were aligned by hand using Se-Al v2 software.

RESULTS & DISCUSSION: A dataset of 44 DMP1 sequences (37 mammals and 7 reptiles) was obtained. The alignment revealed numerous conserved residues (310 millions years of evolution), that appeared to be important for correct DMP1 function.Among these conserved residues, some are suspected to induce recessive autosomal hypophosphatemia when mutated. Moreover, the evolutionary analysis shows: (i) the conservation of SXE and RGD motives that were described also in all members of SIBLING family; and (ii) the conservation of sites known to be clived during proteolytic processing. However, at the beginning of exon 6 a well-conserved region (DDEDDSGDDTF) has been revealed. A functional analysis of this motif by ProSite did not reveal any biochemical function. The role of this DMP1 peptide might be investigated in the next future.

CONCLUSIONS:
Our evolutionary analysis of DMP1 highlights several amino acids that are interesting targets for next investigations aiming to elucidate the role of its various regions. .

REFERENCES: [1] M. Delgado S, Ishiyama M and Sire J-Y (2007) *J Dent Res* **86**(4):326-330). [2] S. Toyosawa, C. O'hUigin, H. Tichy and J. Klein (1999) *Gene* 234, 307-314.[3] S. Toyosawa, A. Sato, C. O'HUigin, H. Tichy and J. Klein (2000) *J Mol Evol* 50, 31-38.

GENETIC PATHWAYS MEDIATING SPECIES-SPECIFIC CRANIOFACIAL VARIATIONS

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The human face shows remarkable variability and because of this it is oftentimes the singular feature used to distinguish and discriminate among individuals. Despite this exclusivity, the structural edifice of the face is so highly conserved that its underlying pattern is shared by nearly all vertebrates. One might then wonder, what forces act to establish the craniofacial bauplan? And what are the driving influences behind the divergence in craniofacial form? As Darwin and many other scientists speculated, the answer to both questions lies in genetics but we still have little notion of how certain genes function in facial morphogenesis, let alone how particular pathways mediate craniofacial variation. In this talk I will present new data that addresses this question, beginning with the identification of key pathways that control local proliferation within the emerging facial prominences and thus lead to species-specific variations in facial form. How much genetic change has to occur in order to generate such craniofacial diversity is still a mystery, but we explore this question by modulating these pathways in an incremental fashion, and observe the resulting variations in craniofacial morphology. In the end, our goal is a detailed understanding the tissue interactions that mediate normal craniofacial morphogenesis because this information provides much needed clues into the developmental steps that underlie craniofacial malformations and defects.

CELL FATE SPECIFICATIONS DURING SUTURE FORMATION: CONSEQUENCES FOR CRANIOSYNOSTOSIS

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INTRODUCTION: At a molecular level we have a good understanding of what regulates the commitment of undifferentiated mesenchymal cells into osteoblasts. However, we know surprising little about the cellular processes that control intramembranous bone growth which accounts for the majority of the growth of the face and calvaria and in the surface periosteum of most bones during modeling and remodeling.

METHODS & RESULTS:: : In this study we have addressed the fundamental question of what cellular mechanisms control the growth of the calvarial bones and conversely, what is the fate of the sutural mesenchymal cells when calvarial bones approximate to form a suture. There is evidence that the size of the osteoprogenitor cell population determines the rate of calvarial bone growth. In calvarial culture we reduced osteoprogenitor cell proliferation by 50%; however, we observed a reduction in parietal bone growth of only 19%. This discrepancy prompted us to study whether suture mesenchymal cells participate in the growth of the parietal bones. We found that proliferation and subsequent differentiation of osteoprogenitors at the osteogenic fronts, although important, is not the only cellular mechanism that contributes to calvarial bone growth. Sutural mesenchymal cells can differentiate into osteoblasts and become incorporated into the growing parietal bones, but only if adjacent to the osteogenic fronts.

DISCUSSION & CONCLUSIONS: The fate of calvarial mesenchymal cells varies depending on their position within the suture. Under normal conditions we demonstrate that a small percentage of sutural mesenchymal cells are incorporated into the growing bones. We can hypothesize that during pathological conditions such as craniosynostosis the relative contribution of recruitment from the mesenchyme into the calvarial bones may be altered.

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NORMAL AND ABNORMAL LOWER JAW DEVELOPMENT

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Functioning of the masticatory and breathing apparatus and harmony of the face are significantly influenced by the size and shape of the lower jaw. Absent or deficient lower jaw growth may lead to life threatening condition because of obstruction in the airway. Prevention and treatment of congenital and acquired mandibular anomalies necessitate understanding of mandibular morphogenesis at the cellular and molecular level.

Mandibular development is complex and peculiar, since it involves three different and essential elements: Meckel's cartilage, intramembraneous bony component and cartilaginous condylar blastema.[1] Meckel's cartilage is thought to be the supportive element of the first branchial arch, however, it may have a crucial role as an inductor for the intramembraneous bone formation of the mandible on its lateral surface. [2] Thus, defect in the neural crest originating Meckel's cartilage may adversely affect jaw morphogenesis. A more serious defect has been documented following FGF8 gene inactivation: the first branchial arch does not develop properly and mandible remains rudimentary. [3]

Genetic experiments in mice have begun to address the role of epithelial-mesenchymal interactions by targeting genes expressed in one tissue layer. It has been shown that Tgfb, Pitx1, Tbx1, Sox9, and Runx2 are necessary for normal lower jaw growth and development.[4-7]

Condylar cartilaginous blastema appears later in the development than primary cartilages. Ihh seems to be very important for the development of the this secondary cartilage and the TMJ.[8] During development the anteriorly expanding cartilaginous blastema fuses with the posteriorly growing bony part, however, process of fusion and defects in this are poorly understood. CTs of patients with hemifacial microsomia have revealed a small bony structure on the buccal side of the mandible not being attached to the ramus.[9] This structure could be condylar blastema, which did not fuse to the mandibular ramus.

During postnatal growth condylar cartilage has a central role in the mandibular growth. Recent studies have increased our understanding on the endochondral bone formation cascade in the condylar cartilage. Large variation in the growth quantity and direction of condylar growth leads to large individual variation in the mandibular shape and also to difference in response to treatment. A recent association study has shown that the variation may be partly due to single nucleotide polymorphism in the growth hormone receptor gene.[10]

Holistic view of the factors associated with pre- and postnatal mandibular growth is poorly understood and the future challenge is to disclose these factors to prevent and treat individuals with lower jaw growth disturbances.

REFERENCES: [1] S.K. Lee et al., (2001) Prenatal development of the human mandible. *Anat Record* 263:314-25. [2] J. Frommer and M.R. Margolies (1971) Contribution of Meckel's cartilage to ossification of the mandible in mice. *J Dent Res* 50:1260-7. [3] A. Trumpp et al. (1999) Cre-mediated gene inactivation demonstrates that FGF8 is required for cell survival and patterning of the first branchial arch. *Gen Dev* 13:3136-48. [4] C. Lanctôt et al., (1999) Hindlimb patterning and mandible development require the Ptx1 gene. *Development* 126: 1805-10. [5] P. Miettinen et al., (1999) EGFR function is necessary for normal craniofacial development and palate closure. *Nature Genet* 22:69-73. [6] I. Satokata et al., (2000) Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. *Nature Genet* 24:391-5. [7] H. Wurdak et al., (2006) DiGeorge syndrome and pharyngeal apparatus development. *BioEssays* 28:1078-86. [8] Y. Shibukawa et al. (2007) TMJ formation and condyle growth require Ihh signaling. *Dev Dyn* 236:426-34. [9] C.E. Huisinga-Fischer et al., (2003) Longitudinal results of mandibular distraction osteogenesis in hemifacial microsomia. *J Craniofac Surg* 14:924-33. [10] J. Zhou et al., (2005) The GHR gene is associated with mandibular height in a Chinese population *J Dent Res* 84:1052-6.

THE FUNCTION OF FGF SIGNALLING DURING EARLY CRANIOFACIAL DEVELOPMENT

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INTRODUCTION: The FGF family of signaling molecules has a central role in the regulation of many aspects of vertebrate embryogenesis and many processes in the adult. In the developing face *Fgf3, 8, 9, 10, 15, 17,* and *18* are expressed in partially overlapping domains in the ectoderm of the early facial region, and *Fgfr1* and *Fgfr2* are both expressed in the underlying neural crest mesenchyme and continue to be expressed in the mesenchyme of the developing palatal shelves [1]. To study the function of *Fgf8* and *Fgfr1* during early craniofacial development we have analyzed the phenotype of mice with a conditional inactivation of these two genes in the facial region.

METHODS: Mice homozygous for a conditional allele of *Fgf8* (*Fgf8*$^{flox/flox}$) were bread to mice carrying a null allele of *Fgf8* and a *cre*-cDNA under control of the *Foxg1* promoter (*Fgf8*$^{-/+}$; *Foxg1cre*$^{/+}$) as described in [2]. In *Fgf8*$^{-/flox}$; *Foxg1cre*$^{/+}$embryos (in the following referred to as *Fgf8* mutant embryos) Cre-mediated recombination leads to a loss of functional *Fgf8* message in the prospective midfacial ectoderm and forebrain by the 12-somite stage. To inactivate *Fgfr1* in neural crest cells mice homozygous for a conditional allele of *Fgfr1* (*Fgfr1*$^{flox/flox}$) were bread to mice carrying the conditional allele and *Wnt1::Cre* (*Fgfr1*$^{flox/+}$; *Wnt1::Cre*). In *Fgfr1*$^{flox/flox}$; *Wnt1::Cre* embryos Cre-activity leads to loss of functional *Fgfr1* protein in migrating neural crest cells [3]. Embryos were isolated from pregnant females between 8 and 18 days post coitum, fixed and processed for analysis using histological stainings, TUNEL and cell proliferation assays, and in situ hybridization with panel of markers of facial development.

RESULTS: *Fgf8* mutant embryos are born with severe brain and midfacial defects and die within an hour after birth. In the facial region, defects first become apparent around E9.5 as a reduction in the amount of mesenchyme. Nile blue sulfate staining and TUNEL assays revealed a dramatic increase in the amount of cell death in the facial mesenchyme at E9.5, and BrdU incorporation assays suggest that cell proliferation is also slightly reduced. In contrast, immigration of neural crest cells into the facial area is not affected.

We also found that in the remaining nasal tissue *Erm*, *Pea3*, and *Tbx2*, genes that we had previously identified as FGF regulated genes [4], fail to be expressed close to the facial midline at E9.5. At E10.5, their expression surrounding the nasal pits is largely unaffected. Further analysis revealed that expression of *Fgf9* and *Fgf10*, which are expressed in an overlapping region with *Fgf8* at E10.5 but not E9.5, is maintained in *Fgf8* mutant embryos. Experiments *in vitro* confirmed that Fgf9 can substitute for Fgf8 in inducing and maintaining expression of these transcription factors when tested in facial explants. Therefore Fgf9 seems to partially compensate for the lack of Fgf8 after E10. In addition to its functions in the facial mesenchyme *Fgf8* is also essential for normal development of the olfactory epithelium.
Facial defects in *Wnt1::Cre; Fgfr1*$^{flox/flox}$ embryos are much milder than in *Fgf8* mutants. Embryos develop midfacial clefts and cleft palate with incomplete penetrance. The midfacial cleft first becomes apparent at E11 as an increased distance of the media nasal processes, which subsequently fail to fuse. Analysis of *Alx3*, *Alx4*, *Msx1*, *Tbx2*, *Fgf8* and *Shh* expression revealed no differences between mutant and wildtype embryos at E10.5 and E11.5. Analysis of secondary palate development revealed normal outgrowth of mutant palatal shelves lateral to the tongue between E12.5 and E14. In contrast, elevation of the palatal shelves in the majority of the mutants was severely disturbed.

DISCUSSION & CONCLUSIONS: Our results demonstrate that Fgf8 is essential for survival and nnormal development of the neural crest derived facial mesenchyme and suggest that other Fgf receptors in addition to *Fgfr1* are involved in the reception of the Fgf8 signal.

REFERENCES: [1]M. Bachler and A. Neubüser (2001) *Mech Dev* **100**:313-6. [2]E.E. Storm, J.L. Rubenstein and G.R. Martin (2003) Proc Natl Acad Sci U S A **100**:1757-62. [3]N. Trokovic, R. Trokovic, P. Mai, et al (2003) *Genes Dev* **17**:141-53. [4]N. Firnberg and A. Neubüser (2002) *Dev Biol* **247**:237-50.

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THE CREATION OF A 3D ATLAS ON HUMAN PRENATAL DENTAL DEVELOPMENT

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INTRODUCTION: Although the development of teeth is one of the most prominent topics in modern craniofacial developmental research, our knowledge in prenatal human dental morphogenesis is relatively sparse. The most recent extensive compilation that shows aspects of 3D-development, dates back to 1965 (Kraus and Jordan 1965). In our own work on selected stages (Radlanski 1993), only single aspects were being addressed (Radlanski and Renz 2005, 2006). Many other dental studies that include the 3D-aspect, deal with non-human teeth, mostly with rodent teeth, e.g. Lesot et al, (1996) and Viriot et al. (1997). Our knowledge of dental development has increased in detail due to progress that has been made in recent years, also in the field of dental tissue engineering. Although it is the common aim to replace lost human teeth eventually, we still lack a reference system of human prenatal dental development in 3D to compare the laboratory achievements with.

MATERIALS, METHODS & AIM: It is the aim of this project to generate 3D reconstructions of developing human dental primordia of the deciduous dentition. The stages that are being covered reach from 19 mm CRL (7^{th} week, lamina stage) up to 270 mm CRL (28^{th} week, mineralization stage). 20 histological series of the Radlanski-Collection are currently reconstructed in 3D (Software Analysis, SIS, Münster, Germany), including not only detailed dental structures (dental follicle, papilla, dentin, enamel), but also the surrounding bone. This way, formation of the early bony compartments, which will in time lead to formation of alveolar bone, will also be covered in 3D.

CONCLUSIONS: With this 3D-atlas of prenatal human dental development we want to present a reference system for any other study that will deal with human and non-human dental development.

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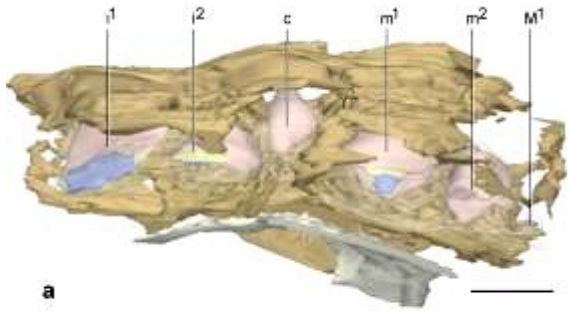

Fig. 1a: Computergraphic 3D-reconstruction of histological serial sections of a human fetus (190 mm CRL). Left half of maxillary region with dental primordia in a 45° anterior and 45° lateral, and 20° caudal view. Bar: 2 mm.

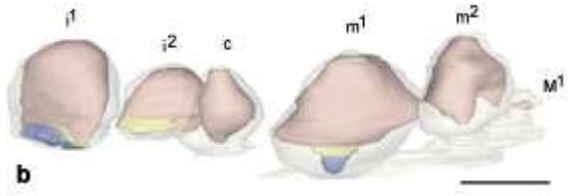

Fig. 1b: Details from Fig. 1a, dental primordia without surrounding bone. Bar: 2 mm.

REFERENCES:

Kraus BS, Jordan RE (1965) The human dentition before birth. Philadelphia, Lea & Febiger.

Lesot H, Vonesh JL, Peterka M, Tureckova J, Peterkova R, Ruch JV (1996) Int J Dev Biol 50: 1017-1031.

Radlanski RJ (1993) Contributions to the Development of Human Deciduous Tooth Primordia., Quintessence Publishing Co. Inc. Carol Stream, Illinois USA, 1993

Radlanski RJ, Renz H, (2005) Archs Oral Biol 50: 199-203.

Radlanski RJ, Renz H, (2006) Eur. J. Oral Sci 114 (suppl 1): 1-6.

Viriot, L, Peterkova, R. Vonesh, JL, Peterka, M, Ruch JV, Lesot H (1997): Int J Dev Biol 41: 679-690.

REGULATION OF THE CONTINUOUS GROWTH OF THE MOUSE INCISOR BY EPITHELIAL-MESENCHYMAL INTERACTIONS

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INTRODUCTION: Interactions between the epithelial and mesenchymal tissue components of developing teeth regulate morphogenesis and cell differentiation and determine key features of dentitions and individual teeth such as the number, size, shape and formation of dental hard tissues. Tissue interactions are mediated by signal molecules belonging mostly to four conserved families: TGFβ, Wnt, FGF and Hedgehog. Recent work from our laboratory has demonstrated that tooth morphology and the capacity of the teeth to grow and renew can be affected by modulating these signal pathways in transgenic mice. The continuous growth of the mouse incisors depends on stem cells which are located in a niche in their proximal ends, called the cervical loop[1]. This stem cell niche bears anatomical similarities to the niches in other epithelial organs, in particular in hairs and intestine.

RESULTS & DISCUSSION: Compared to the niches in other organs, the incisor stem cell niche has a powerful property: the stem cell compartments on the different sides of the tooth, namely the labial (anterior) and lingual (tongue-side) cervical loops vary greatly in size and proliferative capacity. This contributes to the characteristic asymmetric growth and enamel distribution which together maintain the sharpness of the continuously growing incisor.

We have shown that incisor growth is dramatically altered by modulating a network of FGF and two TGFβ signals, BMP and Activin, mediating interactions between epithelium and mesenchyme[2]. This network is responsible for the regulation of the maintenance, proliferation and differentiation of epithelial stem cells which again are responsible for growth and enamel production [2,3].

Interestingly, *Fgf3*, *Bmp4* and *Activin* are all coexpressed in the mesenchyme adjacent to the cervical loop epithelium. This supports earlier evidence indicating that the capacity to regulate epithelial morphogenesis and tooth identity resides in the dental mesenchyme.
Continuously growing teeth are common in many animals and there are variations in the extent of enamel coverage of the teeth. For example, even primates possess ever growing incisors as seen in the *Aye-aye* lemurs, and some rodents such as voles have continuously growing molars in addition to the ever-growing incisors. Work from our laboratory has previously shown that the cervical loops of continuously growing molars are anatomically similar to those of incisors, and components of the same molecular signal network are in place[4] . Hence it is possible that the evolutionary variation in the growth capacity of teeth and the extent of enamel deposition has resulted from fine-tuning of the complex signal network which regulates the maintenance, proliferation and differentiation of the epithelial stem cells. Also, subtle variations in this or related regulatory networks may explain the different regenerative capacities of various organs and animal species.

REFERENCES: [1] Harada, H., Kettunen, P., Jung, H-S., Mustonen, T., Wang, Y.A., Thesleff, I. (1999) Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. *J Cell Biol* **147**: 105-120. [2] Wang, X-P., Suomalainen, M, Felszeghy, S., Zelarayan, L.C., Alonso, M.T., Plikus, M.V., Maas, R., Chuong, C-M., Schimmang, T., Thesleff, I. (2007) An integrated gene regulatory network controls epithelial stem cell proliferation in teeth. *PLoS Biology* **5**: 1324-1333. [3] Wang, X-P., Suomalainen, M., Jorgez, C.J., Matzuk, M.M.,Werner, S., Thesleff, I. (2004) Follistatin regulates enamel patterning in mouse incisors by asymmetrically inhibiting BMP signaling and ameloblast differentiation**.** *Dev Cell* **7**: 719-730. [4] Tummers, M. and Thesleff, I. (2003) Root or crown: a developmental choice orchestrated by the differential regulation of the epithelial stem cell niche in the tooth of two rodent species. *Development* **130**: 1049-1057.

MOLECULAR MECHANISMS OF EARLY TOOTH DEVELOPMENT

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INTRODUCTION: Transcription factors play important roles during cell fate determination and expression of tissue-specific genes that are necessary for normal organ development. Our previous studies revealed that the transcription factor encoded by the Msx1 homeobox gene plays an important role in tooth development [1]. Genetic studies in both humans and mice indicate that loss-of-function of Msx1 gene affects early tooth formation. Epistasis and functional analysis, using the genetically engineered Msx1 mouse mutants, revealed an Msx1-controlled genetic hierarchy, where several families of growth and transcription factors are involved [2].

METHODS: To further understand the role of Msx1 during early tooth morphogenesis, using genomic and high-throughput technologies, we identified novel genes and pathways whose function is important to early tooth morphogenesis. In addition, we tested the hypothesis that Msx1's activity, as a repressor or activator, is modulated by interactions with tooth specific partners.

RESULTS: We have found that Msx1 protein interacts in vitro and in vivo with several transcription factors in a context dependent manner.

DISCUSSION & CONCLUSIONS: The fundamental understanding of how Msx1 regulatory protein functions will provide valuable insight on the transcriptional mechanism regulating tooth development.

REFERENCES: [1] M. Bei (1998) Development 125: 4326-4333. [2] M. Bei (2000) Development 127: 4711-4718.

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SMAD-DEPENDENT TGF-B/BMP SIGNALING IN REGULATING FIRST BRANCHIAL ARCH PATTERNING AND TOOTH DEVELOPMENT

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Smad4 is the central mediator for TGF-b/BMP signals, which are involved in regulating cranial neural crest (CNC) cell formation, migration, proliferation and fate determination. It is unclear whether TGF-b/BMP signals utilize Smad-dependent or –independent pathways to control the development of CNC cells. To investigate the functional significance of Smad4 in regulating CNC cells, we generated mice with neural crest specific inactivation of the Smad4 gene. Our study shows that Smad4 is not required for the migration of CNC cells, but is required in neural crest cells for the development of the cardiac outflow tract. Smad4 is essential in mediating BMP signaling in the CNC-derived ectomesenchyme during early stages of tooth development because conditional inactivation of Smad4 in neural crest derived cells results in tooth development arrested at the dental lamina stage. Furthermore, Smad-mediated TGF-b/BMP signaling controls the homeobox gene patterning of oral/aboral and proximal/distal domains within the first branchial arch. At the cellular level, a Smad4-mediated downstream target gene(s) is required for the survival of CNC cells in the proximal domain of the first branchial arch. Smad4 mutant mice show underdevelopment of the first branchial arch and midline fusion defects. Taken together, our data show that TGF-b/BMP signals rely on Smad-dependent pathways in the ectomesenchyme to mediate epithelial-mesenchymal interactions that control craniofacial organogenesis.

WNT SIGNALING IN TOOTH REPLACEMENT

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Mammals normally develop only two dentitions. After the development of the permanent dentition, there is no additional tooth replacement. However, if the runt-domain transcription factor Runx2 is mutated in humans, the patients develop supernumerary teeth which represent the third dentition (1, 2). Also Wnt signalling has been associated with tooth renewal. In humans when the Wnt inhibitor Axin2 is mutated, the patients show tooth agenesis specifically in the permanent dentition (3). Moreover, we have shown that activated Wnt signalling in dental epithelium leads to continuous tooth generation in mice (4). These and other results suggest that both Runx2 and Wnt signalling regulate mammalian tooth replacement. We are using transgenic mouse models and in vitro studies in order to further examine the roles of Wnt signalling and Runx2. We have studied the regulation of Runx2 in dental mesenchyme in vitro and shown that Wnt induces Runx2. This indicates that Wnt and Runx2 signalling pathways may interact and supports the idea that Wnt signalling plays a central role in tooth renewal in mammals. Furthermore, as mice develop only one dentition, we started to investigate the mechanism of tooth replacement in other animals to gain new insight into the precise morphological and molecular changes that take place during the events of tooth replacement. Observations on tooth replacement in the common shrew, *Sorex araneus*, and the ferret, *Mustela putorius* will be reported.

MOLECULAR REGULATION OF INCISOR FATE IN DEVELOPING DENTITION

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INTRODUCTION: Ectodin is a Bmp-inhibitor which binds to Bmps with high affinity. Bmps themselves induce the expression of *ectodin*, which is absent from the signaling center, enamel knot, and several cell layers around it (Laurikkala et al. 2003). We have previously shown that Ectodin affects the morphology of mouse molars by restricting the size and the placement of enamel knots. In addition, in the Ectodin knock-out mice, an extra molar develops in the location of the premolar primordium, which has been proposed to be an evolutionary rudiment in mice. In this study we explored the role of Ectodin in incisor development.

RESULTS AND DISCUSSION: *Ectodin*-deficiency causes the development of supernumerous incisors in both the mouse upper and lower jaw. These extra teeth appear lingually to the main incisors, resembling the dental pattern present in Lagomorpha.

TUNEL-staining revealed two distinct apoptotic centers in wild type incisor region at e14 stage. In contrast apoptosis was not detected in *ectodin*-deficient mouse. Bmp-target genes, *p21* and *Msx2*, as well as *Shh*, marker for dental placodes, showed expanded expression domains in the *ectodin*-deficient mice, indicating expansion of tooth making potential to areas normally forming oral skin.

Because *ectodin* is normally expressed strongly in the mesenchyme surrounding the incisors, next we examined wild type incisors cultured *in vitro* after removal of most of the surrounding mesenchymal tissue. The results show that extra incisors develop in wild type mice when the main incisor germs were dissected into *in vitro*-conditions before the appearance of apoptosis. Hence, partial removal of mesencymal tissue seems to release the incisor placodes from the tooth-surrounding *ectodin*-expressing mesenchyme resulting in a phenocopy of the *ectodin* mutants. This suggests that the apoptotic disappearance of tooth-forming potential is caused by the mesenchymal Ectodin, which probably restricts the key signalling needed for tooth development to proceed. In *ectodin* mutants, Bmp4-protein treatment-increased *Shh*-expression in the tooth epithelium, indicating that Bmp-4 lies somewhere upstream of *Shh*-inducing pathway. Removal of the surrounding mesenchyme in wild type incisors also increased the stimulation of *Shh* expression by Bmp-4, suggesting that one role for ectodin is to spatially limit the inductive potential of dental mesenchyme.

REFERENCES: [1] J. Laurikkala, et al (2003) *Developmental Biology*, **264**:91-105

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LOSS OF SPROUTY GENE FUNCTION LEADS TO DENTAL ANOMALIES BY RENDERING DENTAL TISSUES HYPERSENSITIVE TO FGF SIGNALING

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The Sprouty gene family encodes intracellular proteins that antagonize Fibroblast Growth Factor (FGF) and other receptor-tyrosine kinase signaling. Our previous studies in mice established that two Sprouty family members, *Spry2* and *Spry4*, are essential for the formation of the normal number of teeth in the molar region (Klein et al., 2006). Based on these studies, we proposed that Sprouty genes modulate two discrete parts of an epithelial-mesenchymal FGF signaling loop, and that loss of Sprouty function leads to upregulation of this signaling loop, resulting in diastema tooth development. In our current work, we are studying the role of Sprouty genes in incisor development. The rodent incisor has evolved two modifications, relative to the ancestral mammalian incisor, which enable it to maintain the sharp incisal edge required by its diet (Wang et al., 2004). The first modification is continuous growth, which is thought to be fueled by the presence of epithelial and mesenchymal stem cells in the proximal part of the tooth. The second modification is the deposition of enamel exclusively on the labial surface of the incisor. The coupling of continuous growth and asymmetric enamel distribution leads to maintenance of a sharp edge as the maxillary and mandibular incisors file each other down. We have found that when Sprouty genes are inactivated in combination, dramatic effects on incisor development result. We demonstrate that mice that are heterozygous for either *Spry1* or *Spry2* and homozygous null for *Spry4* have lingual enamel, and they develop remarkable 'tusk'-like incisors because the presence of the ectopic lingual enamel prevents filing down of the teeth. The lingual enamel resulting from loss of Sprouty function occurs as a consequence of the development of a lingual adult stem cell

population, and this population forms because of hypersensitivity of the dental tissues to FGF signaling. Interestingly, loss of function of only *Spry4* in an animal that is wild-type for *Spry1* and *Spry2* leads initially to the development of a lingual stem cell region. However, a regression of the stem cell-containing region occurs in *Spry4* null mice, indicating that Sprouty genes cooperate to prevent lingual enamel formation. First, *Spry4* functions to prevent development of lingual ameloblast stem cells. Subsequently, *Spry1* or *Spry2* ensure that any stem cells that have developed on the lingual side will regress, thereby providing a second layer of defense against formation of lingual enamel. Together, our studies indicate that Sprouty genes are critical regulators of mammalian tooth development.

REFERENCES: Klein, O. D., Minowada, G., Peterkova, R., Kangas, A., Yu, B. D., Lesot, H., Peterka, M., Jernvall, J., and Martin, G. R. (2006). Sprouty Genes Control Diastema Tooth Development via Bidirectional Antagonism of Epithelial-Mesenchymal FGF Signaling. Dev Cell *11*, 181-190.

Wang, X. P., Suomalainen, M., Jorgez, C. J., Matzuk, M. M., Werner, S., and Thesleff, I. (2004). Follistatin regulates enamel patterning in mouse incisors by asymmetrically inhibiting BMP signaling and ameloblast differentiation. Dev Cell *7*, 719-730.

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MOLECULAR NETWORKS UNDERLYING DENTAL DEFECTS IN THE DIGEORGE SYNDROME

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INTRODUCTION & DISCUSSION: *TBX1* is a key player in the aetiology of the DiGeorge/Velo-cardio-facial syndrome (DGS/VCFS), which is a complex developmental disorder associated with a variety of abnormalities, including facial dysmorphology, submucous cleft palate and incisor hypoplasia or aplasia. Targeted disruption of *Tbx1* in mice results in facial abnormalities that are very similar to those presented in the human DGS. We investigate the role of Tbx1 in normal tooth development and analyse the consequences of Tbx1 deletion on the formation of teeth. *Tbx1* expression is restricted to the epithelial component of tooth primordia and appears to mark the epithelial cells destined to give rise to the enamel matrix producing ameloblasts. Using Tbx1-/- mice we have identify genes whose expression is altered in the absence of Tbx1 in dental structures, as compared to the wild type mice. In addition, some of the Tbx1 activities may be mediated through the control of FGF expression in the developing teeth. Regulators and targets of Tbx1 should unveil genetic networks that are affected in tooth developmental disorders.

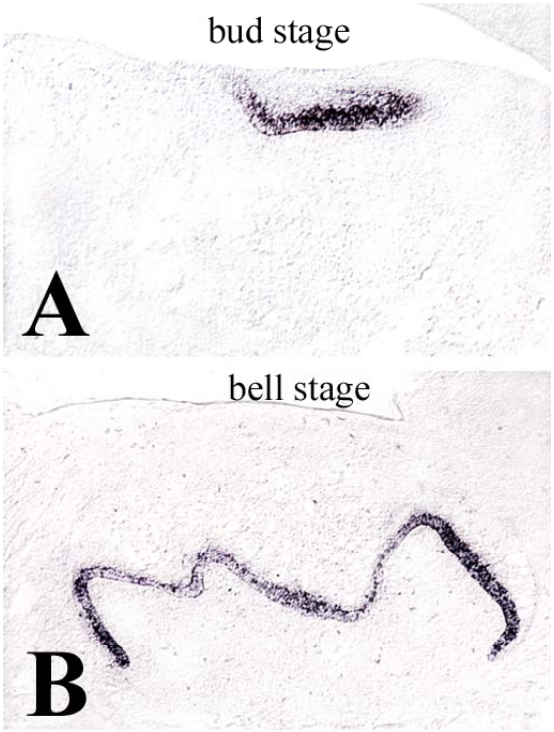

Figure: Expression of Tbx1 in the epithelium of the developing tooth during the bud (A) and bell (B) stages.

REFERENCE: M. Zoupa, M. Seppala, T. Mitsiadis, M. Cobourne (2006). Int. J. Dev. Biol. 50: 504-510.

ODONTOBLASTS AS SENSOR CELLS

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Odontoblasts are post-mitotic cells involved in the dentine formation throughout the life of the tooth and suspected to play a role in tooth pain transmission. They are organized as a single layer of specialized cells along the interface between dental pulp and calcified dentinal tubules into which run a cellular extension (odontoblast process) bathed in a liquid phase. . Interestingly, a primary cilium in the vicinity of the Golgi apparatus has been regularly described at the ultrastructural level and recently antibodies directed against detyrosinated α tubulin specifically identified this structure in human odontoblasts (1). The role of this primary cilium remains unknown but it was suggested that it could constitute, when deflected, a critical link between the transfer of fluid, molecules or ions from dentinal tubules to pulp tissue and odontoblast response to stimuli. Dense sensory unmyelinated nerve fibres surrounded the odontoblast bodies, coiled around the cell processes and give to this complex (nerve/odontoblast) a fundamental role as an active barrier between dentine and pulp following external stimuli (mechanical thermal, electrical, osmotic shock…). Thus, this unique spatial situation of odontoblasts closely related with nerve endings and fluid movements suggest that odontoblasts could convert pain-evoking fluid displacement within dentinal tubules into electrical signals *via* at least mechanosensitive ion channels and bending of the primary cilium. Along this line, two kinds of mechanosensitive K^+ channels have been identified in human odontoblasts: I- TREK-1 channels

belonging to the two-pore-domain potassium channel family and expressed in the plasma membrane of coronal odontoblasts; II- high-conductance Ca^{2+} -activated potassium channels (K_{Ca}) activated by stretch of the membrane as well as osmotic shock (2,3). These findings strengthened by the recent evidence for excitable properties of odontoblasts, concentration of mechanosensitive channels in the borderline between cell extension and bodies and clustering of key molecules at the site of odontoblast-nerve contact strongly suggest that odontoblasts may operate as sensor cells (4).

REFERENCES: [1] H. Magloire, ML. Couble, A. Romeas et al.,(2004), *Cell Biol Int*, **28**: 93-99. [2] B. Allard, ML. Couble, H. Magloire et al. (2000) *J Biol Chem*, **275**:25556-25561. [3] H. Magloire, F. Lesage, ML. Couble et al. (2003) *J Dent Res*, **82**:542-545. [4] B. Allard, H. Magloire, ML. Couble et al. (2006) *J Biol Chem*, **281**:29002-29010.

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DENTIN MATRIX PROTEIN 4 FUNCTIONS IN TERMINAL DIFFERENTIATION OF ODONTOBLASTS

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INTRODUCTION: Knowledge of differentiation of the dental mesenchyme into odontoblasts is rapidly progressing, however, the molecular mechanism responsible for terminal differentiation of odontoblasts remains largely unknown. To identify genes that are synthesized by terminally differentiated odontoblasts, we have used a systematic approach to identify genes synthesized by the odontoblasts during mineralized matrix formation. Suppressive hybridization technique was used to identify novel genes synthesized by the odontoblasts during the terminal differentiation process. Using this approach we have identified dentin matrix 4 (DMP4), a novel gene localized in polarizing odontoblasts. To identify the function of DMP4 we first decided to study the temporo-spatial localization of DMP4 in the developing tooth germs.

METHODS: Cloning of DMP4: The full length cDNA was amplified using the RACE (Rapid Amplification of cDNA Ends) methodology and cloned into pBluescript and used as probe for Northern Analysis and in-situ hybridization.
Northern Analysis: Northern blot analysis was performed as published earlier [1].
In-situ hybridization Analysis: The 3-kb full-length DMP4 cDNA was linearized for in-vitro transcription and used for the synthesis of sense and anti-sense probes.

RESULTS: A full-length cDNA of ~3kb was obtained using the RACE technique. Northern blot analysis performed on total RNA isolated from various tissues identified its presence in liver, brain and kidney however, high expression was observed in calcified tissues of bone and teeth. In-situ hybridization performed on incisor and molar sections obtained from mouse embryos at day 20 and newborns at days 3, 5 and 7 days identified earliest expression of DMP4 in the polarizing odontoblasts at the cuspal region. Interestingly, DMP4 was expressed in the secondary enamel knots of E20 molars. In P3 incisors and molars the expression of DMP4 was observed in all differentiating odontoblasts as well as in the secretory ameloblasts. However, the level of expression decreased in the odontoblasts of P7 mice.

DISCUSSION: Presence of DMP4 in the preameloblasts and preodontoblasts, early during development suggests that DMP4 might facilitate cross-talk between the epithelial and the mesenchymal cells during formation of their respective terminally differentiated state. The presence of DMP4 in the secondary enamel knot is interesting as it implies that such specific molecules could instruct when and where specific phenotypes will emerge in the course of tooth development. The secondary enamel knots correlate with cusp patterns and the presence of DMP4 suggests that it could regulate tooth morphogenesis during early development. We believe that DMP4-mediated early signaling event might be necessary for the terminal differentiation of odontoblasts.

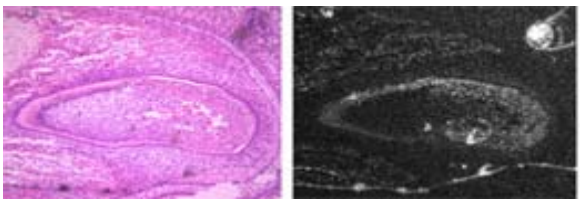

Fig: 1 In-situ hybridization showing the expression of DMP4 in E20 mouse incisor

REFERENCES: [1] J. Hao, K. Narayanan, T. Muni, A. Ramachandran & A. George (2007) Dentin Matrix Protein 4, a novel secretory calcium-binding protein that modulates odontoblast differentiation. J. Biological Chemistry 282: 15357-15365.

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EXTRACELLULAR MOLECULES STIMULATE THE FORMATION OF REPARATIVE DENTIN IN EXPERIMENTALLY EXPOSED ADULT DENTAL PULP OF RATS AND MICE.

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Pulp exposure may occur during the preparation of deep cavities or be the consequence of carious decay. For decades the only biological option have been to heal the pulp by forming a reparative dentinal bridge using calcium hydroxide. Over the past few years, bioactive molecules have been experimentally implanted, and variable results were obtained (Goldberg & Smith, *Crit Rev Oral Biol Med* 2004). We have developed an experimental model using the first maxillary molar in the rat, first, drilling a cavity after gingival electrosurgery, and then pushing the deeper part of the cavity with a steel probe to expose the pulp. We also used a surgical approach for the incisor, allowing implantation of bioactive molecules in the forming zone of the mandibular incisor in rodents. Agarose beads soaked in a culture medium supplemented with the bioactive molecule have been implanted for 8 to 90 days in the molar, and for 10-20 days in the incisor.

In separate experiments, we implanted in the rat molar structural extracellular molecules, which may be also matricellular proteins, including Bone Sialoprotein (BSP), or signaling molecules such as leucine-rich amelogenin peptides (LRAP) produced by ameloblasts, or the low molecular spliced amelogenins that are synthesized by odontoblasts (A+/-4). These procedures induced a slight inflammation at day 8, and stimulated the recruitment of cells involved in the reparative process. After 15 days, reparative dentin started to be formed in the exposure area, appearing as a dentinal bridge or a diffuse mineralization in the coronal pulp. In addition, with A-4, the root canals were totally occluded by reparative dentin. The recruitment of reparative pulp cells may involve 1- either dormant (or latent) adult committed odonto/osteoblast progenitors, or 2- cells issued from the transdifferentiation of pulp fibroblasts; or 3- selected lineage(s) derived from inflammatory cells that de-differentiate and re-differentiate into odontoblast-like progenitors. As shown by PCNA staining, at day 8 labeled cells were seen in the central part of the coronal pulp, near the agarose beads acting as carrier, and at the periphery of the root pulp beneath the Höehl's (sub-odontoblastic) layer. At day 15, proliferation was reduced in the coronal pulp and disappeared in the root. We used RP59, a marker for osteoblast progenitors, and positively labeled cells appeared near and around the carrier beads. Osteopontin (OPN)- positive cells increased in number between 1-3 and 8 days. At day 8, cells located around the beads were strongly OPN positive, whereas they were negative for dentin sialoprotein (DSP) staining, suggesting an early differentiation along the osteoblastic lineage. At day 15, a few cells located near the pulp exposure site were DSP positive, whereas the cells in close contact or around the carrier beads formed a ring immunostained for OPN. Mineralization, which started at day 15, was achieved at day 30, and did not vary much at day 90. The reaction created by Dentonin, a peptide from MEPE, differed from other biomolecules in that though cell recruitment was stimulated, with proliferation and commitments toward early stages of differentiation. The process was then apparently stopped at that point. Terminal mineralization was heterogeneous, some teeth forming reparative dentin whereas in other pulps, there was no evidence for such formation (Six et al. *J Dent Res*, 2007). In the rodent incisor, implantation of A+/-4 revealed at day 10 the formation of bone-like dendritic structures in the central part of the pulp, which increased in size and developed into a diffuse mineralization. The pulp exposure site where beads were implanted was filled firstly by osteodentin and later, with thickening in some areas by reactionary orthodentin.

Altogether, these experimental approaches allow a better understanding of the cascade of events occurring between cell commitment and the terminal differentiation. It also allows screening of the individual effects of bioactive molecules. The specificities of each molecule lead to their reclassification as matricellular, structural and bioactive molecules (growth factors), although most structural proteins appear to be involved as signaling and bioactive molecule.

TGF-ß1 INDUCES APOPTOSIS THROUGH SMAD SIGNALING-PATHWAY IN ODONTOBLASTS OF NFI-C NULL MICE

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INTRODUCTION: The critical roles of transcription factors and growth factors in tooth development especially for crown formation have been well documented. However, the molecular mechanism responsible for root development is not well defined. The NFI gene family encodes the site-specific transcription factors essential for the development of a number of organ systems. There are four NFI gene family members in vertebrates (*Nfia*, *Nfib*, *Nfic*, and *Nfix*). Our previous studies have demonstrated that nuclear factor I-C (*Nfic*) null mice developed short molar roots that contain aberrant odontoblasts and abnormal dentin formation. Based on these findings, studies were performed to uncover the underlying mechanism of *Nfic* function in odontoblasts during root formation.

METHODS: In this study, we investigated the expression of p-Smad2/3 and TGF β-RI in *Nfic* (-/-) mice using immunohistochemistry, RT-PCR and western analysis in vivo and in vitro. Second, we investigated if disruption of *Nfic* gene causes cell growth arrest and apoptosis of odontoblasts. We evaluated the cell proliferation and apoptosis by BrdU staining, MTT assay, flow cytometry, and TUNEL staining. Third, we tried to examine the molecular mechanism of cell growth arrest and apoptosis in *Nfic* (-/-) odontoblasts. Fourth, cDNA microarray was also performed for the identification of *Nfic*–related gene alteration in odontoblasts.

RESULTS: Initial studies demonstrated that disturbance of the *Nfi-c* gene increased both TGFβ-RI and p-Smad2/3 expression in aberrant odontoblasts and pulp cells in the sub-odontoblastic layer *in vivo*, and primary pulp cells from *Nfic* (-/-) mice as well as MDPC 23 cells transfected with dominant negative transgene *in vitro*. Cell proliferation analysis of both pulp cells and HERS of the *Nfic* (-/-) mice revealed a significantly decreased proliferation activity compared to normal. Also, *Nfic* (-/-) primary pulp cells showed increased expression of p21 and p16, but a decreased cyclin D1 and cyclin B1 expression, strongly suggesting a cell growth arrest when the *Nfic* gene function was disturbed. Analysis of apoptotic cells in the sub-odontoblastic layer of the pulp in *Nfic* (-/-) mice exhibited an increased apoptotic activity in *Nfic* (-/-) mice. Futher, *Nfic* (-/-) primary pulp cells and *Nfic*-inactivated MDPC-23 cells increased not only the expression of Fas and FasL but also the activation of caspase-8 and -3, while the cleaved form of Bid was hardly detected. These results indicate that disturbance of the *Nfi-c* gene suppresses odontogenic cell proliferation and induces apotosis of aberrant odontoblasts by up-regulating the expression of TGFβ-RI and its downstream signaling molecules during root formation, contributing to the formation of short roots.

DISCUSSION & CONCLUSIONS: These results are expected to help better understand the molecular mechanism responsible for cell proliferation, differentiation and apoptosis in odontoblasts during tooth root development. However, more study will be needed to determine if *Nfic* is required in odontoblasts, the epithelial component of the root, or both, for root development using the conditional knockout allele of *Nfic*.

REFERENCES: [1]George Steele-Perkins, Kenneth G. Butz, Gary E. Lyons, Margarita Zeichner-David, Heung-Joong Kim, Moon-Il Cho, and Richard M. Gronostajski1 (2003) *Mol Cell Biol* **23**, 1075–1084. [2]Joo-Cheol Park, Yeek Herr, Heung-Joong Kim, Richard M. Gronostajski, Moon-Il Cho (2007) *J Periodontol*. In-press. [3]Tamizchelvi Thyagarajan, Taduru Sreenath, Andrew Cho, J. Tim Wright, Ashok B. Kulkarni (2001) *J. Biol. Chem.* **276**, 11016–11020.

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ODONTOBLASTS SENSE PATHOGENS AND TRIGGER AN IMMUNE RESPONSE IN THE HUMAN DENTAL PULP.

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INTRODUCTION: The human tooth is the target of a substantial number of oral bacterial agents which are responsible for the development of carious lesions. These agents induce demineralization of enamel that normally constitutes an impermeable barrier protecting the underlying dentin and pulp tissues from the oral environment. When the enamel barrier is disrupted, dentin becomes exposed to the oral environment and is degraded by Gram-positive bacteria, including *Streptococcus*, *Lactobacillus* and *Actinomyces* spp. that largely dominate the dentin carious lesion microflora. Bacterial penetration into the disrupted dentin leads to the development of inflammatory and immune events in the underlying dental pulp, the molecular and cellular determinants of which remain largely unknown.

RESULTS: In this study we evidenced by real-time PCR that *in vitro* differentiated odontoblasts expressed genes encoding the pattern recognition molecules TLR1-6 and 9, but not TLR7, 8 and 10. Expression levels of TLR2, 3, 5 and 9 were significantly increased when odontoblasts were stimulated by lipoteichoic acid (LTA), a component of Gram-positive bacteria sensed by TLR2. TLR2 increase was confirmed at the protein level by flow cytometry. Immunostaining showed the localization of TLR2 in the cell membrane of LTA-stimulated odontoblasts, whereas TLR2 was not detected in unstimulated cells. Translocation of the NF-κB transcription factor from the cytoplasm to the nucleus confirmed TLR signalling pathway activation. Gene array and real-time PCR analyses demonstrated that odontoblasts expressed several chemokine-related genes among which CCL2, CCL7, CXCL2 and CXCL10 were up-regulated by LTA. Antibody array analysis revealed a higher level of CCL2 and CXCL10 in culture supernatants from LTA-stimulated odontoblasts than in controls. These supernatants augmented immature dendritic cell migration *in vitro*. Immunohistochemical analysis of human teeth demonstrated that CCL2 was expressed *in vivo* by odontoblasts and blood vessels present under active carious lesions, but not in healthy dental pulps. Finally, real-time PCR analysis revealed that gene expression of major dentin matrix components (type I collagen, dentin sialophosphoprotein) and TGF-β1 was down-regulated in odontoblasts *in vitro* by LTA.

DISCUSSION & CONCLUSIONS: Together these data suggest that odontoblasts activated by LTA are able to initiate an immune response by secreting chemokines that recruit immature dendritic cells, while down-regulating their specialized functions of dentin matrix synthesis and mineralization. These results support a role for odontoblasts in the sensing of pathogens that enter dentin tubules during the carious process and in the triggering of immune events within the pulp tissue.

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REFERENCE: SH. Durand, V. Flacher, A. Roméas, F. Carrouel, E. Colomb, C.Vincent, H. Magloire, M-L. Couble, F. Bleicher, M-J. Staquet, S. Lebecque, and J-C. Farges (2006) *J Immunol* **176**:2880-87.

IN VITRO STUDIES ON BONE TISSUE ENGINEERING

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Physiological regeneration especially of larger bone defects remains an unsolved problem. One of the major challenges in tissue engineering (TE) strategies is the provision of an adequate blood supply, this being the focus of our research. There are many approaches, one of the current ones being the incorporation of a drug or gene delivery system for pro-angiogenic factors (e.g. VEGF), for example, into a suitable scaffold material. Whilst ultimately requiring proof of principle in studies *in vivo* there is a need for suitable *in vitro* methods to delineate underlying mechanisms operating in the complex regenerative niche. Relevant methods must aim to use primary human endothelial cells (EC) in 3D culture systems simulating as closely as possible the situation in vivo during bone healing. In this presentation examples of such assays will be given, using micro- and nanofibre meshes of polymeric scaffolds (silk fibroin, and a blend of starch with poly(caprolactone), SPCL), but porous ceramics and metals have also been used.

Confocal laser scanning microscopy (CLSM) to localize specific surface and intracellular proteins, RT-PCR to study gene expression at mRNA level and scanning and transmission electron microscopy (SEM, TEM) are the principal methods used to study cell-biomaterial interactions. In addition, analytical techniques such as ELISA and cell-EIA are also employed to determine concentrations of soluble factors released into the supernatant or expressed on the cell surface respectively. The EC types are mostly microvascular EC (MEC), as these are the relevant cells for neo-angiogenesis, but endothelial progenitor cells (EPC) from human peripheral blood have also been studied, as one aim would be to recruit these cells from the circulation to the bone regenerative niche.

Generally, protein adsorption with fibronectin is required to permit good adhesion and growth of EC on the biomaterials [1], but a plasma polymerization step has been shown to be equally effective in the case of SPCL [2]. Silk fibroin or SPCL micromeshes provide an excellent scaffold for EC colonization, but the provision of additional nanofibre structures of the material on the micromesh permit a vast increase in the density of EC, these cells adopting the morphology of the angiogenic phenotype. The isolated EPC yield a mature phenotype, called OEC (outgrowth EC), which give vascular structures with lumen formation in interaction with fibroin and type I collagen [3]. Further studies show that it is possible to adapt the 3D angiogenesis assay in a collagen type I hydrogel to study the interface with various metals. No impairment was seen for EC at the interface between collagen and TiO_2, but angiogenesis was disturbed in contact with cobalt-chromium alloys.

Remarkable results have come from co-culture studies of MEC and primary human osteoblasts (pHOB) in the presence of the 3D biomaterial scaffolds. The addition of collagen type I to the co-culture has yielded massive vessel-like branching sprouts segregated from the pHOB, giving a tissue-like self-assembly [4]. Of special significance is the fact that this reaction occurs in the absence of exogenous pro-angiogenic factors and thus provides data questioning the necessity for such additional growth factors. Further studies are being carried out in this system to investigate the effects of the hetero-typic cell interactions on the individual cell types. Other investigations are concentrating on how biomechanical stress affects cell functionality in the co-culture systems.

REFERENCES: [1.] R.E. Unger, M. Wolf, K. Peters, et al. (2004) *Biomaterials* **25**:1069-75. [2.] M.I. Santos, S. Fuchs, M.E. Gomes, et al. (2007) *Biomaterials* **28**:240-8. [3.] S. Fuchs, A. Motta, C. Migliaresi, et al. (2006) *Biomaterials* **27**:5399-408. [4.] R.E. Unger, A. Sartoris, K. Peters, et al. (2007) *Biomaterials* **28**: in press

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CALCIUM MEDIATED DIFFERENTIATION OF AMELOBLAST LINEAGE CELLS *IN VITRO*

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INTRODUCTION: Calcium is a key component of the mineralized enamel matrix. Our previous studies have shown that the morphology of human ameloblast lineage cells is altered by increased calcium in the media. This observation along with the identification of the calcium sensing receptor in enamel organ epithelial-derived cells (1,2), suggest a signaling role for calcium in ameloblast mediated enamel formation. The hypothesis tested in this study was that calcium promotes ameloblast lineage cell differentiation.

METHODS: Primary human ameloblast lineage cells (ALC) were isolated from human fetal tooth buds as previously described (3), and were grown in KGM-2 to passage 2. The cells were grown in 96 well tissue culture dishes, treated with 0, 0.05, 0.3, or 1.8 mM calcium and assayed for proliferation by BrdU immunoassay. Other cells were grown in 10 mm tissue culture dishes, and assayed for mineral formation by Von Kossa staining. Calcium mediated regulation of matrix metalloproteinase 20 (MMP-20) was analyzed by luciferase reporter gene assay.

RESULTS: Increasing concentrations of calcium significantly reduced ameloblast lineage cell proliferation *in vitro* (Fig 1). At 1.0 mM calcium, ameloblast lineage cells began to pile up and form nodules, and in the presence of 3 mM calcium, the cells could form a mineralized matrix (Fig 2).

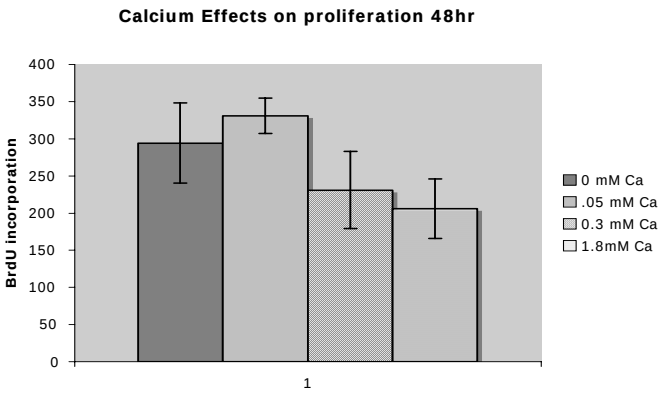

Fig.1 BrdU incorporation in ALC exposed to different levels of calcium for 48 hrs. Proliferation was significantly reduced in cells exposed to 1.8 mM calcium as compared to 0.5 mM calcium, *p<0.05.

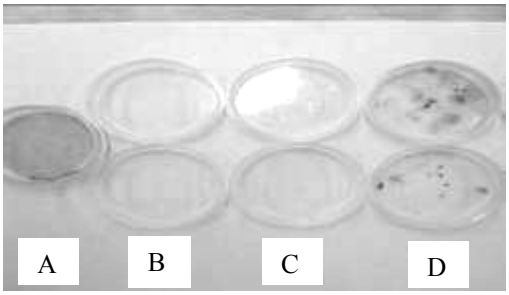

Fig 2. von Kossa staining: A) Positive control, dental pulp cells grown in 3 mM Ca^{2+} showed generalized staining. B-D) ALC grown in 0.0, 0.3 and 3.0 mM Ca^{2+} respectively, showed mineralization nodules forming only at 3 mM Ca^{2+}.

MMP-20 reporter gene assays showed that calcium promoted upregulation of the MMP-20 promotor through AP-1 binding sites.

DISCUSSION & CONCLUSIONS: These results show that calcium promotes ameloblast lineage cell differentiation. Calcium may regulate gene expression, resulting in upregulation of MMP-20 through AP-1 binding sites.

REFERENCES: [1] Bawden, W. J. *et al* (2000). "Immunohistochemical localization of Gqα,PLCβ, Giα, PKA, Endothelin B, and extracellular calcium sensing receptor during early amelogenesis." *Journal of Dental Research* 79:1896-1909. [2]Mathias, R. *et al* (2001). "Identification of calcium sensing receptor in developing tooth organ." *J Bone Miner Res*12: 2238-2244. [3]DenBesten, P K. et al (2005). "Characterization of human primary enamel organ epithelial cells in vitro" *Arch Oral Biol*: 689-694.

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DLX HOMEOPROTEINS IMPLICATION IN AMELOBLASTS ORGANIZATION AND FUNCTION.

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INTRODUCTION: Homeobox genes of the *Dlx* family participate in early tooth development through their implication in two major events: tooth initiation and morphogenesis (1). The expression of one member of the family, *Dlx2*, was also reported during amelogenesis, a late stage in tooth development (2). Indeed, using a *Dlx2/LacZ* transgenic mouse model, stage specific expression of *Dlx2* in ameloblasts was observed in mouse continuously growing incisors. A linear inverse relationship between enamel thickness and *Dlx2* expression was also established (2), raising the question of *Dlx* implication in enamel morphological control.

METHODS: In this study, we investigate whether DLX acts as a transcriptional regulator of amelogenin, the major component of enamel organic matrix, using *Dlx2/LacZ* transgenic mice, amelogenin immunohistochemistry, vitamin D receptor mutant mice and amelogenin promoter analysis techniques. We also investigate whether DLX are necessary to ameloblasts organization using *Dlx2* and *Dlx1/Dlx2* mutant mice.

RESULTS: We report, using *Dlx2/LacZ* transgenic mice, that *Dlx2* and amelogenin have complementary expression patterns during both molar and incisor amelogenesis. We also evidence that both amelogenin and *Dlx2* expressions are jointly perturbed in dental cells during rickets using vitamin D receptor KO mice. Sequence analysis of the amelogenin gene promoter revealed five potential response elements for DLX that were shown to be functional for DLX2 in *in vitro* experiments. Moreover, we show that *Dlx2* with *Dlx1* are essential to achieve normal molar ameloblast functional organization (Figure 1) using *Dlx2* and *Dlx1/Dlx2* mutant mice.

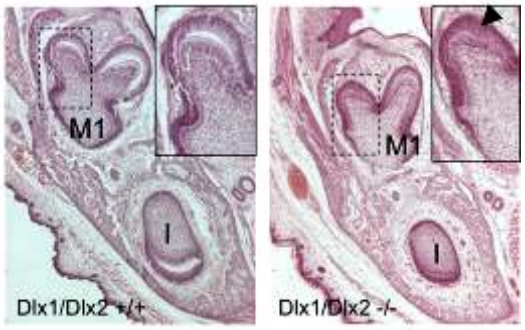

Fig. 1: Dlx1/Dlx2 null mutation impact on mouse molar ameloblasts organization. Ameloblasts of newborn first molar buccal part appear badly organized in double mutant (right) while normal in wild-type mice (left).

DISCUSSION & CONCLUSIONS: This study establishes supplementary functions of *Dlx* family members during tooth development. These functions are a participation in the functional organization of the dental epithelium that remain to be analyzed at molecular level, and the control of enamel morphogenesis via the regulation of amelogenin expression.

REFERENCES: [1]B. Thomas, AS. Tucker, M. Qiu M et al (1997) *Development* **124**:4811-18. [2]F. Lezot, B. Thomas, D. Hotton et al (2000) *J Bone Miner Res* **15**(3):430-41.

ACKNOWLEDGEMENTS: Authors thank Dr Shigeaki Kato and Pr Satoshi Sasaki for the gift of the VDR mutant mouse line and the amelogenin antibody, and Dr. William Abrams for technical assistance.

LOCALIZATION AND FUNCTION OF THE ANIONIC EXCHANGER AE2 IN DEVELOPING RODENT TEETH

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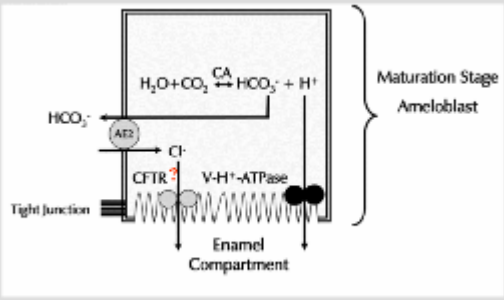
INTRODUCTION: The presence of cytoplasmic carbonic anhydrase II and V-ATPase in the distal cell membranes of maturation stage ameloblasts[1,2] suggests that these cells produce and secrete protons into the enamel space. Proton secreting cells have to regulate intracellular pH to maintain electroneutrality. One candidate for regulating intracellular pH is the anionic exchanger Ae2 that exchanges bicarbonate for chloride. We tested the hypothesis that the anion exchanger-2 (Ae2) is involved in pH regulation in maturation stage ameloblasts.

METHODS: Paraffin sections of jaws of 2-6 days old hamsters and adult wild type and Ae2 knockout mice were used for immunohistochemistry. MMA embedded tissue blocks of wild type and Ae2 knockout mice were subjected to microprobe analysis

RESULTS: Strong immunostaining for Ae2 was detected in basolateral membranes of maturation stage ameloblasts whereas weak staining was seen in the Golgi area of secretory ameloblasts. Staining was also seen in Golgi area of young odontoblasts, osteoblasts, and osteocytes.

Gene targeting of three of the five isotypes of Ae2 in mice affected the structure of maturation-stage but not secretion-stage ameloblasts. It abolished the immunostaining for Ae2 in ameloblasts and reduced the mineral content of maturation stage enamel along with a slightly higher protein content. Secretory stage enamel, dentin and bone in the transgenic mice were not structurally altered. Mineral content of dentin and alveolar bone in the transgenic mice was not different from wild-type littermates. Incisor enamel was more severely affected than molar enamel. The enamel from transgenic mice wore down much faster than that from wild type littermates.

Fig.1 Proposed model for Ae2 function in maturation stage ameloblasts. CA: carbonic anhydrase-II, Ae2: anion exchanger 2; CFTR: cystic fibrosis transmembrane regulator[3]



DISCUSSION & CONCLUSIONS: The presence of anionic exchanger Ae2 associated with the basolateral membranes of maturation stage ameloblasts is essential for their function. Ae2 may regulate intracellular pH in maturation phase ameloblasts (Fig.1). Without Ae2 the mineral growth in maturation stage enamel is impaired and eventually the enamel that enters the oral cavity is much softer.

REFERENCES: [1] H.M. Lin., H. Nakamura, T. Noda and H. Ozawa H. (1994) *Calc Tissue Int,* **55**: 38-45. [2] S. Toyosawa S., Y. Ogawa, T. Inagaki and N. Ijuhin (1996) *Cell Tissue Res* **285**: 217-225. [3] W. Sui, C. Boyd and J.T. Wright (2003) *J Dent Res* **82**: 388-392.

ACKNOWLEDGEMENTS:
This work was supported by NIH grant DE13508-06 (DL and AB) and by a grant from The Netherlands Organization for Scientific Research (NWO), program 912-02-073 (ROE). We furthermore acknowledge the support of COSTB23.

REUPTAKE OF EXTRACELLULAR AMELOGENIN BY DENTAL EPITHELIAL CELLS RESULTS IN INCREASED LEVELS OF AMELOGENIN mRNA THROUGH ENHANCED mRNA STABILIZATION

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INTRODUCTION: In this study, we focused on effects of amelogenin on the differentiation of dental epithelial cells. We produced recombinant mouse amelogenin in baculovirus insect cell expression system and administered Amelogenin protein to dental the epithelial cell line (HAT-7). Our results indicated that amelogenin protein induces increases in the quantity of amelogenin mRNA through enhancing mRNA stability. Here, we describe a unique role for amelogenin protein regulating amelogenin mRNA quantity at the post-transcriptional level.

METHODS: The mRNA levels of differentiation-related marker genes were determined by quantitative real-time PCR as described previously (1-3). A rat amelogenin promoter driving luciferase reporter plasmid was constructed as described in previous study (4) according to the genomic database information of rat (NW_048039). Transient tranfection luciferase assays were performed with Lipofectamine 2000 (Invitrogen). 200 µg of Amelogenin protein was precipitated and re-dissolved in 200 µl of 0.1 M bicarbonate buffer (pH 9.5). Then, 20µl of 1 M bicarbonate buffer (pH 9.0) was added. Next, 20 µl of FITC solution (10 mg/ml) was added and incubated with stirring for 1 h on ice. The reaction mixture was passed through a G-50 column to remove excessive FITC. For the study of uptake and localization of exogenous amelogenin, FITC-Amelogenin was added exogenously to cells at a concentration of 10 µg /ml and followed by a 4-hour culture period. The cells were fixed in paraformaldehyde and the nuclei were stained with Hoechst dye. Coverslips were mounted and cells were observed under laser confocal microscope (Zeiss, LSM 510).

RESULTS: Recombinant mouse Amelogenin enhanced expression of endogenous amelogenin mRNA in a cultured dental epithelial cell line (HAT-7), despite of a lack of increased amelogenin promoter activity. To solve this discrepancy, we analyzed effects of Amelogenin protein on stability of amelogenin mRNA. The half-life of amelogenin mRNA is extremely short, but in the presence of Amelogenin protein its half-life was extended four times longer than control. Furthermore, we showed entry of exogenous FITC-conjugated Amelogenin protein into the cytoplasm of HAT-7 cells (Fig.1). It follows from our results that exogenous amelogenin increases amelogenin mRNA levels through stabilization of mRNA in the cytoplasm of HAT-7 cells. Here, we speculate that during differentiation, dental epithelial cells utilize a unique mechanism for increasing the production of amelogenin, the reuptake of secreted amelogenin.

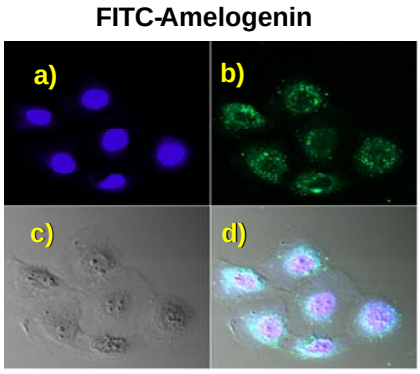

Fig. 1. Uptake and localization of Amelogenin by HAT-7 cells. a) nuclear stain, b) Split XY image of FITC-Amelogenin, c) DIC image and d)composite of the three images, and ortho image of Z-section of composite of FITC-Amelogenin.

DISCUSSION & CONCLUSIONS: In this study, we showed that dental epithelial cells take extracellular amelogenin into the cytoplasm, and increases stability of amelogenin mRNA. It could be speculated that *in vivo*, ameloblasts are able to, in an autocrine fashion dramatically increase production of amelogenin.

REFERENCES: [1] L.Xu, H Harada, A Taniguchi et al., (2006) *J. Biol. Chem.* **281**,2257-2262. [2] L.Xu, H Harada, A Taniguchi et al., (2006) *J. Biol. Chem.* **281**, 32439-32444. [3] L.Xu, H Harada, A Taniguchi et al., (2007) *Open Biotechnol. J.* 1, 18-20. [4]Zhou, Y.L., and Snead, M.L. (2000) *J. Biol. Chem.* **275**, 12273-12280

REVISITED PHENOTYPE OF BONE AND DENTAL CELLS – THE PHYSIOLOGICAL QUESTION OF SITE-SPECIFIC BIODIVERSITY

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INTRODUCTION: Recently, mineral-related genes emerge as specific modules driven by several transcription factors depending on tissue identity. Our laboratory raises another question in cell phenotype, their anatomical diversity. Our goal is to provide some clues on the relationships between anatomical shapes, site-specific homeostasis and regional gene regulation. The strategy used Msx2 as a candidate developmental gene which would play a part in physiology, based on its established roles at various regulatory levels from organs, to cells and genes.

METHODS: Wild-type and transgenic Msx2 knock-in mouse mandibles were studied. Molar and alveolar bone growth was analysed from birth to 28 days. 3 month-old mice enabled the study of adult homeostasis (basal bone, molars and their alveolar bone). The incisors were used for continuous tooth and alveolar bone growth. Dental (epithelial enamel-related and mesenchymal dentin related) and oral bone secretory cells were studied by real time RT-PCR. Triplicate samples were microdissected from 3 month-old Msx2 +/+, Msx2 +/- and Msx2 -/- mice. Amelogenins (AMG), amelin (AMB) and type I collagen α1 chain (Co1A1) were analysed. Microradiographical and morphological studies were done at all stages. Guided by RNA measurements, AMG and AMB immunoperoxydase labeling was performed with sequential dilutions. Bone resorption was studied by tartrate-resistant acid phosphatase (TRAP) histoenzymology. RANK, RANK-ligand and osteoprotegerin (OPG) RNAs were also measured by RT-PCR in the same samples than the ones used for matrix protein RNAs.

RESULTS: AMG and AMB RNAs were expressed in all mineralized tissues, while Co1A1, only in bone and dentin samples. Their relative abundance was higher in enamel versus all mesenchymal mineralized tissues in wild-type mice. Measurements showed statistically significant site-specific reverse up- or down-regulation of their steady levels in Msx2 +/-, Msx2 -/- and control mice. *In situ* studies could indicate an "ectopic" expression but sequential dilutions evidenced affected ratios in epithelial dental cells. Supra-ameloblastic and root sheath cells expressing lowest levels in wild-type mice were reversely the ones showing the highest levels in Msx2 -/- mice. An irregular amelogenesis imperfecta in Msx2 -/- and regional osteopetrosis involving exclusively the alveolar region were evidenced on microradiographs, tissue sections and by TRAP assays on growing teeth. This phenotype was related to a RANK-ligand (but not RANK) decrease in dental and in alveolar bone cells where it was statistically significant. OPG variations were not significant.

DISCUSSION & CONCLUSIONS:
Our data support previous studies on AMG and AMB in bone tissue and cells. Low dilutions in immunoperoxidase assays provide a diffuse pattern in the mandible. They diverged from ameloblast-specific pattern which may be generated by high dilutions but do not correspond to the relative expression levels of RNAs shown here. Msx2 is instrumental in the regulation of AMG and AMB expression in vivo, in a cell-specific manner. Msx2 -/- phenotype is a physiological example of AMG/AMB overexpression in relation with alveolar osteoclast impairment. More refined cell sampling is required to decipher the suspected signaling cascades in physiological cell networks, complex when compared to early development. The most fascinating finding of our strategy was that in physiology and anatomy, there exists molecular fields, delineated by Msx1 and Msx2. Developmental transcription factors could decipher physiopathological pathways, linking specific target-genes (matrix proteins) and -cells within anatomical sites.

ACKNOWLEDGEMENTS: Knock-in mice were provided by B. Robert - France and AMG and AMB antibodies, by M. Fukae - Japan.

SONIC HEDGEHOG AND FGF SIGNALING ARE IMPORTANT FOR TOOTH ROOT DEVELOPMENT

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INTRODUCTION: The cell-therapy for the tooth root tissue regeneration combined with tissue-stem cells and tissue-engineering technology would be a very powerful tool in the future, but there is not enough accumulation of basic knowledge about tooth root development. In fact, several signalling molecules in the Shh, FGF, BMP and Wnt families appear to regulate the developmantal steps of tooth morphogenesis. Here, we examined the biological effects for the root development of typical signaling pathway, SHH[1] and FGF[2].

METHODS: Mice C57BL/6 wild-type and heterozygous *Ptc*^{mes} mutant (Makino *et al*., 2001) mice were used for the experiments.. **Immunohistochemistry.** Immunohistochemistry was carried out essentially according to the manufacturer's methods(Roche). **In situ hybridization and Realtime RT-PCR** In situ hybridization and realtime RT-PCR were performed according to the standard protocol. **Recombinant protein, Protein-beads , and Kidney capsule grafting** P5 mandiblar molars were dissected just before root formation and recombinant proteins were added with beads in attempts to examine the functions in tooth root formation. We examined SHH (50 μg/ml) protein and FGF2 and FGF18 (250μg/ml).

RESULTS: Disturbance of Tooth Eruption and Root Formation in Homozygous *Ptc*^{mes} Mutants. All lower molars had finished eruption in control littermates by P28 (Fig. 1A; n = 11/11), whereas in homozygous mutants, all molars showed delayed eruption (Fig. 1B; n = 7/7). In addition, all tooth roots of mutants (right tooth in Figs. 1C-1E) were shorter than controls (left tooth in Figs. 1C-1E), especially the third molars (white arrow in Fig. 1E). The length of all tooth roots (asterisks in Fig. 1F; $P<0.001$). We examined gene expressions of several molecules involved in SHH pathway and FGF family by realtime RT-PCR (data not shown). Interestingly, *Fgf18* was significantly repressed in the mutants. Therefore, we examined a functional assay of signaling molecules in developing tooth root using recombinant proteins (Fig.2) and observed that FGF2 and FGF18, but not SHH, significantly stimulated tooth root elongation (data not shown).

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Fig. 1: Disturbance of tooth eruption and root formation in homozygous Ptcmes mutants at P28. Wild type (left) vs. Ptcmes (right).

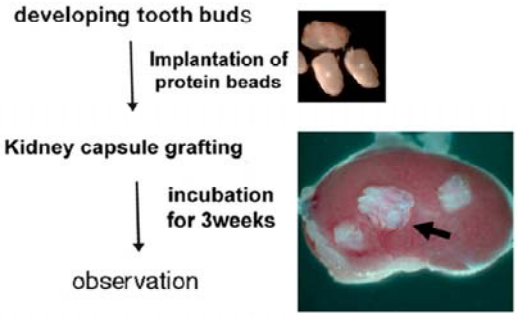

Fig. 2: functional assay of signaling molecule in developing tooth root.

DISCUSSION & CONCLUSIONS:
Our findings indicated that SHH signaling pathway is important for tooth root development and FGFs effectively promote the tooth root elongation and periodontal tissue formation.

REFERENCES: [1]Nakatomi M. et al. (2006) *J. Dent Res*. 85:427-31. [2]Ota M. et al. (2007) *J. Oral Tissue Engn* 4:137-142.

ACKNOWLEDGEMENTS: We thank Dr. T. Shiroishi for permission to use heterozygous Ptcmes mutant mice and RIKEN Bioresource Center for providing them.

AMELOGENIN SIGNALING IN TOOTH ROOT FORMATION

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Introduction: Tooth root development is a complicated process likely involving the interaction of both epithelial and mesenchymal tissues in a similar fashion to tooth crown formation. A porcine enamel matrix protein preparation (EMD), predominantly composed of amelogenin proteins, has been in clinical use to regenerate periodontal tissues. To examine the effects of specific amelogenin proteins ([A-4]/LRAP/M59 and [A+4]/M73) on root development we employed 2 models, an ex vivo murine mandible organ culture system was employed following that described by Fujiwara (Fujiwara, Tabata, Endoh, Ishezeki, and Nawa. 2005, *Cell Tiss Res* Vol 320:69-75), and 3-dimensional cell pellet *in vitro* culture system. Three-dimensional pellet culture has been used to examine the development of chondrocytes, osteoblasts, and dental pulp cells.

Methods: Hemi-mandibles were dissected from post-coital day 24 (PCD) CD-1 mice. Explants were cultured using a modified Trowell technique with A+4 or A-4 as experimental conditions for 1 week, then processed for histology. For the pellet cultures, $1X10^6$ OCCM (immortalized cementoblast cell line) cells were suspended in DMEM/10%FBS into 15 ml tubes. These tubes were spun to pellet the cells. The next day, the cell masses in each tube had formed a 3-D pellet. The media was replaced with 3 mls of either DMEM /5%FBS as a negative control, DMEM/5%FBs with 50ng/ml BMP2 as positive control, or DMEM/5%FBS and 20ng/ml of either A+4 or A-4 as experimental conditions and cultured for 21 days changing the media every 3rd day. Following the culture period, the samples were fixed and processed for histology. We also developed a murine dental follicle cell line, derived from micro-dissected dental follicle of PCD 24 CD-1 mice. This cell line was also used in the pellet culture system as described above. Here, in addition to samples used to prepare histological slides, from additional samples RNA was isolated and cDNA prepared for use in real time PCR.

Results: Microscopic examination revealed that the root explant samples treated with either A+4 or A-4 had an increase in root dentin formed along Hertwig's epithelial root sheath compared to the control conditions. In the OCCM pellet culture, staining with H&E revealed an abundant production of eosinophilic extracellular matrix (ECM) in the amelogenin treated samples, which stained blue with Masson Trichrome indicating this was Collagen. The results of H&E staining the follicle cell pellet cultures revealed the BMP2 samples as well as the amelogenin samples having produced ECM, with BMP2 the most effective. This result was mirrored in the RT-PCR assay for Collagen I RNA expression. When assayed for BSP via RT-PCR, the BMP2 sample had increased expression above control, while the amelogenin treated samples had a reduction in BSP expression, in line with previous results (Viswanathan, Berry,Foster, Gibson, Kulkarni, Snead, Somerman. 2003, *J. Perio*; Vol 10:1423-31)

Conclusion: The amelogenin proteins A+4 and A-4 demonstrated positive effects on root development in both a murine root explant model and cementoblast/follicle cell line pellet culture. These and/or other amelogenin isoforms may play important roles in tooth root development.

A ROLE FOR α11β1 INTEGRIN IN MOUSE INCISOR ERUPTION

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α11β1 integrin is a major collagen receptor in fibroblasts originating from ectomesenchyme and mesodermal structures. We have recently shown that *α11-null* mice show disturbed incisor eruption. In mice lacking α11β1 upper and lower incisors are typically shorter and have altered shape when compared to incisors from wild type animals. Immunohistochemical analysis showed increased collagen in the soft connective tissue placed between the teeth and the alveolar bone, a structure called the periodontal the ligament (PDL). PDL tissue is known to have a high collagen turnover and as a major cell population contain fibroblasts which are assumed to be responsible for regeneration and maintenance of PDL width. We found that α11β1 integrin is the only collagen-binding integrin expressed in PDL fibroblasts and it is also a major integrin in cultured incisor PDL fibroblasts. In order to identify possible cellular mechanism resulting in the observed PDL phenotype in *Itga11* -/- mice we analyzed fibroblasts from these mice in cell attachment, cell spreading, cell proliferation, cell migration and collagen lattice reorganization. Our results support the assumption that the collagen remodeling properties of PDL fibroblasts as well as regulation of incisor eruption occurs in an α11β1 dependent manner, possibly involving MMP-13 and MMP-14.

THE FLEXIBLE MODULATION OF EPITHELIAL DIFFERENTIATION IN THE CERVICAL LOOP SHEDS NEW LIGHT ON THE RELATIONSHIP BETWEEN ROOT FORMATION AND CONTINUOUS GROWTH.

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INTRODUCTION: In the classic literature the first sign of root formation is a structural change in the apical epithelium at the tip of the root, the cervical loop. The central epithelial stellate reticulum and stratum intermedium of the cervical loop disappears leaving a double layer of basal epithelium known as Hertwig's epithelial root sheath (HERS) at the tip of the root. The root extends from the crown and the HERS epithelium fragments. This gives rise to another typical epithelial root structure: the epithelial cell rests of Malassez which consists of small islands of epithelial cells above the HERS. It is also sometimes thought that the maintenance of the cervical loop prevents root formation.

Molar tooth germs of the bell stage of development were grown for up to six weeks in a Trowell-type *in vitro* tissue culture system. The tooth germ developed a root on a macroscopic level. On a microscopic level there were interesting peculiarities. The epithelium making contact with the filter on which the tooth germ was cultured resembled cuboidal root epithelium of the Hertwig's epithelial root sheath without producing epithelial cell rests of Malassez. On the opposing side the tissue was exposed to the air and no epithelium formed. However, the epithelium on the lateral sides maintained all the characteristics of the cervical loop of a continuously growing tooth. The *in vitro* molar culture resulted in a tooth composed of elements of continuously growing and non-continuously growing teeth, showing the potential for the molar to acquire the continuously growing fate under the influence of changes in environmental cues.

This flexibility in the modulation of the epithelium is exemplified by the existence of continuously growing roots, such as the sloth molar. This molar lacks a Hertwig's epithelial root sheath despite producing a root phenotype and at the same time has maintained its cervical loop structure, the epithelial stem cell niche of the tooth. This phenotype can be reconstructed in transgenic models such as the incisor of the k14-eda transgenic mouse. The fate of the labial side has changed from crown analogue to root analogue changing the entire incisor into a continuously growing root. And also here the cervical loop is maintained, indicating that the transition from crown to root epithelium is not directly linked to the fate of the epithelial stem cell compartment into HERS and that the cervical loop acts as a stem cell niche in the entire circumference of the tooth instead of one localization.

ESTABLISHING A 3D *EX VIVO* CULTURE SYSTEM FOR INVESTIGATIONS OF BONE METABOLISM

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Introduction: Mechanical load can act as an anabolic stimulus on bone. Disuse of bone (e.g. immobilisation) can cause a severe loss of mass and strength. Commonly, 2D cell cultures are used to investigate bone related questions. These systems lack mechanical load and the complex 3D interplay between the different cell types. The Zetos[1] (Fig.1) load-providing culture system has been validated with ovine, bovine and human samples to keep 3D cancellous bone tissue cores viable *ex vivo*. Bone integrity and activity was demonstrated during 2 to 3 weeks of culture. Viable osteocytes and bone specific markers could be identified. Culture conditions were improved using a serum free medium containing TGF-β3.

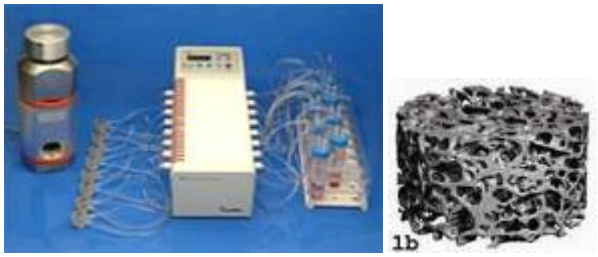

Figure 1. *Ex vivo* Zetos culture system. Microprocessor controlled pump allows perfusion of fresh media through the chambers. The bone cores (1b) are stimulated daily.

Methods: The processing of each bone includes cutting 7 mm thick slices with the use of an Exakt 300 band saw, cores of 9.5 mm diameter are bored from the sections with a Synthes drill bit. Cores are cut parallel to 5 mm height with a Leica annular saw [2]. After removing debris with repeating washing steps, each core is placed in its individual culture chamber and perfused with its individual culture medium with a flow rate of 0.1 ml/min. Mechanical loading can be applied with the bioreactor by inserting a chamber into the loading device. The loading procedure used is performed daily for a duration of 5 min using a complete jump wave form (300 cycles,1 Hz, 4000 μstrain). After long term culture cores can be harvested and analysed in order to answer the specific question.

After culture, cores can be either fixed in 70% ethanol prior to dehydration and Technovit 9100 embedding [3] for histochemical analysis; or fixed and decalcified prior to cryosectioning, to perform immunohistochemical evaluation on 6-12 μm sections.

Investigating bone viability of 3D bone explants after culture can be difficult. The bone matrix autofluorescence interferes with many fluorescence labelling techniques. Radioactive labelling of protein synthesis has inherent risks and can take many weeks to develop images. Both of these methods require embedding or decalcification procedures prior to cutting. We have focused on using a Lactate dehydrogenase assay (LDH) to detect an essential cytoplasmatic enzyme. The high stability of LDH allows cutting of fresh cores prior to staining. After fixation, visualisation of purple stained viable cells can be performed. We could quantify osteocyte viability per area of bone matrix with an optimised method using the natural autofluorescence of the bone matrix to enhance the staining contrast[4].

RESULTS: Histological sections (6 μm thick) of bovine, ovine and human material after culture are comparable to fresh bone concerning matrix and cell integrity, as demonstrated by a variety of stains. The presence of noncollagenous proteins, such as osteopontin, osteonectin and osteocalcin could be localised immunohistochemically.

Using the LDH viability method on human cancellous bone cores, it could be demonstrated, that a daily applied load could maintain higher osteocyte viability after 7 and 14 days of culture in comparison to unloaded control cores. Therefore the bioreactor itself improves 3D culture conditions.

Conclusion: The Zetos system gives the opportunity to study bone biology, hormonal effects as well as biomaterial interactions *ex vivo* prior to animal studies. It also has advantages in reducing the variability, cost and ethics behind *in vivo* studies.

References: [1] Jones D.B. *et al.,* (2003). Eur Cell Mater 5: 48-60. [2] Davies *et al.,*(2006) Eur Cell Mater 11 57-75. [3] Yang R. et al., (2003) Eur Cell Mater (6) 57-71. [4] Stoddart et al., (2006) Eur Cell Mater 12, 16-25.

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CROWN FORMATION DURING TOOTH DEVELOPMENT AND TISSUE ENGINEERING.

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INTRODUCTION: Tooth crown morphogenesis involves epithelial histogenesis, cusps development and cell differentiation. All these steps were achieved when E14 molar epithelial/mesenchymal cell-tissue or even cell-cell reassociations were grown in vitro (1). Although cell position in the epithelial compartment was lost, epithelial histogenesis was rapidly restored. In cultured cell-cell reassociations, crown morphogenesis appeared more difficult to achieve. This raised the question of the control of primary and secondary enamel knots formation and fate. To address and restrict this broad question, attempts were made 1) to determine whether the mesenchyme can control the fate of PEK cells, by using heterotopic reassociations, and 2) to analyze the situation when BMC are reassociated with a dental epithelium (2).

METHODS: Histology and immunostaining have been performed on tooth germs, cultured heterotopic reasssociations between dental epithelia from molars or incisors with converse mesenchymes, and reassociations between molar epithelium and bone marrow cells (BMC) (2). Dental tissues were obtained by trypsin dissociations of mouse tooth germs from ED14 and ED13. BMC were prepared and cultured according the protocol of Ohazama et al., (2). Immunostainings for cadherin and ssDNA were performed as described previously (3).

RESULTS: In heterotopic tissue reassociations between molar and incisor tissues, the formation and fate of a primary enamel knot was compared to what was observed when culturing intact teeth. As expected, the reassociations, the fate of the PEK is directed of the mesenchyme with a complex pattern of segregation of the non-dividing IDE cells of the PEK when controlled by a molar mesenchyme.
Tooth tissue engineering requires the use of non-dental cell sources. When reassociating a ED14 molar epithelium with BMC cells instead of a dental mesenchyme, teeth developed but a molar shape with several cusps could not be achieved.

DISCUSSION & CONCLUSIONS: Tooth functionality depends on crown morphology. Not only the shape of the epithelial-mesenchymal junction (EMJ), but also the progressive gradients of odontoblasts and ameloblasts differentiation, must be preserved in order to allow the final shaping of the mineralized crown. A major constraint for tooth tissue engineering is linked to the growth of the organ, which is hardly compatible with the use of scaffolds to direct morphology. A PEK formed in cultured heterotopic dental tissue reassociations. The mesenchyme controls the fate of the EK cells to drive single/multiple cusps tooth development. By allowing the differentiation of odontoblasts (2) or ameloblasts (4), BMC obviously represent an attractive cell ressource for tooth engineering. Their use to replace the mesenchyme indeed allows the development of teeth. However, a correct morphology could not be achieved. In enamel organ/dental papillae cells reassociations, the number of mesenchymal cells appeared to be a critical parameter. Similar reassociations with a very large excess of BMC did not improve the results, suggesting a limitation in crude BMC, possibly linked to their ability to store signaling molecules.

REFERENCES: [1] Hu B, Nadiri A, Bopp-Kuchler S, Perrin-Schmitt F and Lesot H. (2005) Dental Epithelial Histomorphogenesis in vitro. J Dent Res., **84** :521-525. [2] Ohazama A, Modino SA, Miletich I and Sharpe PT., (2004), Stem-cell-based tissue engineering of murine teeth. J Dent Res., **83** :518-522. [3] Obara N and Lesot H (2004) Subcellular localization of beta-catenin and cadherin expression in the cap-stage enamel organ of the mouse molar. Histochem Cell Biol. **121** :351-358. [4] Hu B, Unda F, Bopp-Kuchler S, Jimenez L, Wang XJ, Haikel Y, Wang SL, and Lesot H., (2006), Bone marrow cells can give rise to ameloblast-like cells. J Dent Res., 2006, **85**, 416-421.

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EGF PREVENTS FORMATION OF HERTWIG'S EPITHELIAL ROOT SHEATH DURING DEVELOPING MOUSE MOLAR TOOTH *IN VITRO*

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INTRODUCTION: Tooth root formation starts after the completion of crown morphogenesis. However, little is known about the molecular mechanisms of the transition from crown to root. The cuff of enamel organ referred to as cervical loop, consisting of inner (IEE) and outer enamel epithelium (OEE), stratum intermedium and stellete reticulum (SR), contributes to the promotion of crown morphogenesis via the proliferation of IEE cells. The transition is seen as the formation of Hertwig's epithelial root sheath (HERS), consisting of two epithelial layers. We designed the new culture system to observe the process of root formation and examined EGF effects in the transition.

METHODS: Mandibular first molars of embryonic and neonatal mice were used. The specimens for light microscopy were fixed and decalcified by conventional methods prior to embedment in paraffin or Epon mixture for preparing sections. For the immunohistochemical procedure, paraffin sections were processed by using a monoclonal antibody to EGF receptor (diluted 1:20; Epitomics, Burlingame, CA, USA). The procedures for organ culturing were carried out as described previously (Fig. 1) [1], and 100 ng/ml of mouse EGF (Upstate Biotechnology, Lake Placid, NY, USA) was added to the culture medium for experiments.

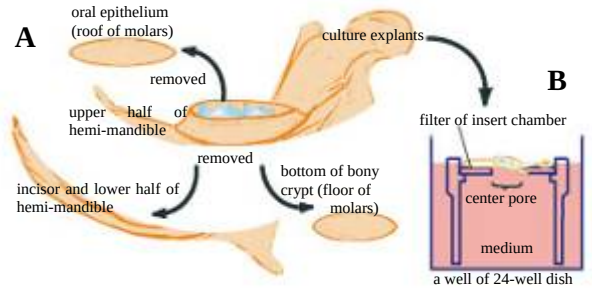

Fig. 1: Schematic representation of culture system

RESULTS: The expression of epidermal growth factor (EGF) around the cervical loop was decreased gradually during the transition [2]. Additionally, immunohistochemical study showed that EGF receptor (EGFr) was expressed in IEE, OEE and SR, as well as a previous paper [2], between embryonic day 18 and neonatal day 0. To prove a relationship between the HERS formation and the disappearance of EGF expression, we designed the organ culture system to observe the root formation *in vitro* and examined the effects on gain and loss-function of EGF. In the culture system exogenous EGF stimulated the expansion of SR residing between the inner and outer epithelial layers in HERS (Fig. 2) and inhibited the formation and growth of HERS formation, and consequently root formation. In contrast, inhibitor of EGFr kinase, tyrphostin resulted in the HERS formation and the transition from crown morphogenesis to root formation.

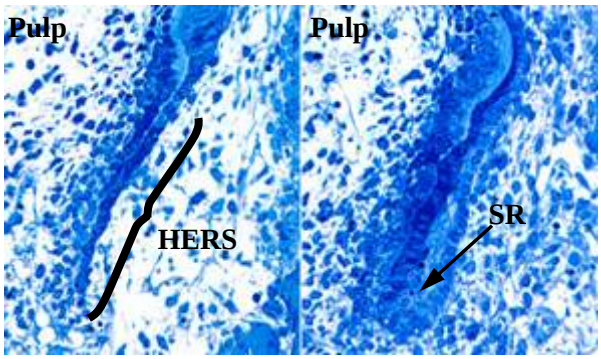

Fig. 2: Histology of HERS formation in the first molars in vitro.
The specimens were prepared form 5-day-old mice and cultured for 4 days in control medium (control, left) and EGF added medium (EGF group, right). Root development is observed in control, but HERS formation of EGF group is not succeeded, SR (arrow) between IEE and OEE is observed.

DISCUSSION & CONCLUSIONS:
Disappearance of SR area may be key events to control the timing of onset of HERS formation and to contribute successful root formation. Ectopic EGF induce expansion of SR, and prevent the HERS formation. EGF may be one of regulatory factors to HERS developed from cervical loop.

REFERENCES: [1]N. Fujiwara, M.J. Tabata, M. Endoh, et al (2005) *Cell Tissue Res* **320**:69-75. [2]G.E. Wise, F. Lin, and W. Fan (1992) *Dev Dyn* **195**, 121-126

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CHARACTERIZATION OF MINERALIZED–TISSUE-FORMING CELLS IN CULTURE OF HUMAN PERIODONTAL CELLS STIMULATED BY ENAMEL MATRIX DERIVATIVES USING FLOW CYTOMETRY

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INTRODUCTION: Emdogain©, which contains Enamel Matrix Derivatives (EMD), is used for stimulating the regeneration of periodontal tissues lost during periodontitis [1]. This process is initiated in the remaining periodontal ligament (PDL), a non-mineralized connective tissue that harbours progenitors for cementum and bone. Several in vitro studies have reported that EMD stimulates proliferation of PDL cells and induces tissue mineralization [2-3]. However little is known about the responsive cell fraction, its phenotype and whether these cells turn into cementoblasts or osteoblasts.

Consequently, in the present work, flow cytometry has been implemented in order to study PDL cell response to EMD. This technique has the unique advantage to analyse cells individually for different parameters and can discriminate between subpopulations of cells.

METHODS: The distribution and expression of bone/liver/kidney isoform of alkaline phosphatase (ALP) and osteocalcin (OC), two markers associated with tissue mineralization as well as STRO-1, a mesenchymal stem cell marker recently identified on PDL cells [4] have been analyzed. PDL cells were treated with EMD for 7 days in absence or presence of 1.25(OH)2VitaminD3 (VitD3) used as osteoinductive factor.

RESULTS: It has been found that EMD strongly upregulated ALP expression in PDL cells costimulated with VitD3 in a dose-response manner. ALP expression was only slightly up-regulated by EMD or VitD3 alone suggesting that VitD3 and EMD had a synergistic effect on this marker. Furthermore, these results corroborated well ALP activity measured in an enzymatic assay. We also succeeded in detecting OC and its expression showed to be up regulated by EMD in a dose dependent manner but independently of the hormonal treatment. STRO-1 was detected on 15-20% PDL cells in subconfluent cell cultures and its expression was up-regulated by VitD3 alone. Costaining for STRO-1 and ALP showed that there was an inverse association between the two markers in which the up-regulation of ALP expression induced by EMD was associated with a strong decrease of STRO-1 expression.

DISCUSSION & CONCLUSIONS: It has been shown for the first time that the differentiation of PDL cells into mineralized-tissue forming cells can be analyzed by flow cytometry and that this technique is a complementary tool to traditional methods. We have found that the treatment of PDL cells with EMD stimulates the expression of ALP and OC in a dose-dependent manner while it down regulates the stem cell marker STRO-1. This technique opens new perspectives of research on periodontal regeneration since it is a unique tool to characterize further subpopulations of progenitors present in the PDL tissue, their response to EMD and their differentiation into bone and cementum forming cells.

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REFERENCES:

[1] A. Sculean, F. Schwarz, J. Becker and M. Brecx (2007) *Med Princ Pract.* 16:167-80. [2] S. Gestrelius, C. Andersson, D. Lidström, L. Hammarström and M. Somerman (1997) *J Clin Periodontol.* 24:685-692. [3] M. T: Van der Pauw, T. Van den Bos, V. Everts and W. Beertsen (2000) *J periodontol* 71:31-43. [4] BM. Seo, M. Miura, S. Gronthos, P. M. Bartold, S. Batouli, J. Brahim, M. Young, P. G. Robey, CY. Wang and S. Shi (2004) *The Lancet* 364:149-155.

HUMAN NEURAL CREST-DERIVED CELLS FROM ADULT DENTAL TISSUES: AN EXTRAORDINARY NICHE OF EMBRYONIC STEM CELLS IN THE ADULT BODY

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During the human embryonic development, at the end of the first month, neuroectodermic neural crest cells start to migrate from the dorsalmost region of the neural tube to the head, neck and other regions of the human body. These cells, in the craniofacial region, penetrate the mesenchyme, reinforcing it and, then, differentiate into cartilage, bone, neurons, glial cells and connective tissues. Odontogenic tissues, deriving from the neural crest, remain quite undifferentiated till adult age, when they develop into dental and periodontal structures of the secondary dentition. This research was focused to study the follicle enveloping the dental germ, due to its direct origin from neural crests. Fifty dental folliculi were collected during impacted wisdom tooth extraction of human subjects aged 18 to 45. After tissue digestion, isolation and expansion, these cells were observed at the FACsorter. They were analyzed for CD90, TRA1-60, TRA1-81, OCT-4, CD133 and SSEA-4. RT-PCR analyses showed that RNA transcripts for Nanog and Rex-1 were highly expressed. Due to their "embryonic" stage, we challenged these cells in order to observe their differentiation toward several cell types, including smooth muscle, osteoblasts, cartilage, neurons, glial cells and adipocytes. In addition, we performed cell injections into murine blastocysts, in order to evaluate chimera formation. Our results confirm that this cell population, thanks to their pluripotency, may represent a great advancement for clinical applications, providing for the first time a neural crest embryonic cells source directly from human adults. In addition, these cells can be stored after cyopreservation to be used for transplantation.

THE DEVELOPMENT AND *IN VIVO* TRANSPLANTATION OF AN ARTIFICIAL TOOTH GERM RECONSTITUTED BY THE BIOENGINEERED ORGAN GERM METHOD

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INTRODUCTION: The ultimate goal of regenerative therapy is to develop bioengineered tissue that can replace lost or damaged organs following disease, injury or aging. The development of three-dimensionally reconstructed bioengineered organs from dissociated single cells *in vitro* is therefore a goal of this technology. In this study, we developed a novel method for the reconstitution of three-dimensionally organ germ, using completely dissociated epithelial and mesenchymal cells.

METHODS: The bioengineered tooth germ was reconstituted with the cell compartmentalization between epithelium- and mesenchyme-derived single cells isolated from incisor tooth germ at cap stage from the lower jaw in ED14.5 mice at high-cell density ($5x10^8$ cells/ml) within a collagen gel drop. The development of the bioengineered tooth germ was analysed by the transplantation into a subrenal capsule or a tooth cavity after the extraction of a mandibular incisor in an 8-week-old adult mouse.

RESULTS: The bioengineered tooth germ generates a structurally correct tooth following both transplantations under a subrenal capsule *in vivo* and also in *in vitro* organ cultures with a high frequency. Plural teeth containing natural tooth materials and correct cell placement were observed to develop from each primordium, give rise from the periphery of the boundary surface between epithelial and mesenchymal cells after 2-3 days growth. By *in situ* hybridization analysis, it was indicated that our current tooth germ model reproduced the interaction between epithelial and mesenchymal cells in early tooth organogenesis. Treatments of both dissociated epithelial and mesenchymal cells by neutralizing anti-CD29 monoclonal antibodies inhibited the tooth formation in a subrenal capsule assay at the frequency of 90% (9/10) and the mRNA expressions of DSP and amelogenin could not be detected. We additionally generated a tooth cavity by extraction of a mandibular incisor in adult mice and find that a complete tooth structure, showing penetration of nerve fibers, can be successfully developed following the engraftment of bioengineered primordium isolated from cultured bioengineered tooth germ. The lengths of the bioengineered tooth after the transplantation for 14, 51 and 73 days in a tooth cavity developed from a single primordium were found to be 2.0±0.5 mm (8.0-fold increase in length), 3.6±0.5 mm (14.4-fold increase in length) and 4.4±0.6 mm (17.6-fold increase in length), respectively. Periodontal ligaments could be detected in the areas around the dentin in the explants after 73 days transplantation.

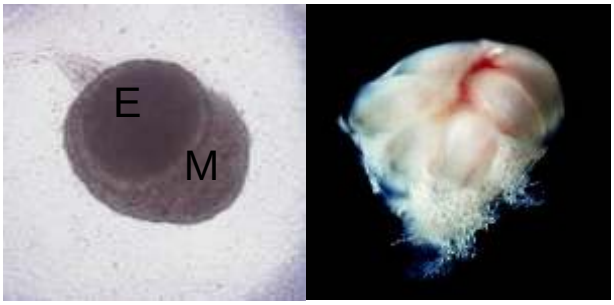

Fig. 1: Representative phase contrast images showing a bioengineered tooth germ (left) and teeth (right) developed in a subrenal capsule for 14 days.

DISCUSSION & CONCLUSIONS: Our data suggests that the replacement of biological and functional teeth would be possible by reconstitution in the tooth cavity of adult animal. These results also represent a significant advance in the development of bioengineered organ replacement strategies and regenerative therapies.

REFERENCES: Nakao K., Morita R., Saji Y., Ishida K., Tomita Y., Ogawa M., Saitoh M., Tomooka Y. & Tsuji T. (2007) *Nature Method* **4**: 227-230.

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ENAMEL-DENTIN AND DENTIN-CEMENTUM COMPLEX STRUCTURE FORMATION IN TOOTH-TISSUE REGENERATION

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INTRODUCTION: Previous studies have demonstrated tooth-tissue regeneration with porcine tooth cells in the early stage of crown formation seeded onto scaffolds [1, 2]. However, the formation of a tooth with a normal shape was infrequent as most tissue-engineered teeth exhibited a round enamel-dentin complex or a stick-shaped dentin-cementum complex. This study aimed to investigate the mechanism underlying tooth-tissue reconstitution to identify why only enamel-dentin or dentin-cementum structures were formed in the scaffold.

METHODS: Tooth buds from 6-month-old pigs were separated into six compartments at the early stage of crown formation: 1) enamel organ without cervical loop region, 2) cervical loop region, 3) dental pulp horn within dental cusp, 4) dental pulp cone in apical side of pulp, 5) dental follicle upper enamel organ, and 6) dental follicle beneath dental papilla. These segment tissues were dissociated into single cells by enzyme digestion. Single-cell populations were recombined with other single-cell populations on a collagen sponge scaffold then implanted into the omentum of immuno-deficient rats. Specimens were collected and examined histologically 15 weeks after transplantation. In addition, semi-quantitative RT-PCR was performed to examine the characteristics of cell populations in their segment tissues.

RESULTS: Enamel-dentin complex structures were reconstituted by the cervical loop region cells (CLC) in recombination with dental pulp corn cell population (PCC; Figure 1A, Table 1). In contrast, dentin-cementum complex structures were reconstituted by cervical loop region cells in recombination with the dental pulp horn cell population (PHC) within the dental cusp (Figure 1B, Table 1). A complete tooth was not observed using this recombination technique.

In addition, RT-PCR analysis revealed different characteristics between dental pulp horn and dental pulp cone cells. The dental pulp cone cell population was more immature than the dental pulp horn cell population.

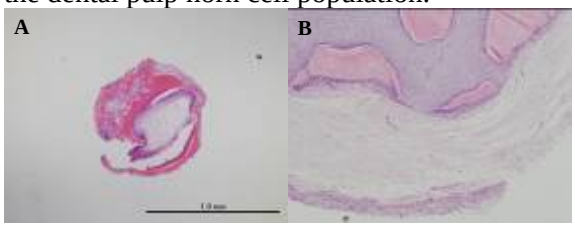

Figure 1: A. Enamel-dentin complex structures. B. Dentin-cementum complex structures.

Table 1:
Relative amounts of complex structures in reconstituted tooth tissue.

	CLC and PHC	CLC and PCC
Enamel-dentin	None	High
Dentin-cementum	All	Low

DISCUSSION & CONCLUSIONS: These results suggest that cervical loop cells play a key role in the reconstitution of tooth structures. In addition, dental pulp cells have diverse abilities depending on their location in the reconstituting tooth-tissue. We have also identified the mechanism by which enamel-dentin and dentin-cementum tissues form in the scaffold using recombinant methods. However, further investigation is required to identify the mechanism behind complete normal tooth regeneration.

REFERENCES:

[1] Honda MJ, Sumita Y, Kagami H, Ueda M. Histological and immunohistochemical studies of tissue engineered odontogenesis. Arch Histol Cytol 2005; 68: 89-101.

[2] Honda MJ, Tsuchiya S, Sumita Y, Sagara H, Ueda M. The sequential seeding of epithelial and mesenchymal cells for tissue-engineered tooth regeneration. Biomaterials 2007; 28: 680-9.

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CLONAL CELL LINES ISOLATED FROM MOUSE DENTAL PULP BEHAVE AS EITHER MONOPOTENT OR MULTIPOTENT PROGENITORS IN VITRO AND CONTRIBUTE TO REPARATIVE DENTIN FORMATION AFTER IMPLANTATION IN THE MOUSE INCISOR

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Tooth formation depends on interactions between epithelial and mesenchymal cells of the dental papilla originating from the neural crest. These interactions stimulate a subpopulation of mesenchymal cells to differentiate into odontoblasts which will synthesize the primary dentin. In adult teeth, reparative dentine can be formed by odontoblast-like cells in response to trauma or carious lesions. These cells are thought to arise from the recruitment, proliferation and differentiation of a precursor cell population residing somewhere within the pulp. Despite all the data available on tooth development, still little is known on the characteristics and properties of these precursor cells. A precise understanding of the nature of these cells and of the molecular mechanisms underlying their differentiation would greatly facilitate the development of cell therapies which could be in the near future, a valuable alternative to the existing unsatisfactory treatments of dental pulp lesions.

We have recently described the isolation of a series of dental pulp clonal precursor cells from mouse ED18 first molar (1). These clones can be induced towards an odontoblastic differentiation program in vitro (2). In the present study, we have evaluated whether these odontoblast precursor clones could behave as progenitors capable of differentiating towards various lineages in vitro and in vivo. We show that, in vitro, some of the dental pulp clones behave as "monopotent" odontoblast progenitors whereas others correspond to multipotent mesenchymal-like progenitors. Indeed, the latter can engage into osteogenesis, chondrogenesis or adipogenesis in the presence of specific inducers, and express the corresponding differentiation markers (alkaline phosphatase and osteocalcin for osteogenesis, sox9, type IIA and X collagen for chondrogenesis, and PPAR and lipoprotein lipase for adipogenesis). In vivo, after implantation in an adult mouse incisor, all the pulp clones are able to contribute to the formation of reparative dentin which, depending on the progenitor clone, is of the orthodentin or osteodentin type (Fig 1). Implanted in the calvaria after formation of a critic defect, the pulpal cells contribute to new bone formation and defect repair. Altogether, these data demonstrate the presence of "monopotent" and multipotent mesenchymal-like progenitors within the mouse dental pulp. Both types of progenitors can efficiently contribute to reparative dentin formation after implantation in the pulp. These progenitor cell lines therefore constitute novel tools to pave the way towards a stem cell-based therapy of pulp lesions.

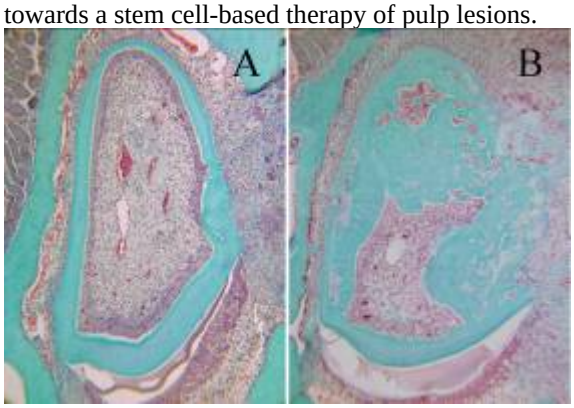

Fig.1: Neodentin formation 10days after implantation in the pulp of a mouse incisor:A) Sham B) Pulpal progenitor cells

References

[1] F. Priam, Ronco V., Locker, Bourd K., Bonnefoix M., Duchêne T., Bitard J., Wurtz T, Kellermann O., Goldberg M. and Poliard A. (2005)*Archs Oral Biol*: 50, 271-277. [2] Lacerda-Pinheiro S, Jegat N, Septier D, Priam F, Bonnefoix M, Bitard J, Kellermann O, Tompkins K, Veis A, Goldberg M, Poliard A.(2006) *Eur J Oral Sciences* 114 : 232-254

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ISOLATION, CULTURE AND DIFFERENTIATION OF HUMAN EPITHELIAL SALIVARY CELLS - A STEP TOWARDS SALIVARY GLAND REGENERATION

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INTRODUCTION: The two most common causes of oral dryness include autoimmune disorders such as Sjögren's syndrome and therapeutic irradiation in the treatment of head and neck cancers. In these disorders the electrolyte, fluid and protein secretory function of salivary glands is lost because of the destruction of acini. To restore this function, either acinar cell renewal should be achieved or the function of the remnant ductal cells should be altered from an absorbing epithelium into a secretory epithelium. In the present work we aimed to achieve acinar differentiation by basal membrane extract BME and to grow huSMG cells to polarized monolayer with high transepithelial resistance on Transwell filters.

METHODS: Human submandibular (huSMG) gland samples were obtained from patients undergoing neck dissection involving the submandibular gland. The cells were isolated and cultured similar to a recently published protocol (Tran S et al., Tissue Eng 11: 172-181, 2005). Cells were cultured on plastic in Hepatostim (BD Bioscience) medium supplemented with 10% FCS or in MEM (Sigma-Aldrich) with or without BME. Change in morphology was registered by phase-contrast microscope. Confocal microscope was also used with immunohystochemistry for amylase and aquaporin 5 (AQP5). Cell proliferation rate was estimated. Amylase, claudin , claudin 3, kallikrein 1 and vimentin mRNA expression was measured by real time PCR. In some experiments cells were plated on Transwell membranes, transepithelial resistance (TER) and electrolyte transport were estimated.

RESULTS: Isolated huSMG cells, cultured these primary cells for up to 3 passages, became undifferentiated during the early course of culturing. These cells cultivated on a plastic surface grew as an epithelial monolayer with cobblestone appearance surrounded by fibroblast like cells. A weak amylase signal in cells grown on plastic could be still detected. When cells were plated onto Transwell membranes, their morphological pictures showed polarization and ZO-1 immunopositivity was observed between the cells. Transepithelial resistance increased considerably, usually by more than 250 ohm cm^2 over basal value within two weeks. BME induced similar morphological changes: formation of acino-tubular structures was observed. Polarized membranes showed variable levels of transepithelial electrolyte movements. Consecutive videomicrographs clearly showed the rearrangement of cell distribution of huSMG cells on BME and the formation of acino-tubular complexes developed in the first 24 h on BME. AQP5 immunostaining in acino-tubular complexes could also be observed. While a weak amylase signal was observed in cells grown on plastic, a much stronger, but unevenly distributed amylase staining was seen in cell clusters on BME. The proliferation rate of huSMG cells on BME decreased in all samples compared to cells grown on plastic. Amylase claudin 1, claudin 3, kallikrein and vimentin expression variably changed in response to BME.

DISCUSSION & CONCLUSIONS: In the present work we report the successful isolation, culture and acinar redifferentiation of human submandibular gland cells. Our data clearly show that basal membrane components play a crucial role in the ductal-acinar transdifferentiation not only in transformed but also in normal human salivary cells. The BME model offers unique experimental system to study the molecular mechanisms regulating epithelial differentiation processes. We were also able to construct epithelial monolayers of these cells on Transwell membranes with high transepithelial resistance suitable for studying transepithelial secretory processes.

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GENETIC CHANGES IN SPORADIC KERATOCYSTIC ODONTOGENIC TUMOUR (ODONTOGENIC KERATOCYST)

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INTRODUCTION: Little is known about the genetic background of keratocystic odontogenic tumour (KCOT, odontogenic keratocyst). Our aim was to characterize genomic aberrations in sporadic KCOT using cDNA-expression arrays and array-comparative genomic hybridization.

METHODS: For cDNA-expression arrays, 10 KCOT specimens and 20 fetal tooth germs were studied. Quantitative real-time reverse transcription-polymerase chain reaction and immunohisto-chemical studies were also undertaken.

RESULTS: Several genes were over-expressed in 12q13 including cytokeratin 6B (KRT6B) (≈10 fold), epidermal growth factor receptor ERBB3 (~ 4.7 fold) and glioma-associated oncogene homologue 1 (GLI1) (~ 5–12-fold). One amplicon (~0.7 Mbp), covering several genes involved in regulation of cell growth was found in 12q13.2. Deletions were found in 3q13.1, 5p14.3 and 7q31.3 including cell adhesion-related genes cadherin 18 (CDH18) and leukocyte cell adhesion molecule (ALCAM, MEMD).

DISCUSSION & CONCLUSIONS: Over-expressed and amplified genes in 12q13, also reported in several other tumours and cell lines, may contribute to the persistent growth characteristics of KCOT.

KEY WORDS: genomic aberrations; gene expression; keratocystic odontogenic tumour; odontogenic keratocyst.

REFERENCES: K. Heikinheimo, K.J. Jee, P.R. Morgan, B. Nagy, S. Knuutila, and I. Leivo: **Genetic Changes In Sporadic Keratocystic Odontogenic Tumour (Odontogenic Keratocyst)** *J Dent Res* **86(6)**:544-549, 2007.

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PHENOTYPIC CHARACTERIZATION OF A BRAZILIAN FAMILY WITH DOMINANT AUTOSOMAL ANKYLOGLOSSIA

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INTRODUCTION: Ankyloglossia (OMIM 106280) is a congenital oral anomaly characterized by presence of a hypertrophyfic lingual frenulum. The shortened frenulum may restrict movement of the tongue affecting breastfeeding, speech and can influence the social behavior and self-confidence of children and adolescents. Ankyloglossia can be observed as part of a syndrome or as an isolated trait. Little is known about the pathogenesis of ankyloglossia. Cleft palate with ankyloglossia (CPX;OMIM 303400) with a dominant X-linked mode of inheritance has been reported in the literature and more recently, mutations in the transcription factor TBX22 gene have been identified in some of these families [1,2]. The aim of the present study was to report the phenotypic characterization of a Brazilian family with a dominant autosomal ankyloglossia associated to tooth numbers anomalies.

RESULTS: Twelve individuals of three generations were submitted to physical, oral and radiographic examinations. Eight individuals, had ankyloglossia with no other apparent physical anomaly, suggesting a dominant autosomal mode of inheritance in this family (*Fig.1*).

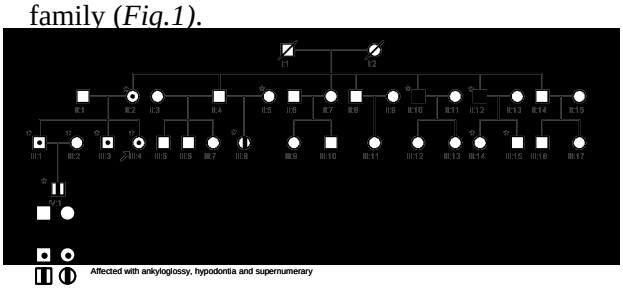

Fig. 1. Pedigree of the studied family (Index case is indicate by the black arrow).

The intraoral examination of the proband revealed absence of three lower incisors and a severely hypertrophic lingual frenulum (Fig. 2). The other family affected individuals showed variable degrees of ankyloglossia and hypertrophy of the labial frenulum. Five of them had dental anomalies in the lower incisor region; all of the individuals had hypodontia in the region of the frenulum insertion and two individuals also had supernumerary teeth.

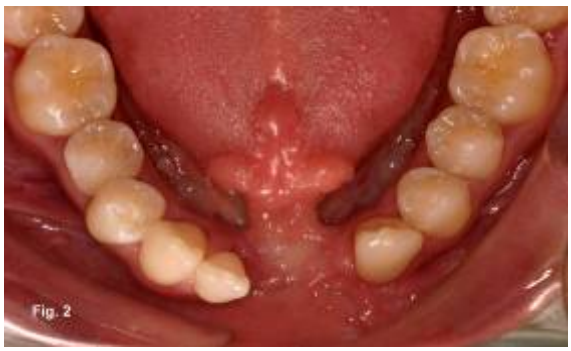

Fig. 2. Lower occlusal view of ankyloglossia and hypodontia in the proband.

CONCLUSIONS: Molecular studies are necessary in order to better understand the pathogenesis of ankyloglossia in this family. The association with tooth anomalies such as tooth agenesis and supernumerary teeth needs to be further investigated.

REFERENCES: [1]Braybook C., Doudney K., Marçano A.C., Arnason A., Bjornsonn A., Patton M.A., Goodfellow P.J., Moore G.E., Stanier P (2001) The T-box transcription fator gene TBX22 is mutated in X-linked cleft palate and ankyloglossia. Nat Genet; 29:179-183. [2]Braybook C., Lisgo S., Doudney K.,Henderson D., Marçano A.C.B., Strachan T., Patton M.A., Villard L., Moore G.E., Stanier P., Lindsay S (2002) Craniofacial expression of human and murine TBX22 correlates with the cleft palate and ankyloglossia phenotype observed un CPX patients. Hum Mol Genet; 11: 2793 – 2804.

MAMMALIAN ENAMELINS: IDENTIFICATION OF CONSERVED REGIONS, EVOLUTION MODE AND MADE USE OF FOR VALIDATION OF MUTATIONS LEADING TO AMELOGENESIS IMPERFECTA

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INTRODUCTION: The uniqueness of dental enamel as a mineralized tissue is reflected in the tissue specificity of its principal matrix constituents, amelogenin (AMEL), enamelin (ENAM) and ameloblastin (AMBN). ENAM is the largest and least abundant non-amelogenin protein in the developing enamel matrix. It represents roughly 1 to 5% of total matrix protein. ENAM is known to possess a SXE motif that is supposed to be a phosphorylation site and a RGD motif that allows protein-cell membrane binding. Intact ENAM (186 kDa) is found only in the surface enamel at the mineralization front near Tomes' process. Among the ENAM cleavage products, the 32 kDa (amino acids 136-241) is the most characterized. It represents up to 1% of total enamel protein in late stage of enamel maturation, and it accumulates in the deeper rod and inter-rod enamel, where it is hypothesized to bind the sides of developing enamel crystals and regulate their shape. The importance of ENAM for proper enamel formation is manifested in the autosomal-dominant form of *amelogenesis imperfecta* (AIH2), displayed by individuals with defective ENAM alleles.[1, 2]

A recent study of AMEL has shown that evolutionary analysis is a useful approach to understand protein evolution, reveal conserved regions, which have certainly important functions, and highlight residues that could lead to a genetic disease if modified. [3, 4]. We have performed an evolutionary analysis of ENAM in mammals (i.e., 250 millions years of evolution) with these objectives in mind.

METHODS: ENAM sequences were obtained in 34 representatives of the main mammalian lineages by blasting complete and raw sequenced genomes in databases. Sequence alignment was done by using Se-Al v2.0 program.

RESULTS & DISCUSSION: The evolutionary analysis reveals that only the SXE motif is conserved in mammalian ENAM. The RGD motif lacks in some sequences suggesting that it would not be of great importance for the normal protein function. A proline-glutamine rich region, encoded by exon 7 has been identified. This region is homologous to the proline rich region described in AMEL and AMBN sequences. This finding confirms our previous hypothesis of evolutionary relationships of enamel matrix proteins. [4]

The analysis of the 32 kDa ENAM fragment, which is degraded by kallikrein 4 during the late stage of maturation shows that three out of five cleavages sites of this proteinase are conserved, as well as, one asparagine glycosylation site and two serine phosphorylation sites. In addition, two motifs are also well conserved (PYYSEEM and SNExGGNP). Such a long lasting conservation indicates an important function for this region. Sequence comparison allows identifying more than 70 amino acids, which were conserved during mammalian evolution. Without doubt, each of them plays an important role for ENAM function, and is a strong "candidate" for AIH2 if it is modified.

CONCLUSION: The evolutionary analysis is an efficient approach for predicting possible function of ENAM. Such a prediction is particularly important as numerous gene sequences are generated with little or no accompanying experimentally determined functional information. In addition our ENAM sequence dataset will be highly useful to highlight residues that could lead to (AIH2) if modified.

REFERENCES [1] JCC. Hu and Yamakoshi, (2003) *Crit Rev Oral Biol*, **14**(6):387-98. [2] J. Kim, F. Seymen, B. Lin, et al. (2005) *J Dent. Res*, **84**:278-82. [3] S. Delgado, M. Girondot and JY. Sire (2005). *J Mol Evol.* , **60**(1):12-30 [4] JY. Sire, S. Delgado and M. Girondot (2006) *Eur. J Oral Sci* 114:64-77.

GROWTH HORMONE STIMULATES PROLIFERATION AND DIFFERENTIATION IN M2H4 ODONTOBLASTS

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INTRODUCTION: Growth hormone (GH) is a peptide secreted by somatotropic cells in the anterior lobe of the pituitary gland. Many authors have studied the effect of GH on bone metabolism. In spite of the similarities between bone and dental tissues, few studies describe the role of GH in the craniofacial growth and in the dental development. The biological effects of GH are mediated by a specific receptor (GHR) located on the surface of target cells. The presence of GHR was described on ameloblast, odontoblasts and cementoblasts at various stages of dental development in the rat, suggesting a role for GH in tooth development and in dentinogenesis (1). Recently GH status was found to influence crown width, root length, and dentin thickness in mice (2). However, in human, no dentin structure anomaly was described in the cases of GH deficiency or excess. To better understand the role of GH in dentinogenesis we study the *in vitro* effect of GH on the odontoblastic M2H4 cell line.

METHODS: For all experiments M2H4 were seeded at a final density of 6000 cells/cm^2 and grown in MEM containing 10% SVF, 1% antibiotics, 1% glutamine, 5 to 500 ng/ml of GH or its vehicle and 1µM U0126 or its vehicle. The presence and the functionality of GH receptor were studied by RT-PCR and Western Blot analysis. The effect of GH on cellular proliferation was assessed by cell counting after Trypan blue staining. The effect of GH on M2H4 differentiation was assessed by real-time RT-PCR analysis of IGF-1, Runx2 and DMP-1 expression.

RESULTS: RT-PCR analysis indicated that M2H4 express GHR transcripts. GH treatment of M2H4 induces a rapid phosphorylation of STAT5, as well as a significant increase in IGF-1 expression. In addition GH stimulates cellular proliferation in a dose-dependant manner and this effect was blunted by adding U0126, an inhibitor of the ERK pathway. Finally GH enhanced the expression of Runx2, a transcription factor involved in odontoblast differentiation. GH also stimulated the expression of DMP-1, one of the major odontoblastic markers.

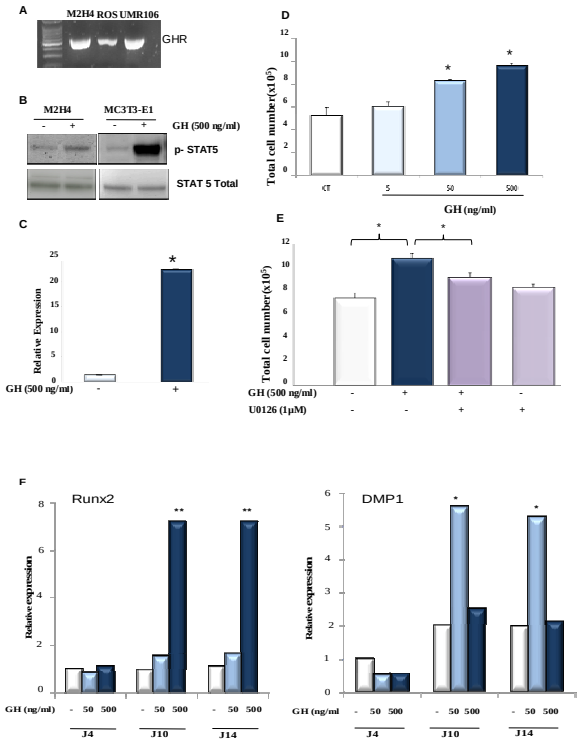

*Figure. **A**: Expression of GHR transcripts by RT-PCR. **B**: Western Blot analysis of STAT5 activation after GH treatment. **C**: Expression of IGF-1 transcripts by real time RT-PCR. **D**: cell counting after 3 days GH treatment. **E**: cell counting after 3 days in the presence of 1µM U0126. **F**: Expression of Runx2, DMP-1 by real-time RT-PCR.*

DISCUSSION & CONCLUSIONS: This study demonstrates for the first time that (i) M2H4 express functional GHR and (ii) GH stimulates both proliferation and differentiation of M2H4. The effect of GH on proliferation likely involved the ERK 1/2 pathway and the effect of GH on cellular differentiation could be direct or mediated by IGF-1. All these data suggest that GH could play a role in dentinogenesis.

REFERENCE: (1) Zhang, C.Z. et al. Calcif Tissue Int,1992, 50, 541-546. (2) Smid JR et al. J Dent Res. 2007 May;86(5):463-8.

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TGF-β1 INDUCES HUMAN PULP FIBROBLASTS TO EXPRESS α-SMOOTH MUSCLE ACTIN IN VITRO

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Transforming growth factor-beta 1 (TGF-β1) has been related to induce the expression of α-smooth muscle actin (α-SMA) in fibroblasts during connective tissue repair. Since pulpal fibroblasts seem to be somewhat different from other fibroblasts, the present study investigated *in vitro* whether TGF-β1 enhances the expression of α-SMA in human pulpal fibroblasts. TGF-β1 was added in doses between 5-10 ng/ml to cultures of both dental pulp and gingiva human fibroblasts. The expression of α-SMA was analyzed by immunofluorescence and western blot, while the ultrastructure was evaluated by transmission electron microscopy. In addition, the immunoexpression of tenascin, osteonectin, and vimentin was also investigated. Both fibroblast types were immunoreactive for α-SMA even without TGF-β1. When TGF-β1 was added to cell cultures, the expression of α-SMA increased dramatically in pulpal fibroblasts independent of the concentration used. It was confirmed by the western blot analysis. Ultrastructure revealed myofilaments and indented nuclei in both fibroblasts treated with TGF-β1. Tenascin and ONEC were only immunolabeled in pulpal fibroblasts treated or not with TGF-β1. Both fibroblast types were positive for vimentin. The present findings showed that TGF-β1 up-regulated the expression of α-SMA thus inducing pulpal fibroblasts to acquire the myofibroblast phenotype.

Grants from FAPESP (# 03/06996-9 and 06/50153-4) and CNPq, Brazil

ALKALINE PHOSPHATASE-INDUCED MINERAL DEPOSITION TO ANCHOR COLLAGEN FIBRILS TO A SOLID SURFACE

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INTRODUCTION: Reconstruction of load-bearing tissues requires proper attachment of the tissue-engineered construct to surrounding tissues. Anchoring of collagen-rich soft connective tissues, including tendons and ligaments, to mineralizing tissues is accomplished *in vivo* by embedding collagen bundles into a mineralizing layer. This layer is partially the result of high local activity of alkaline phosphatase (ALP), an enzyme that facilitates mineral deposition both *in vivo* and *in vitro* [1, 2]. A problem of tissue engineering of a collagenous network seeded with fibroblasts *in vitro* is that a collagen gel is contracted by the fibroblasts resulting in a reduced gel volume and detachment from the surrounding solid surface. We tested whether mineral deposition induced by ALP at the interface of collagen gels and culture well surfaces can prevent gel detachment by human periodontal ligament (PDL) fibroblasts *in vitro.* In addition, the effect of these mineral-inducing conditions on collagen I, Runx2, BSP, OPN and DMP1 gene expression by the fibroblasts was studied.

METHODS: Wells were coated with intestinal ALP prior to addition of collagen gels harboring human PDL fibroblasts. Cultures were performed in the presence of the enzyme substrate β-glycerophosphate (β-GP). Gene expression by the fibroblasts was analyzed after culture for 1 (initial mineral deposition) and 3 days (increased mineral deposition) by Quantitative Polymerase Chain Reaction (QPCR).

RESULTS: Coating of culture wells with ALP prevented detachment of gels from the culture well surfaces. Mineral deposition was observed predominantly at the interface between gels and wells. Contraction of free-floating gels was not influenced by the combined presence of ALP and β-GP. Collagen I gene expression was significantly decreased under mineral-inducing conditions after culture for 1 day, whereas DMP1 gene expression was detected after culture for 3 days. Runx2, BSP and OPN gene expression were not affected under these conditions. Blocking of intracellular phosphate entry into the fibroblasts by foscarnet decreased DMP1 gene expression after culture for 3 days. Addition of phosphate induced DMP1 gene expression in the absence of exogenous ALP but the expression was prevented by blocking the phosphate transporter. DMP1 protein was associated with some fibroblasts in mineralizing gels only.

DISCUSSION & CONCLUSIONS: Coating a solid surface with ALP and providing its substrate to allow mineral deposition can prevent detachment of collagen gels by PDL fibroblasts [3]. This anchoring of collagen fibrils to solid surfaces may be used for reconstruction of load-bearing tissues and temporary attachment of collagenous ligaments to implants. As a consequence of the induction of mineral the fibroblasts are stimulated to differentiate into the osteogenic lineage. This differentiation appears to be mediated by intracellular inorganic phosphate.

REFERENCES: [1] W. Beertsen and T. Van den Bos (1992) *J Clin Invest* **89**:1974-80. [2] T. Van den Bos, J. Steinfort and W. Beertsen (1993) *Bone Miner* **23**:81-93. [3] A.D. Berendsen, T.H. Smit, K.A. Hoeben, et al (2007) *Biomaterials* **28**:3530-6.

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DIVERSITY OF THE MOLAR RADICULAR COMPLEX IN MURINE RODENTS: INVESTIGATING THE DARK SIDE OF THE TOOTH

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INTRODUCTION: Among murine rodents (Myomorpha, Rodentia), many taxa display molars with so similar crown morphology that it is difficult to distinguish between species only using occlusal characters. Consequently and as many fossil species are only known after their teeth, non-occlusal dental characters have to be explored. In this context, the present study aims to investigate the diversity of the molar radicular part in extant and extinct Murinae.

MATERIALS & METHODS: The sample was composed of 42 adult skulls belonging to 32 living murine species. A study of the intraspecific variability was conducted on 11 specimens of *Arvicanthis niloticus*. Fossil murine molars from the Mio-Pliocene hominid sites of Chad (Toros Menalla, Kollé) and Ethiopia (Hadar) included molars of *Saidomys afarensis*, *Golunda gurai*, *Oenomys sp.*, *Arvicanthis sp.*, and undetermined material. Virtual sections of the radicular complex were obtained by X-ray microtomography. Voxel size ranged from 25 to 60 µm. Virtual slices were performed parallel to the cervix plane at the third and the half of the cervix-apex distance. Morphometric analyses were made on virtual slices of pulpar canals. PCA were carried out over Procruste residuals. UPGMA dendrograms were obtained from scores of the specimens in these PCA shape space. Dendrograms were then compared with a molecular phylogeny [1].

RESULTS: Morphometric analyses of first upper molars of species belonging to the '*Arvicanthis* group', a clade of African rats with a high dental root number, led to the clustering of species of the same genera. Thus, the shape of the radicular complex revealed to have a taxonomic significance. Three murine groups were determined from the root number and position in the first lower molars (Fig. 1). These groups were in agreement with current molecular phylogeny [1]. Radicular pattern confirmed that the fossil genus *Saidomys* could be placed into the '*Arvicanthis* group'. Beside this, the shape of the radicular complex in various species of *Oenomys* demonstrated that this genus evolved through at least two separated African lineages.

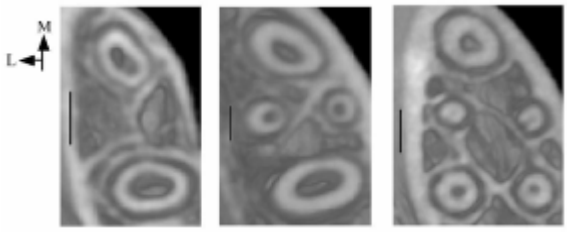

Fig. 1: Virtual slices in the first lower molars representing the three murine groups (2 roots, 4 roots and 5 roots). Arrows point toward mesial (M) and (L) lingual directions. Scale bars: 500µm.

DISCUSSION & CONCLUSIONS: Whereas Herold [2] stated that the number of roots was decreasing in the course of evolution of murine molars, Gállego [3] concluded in an increase of this number. The present study clearly showed a global increasing trend in each of the main murine clades. The primitive pattern has been established in *Potwarmus* which is the ancestor of all murines. It consisted of 3 roots in each upper molar and 2 roots in each lower molar. From this, an increase in the number of roots of some or all the molars was observed. Nevertheless, a secondary evolutionary trend in various genera of the '*Arvicanthis* group' was marked by a decrease in the number of roots.

REFERENCES: [1] S.J. Steppan, R.M. Adkins, P.Q. Spinks and C. Hale (2005) *Mol. Phylogenet. Evol.* **37**:370-388. [2] W. Herold (1957) *Wiss. Z. Humb. Univ. Berlin* **VI**:237-244. [3] L. Gállego (1972) *Bol. R. Soc. Española Hist. Nat. (Biol.)* **70**:321-337.

ACKNOWLEDGEMENTS: We thank J. Cuisin (MNHN, Paris), P. Jenkins, D. Hills and L. Tomsett (NHM, London) for lending of extant specimens. We are also grateful to B. Marandat (ISEM, Montpellier) for allowing access to Hadar fossils. Thanks to V. Lazzari for 3D images of *Potwarmus*. Investigations on the fossil material have been financed by the NSF/RHOI.

SERIAL ANALYSIS OF GENE EXPRESSION DURING MOUSE TEETH DEVELOPMENT AT DAY E 14.5 THROUGH THE EUREXPRESS INITIATIVE

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INTRODUCTION: Odontogenesis is under strict genetic control. Genes that regulate tooth development are being identified with increasing speed. More than 300 genes are included in the graphical database illustrating the gene expression patterns during tooth development (http://bite-it.helsinki.fi). Ascertaining when and where genes are expressed is of crucial importance in order to understand the physiological role of a given gene/protein and the interactions between them. In addition, the normal expression patterns can then be compared to those observed in a variety of pathological conditions to identify pathological hallmarks of gene expression. This has helped understanding how the corresponding genes regulate tooth formation, and how aberrant functions of specific genes cause dental defects.

METHODS: To accelerate the discovery of new genes involved in tooth development we have undertaken a systematic approach using the EURExpress database detailing expression patterns of genes during mouse teeth (molars and incisors) development at the cap stage (E14.5). This stage is characterised by histomorphogenesis of the enamel organ, formation of signalling centres (the primary enamel knots) and condensation of the underlying ectomesenchyme. It is potentially a critical stage regarding anomalies of teeth number, size, shape and structure with the individualisation of the inner dental epithelium from which ameloblasts originate. EURExpress (http://www.eurexpress.org) an integrated project funded by the EU under the 6th Framework Program, is implementing a transcriptome-wide atlas of gene expression patterns by means of in situ hybridisation (ISH) with non-radioactive probes, which is implemented in real time in a freely accessible web-linked database. The final goal of the project is to analyse expression data of > 20,000 genes by RNA in situ hybridisation on serial sagittal sections from E14.5 wild type C57B16 murine embryos. These data will result in a detailed description (at a cellular level) of gene expression patterns in the developing mouse. The "transcriptome atlas" is generated using a newly developed automated RNA in situ hybridisation system.

RESULTS: We created a database for "Odontogenesis", allowing automatic recollection of expression patterns within tissues of interest whether epithelial or mesenchymal. Are considered localisation, intensity and homogeneity of ISH signals. A differential comparison is made with other neighbouring regions of the embryo. An ubiquitous distribution of transcripts or absence of signals are also recorded. Selected images are integrated in the database allowing a quick identification of the expression patterns within incisor and molar tissues. Today more than 400 genes have been analysed. Eventually the database will include the expression data of all the mouse genes analysed through EURExpress.

DISCUSSION & CONCLUSIONS: Data will be scrutinised to discover new genes involved in tooth development, and pinpoint signalling pathways and synexpressions groups. We will also compare the teeth expression data with eye and ear expression data analysed by other members of the research group using similar approaches. This comparative approach is similar to a syndromic phenotyping approach gathering data from different systems and organs to facilitate diagnosis of syndromes combining teeth, eye and ear features.

ACKNOWLEDGEMENTS: We acknowledge support from COST B23, Orofacial Development and Regenera

MORPHOLOGY OF THE LOWER JAW INCISOR IN SPRY NULL MICE

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INTRODUCTION: Incisor development is dependent on a series of interactions between dental epithelium and neural crest-derived ectomesenchyme [1]. Signaling molecules including FGF are involved in mediating the tissue interactions [2]. Members of the *Sprouty* (*Spry*) family encode negative feedback regulators of FGF and other receptor tyrosine kinase signaling [3]. Loss of *Spry* function has been shown to stimulate revival of rudimental diastemal buds, leading to origin of a supernumerary cheek tooth in adult mice [4].
We aimed to investigate the effect of the loss of *Sprouty* function on the morphogenesis and prenatal growth of the lower mouse incisor.

METHODS: The lower incisor was investigated in *Spry4-/-;Spry2+/-*, *Spry4-/-*, and *Spry2-/-* mice and wild-type (WT) controls of similar body weight during embryonic days (ED) 14.5, 16.5, 18.5 and in newborn mice at postnatal day (PND) 0. We used serial 7 µm thick frontal histological sections, computer-aided 3D reconstructions, and morphometry of the incisor enamel organ on sections (Fig. 1).

RESULTS: In comparison to WT mice, the lower incisor enamel organ of *Sprouty* null animals showed a different morphology on 3D reconstructions during late prenatal period. In *Spry4-/-;Spry2+/-*, the antero-posterior length of the incisor enamel organ was decreased during whole period under observation (ED 14.5 – PND 0). The antero-posterior length of the incisor enamel organ was decreased in *Spry2-/-* and *Spry4-/-* only at ED 14.5 and 16.5. Width of the enamel organ was markedly larger in *Spry4-/-;Spry2+/-* and *Spry4-/-* at ED 18.5 compared to WT.

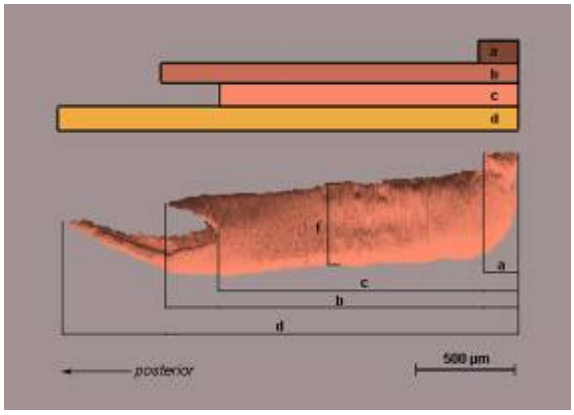

Fig. 1: The 3D reconstruction of the lower incisor enamel organ of Spry4-/-;Spry2+/- at ED 18.5. The measured antero-posterior dimensions (a, b, c, d) and the width (f) are indicated.

DISCUSSION & CONCLUSIONS: The present data documented that *Spry* genes influence not only cheek teeth development [4], but also incisor growth and development. Isolated loss of *Spry2* or *Spry4* resulted in a transient prenatal decrease in the antero-posterior growth of the incisor enamel organ. Loss of *Spry4* combined with the decrease of *Spry2* had important effect on the growth of the dental epithelium during ED 14.5 – PND 0 and resulted in a shorter and thicker incisor enamel organ.

REFERENCES: [1]S. Amar, V. Karcher-Djuricic, J. M. Meyer, et al (1987) *Arch Anat Microsc Morphol Exp* **75**:229-39. [2]M. Seppala, M. Zoupa, O. Onyekwelu, et al (2006) *Dent Update* **33**(10):582-4. [3]A. Reich, A. Sapir, and B. Shilo (1999) *Development* **126**:4139-47. [4]O. D. Klein, G. Minowada, R. Peterkova, et al (2006) *Dev Cell* **11**:181-90.

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XENOPUS TROPICALIS: A MODEL ORGANISM TO STUDY TOOTH REPLACEMENT

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Looking at continuous tooth replacement, one is limited to studying non-mammalian vertebrates (fish, amphibians, reptiles), since only these show tooth replacement during their entire life. Morphological studies of the process have been carried out in several species, but if one is interested in the molecular control of the process, a thorough knowledge of the genome of the species under consideration, and the availability of a set of molecular tools, represent an unquestionable advantage. Taking this into account, the range of possible model species narrows down to a small number of species.

The pipid frog *Xenopus tropicalis* is increasingly being used as a model organism, especially in studies on embryonic development and cell biology. Contributing to this are two factors: *X. tropicalis* has a very small genome and it is the only *Xenopus* species that is diploid, greatly simplifying genetic studies. Currently a whole genome sequencing project is being undertaken (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html**).** Furthermore a set of advanced molecular tools has been developed, such as microarrays and specific antibodies.

This makes *X. tropicalis* a promising model in studies on tooth replacement. Yet, although its relative *X. laevis* has been the subject of morphological studies of tooth renewal [l-4], morphological aspects of tooth replacement in *X. tropicalis* have not been documented. They are the subject of this study.

Preliminary results suggest that there is a great similarity between the two *Xenopus* species. The dentition is homodont, with a single row of monocuspid teeth on the maxillae and premaxillae. Unlike most lissamphibians, teeth are non-pedicellate, as there is no dividing zone between the tooth and the supporting bone. Teeth are attached to the bone by a ring-shaped attachment bone. An important difference between *X. laevis* and *X. tropicalis* is the presence, resp. absence, of a stellate reticulum between the inner and outer dental epithelium.

Although the teeth in adult erupt it is hard to assign them an important function in feeding, because even when fully erupted the tooth tip hardly projects into the mouth. On the lingual side of the erupted tooth, two tooth germs are usually present, connected to each other and to the functional tooth by the dental lamina.

We found that larval teeth are formed in two series: a first series in the even-numbered positions and a second in the odd-numbered positions. This finding is similar to what Shaw (1979) described in *X. laevis.* In adults, no regular patterning could be found, suggesting that development and replacement of teeth is regulated independently for each locus.

These data provide the morphological basis necessary to embark on studies revealing the genetic pathways that are controlling the process of tooth replacement in this species.

REFERENCES: [1] J.C. Cambray, (1976). *Ph.D. Thesis*, Université Paris VII. [2] Shaw, J.P. (1979). *J Anat* **129**:323-342. [3] Shaw, J.P. (1985). *J Zool Lond* **207**:171-179. [4] H. Chisaka, *unpublished results*

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CHICK TOOTH' REVISITED

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Teeth have been missing in Aves for more than 80 million years. However, it is believed that the avian oral epithelium retains the molecular signaling required to induce odontogensis. In this study, we produced teeth as previous report with chick oral epithelia combined with mesenchyme from mouse molar teeth. The size of these recombinant teeth is smaller than that of the mouse molars. The single cusp pattern of these teeth, which may be determined by the epithelial factors, is similar to that of the avian tooth in late Jurassic. Perfect structures of both the ameloblasts and enamel were found in these teeth with showing similar histological characteristics as those of mice. Moreover, to investigate the development of enamel, both the level and distribution of mouse *Amelogenin* (m*Am*) were examined in the developing recombinants by RT-qPCR and *in situ* hybridization. To date, our findings consistent with the previous report that odontogenesis is initially directed by species-specific mesenchymal signals interplaying with common epithelial signals. Furthermore, chick enamel protein is expected to elucidate.

MORPHOLOGICAL DIVERSITY OF UPPER CHEEK TEETH IN THE TABBY MOUSE

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INTRODUCTION: The mutant Tabby mice display developmental anomalies in organs with an ectodermal origin such as hair, glands and teeth [1]. In mouse, disruptions in tooth shape, size and number have already been linked with the *Ta* mutation and a high variability of these parameters has been reported in lower molars [2,3]. The aim of the present work was to study the number and shape variations of upper cheek teeth in both heterozygous and homo/hemizygous Tabby mice. We also studied the relations between the length of the jugal tooth row, the tooth size and the tooth shape.

METHODS: A sample composed of 85 heterozygous females, 23 homo/hemizygous Tabby females, 57 hemizygous Tabby males, and 40 control females (Wild-type) was analyzed. The specimens were grouped according to the morphotypes of their jugal tooth rows. Width and length of the cheek teeth of each mouse were measured. Fourteen upper jaws were selected as a representative panel covering the totality of morphotypes and the rare molar morphologies. Their upper tooth rows were imaged by X-ray synchrotron µCT at the European Synchrotron Radiation Facility (Grenoble, France). This allowed to study in details the dental roots number and position in addition to the crown.

RESULTS: Results showed that the morphological variations in heterozygous mice were more important than in homozygous specimens (Fig. 1). An asymmetry occurred frequently in heterozygous mice in which nearly half of the specimens exhibited different tooth row patterns on the right versus the left side. Measurements of jugal tooth rows indicated two statistically different groups composed of various tooth row morphologies. Inside those two groups, morphotypes differed by the length of the jugal teeth. Dental roots displayed many variations clearly linked with the occlusal surface of the crown phenotypes.

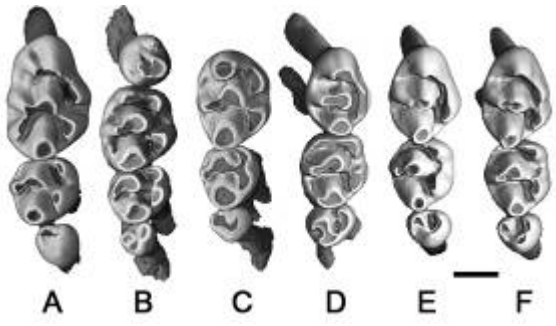

Fig. 1: Major abnormal morphologies of upper jugal cheek teeth in heterozygous Tabby mice (A to D) and homo/hemizygous Tabby mice (E and F). Scale bar: 1mm.

DISCUSSION & CONCLUSIONS: The rare occurrence of a supernumerary tooth was accompanied by a reduction of the mesial part of the first molar. The high level of asymmetry in Tabby heterozygous females might be related to the presence of one X chromosome carrying the mutation and the other one with the non-muted allele. Measurements indicated that a first consequence of the Tabby mutation was the shortening of the upper jugal tooth row. A second consequence was a modification of the segmentation, resulting in a high variability of the dental row morphology.

REFERENCES: [1] H. Grüneberg (1971) *Proc. R. Soc. Lond.* **179**:139-156. [2] J.A. Sofaer (1969) *J. Embryol. exp. Morph.* **22**:181-205. [3] P. Kristenová, M. Peterka, S. Lisi et al (2002) *Orthodont. Craniofacial Res.* **5**:205-214.

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IDENTIFICATION OF PEPTIDES RESULTING FROM DMP1 DEGRADATION BY MMP-2 TO PROMOTE STEM CELL DIFFERENTIATION.

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INTRODUCTION: Historically, matrix metalloproteinases (MMPs) are multidomain proteins that degrade structural components of the ECM. It is now broadly acknowledged that MMPS can produce specific substrate-cleavage fragments that have different biological activities from their precursors. Several MMPs have been identified in dentin and pulp [1]. Among NCPs of dentin, DMP1 has been shown to play a central role in dentin mineralization. The C-terminal domain rich in glutamic acid and serine has been implicated to have a functional role in the nucleation of HAP [2]. DMP1 also acts as a signaling molecule that might induce cytodifferentiation of undifferentiated pulp cells after an insult or trauma of the tooth[3]. Recent data had shown that DMP1 could act as a transcription factor when transported to the nuclear compartment. Furthermore, DMP1 has the capacity to regulate the DSPP gene transcription during early odontoblast differentiation by binding to the promoter of this gene through its carboxyl end [3].
The objective of our work was (1)- to study the cleavage of DMP1 by MMP-2 - and (2) -to analyze if the resulting peptide (s) could act as signaling molecules on dental pulp stem cells (DPSCs).

METHODS: Activated MMP-2 (1 µg/ml) was added to recombinant rat DMP1 at 37°C for 0 to 48 h in 100 mM Tris buffer pH 7.2. The resulting supernatant was run on 12% SDS-PAGE for visualizing peptides by Coomassie blue or stains-all. The bands were excised from the gel and analyzed by mass spectrometry (MS) after trypsin digestion. Human DPSCs were cultured with N and C recombinant polypeptides closely matching limited cleavage products of DMP1 by MMP-2. Proliferation and differentiation were assessed.

RESULTS: MMP-2 at short time points (1 to 6 h) and for both concentrations cleaved DMP1 into two fragments; a major fragment (residues 1-382, 35 kDa) and a second one corresponding to the C terminus (residues 383-489) which could not be visualized neither on various gels nor by western blot. At longer time points (24 to 48 h), the N fragment was processed into 3 smaller fragments and all could be identified by MS. Results from the activity of 2 recombinant polypeptides mimicking the limited cleavage of DMP1 demonstrate that the N polypeptide did not display any significant effect on DPSCs, whereas the C polypeptide enhanced cell proliferation initially. This fragment also had an effect on cell differentiation with an increased expression of Dentin sialoprotein (DSP) and DMP1.

DISCUSSION: Future work including the synthesis of smaller polypeptides with respect to the MMP-2 cleaved fragments will be necessary to determine the precise region within the DMP1 molecule responsible for its cell proliferation and differentiation activity

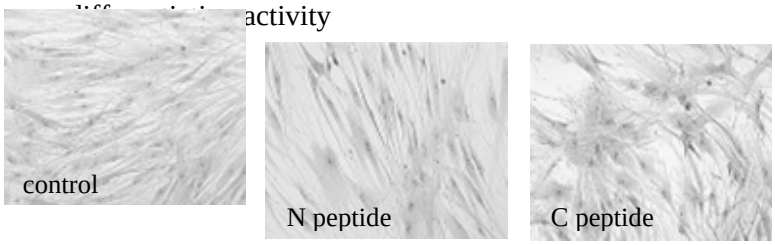

Fig: 1 Effect of DMP1 polypeptides on cell morphology at 48 h

REFERENCES: 1: Goldberg M, Septier D, Bourd K, Hall R, George A, Goldberg H, Menashi S. Immunohistochemical localization of MMP-2, MMP-9, TIMP-1, and TIMP-2 in the forming rat incisor Connect Tissue Res. 2003;44:143-53
2: Gajjeraman S, Narayanan K, Hao J, Qin C, George A. Matrix macromolecules in hard tissues control the nucleation and hierarchical assembly of hydroxyapatite. J Biol Chem 2007;282:1193-204.
3: Narayanan K, Gajjeraman S, Ramachandran A, Hao J, George A. Dentin matrix protein 1 regulates dentin sialophosphoprotein gene transcription during early odontoblast differentiation. J Biol Chem 2006 ;281:19064-71.

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MESENCHYME IS RESPONSIBLE FOR TOOTH SUPPRESSION IN THE MOUSE LOWER DIASTEMA

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INTRODUCTION: Diastema is a toothless gap () between the incisor and molars in mice. Diastema might contain vestigial tooth primordial, but diastemal buds disappear via apoptosis, whereas the molar buds develop into complete teeth during odontogenesis. The reason of tooth suppression in the mouse diastema is ambiguous. To reveal tooth regression in the mandibular diastema is attributed to epithelium or mesenchyme or both, the odontogenic capacity of the epithelium and mesenchyme of the lower diastema at E11.5 and E13.5 was investigated by heterotypic tissue recombinations and compared with that of the lower molar.

MATERIALS & METHODS: Five types of experimental tissue recombinations were prepared as follows: Recombinations = E11.5 diastemal epithelium + E10.5 second branchial mesenchyme; Recombinations = E11.5 diastemal mesenchyme + E10.5 first branchial epithelium; Recombinations = E13.5 diastemal epithelium + E13.5 molar mesenchyme; Recombinations = E13.5 diastemal mesenchyme + E10.5 second branchial epithelium; Recombinations = E13.5 diastemal mesenchyme + E10.5 first branchial epithelium. The epithelium and mesenchyme of the lower molar at E11.5 and E13.5 was used in parallel as controls.

The recombinants were cultured following the Trowell technique for 24 hours and then transplanted to kidney capsules of adult male mice. After subrenal culture for 2 weeks, the resulting tissues were harvested and processed with Azan dichromic staining for histological observation of tooth formation.

RESULTS: Teeth were formed in all types of the experimental recombinants except type . The percentages of tooth formation in the five types of experimental recombinants were 3/8, 4/9, 8/9, 0/9, 5/9, respectively. Meanwhile, teeth were developed in all kinds of the control recombinants. The percentages of tooth formation in the five types of control recombinants were 4/5, 5/5, 8/9, 7/8, 4/5, respectively.

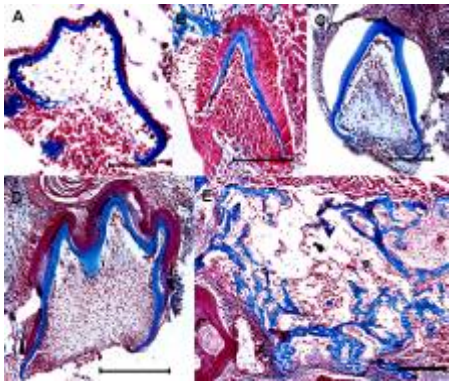

Figure. Recombinants of type (A), (B), (D), (C) yielded teeth and type (E) formed bone and keratinizing cysts without teeth. Scale bars: 100μm (A, B), 200μm (C-E).

DISCUSSION & CONCLUSIONS: Our results demonstrated that at E11.5, the lower diastemal epithelium and mesenchyme possessed the odontogenic potential and competence, respectively; at E13.5, both the lower diastemal mesenchyme and epithelium had odontogenic competence, while the lower diastemal mesenchyme did not possess odontogenic potential. Consequently, on the basis of comparison of the odontogenic capability between the diastemal and molar tooth primordia, our results support that E13.5 diastemal mesenchyme devoid of odontogenic potential is responsible for tooth suppression in the mouse lower diastema.

ACKNOWLEDGEMENTS

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TEMPOROSPATIAL LOCALIZATION OF ACETYLCHOLINESTERASE ACTIVITY IN THE DENTAL EPITHELIUM DURING MOUSE TOOTH DEVELOPMENT

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INTRODUCTION: Acetylcholinesterase (AChE), a principal modulator of cholinergic neurotransmission, also has been demonstrated in non-neuronal cells. Furthermore, AChE has been shown to be associated with the developmental processes of non-neuronal tissue such as bone and cartilage [1-2] and is suggested to be a multifunctional molecule with morphogenetic properties during development. To date, although the morphogenic potential of AChE has been postulated in the various tissues, little information is available on its morphogenic roles in the tooth development. Recently, cholinesterase activity has been identified in the enamel organ of continuously erupting teeth of the guinea pig [3]. However, it is not clear whether AChE is actually involved in the morphogenic process during tooth development. Therefore, we followed the temporospatial appearance of AChE activity in the developing mouse tooth.

MATERIALS & METHODS: To identify the AChE activity, direct coloring method [4] was performed on the mouse embryos (E13, E14, and E18) and on the incisors and molars of the neonatal mouse (P10). For blocking the other esterase activities, iso-OMPA, BW248C51 and eserine were added in the substrate medium, respectively.

RESULTS: In the developing mandibular first molar of mouse embryos, AChE activity was not found in the dental epithelium at E13 (bud stage). AChE activity was first appeared in the developing cervical loops of enamel organ at E14 (cap stage), but was not found in the enamel knot (Fig. 1A). At E18 (bell stage), AChE activity was restrictedly localized in the inner enamel epithelium except cervical loop area (Fig. 1B). In the incisors and molars of neonatal mice (P10), AChE activity was exclusively localized in the inner enamel epithelium of cervical loop (Fig. 1C) and the cells of enamel-free area (Fig. 1D), respectively.

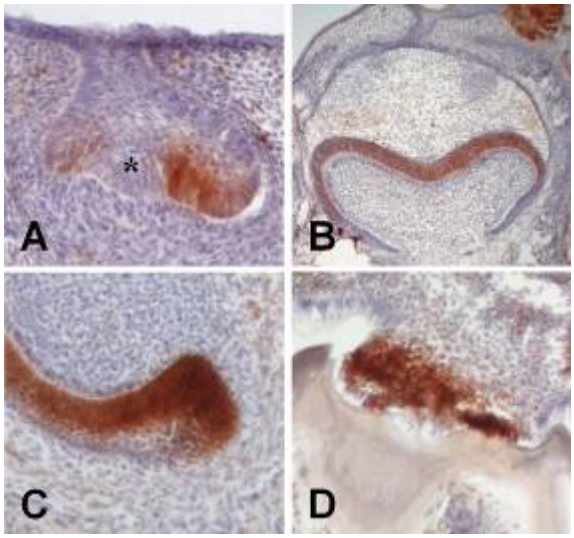

Figure 1. Localization of AChE activity in the dental epithelium of developing mouse tooth. AChE activity is appeared in the dental epithelium of the cervical loop except enamel knot (asterisk) at cap stage (A). At bell stage, AChE activity is found in the inner enamel epithelium (B). AChE activity is also found in the cervical loop epithelium of incisor (C) and enamel-free are of molar (D).

DISCUSSION & CONCLUSIONS: We have shown that AChE activity is temporospatially localized in the differentiating dental epithelium during mouse tooth development. The results suggest that AChE may regulate the histo- and cytodifferentiation of the enamel organ into the inner enamel epithelium and presecretory ameloblasts.

REFERENCES: [1]P.G. Genever, M.A. Birch, E. Brown et al (1999) *Bone* **24**:297-303. [2]Y. Umezu, N. Nagata, Y. Doi et al (1993) *Arch Histol Cytol* **56**:217-224. [3]C.K. Jayawardena, and Y. Takano (2004) *Anat Embryol* **208**:281-287. [4]M.J. Karnovsky, and L. Root, (1964) *J Histochem Cytochem* **12**:219-221.

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SPECIFICITY IN ERK ACTIVATION AND FUNCTION DURING TOOTH DEVELOPMENT

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The ERK is intercellular signalling pathways that a pivotal role in many essential cellular processes including acute responses to major developmental changes and many domains of ERK activation were FGFR-dependent. Although there is some information about how ERK pathway is regulate during mouse embryogenesis, the way in tooth development is unknown. Here, we focus on ERK pathway and compare its location with related molecules and proteins in early tooth development. As our results, ERK detected specific regions and time during craniofacial development. ERK can be used as a marker of when and where signaling is active during tooth development. Taken together, we investigated ERK and PTEN pathway though the FGFR and BMPs using pharmacological inhibitors during in vitro culture system and quantitative real-time PCR. These finding could be first evidence of mutual interactions between ERK and PTEN pathway required for morphogenesis, proliferation, and gene expression during early tooth development.

PATTERNING OF TOOTH IN RODENTS

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INTRODUCTION: Ectodermal organs such as tooth, hair, mammary gland and feather share common morphological features, which develop from epithelial-mesenchymal interactions during the early stages of morphogenesis. The epithelial-mesenchymal interaction in tooth development has been widely studied with the recombination and reaggregation methods [1]. However, the roles of the epithelium and mesenchyme in the patterning of teeth have not been studied sufficiently. The patterning of an organ could be divided into macro- and micropatterning [2]. In this study, we divided the tooth patterning into the macropatterning and micropatterning. The macropatterning includes the tooth size and tooth number in maxilla or mandible, while the micropatterning of teeth includes the cusp size and cusp number in an individual tooth [3].

METHODS: Mice and rats, two species of rodents, show some dental similarities such as tooth number and cusp number, and differences such as tooth size and cusp size. In this study, we did recombination and reaggregation between mouse and rat tooth germs. The factors regulating the tooth patterning were investigated in the heterospecific recombinatnt teeth. The patterning of various recombinant teeth in the present study was discussed in terms of a reaction diffusion mechanism as previous studies on tooth patterning.

RESULTS: The mouse and rat dental epithelium forms the mouse cusp-sized cusp and the rat cusp-sized cusp respectively. The mouse and rat dental mesenchyme forms the mouse crown-sized crown and the rat crown-sized crown respectively. These results mean that tooth size and cusp size are determined by the mesenchyme and epithelium respectively. Furthermore, cusp number is co-regulated by the tooth size and cusp size. Additionally, tooth size is not affected by artificially changing dental mesenchymal cell number. On the other hand, the number of teeth is regulated by mesenchymal cell number.

DISCUSSION & CONCLUSIONS:
our results suggest that the tooth size and cusp size are determined by the dental mesenchyme and dental epithelium respectively and co-regulate cusp number by the epithelial – mesenchymal interactions. Tooth number as well as cusp number can be modeled as a reaction diffusion mechanism, in which the key molecules such as activators and inhibitors may determine the micropatterning and macropatterning of teeth. It is necessary to elucidate these key molecules in order to adjust tooth size and cusp size in the future bioengineered teeth.

REFERENCES:

[1] M. Mina, E.J. Kollar. (1987) *Arch. Oral Biol.* **32**, 123−127. [2] T.X. Jiang, H.S. Jung, R.B. Widelitz, C.M. Chuong (1999) *Development* **126**, 4997 – 5009. [3] I. Salazar-Ciudad, J. Jernvall. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 8116–8120.

IMMUNOLOCALIZATION OF INTEGRIN •4 SUBUNIT DURING THE AMELOGENESIS

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Introduction

Tooth development involves many interactions between cells and extracellular matrix, these interactions are required for cell survival and differentiation. During early stages of odontogenesis, a basement membrane is located between the inner dental epithelium and the underlying mesenchyma. This basement membrane gives the structural support to the developing ameloblasts and the signals necessary for cell survival and differentiation. However, the basement membrane disappears during the secretory stage. Moreover, the mechanism of adhesion and signaling of ameloblast to enamel matrix is not clearly known.

Integrins are the major adhesion molecules to extracelular matrix, consistent of covalently linked subunits a and b. The a6b4 is a hemdesmosome associated integrin, which is expressed in many epithelial cells, in wich also transmit mechanical and chemical signals. The main ligand for integrin a6b4 is laminin 332 (also known as laminin 5). Previous reports have described the presence of laminin 332 in the basement membrane of inner dental epithelium and in the junctional epithelium in adult mice. Integrin a6b4 is expressed in mouse junctional epithelium, and a6 subunit is expressed in presecretory amelolasts, but the presence of integrin a6b4 during amelogenesis have not been reported.

The present study was designed to probe the hypothesis that integrin b4 subunit is localized in the interface secretory ameloblasts-enamel matrix during the amelogenesis in the mouse.

Materials and Methods

Ten newborn mice of 0, 2, 4, 6, and 8 days and eight adult mice were used for this study. The animals were killed by decapitation, the maxilar and mandibular segments were immediately fixed in buffered formaldehyde for 24 hours. The specimens were demineralized in 10% formic acid for tree days, dehydrated and embedded in paraffin. An indirect immunofluorescent technique, or a streptavidin-biotin-peroxidase method was applied on serial sections of 5µm thickness. A rat monoclonal anti-mouse b4 integrin antibody was used (346-11A, BD Pharmingen). The observations were made using epifluorescent microscopy and light microscopy.

Results

In mouse incisor basement membrane of outer dental epithelium, and oral epithelium were strongly stained. However, the presecretory amelolasts where negative for the immunoreaction. Surprisingly the expression of integrin β4 subunit started in the apical pole of secretory ameloblast, and become more intense in maturative and protective ameloblast. This resuts where confirmed with the study of mouse molars, which show intense immunolabeling of Tomes processes of ameloblasts during the secretory and madurative phase of amelogenesis. The immunostaining remain in reduced ameloblasts and junctonal epithelium, the late phases of ameloblast life cicle.

Discussion

Our results confirm the thesis that the ameloblasts express adhesion molecules in their apical pole, this glicoproteins remained during enamel maturation and tooth eruption. The main ligands for integrin a6b4 are laminin 322 and laminin 511/521, we suggest that these glicoproteins must be present in the enamel matrix, confirming previous reports that proposed that the enamel matrix have an adhesive role during amelogenesis.

MODULATION OF BMP ACTIVITY AFFECTS SIZE AND SHAPE OF INCISOR MORPHOGENESIS

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INTRODUCTION: Teeth are typical examples of epithelial appendages, where sequential and reciprocal interactions between oral epithelium and neural crest derived mesenchyme play a pivotal role in their development. The repeating crosstalk via different members of the BMP, FGF, Wnt and Hedgehog families is responsible for their patterning, morphogenesis and differentiation. Mutations in critical components of these pathways profoundly affect tooth development. The importance of different BMP inhibitors for tooth developmental program has been illustrated earlier, and it is possible that they have redundant functions. Therefore the purpose of this study was to determine how the collective absence of two inhibitors i.e. *ectodin* and *follistatin* affects incisor morphogenesis and differentiation.

MATERIALS & METHODS: All procedures using animals were approved by the Animal Care and Use Committee of University of Helsinki and tissues were obtained in accordance with the guidelines. The generation of *follistatin* and *ectodin* mice lines have been reported earlier and double knockout mice were generated in-house by mating heterozygous *ectodin* and *follistatin* mices. Wild-type, heterozygous, and homozygous ectodin and follistatin screening was done by PCR. In situ hybridization and immunohistochemistry have been preformed on serial saggital sections obtained either from E14 to E17,5 stage of development or from kidney transplanted incisor. For organ culture experiments, the lower incisor teeth from day 14 mouse embryos were dissected and cultured. Cell proliferation assays have been also applied to define the cell proliferation index.

RESULTS & DISCUSSION: In all compound null mutants overgrown lingual cervical loops with numerous BrdU positive cells were obvious together with an extra coronal invagination, formed by the inner enamel epithelium. As it shown earlier Fgf3, Fgf9 participate PEK regulation, BMP down-regulate Fgf3, indirectly Fgf9 and both *follistatin* and *ectodin* is a negative regulator of BMP. Taken together *follistatin* and *ectodin* may effect -inhibit- incisor enamel knot function, indirectly resulting the observed invagination. In situ hybridization (MMP20, Shh, TBX-1) and immunohistochemical analysis (amelogenin) indicated that these cells later differentiated into enamel secreting ameloblasts suggesting that *follistatin* and *ectodin* not only regulates the early morphological event of incisor development but also effect ameloblast differentiation. Tissue culture experiments indicated that similar to molar morphogenesis, the development of wild type incisor is relatively robust against excess BMP in contrast to inhibitor knockout, where the sensitivity of tooth germs to BMP resulted in a more advanced stage of ameloblast differentiation. Moreover, when the compound mutant tooth germs were cultured as transplants under adult mouse kidney capsules, a dramatic stimulation of matrix secretion and abnormal incisor phenotype was evident.

CONCLUSIONS:

Our study further supports the hypothesis that BMP activity is regulated at different molecular levels and by several inhibitors, which play an important role in ameloblast maturation and matrix formation.

ACKNOWLEDGEMENTS: Grants from the Academy of Finland (to I.T.), and EU Marie Curie Fellowship (to Sz. F) is gratefully acknowledged. We thank Martin Matzuk (Baylor Collage of Medicine, Huston, USA) for *Follistatin* $^{-/-}$ mice; Nobuyuki Itoh (Department of Genetic Biochemistry, Kyoto University, Kyoto, Japan) for *ectodin* $^{-/-}$ mice and Merja Mäkinen, Riikka Santalahti for technical assistance.

EXPRESSION PROFILING AND BIOCHEMICAL CHARACTERIZATION OF MOUSE AMELOTIN

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INTRODUCTION: Dental enamel is formed in a biomineralization process under the influence of ameloblast-specific proteins, mainly amelogenins, which orchestrate the crystallization of hydroxyapatite ribbons, resulting in the hardest known bioceramic material. The roles of several additional structural (e.g. ameloblastin, enamelin) and proteolytic (enamelysin/MMP-20 and kallikrein-4) ameloblast proteins are currently being deciphered. We have recently discovered a novel ameloblast-specific gene, **amelotin** (AMTN[1]), whose mRNA is highly and transiently expressed in maturation-stage ameloblasts. AMTN appears to be secreted and was reported in the junctional epithelium and the basal lamina in erupted rodent teeth[2], suggesting a potential involvement in soft tissue attachment to enamel. However, the AMTN expression profile during pre-eruptive tooth development has not been determined and the protein has not been characterized further.

METHODS: A polyclonal anti-AMTN antibody was raised in rabbits and affinity purified. After confirming its specificity for native AMTN protein in Western blots, it was used for immunohistochemical and immuno-gold TEM staining in mouse molars and incisors from various post-natal stages.

RESULTS: In these analyses AMTN shows a specific, transient and unique expression pattern: AMTN expression was first detected at postnatal day 1 (D1) in cells of the developing incisor tip and cuspal surfaces of molars. Expression levels increased to a maximum around D10, then declined until tooth eruption In incisors, the protein was detected from D1 onwards and persisted until after eruption in a zone extending from the late secretory through the entire maturation stage to the zone of reduced enamel epithelium. The majority of the protein was found - in molars and incisors - at the interface between maturation stage ameloblasts and dental enamel and – to a lesser extent - in the enamel. AMTN is predicted to be post-translationally modified by O-glycosylation and/or phosphorylation. To confirm this, we have expressed the recombinant AMTN protein from mouse and human in bacteria and compared it with native protein extracted from mandibular molars and incisors. SDS-PAGE comparison of the respective molecular weights has shown that the native protein is approximately 9kD larger than its recombinant counterpart. Immunostaining with specific antibodies to O-linked carbohydrate moieties has indicated that AMTN is a glycoprotein.

DISCUSSION & CONCLUSIONS: This work has shown that AMTN protein is highly and specifically expressed in ameloblasts, transiently in molars, and continuously in incisors. The protein accumulates at the ameloblast/enamel interface, and some AMTN is found deeper in the enamel. The murine protein appears to be post-translationally modified at least by O-glycosylation. The AMTN expression pattern is distinct from that of known structural and proteolytic enamel proteins and suggests a unique role for AMTN in cell-to-enamel attachment and/or enamel maturation.

REFERENCES: [1]K Iwasaki, E Bajenova, E Somogyi-Ganss et al. (2005): Amelotin - a Novel Secreted, Ameloblast-specific Protein. *J Dent Res*. 84(12):1127-32. [2] P Moffatt, CE Smith, R St-Arnaud et al. (2006): Cloning of rat amelotin and localization of the protein to the basal lamina of maturation stage ameloblasts and junctional epithelium. *Biochem J*. 399(1):37-46.

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GENERATION AND CHARACTERIZATION OF CONDITIONAL ALLELES FOR BMP-2 AND BMP-7

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INTRODUCTION: Bone Morphogenetic Proteins play multiple and important roles in embryonic development and homeostasis and tissue repair in adult tissues. In the adult or later stage embryos, systematic functional studies have so far been hampered by lack of mice that would allow conditional gene ablation, a prerequisite to overcome the early or complex phenotypes of straight null alleles.
We present here the generation of conditional alleles for BMP-2 and BMP-7. Both molecules are highly conserved across mammalian species. Thus allele design is of prime importance, as particular care has to be taken not to create hypomorph alleles. For this reason, for targeting construct assembly we used Bacterial Homologous Recombination (BHR) on Bacterial Artificial Chromosomes (BAC), a technology that allows genome manipulation with base-pair precision and enabled us to take locus idiosyncrasies into consideration. For the generation of the conditional alleles we have adapted the classic two loxP-site strategy to BHR. Functional analysis in mice carrying these conditional alleles showed that they behave like null alleles following Cre-mediated recombination, making them suitable tools for *in vivo* studies.

METHODS: Bacterial Homologous Recombination (BHR) on Bacterial Artificial Chromosomes was used for targeting construct assembly. Manipulated BACs were directly targeted to C57Bl6/129 F1 ES cells. Mouse lines were established by blastocyst injection. Resulting mice were bred to a germline Cre-deleter to create wt/Δ mice. Embryos from timed matings were collected to assess Δ/Δ phenotype.

RESULTS: The 2-loxP strategy for generation of conditional mouse alleles was adapted to BHR on BACs. Before targeting construct assembly, careful comparative analysis of human and mouse BMP-2 and BMP-7 loci was performed to identify suitable insertion sites for the loxP cassettes according to the following criteria: integration site does not fall into a region conserved between the two species and does not disrupt a potential functional element, and removal of the intervening exon(s) is predicted to result in a null allele. Generic cassettes suitable for BHR were prepared for both loxP sites and the targeting constructs assembled. Mice carrying the resulting alleles in homozygocity (flx/flx) appear normal and breed well.

To assess the characteristics of the alleles following deletion, recombination was induced in the germline (wt/Δ mice). No live Δ/Δ pups were recovered for both BMP-2 and BMP-7. Analysis of e9.5 BMP-$2^{\Delta/\Delta}$ embryos revealed phenotypic manifestations identical to the BMP-2 ko embryos[2]. Analysis of e16.5 BMP-$7^{\Delta/\Delta}$ embryos revealed the presence of phenotypic changes as published[3]: missing eyes and abnormal kidney development (fully penetrant), polydactyly of the fore limbs (variable penetrant).

Crossing the alleles to a variety of cell type specific Cre-drivers indicated that both alleles efficiently recombine *in vivo.*

DISCUSSION & CONCLUSIONS: BMP-2 and BMP-7 are two members of the BMP superfamily that have been implicated in multiple aspects of embryonic, and specifically of craniofacial and tooth development. The generation of conditional alleles opens the way for functional dissection of BMP-2 and BMP-7 in later embryonic or adult stages by enabling to study their roles in a cell or tissue type specific manner thereby overcoming complex and complicated phenotypes.

REFERENCES: [1]D.M. Valenzuela et al (2003) *High-throughput engineering for the mouse genome coupled with high-resolution expression analysis*. Nat Biotechnol. **21**:652-9. [2] A.T. Dudley, K.M. Lyons, E.J. Robertson (1995) *.A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye.* Genes Dev.;**9**:2795-807. [3]H Zhang, A Bradley (1996) *Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development*. Development. **122**:2977-86.

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SPATIAL-TEMPORAL EXPRESSION OF AMELOGENIN IN THE DEVELOPING EMBRYONIC CRANIOFACIAL COMPLEX

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INTRODUCTION: Amelogenin has long been considered the major marker of developing and mineralizing ectodermal enamel. Recent data suggest other roles for amelogenin beyond regulation of mineral crystal growth. We previously showed that in addition to the expression of amelogenin in ectodermal developing enamel, it was also expressed in the adult dog, rat and mouse, mesenchymal dentin, cementum and periodontal ligament (PDL) cells, in cells of the alveolar bone (normal and regenerating) and in long bone cells (osteoblasts, osteocytes, osteoclasts and some of the bone marrow cells including mesenchymal stromal cells), in cells of chondrocytes in articular cartilage, differentially expressed in cell layers of the epiphyseal growth plate, in cells surrounding blood vessels, in salivary glands, in the brain, specifically in the glia cells, and in some of the hematopoietic cells such as megakaryocytes and macrophages (Deutsch et al. 2006, Haze et al. 2007).
The purpose of the present study was to look at the spatial-temporal expression of amelogenin and its possible role(s) in the mouse developing embryonic craniofacial complex between E10.5-E16.5.

METHODS: Immuno-histochemistry, Western blot analysis, and RT-PCR followed by DNA sequencing.

RESULTS: We have found, for the first time, that amelogenin is expressed in the embryonic craniofacial complex already at E10.5. It is expressed in cells of the embryonic developing, brain, eye, tongue, and bone before it is expressed in the developing tooth germ. The results also revealed the presence of amelogenin in elongated fiber-like structures surrounded by cells. Elongated structures which reacted with the amelogenin antibodies were also observed in the adult mouse brain.

DISCUSSION & CONCLUSIONS: The present results, and our previous results, suggest that amelogenin might have an important role during craniofacial development, not related to its known function in enamel formation.

REFERENCES: (1) Deutsch et al., Eur J Oral Sci 114 (Suppl. 1): 183-189, 2006 (2) Haze et al., The Anatomical Record 290: 455-460, 2007.

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THE EARLY PRENATAL DEVELOPMENT OF THE DENTITION AND VESTIBULUM ORIS IN HUMANS

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INTRODUCTION: Our previous study has shown that the upper part of the oral vestibule does not develop from a continuous horseshoe-shaped vestibular lamina presented by embryological textbooks (Fig. 1A), but from a series of ridges and bulges, and that it has a different origin in the lip and cheek regions (Fig. 1B) [1]. **Aim:** To investigate the early morphogenesis and developmental relationships of the dental and vestibular epithelia in the human lower jaw, and to compare and synthesize these data with the upper jaw.

METHODS: The development of the human dental and vestibular epithelia was examined in 60 series of 10µm thick frontal and sagittal histological sections during prenatal weeks 6-9. A three-dimensional (3D) analysis of the dental and vestibular epithelia in 9 embryos has been performed [1, 2].

RESULTS: The early dental epithelium looked like a long mound in 3D reconstructions with swellings corresponding to single teeth. Both the swellings and mound showed a bud shape on frontal sections. That is why the mesial and distal limits of a tooth bud were not apparent.

In contrast to the generally accepted scheme (Fig. 1A), a continuous anlage of the oral vestibule existed neither in the upper (Fig. 1B) nor in the lower jaw (Fig. 1C).

In the upper jaw (Fig. 1B), the dental and vestibular epithelia originated separately. In the lip region, the series of bulges fused into the canine vestibular ridge, which joined the dental mound distally to the canine. Other vestibular ridges joined the dental mound behind the deciduous first and second molars.

In the lower jaw (Fig. 1C), the dental and vestibular epithelia had a common origin in the incisor region. Two bulges of the dento-vestibular epithelium were present there. The lingual parts of the bulges gave rise to the respective deciduous central and lateral incisor. The labial parts differentiated into vestibular epithelium. In the canine and molar regions, the dental and vestibular epithelia developed separately.

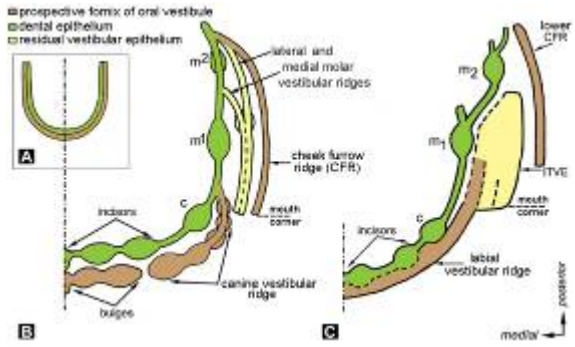

Fig. 1: A scheme of the developmental relationship of the dental (green) and vestibular (yellow and brown) epithelia. (A) According to the embryological textbooks, the dental lamina (anlage of dentition) and vestibular lamina (anlage of the oral vestibule) develop as parallel structures. Updated concept (B) on the upper jaw [1] and (C) on the lower jaw [2]. (ITVE – the irregularly thickened vestibular epithelium).

DISCUSSION & CONCLUSIONS: There is no common scheme of the early developmental relationship of the dental and vestibular epithelia.

Specific differences exist between the upper and lower jaws, as well as between the lip and cheek regions of each jaw [1, 2].

In each jaw, the prospective fornix of the oral vestibule has a separate developmental base in the lip and in the cheek region (Figs 1B, C).

Furthermore, a composite origin of the upper lateral incisor has been documented on 3D reconstructions that may cause a high developmental vulnerability of this tooth [3].

REFERENCES: [1] M. Hovorakova, H. Lesot, M. Peterka, et al (2005) *Anat Embryol* **209**:303-313. [2] M. Hovorakova, H. Lesot, J.L. Vonesch, et al (2007) *Eur J Oral Sci* **115**:1-9, *in press*. [3] M. Hovorakova, H. Lesot, R. Peterkova, et al (2006) *J Dent Res* **85**(2): 167-171.

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BONE CELL TRANSPLANTATION IN RECONSTRUCTIVE SURGERY IN OROFACIAL REGION

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Introduction: Defects of facial bones have a variety of causes(trauma,cysts,benign osteodestructive tumors,malignant tumors,atrophic loss of bones,congenital deformities).The aim of this study is to evaluate possibilities of reconstruction of this defects.

Materials and Methods: There are three types of bone grafts used in reconstruction surgery of hard tissue defects in orofacial region.Autogenous grafts,which are composed of tissues of the same individual.Allogenic grafts,which are taken from another individual of the same species.Xenogenic bone grafts,taken from one species and grafted to another,are not very frequently used in human medicine.In the period within 1998-2006,210 patients were treated at our clinic.In 112 cases autografts were used.In In 98 cases the allografts were used.Xenogenic bone grafts were not used.In 69% of cases supporting of osteointegration with autogenic osteoblasts was used.

Results: Time after operation was in range 6-48 months.The healing success was in 73% of cases,but in combination with autogenic osteoblasts was more than 91% and the period of healing activated and time decrease to 32%.
Conclusion and Discussion: Using a bone grafts in hard tissue reconstruction surgery in orofacial region is very popular in these days.The correct choice of type of bone graft depends on many factors(evaluation of the defect,type of surgical intervention,possibility of supportive therapy using a autogenic osteoblasts).One of the biggest disadvantages of autografting is the mutilation of the patients.But allographting is linked with technical problems and the healing phase is longer and healing success is not as big as in autographting.

References: **1.**Cato T Laurencin,MD, PhD.Yusuf Khan, BA, MS, Jonathan Black,PhD,Francisco Talavera, Pharmd, PhD, Michael Yasezmski, MD, PhD,Dinesh Patel,MD.Jason H Calhoun, MD,FAAOS.Bone Graft Substitute Materials.
2.Jenca A, Jencova V, Jencova J,El Sakka H,Festenburg W.Department of Stomatology and Maxillofacial Surgery, Medical Faculty and University Hospital UPJS Kosice,Slovakia,Associated tissue bank,Medical Faculty Hospital UPJS,Kosice
3.Jenca A, Jencova V, Jencova J, El Sakka H, Department of Stomatology and Maxillofacial Surgery, Medical Faculty and University Hospital UPJS Kosice,Slovakia,ISSN 1335-2393 Acta mechanica slovaca,roc.6,2/2002, p.30-38
4.Rickard Branemark,P-1 Branemerk,B.Rydevik,R.Myers.Department of Orthopaedics,Gothenburg university and the Institute for Applied Biotechnology,Sweden and The AA San Diego Healthcare System.Journal of Rehabilitation Research and Development,Vol 38,No.2 March/April 2001
5.Thomas A, Einhorn, MD, Professor and Chairman,Department of Orthopaedic surgery Boston,Massachusetts,Weyth,Basic sciences of bone graft substitutes.Copyright 2003

Keywords: Demineralised bone matrix; Bone cell transplantation; Osteoblasts; Bone graphting;

INVOLVEMENT OF EPIPROFIN/SP6 TRANSCRIPTION FACTOR IN TOOTH MORPHOGENESIS AND AMELOBLAST AND ODONTOBLAST DIFFERENTIATION

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Epiprofin (Epfn/Sp6/KLF14) is a zinc-finger transcription factor that is expressed during development of certain ectodermal tissues. In the first branchial arch, Epfn was expressed in the oral ectoderm of presumptive molars and incisors and then in the dental lamina at the bud stage of odontogenesis. At the cap stage, Epfn was detected in the inner enamel epithelium, suggesting a role in tooth morphogenesis. The development of Epiprofin/Sp6 mutant mouse teeth showed a clear reduction of dental cusps, malformed teeth and induction of extra molars and incisors, which were formed from just one dental lamina. Lef1 expression was upregulated in the dental epithelium and mesenchyme of mutant teeth, but Dkk1, Bmp4, and Shh had reduced expression in mutant molars. At the bell stage, Epfn was found in ameloblasts and odontoblasts, which indicates that this transcription factor could be involved in differentiation of these cells and in enamel and dentin formation. In mutant mice, the development of the inner enamel epithelium was inhibited these cells remained undifferentiated, and enamel was not formed. These results indicate that Epfn is required for ameloblast formation. Mutant molars and incisors showed differentiated odontoblasts and dentin, but odontoblast polarization was altered, and dentin secretion was delayed and malformed. In this context, DSP/DPP and Collagen-I were reduced, and enamel proteins, such as ameloblastin and enamelin, were not detected. Finally, we observed that Epfn promotes differentiation of dental epithelial cells in vitro. In conclusion, our work shows that Epfn is a transcription factor essential for tooth morphogenesis, ameloblast differentiation, and dentin matrix secretion by different mechanisms of action involving signalling factors.

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A ROLE OF WNT5A IN CONTINUOUSLY GROWING MICE INCISORS

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INTRODUCTION: Tooth growth depends on precise control of basic cellular processes such as cell proliferation and differentiation. Mice incisors are regenerative tissues, which grow continuously throughout life. Some intercellular signalings including fibroblast growth factor (FGF) are essential for the maintenance of the incisor growth [1]. Wnt molecules have been implicated in the regulation of tooth development. *Wnt5a* gene expresses in the dental papilla and follicle [2]. However, little is known about the precise role of Wnt5a signaling during tooth development. To study the functions of *Wnt5a* in growing incisors, we examined the morphological features of incisors in *Wnt5a*-deficient mice.

METHODS: The wild-type (C57BL/6) and *Wnt5a*-deficient mice tooth germ (E-18) specimens which were embedded in paraffin were kindly provided by Dr. Changgong (University of Southern California, USA). To estimate the proliferative activity, they were sectioned and immunostained by Ki67. Rat dental epithelial cell line, HAT-7 and primary mouse dental papilla cells were cultured in the presence/absence of recombinant Wnt5a (R&D Systems, INC, Minneapolis, MN, USA) for 7days. Cell proliferation was measured by MTS assay (Cell Titer 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA). Total RNA was extracted from primary mouse dental papilla cells at 5d, and the expression of *Fgf3* was analyzed by RT-PCR.

RESULTS: *Wnt5a*-deficient mice incisors were much shorter than wild type, the length of inner enamel epithelium as cell proliferation region was very short. On the other hand, morphological defect was not seen in the molars. The morphological features of mutant incisors were similar to those of *Fgf10*-deficient mice. But apical buds were observed clearly in *Wnt5a*-deficient mice incisors. Immunostaining analysis of Ki67 showed a decrease in the number of proliferating cells in *Wnt5a*-deficient mice incisors. To indicate the direct effect of cell proliferation in the dental epithelial cells and mesenchymal cells, we carried out MTS cell proliferation assay by HAT-7 and primary culture of mesenchymal cells. However, recombinant Wnt5a did not have any effect on the proliferation of these cells. We investigated the relationship between *Wnt5a* and *Fgf3* expression in the incisors by the cell and the organ culture of mesenchyme. Recombinant Wnt5a prevented the *Fgf3* expression in the msenchymal cells from disappearing.

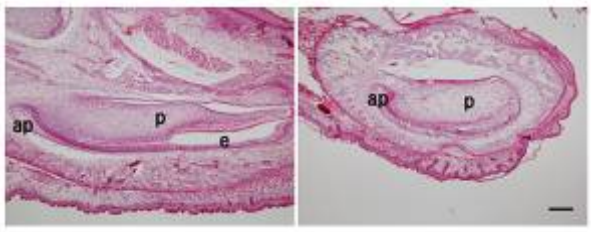

Fig. 1: Histology of Wnt5a-null mouse incisor: wild type (left) vs. Wnt5a-null (right). ap;apical bud, e;enamel, p;pulp, bar;200μm

DISCUSSION & CONCULUSIONS:
The morphological features of *Wnt5a*-deficient mice incisors were similar to those of *Fgf10*-deficient mice. It suggests that Wnt5a plays a role in proliferation of dental epithelium and/or mesenchyme in growing incisors. However, Wnt5a does not have a direct effect on cell proliferation in the cultured epithelial and mesenchymal cells of incisors. Furthermore, apical buds were seen in *Wnt5a*-deficient mice incisors. *Fgf3* expression was maintained by recombinant Wnt5a. These results suggest that Wnt5a plays a role in proliferation of inner enamel epithelial cells in continuously growing incisors through the induction and/or maintenance of *Fgf3* expression.

REFERENCES: [1] H. Harada, T. Toyono, K. Toyoshima, et al (2002) Development **129** 1533-1541. [2] L. Sarkar and P.T. Sharpe (1999) *Mech Dev* **85** 197-200.

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INVESTIGATION OF PRIMITIVE CHARACTERISTICS OF HUMAN PERIODONTAL LIGAMENT CELLS

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Studies have been demonstrated that human periodontal ligament (PDL) comprises multipotent stem cells. To further delineate the primitive phenotypes of PDL cells, we investigated its morphology, cell surface antigens' expression, and dye efflux activity. After obtaining approval from the Ethical Committee, intact permanent teeth were collected from healthy patients who were undergoing orthodontic treatment at Okayama University Hospital. The PDL cells isolated from these teeth were cultured, then were subjected to following analyses. Primary human PDL cells used in this study showed fibroblastic spindle shape and capillary network formation. Flow cytometric studies demonstrated that PDL cells express mesenchymal stem cell markers, including CD9, CD13, CD29, CD44, CD73, CD90, CD105 and CD166, however, they are negative for many hematopoietic markers (CD2, CD3, CD4, CD8a, CD14, CD16, CD19, CD20, CD24, CD33, CD34, CD38, CD41a, CD45, CD66b, CD117, CD133 and CD235a), endothelial cell markers (Flt-1, Flk-1 and von Willebrand factor), and an epithelial cell marker (cytokeratin 5/8). Integrins positive for PDL cells were CD29 (β1), CD41a (α1) and CD51 (αv). Positive cytokine receptors were CD71 (Transferrin R), CD105 (endoglin), CD119 (IFNγ R) and CD120a (TNF RI), and negative were CD117 (c-kit), CD121a (IL-1 R) and CD124 (IL-4 Rα). PDL cells expressed matrix receptors such as CD54 (ICAM-1) and CD166 (ALCAM), but lacked CD31 (PECAM-1), CD56 (NCAM) and CD106 (VCAM-1). Human leukocyte antigen (HLA) expressed in PDL cells was HLA-ABC, however, HLA-DR expression was not detected. No reactions of STRO-1 and CDCP-1 (CUB-domain-containing protein 1) were observed in PDL cells. Hoechst dye efflux assay showed that PDL cells include 3.9% SP cells. This SP profile disappeared in the presence of verapamil (50 μM) or reserpine (5 μM). Only ABCG2-expressing cells (0.25%) were detected in PDL cells by flow cytometric

HUMAN DENTAL FOLLICLE CELLS ACQUIRE CEMENTOBLAST FEATURES UNDER BMP-2/-7 AND ENAMEL MATRIX DERIVATIVES (EMD) STIMULATION IN VITRO.

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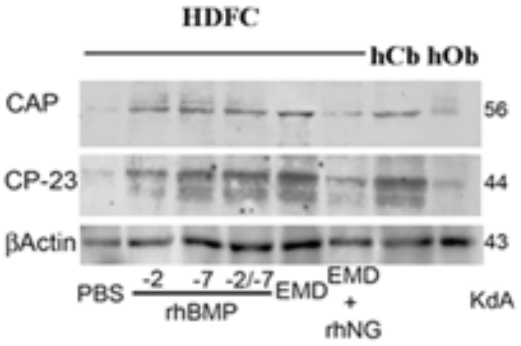
INTRODUCTION: The dental follicle (DF) surrounding the developing tooth germ is an ectomesenchymal tissue composed of various cell populations derived from the cranial neural crest. It is believed that human dental follicle cells (HDFC) contain precursor cells for cementoblasts, periodontal ligament cells, and osteoblasts[1]. Bone morphogenetic proteins (BMPs) produced by Hertwig's epithelial root sheath or present in enamel matrix derivatives (EMD) [2] seem to be involved in the control of DF cells differentiation, but their precise function remains largely unknown. In this study, mesenchymal progenitor properties of human DF cells were first investigated and their ability to be triggered by BMPs evaluated. To test the potential of HDFC to undergo cementoblastic differentiation under EMD and BMP treatments, we assessed the mineralized-cell and specific markers expression in both *in-vitro* and *ex-vivo* culture systems.

METHODS: HDFC isolated from whole DF surrounding third molars before the onset of root development, were submitted to multilineage differentiation culture systems and to FACS analysis for STRO-1, a marker of multipotential mesenchymal progenitor cells. DF tissues and cells were then stimulated by rhBMP-2 and -7 or EMD +/-rhNoggin. Immunostaining were used to localize STRO-1, BMP receptors (BMPR), and cementoblast markers on cell and tissue cultures. Alkaline phosphatase activity was quantified. Expression of two markers, highly expressed in cementoblasts, the Cementum Attachment Protein (CAP) and Cementum Protein 23 (CP-23) [3-4], were also analyzed by Western-blot and Immunohistochemistry.

RESULTS: STRO-1 and BMPR were immunolocalized in the DF *in vivo*. In culture, a mean of 10.75% of HDFC expressed STRO-1 and HDFC exhibited multilineage properties. Cells submitted to EMD demonstrated increased differentiation rates, effects largely dependent on the presence of BMP-2 and -7 in it. EMD and BMP-2 and -7 significantly increased the CAP and CP-23 expression by EMD- stimulation (fig.1), suggesting a specific effect of these compounds to commit HDFC towards the cementoblast phenotype. In addition, rhNoggin partially inhibited the effects of EMD implying that they also exert BMP-independent effects on HDFC.

Fig. 1: EMD, BMP-2 and/or -7 -stimulated HDFC strongly expressed CAP and CP-23 proteins, two putative cementoblast markers. Their expression decreased when rhNoggin was added to EMD . Both markers were strongly expressed in hCementoblast (hCb), but weakly in hOsteoblast (hOb), used as positive and negative controls, respectively.



DISCUSSION & CONCLUSIONS: Our results regarding the effect of EMD on periodontal progenitor cells point out the potentiality of using HDFC or other mesenchymal precursors as cementoblast progenitors, offering new perspectives in periodontal regeneration.

REFERENCES: [1]Morsczeck, C., Gotz, W., Schierholz, J., Zeilhofer, F., Kuhn, U., Mohl, C., Sippel, C., and Hoffmann, K. H. (2005). Matrix Biol *24*, 155-165. [2]Kemoun P, Laurencin-Dalicieux S, Rue J, Vaysse F, Romeas A, Arzate H, Conte-Auriol F, Farges JC, Salles JP, Brunel G. Tissue Cell. 2007 Aug;39(4):257-266. Epub 2007 Jul 26. [3]Alvarez-Perez, M. A., Narayanan, S., Zeichner-David, M., Rodriguez Carmona, B., and Arzate, H.(2006). Bone *38*, 409-419. [4]Arzate, H., Jimenez-Garcia, L. F., Alvarez-Perez, M. A., Landa, A., Bar-Kana, I., and Pitaru, S.(2002).

DUAL MOLECULAR MECHANISMS IN CVP MORPHOGENESIS

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In rodents, a circumvallate papilla (CVP) develops with dramatic changes in epithelial morphogenesis during early tongue development. Molecular and cellular dissections of CVP development revealed that there are two different mechanisms in the apex part and the trench wall forming regions of CVP epithelium. Expression patterns of *Wnt* and *Shh* showed that there are two significant different molecular mechanisms in CVP morphogenesis. *Wnt*, well known key molecule to initiate taste papillae, would govern Rho activation and cytoskeleton formation in apex epithelium of CVP. On the contrast, *Shh* would regulate the cell proliferation to differentiate taste buds and to invaginate the epithelium for development of von Ebner's gland (VEG). Furthermore, *in vitro* organ culture of tongue with various inhibitors confirmed that these *Wnt* and *Shh* would play important roles through different molecular signaling cascades in specific morphogenesis and taste bud patterning of CVP during early mouse embryo development.

ANALYSIS OF GENE EXPRESSION IN ROOT REGION OF DEVELOPING TOOTH USING LASER CAPTURE MICRODISSECTION

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INTRODUCTION: Studies of tooth root development have been limited due to numerous difficulties in access to developing root region in mineralized tissue[1]. Laser-capture microdissection (LCM) has been used on analysis of gene expression in mineralized skeletal tissues. However, the amount of total RNA of tissues in these reports was very low after decalcification[2,3]. Thus, in this study, we described an approach of LCM without decalcification to identify various factors related with tooth root development. RT-PCR analysis was confirmed that the total RNA from two captured areas was sufficient in quantity and quality.

METHODS: Fresh mouse heads at E17.5 were dissected, embedded in TissueTek COT medium, and then frozen in liquid nitrogen. The tissue were sectioned at 7 μm in a cryostat. As shown in Fig.1, the target areas (cervical loop and surrounding tissues) in the fresh sections were microdissected with a LCM (Arcturus Pixcell II). Section and staining conditions were assessed for the optimal retrieval of total RNA from microdissected cells. The mRNA expression levels of various signalling molecules were determined by RT-PCR.

RESULTS: Using the laser microdissection method, we dissected the cervical loop and a part of dental papilla from mouse tooth germs in both early and late bell stage. RNA was extracted from the dissected tissues, and RT-PCR for *Shh, Bmp4, Fgf10, Wnt10b, and β-catenin* was carried out. *Shh, Bmp4, Fgf10, Wnt10b, β-catenin* mRNA were expressed in the cervical loop and a part of dental papilla. The levels of *β-catenin* and *Bmp4* mRNA were much higher than those of other mRNAs in and around cervical loop. *Wnt10b* mRNA was weakly expressed in both areas.

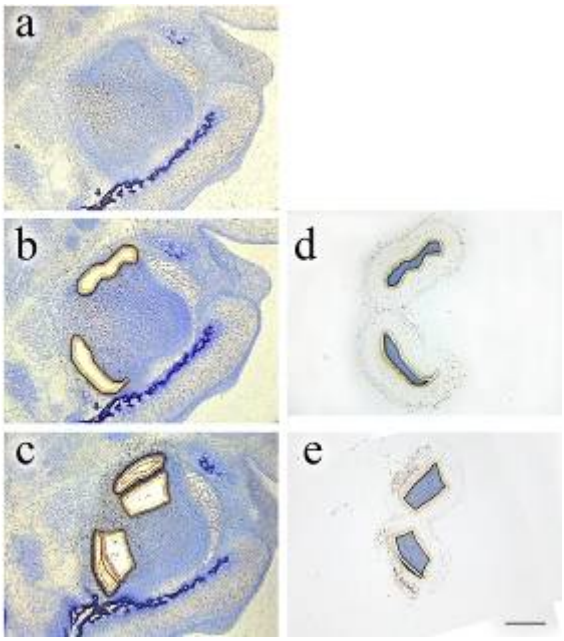

Fig. 1: Cells of the tooth germ at the late bell stage before (a) and after (b,c,d,e) laser microdissection (scale bar, 200 μm). The white areas in fig.1b, c are areas that had been microdissected. Fig.1d,e are capture images.

DISCUSSION & CONCLUSIONS: We have succeeded in isolating total RNA by LCM method without decalcification and confirming the expression levels of several genes in order to characterize cervical loop and HERS during tooth root development. These findings showed the LCM-RT-PCR technique allowed study of gene expression in mineralized tissues such as tooth without decalcification.

REFERENCES: 1 M Aida, T Irié, T Aida, and T Tachikawa (2005) J Dent Res. 84(3):234-9. 2 YY Shao, L Wang , DG Hicks, RT Ballock (2006) Lab Invest. 86(10):1089-95. 3 S Yao, S Ring, WG Henk, GE Wise (2004) Archives of Oral Biology 49:451-6.

ENAMEL AND DENTIN HISTOLOGY OF SYNDROME TEETH

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INTRODUCTION: In the present study we present a preliminary survey of enamel and dentin histology in a collection of teeth from syndrome patients consulting the TAKO-Centre in Oslo, Norway.

METHODS: The methods used were scanning electron microscopy (SEM), light microscopy (LM), and microradiography (MR).

RESULTS: In the following list of syndromes the supposedly affected gene, the number of patients, the number of available teeth, and available genetic analysis (+, corroborates clinical diagnosis, ÷, does not, 0, not available) are given in parentheses. P = permanent teeth, T = temporary teeth.

Amelogenesis imperfecta (AMELX, 1, 4^{P}, 0). Enamel: Variably hypomineralized; less hypomineralized in narrow zone adjacent to EDJ, in superficial enamel and at accentuated Retzius lines; prism structure appears intact. Dentin: The cemento-dentinal junction seemed weaker than usual.

Apert syndrome (FGFR2, 1, 1^{T}, +). Enamel: Incisal hypoplasia. Dentin: Not examined.

Crouzon syndrome (FGFR2, 1, 1^{T}, ÷). Enamel: Cuspal hypoplasia at neonatal line; prenatal enamel somewhat hypomineralized, zones of hypomineralization in postnatal enamel; distinct change in enamel structure at neonatal line. Dentin: Normal.

Cleidocranial dysplasia (RUNX2=CBFA1, 1, 9^{T}, +). Enamel: Extensive regions of moderate hypomineralization. Dentin: The majority of the teeth had large amounts of tertiary dentin in the pulp chamber, i.e. the pulp chambers were partly or completely obliterated.

Fibrodysplasia ossificans progressiva (ACVR1, 1, 2^{T}, 0). Enamel: Normal. Dentin: Extensive internal resorption.

Mucolipidosis II (GNPTAB, 1, 1^{T}, +). Enamel: Large areas of variable hypomineralization, partly along neonatal line. Dentin: Considerable amounts of interglobular dentin at the transition between mantle and circumpulpal dentin.

Goldenhar syndrome (HFM, 1, 4^{T}, 0). Enamel: Some teeth with hypoplasia. Dentin: Pulpal resorption and some tertiary dentin formation.

Moebius syndrome (MBS1, 1, $4^{T,P}$, 0). Enamel: High frequency of prism-in-prism configuration. Dentin: Considerable amount of interglobular dentin, particularly at the mantle/circumpulpal dentin interface.

Solitary median maxillary central incisor (SHH, 1, 1^{T}, 0). Enamel: Normal (in a mandibular first deciduous molar). Dentin: Considerable amount of tertiary dentin.

Penta X syndrome (49,XXXXX, 1, 1^{T}). Enamel: In one area H-S bands terminate abruptly at neonatal line; hypomineralized area incisally. Dentin: Some interglobular and tertiary dentin.

DISCUSSION & CONCLUSIONS: Some of these syndromes are studied at the dental histology level for the first time. Diagnoses were initially clinical, some were confirmed by genetic analysis, one was not (Crouzon), for some genetic analysis was not available. The effect of the genetic defects on enamel is relatively minor, except for Amelogenesis imperfecta, where the major structural protein of enamel, amelogenin, is affected. Generally, it seems that enamel hypomineralization and hypoplasia are more prevalent than changes in enamel structure. This implies that the spatial organization and morphological configuration (Tomes' process) of the ameloblasts are rather robust features, while maturation processes and life span are more easily affected. Also affects on dentin were minor. Of interesting findings we mention the frequent occurrence of prism-in-prism configuration in Moebius, and to a lesser degree in Cleidocranial dysplasia, the distinct difference in degree of mineralization between pre- and postnatal enamel in Crouzon, obliterated pulp chambers in Cleidocranial dysplasia, interglobular dentin in Fibrodysplasia ossificans progressiva and Moebius, internal resorptions in Fibrodysplasia ossificans progressiva and Goldenhar. However, it should be emphasized that the material is rather limited.

ON THE ENAMEL EVOLUTION AND ITS CYTOLOGICAL BACKGROUND

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The author proposes the hypothesis of ameloblast mobility 'Grouping and Dancing' causes the enamel evolution. It is examined by the tooth development and the immunohistochemistry on next points; 1) The correlation between the ameloblast and enamel crystals, 2) The direct relationship between the ameloblast and enamel structures, 3) The origin and development of the mobility, 4) The cytological background.

1) Calcification. The enamel crystal seeds and develops in nano-tube enamelins. It arranges almost perpendicular against the cell membrane of Tomes process with C-terminal affinity. Thus the crystal orientation is decided. 2) It is observed both the enamel and the ameloblast as double layer in thick tangential sections. Each ameloblast group corresponds to a zone of Schreger bands on the developing enamel. The grouped ameloblast is a part of the cluster of enamel organ. It suggests the whole enamel organ moves 'Grouping and Dancing '. 3) The initial group arises in early developing inner enamel epithelium. The stratum intermedium cells develop on these mass of the inner enamel epithelium cell and connects to the outer enamel epithelium cell groups through the enamel cord. These shows the group is associated by other cells of whole enamel organ, having 'Grouping and Dancing'. 4) The anti-actin reaction shows the ameloblast groups which corresponds to zone of Schreger bands. The anti-actin reaction cell groups different from the anti-keratin reacted cell groups on the enamel organ. There are also no reacted cell groups against the keratin. It suggests the keratin and the actin alternately and rhythmically changes the reaction in the enamel organ. Tubulin reaction suggests the ameloblast plays the periodical and the rhythmical secretion. Desmoplakin reaction shows from the stratum intermedium side of the ameloblast layer to the outer enamel epithelium. It shows these areas softly fixes from the enamel organ mobility.

It is concludes that the ameloblast have 'Grouping and Dancing', forming enamel structure, under rhythmical moves and functions of whole enamel organ along with the development of tooth germ.

IDENTIFICATION AND CHARACTERIZATION OF EPITHELIAL STEM CELLS DURING MOUSE INCISOR DEVELOPMENT

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INTRODUCTION: The apical bud, an epithelial protrusion at the apical end of the rodent incisor, has been reported to contain epithelial stem cells that enable continuous growth of the incisor[1]. Recently, there have been studies on the apical bud region and the relation with epithelial stem cells[2]. However, the epithelial stem cell during incisor development is not completely studied. Bromo-deoxyuridine (BrdU) is a thymidine analog that incorporates DNA of dividing cells during the synthetic phase of the cell cycle. Therefore BrdU incorporation method can be useful for localizing stem cells which are slow-cycling cells. Here, we identified and characterized the epithelial stem cells during mouse incisor development.

METHODS: Incisor tooth germs of the mouse embryos from BrdU-injected mice were dissected after different lengths of post-injectional chasing period. They were examined by BrdU staining in order to localize label-retaining cells (LRCs; slow-cycling cells) in the incisor tooth germ. The developing incisor tooth germs were analyzed via immunohistochemistry to label stem cells within the apical bud. Stem cell markers were used as primary antibodies. After dissecting the developing incisor tooth germs, they were divided into the apical bud, inner enamel epithelium, and the mesenchyme surrounding the apical bud. In order to investigate the mRNA expression of these tissues, real-time PCR was performed. Relative abundance of stem cell markers such as *ABCG2*, *Bmi-1*, *CD133*, *p63*, *c-kit*, *Oct4*, *Sca-1*, and *SSEA1* in the apical bud, inner enamel epithelium, and the mesenchyme surrounding the apical bud were verified.

RESULTS: BrdU label-retaining cells which indicate slow cycling cells within the developing tooth germ were localized in the apico-labial part of the apical bud, including the stellate reticulum layer and the surrounding basal layer of the epithelium, and also in the apical and labial part of the mesenchyme tissue surrounding the apical bud. Bmi-1 was detected in the stellate reticulum layer of the apical bud. Sca-1 and ABCG2 was detected in the mesenchyme surrounding the apical bud. *Bmi-1* and *Oct4* showed increased mRNA expression in the apical bud.

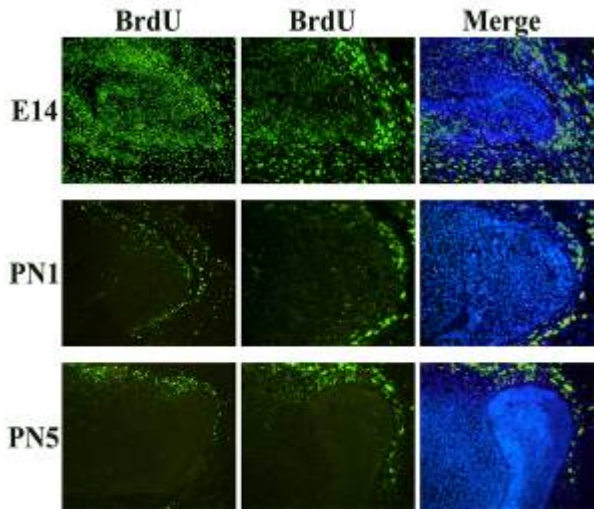

Fig 1: BrdU Label-retaining cells within the developing incisor tooth germs from different developmental stages

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DISCUSSION & CONCLUSIONS: The location and markers of epithelial stem cells in the developing mouse incisor tooth germ were confirmed by BrdU pulse-chase analysis, immunohistochemistry, and real-time PCR. The results correspond with the current researches on stem cells of rodent incisors and suggest specific markers of epithelial stem cells.

REFERENCES: [1] H. Harada, P. Kettunen, H.S. Jung, T. Mustonen, Y.A. Wang, I. Thesleff (1999) *J Cell Biol* **147**:105-120. [2] H. Ohshima, N. Nakasone, E. Hashimoto, H. Sakai, K. Nakakura-Ohshima, H. Harada (2005) *Arch Oral Biol* **50**:153-157.

MICROWEAR PATTERN, TOPOGRAPHICAL MAPS, AND THE MORPHO-FUNCTIONAL INTERPRETATION OF THE MURINE DENTAL PATTERN ORIGINATION

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INTRODUCTION: The evolution of the molar crown morphology in mammals is influenced by three different factors: phylogenetic inheritance, odontogenesis and selection in relation with functional occlusion [1]. Murine rodents (Muroidea, Rodentia) display a highly distinctive upper molar pattern made up by three longitudinal rows of cusps, deriving from the primitive cricetine pattern (only two cusp rows). Upper and lower murine molars together constitute antagonistic rasps that function in a mesio-distal (propalinal) direction. The homoplasic acquisition of the murine molar plan [2] constitutes a good model to study the contribution of the three factors to innovations in the dental pattern since recently, knowledge about both the origin of Murinae [3] and the mouse odontogenesis [4] considerably progressed. Nevertheless, analyses of microwear pattern set a non-elucidated functional problem: the murine dental pattern constitutes a unique case of association of propalinal chewing with preservation of cuspidate crowns [1] while all other rodents with propalinal chewing systematically display a planation of the molar crown [1]. Which morphological changes allowed such a transition?

METHODS: In the present study, the direction of chewing has been inferred on fossil rodents from the direction of the microscratches on their tooth wear facets (Fig. 1B), a character independent of the tooth morphology. The crown morphology of the same samples has then been studied using topographical slope maps (Fig. 1A) elaborated from teeth digitized by X-ray synchrotron microtomography in ESRF (Grenoble, France).

RESULTS: The slope maps revealed that modifications of cusp shape (FIG. 1A) were correlated to changes in the mastication direction (Fig. 1B). In cuspidate teeth, cusps enclose inter-cusp gutters in which cusps of the opposite tooth slide during occlusion (Fig. 1C).

DISCUSSION & CONCLUSIONS: The acquisition of a propalinal chewing in murine rodents appears thus linked to a rotation of these gutters in association with modifications of the cusp shape as the rotation of the lowest cusp slopes (Fig. 1A). Functional aspects of the murine dental pattern evolution could then be related to changes in mechanisms which regulate the cuspidogenesis.

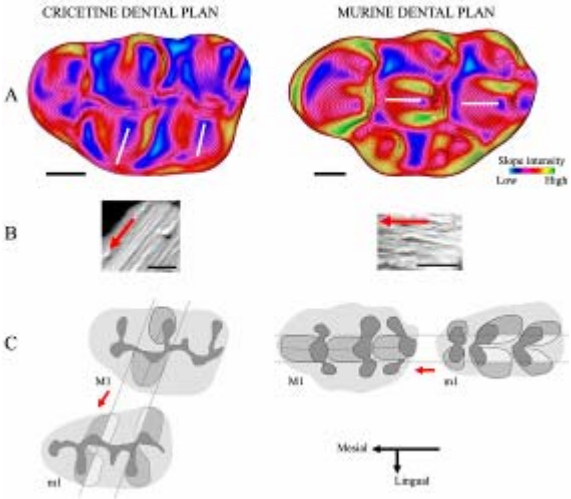

*Fig. 1: Crown topography and microwear pattern in cricetine plan (*Megacricetodon*) and murine plan (*Progonomys*). A: Slope maps with contour lines of left M1. White bars indicate the direction of the protocone and hypocone lowest slopes. Scale bars: 0.25 mm. B: labial protocone wear facet. Red arrows indicate the direction of chewing inferred from microscratches. Scale bars: 50 µm. C: Scheme indicating the trajectory of the M1 cusp row that includes protocone and delimitating the m1 gutter where this row slides, with the cusps which frame it.*

REFERENCES: [1] P. M. Butler (1985) in *Evolutionary Relationship Among Rodents* (eds W. P. Luckett and J.-L. Hartenberger) Plenum Press, pp 381-401. [2] S.A. Jansa and M. Weskler (2004) *Molecular Phylogenetics and Evolution* **31**:256-276. [3] L. L Jacobs and L. J. Flynn. (2005) in *Interpreting the past: essays on Human, Primate, and Mammal evolution in honor of David Pilbeam* (eds D. E. Lieberman, R. J. Smith and J. Kelley) Brill Academic Publishers, pp 63-80. [4] J. Jernvall, S. Keränen and I. Thesleff. (2000) *P. Natl. Acad. Sci. USA* **97**:14444-14448.

WNT11/FGFR1B CROSS-TALK MODULATE THE FATE OF CELLS IN PALATE DEVELOPMENT

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INTRODUCTION: Cellular and molecular events like elevation and fusion of the developing palate occur during embryonic development; these complex mechanisms are known to be mediated by cellular modulations[1,2]. In addition, convergent extension, playing an important role in embryogenesis, was observed in palatogenesis[3]. Some signalling molecules, such as *Wnt11* and *Fgfr1*, are closely associated with convergent extension movement during other organogenesis, including mouse kidney development[4]. To investigate the molecular interactions between *Wnt11* and *Fgfr1b*, we employed an over-expression method as a gain of function and treatment with pharmacological inhibitors as a loss of function with an *in vitro* palate culture system. Moreover, we treated *Wnt11* siRNA during *in vitro* palate culture to confirm the functional significance of *Wnt11* in regulating palatal fusion.

METHODS: Electroporation of organ cultures; The Wnt-11 expression construct (1 μg/μl) in PBS buffer was injected into the palatal mesenchyme using a microcapillary needle, and 20 ms current pulses of 25 volts were applied using an electroporator.

Small Interfering RNA (siRNA) treatment; Diluting a siRNA stock (50 μM) with DMEM/F12 medium containing transfection reagent (siPORT™*NeoFX*™, Ambion) then incubated for 10mins at room temperature. Scrambled control siRNA (Silencer® Negative-control siRNA; Ambion) and *Wnt11* siRNA (Silencer® Pre-designed siRNA; siRNA ID, 65306; Ambion) were treated at final concentrations of 500 nM[5,6].

RESULTS: Concerning specific morphological phenomenon, we examined expression patterns of *Wnt11* and *Fgfr1b*, which are believed to be key factors in convergent extension, in mouse palate development. *Wnt11* and *Fgfr1b* expression patterns suggest their fundamental importance in palatal growth and fusion. Wnt-11 over-expression and SU5402 as a Fgfr1 inhibitor, containing bead implantation were employed in *in vitro* organ culture. Results revealed that interactions between *Wnt11* and *Fgfr1b* may be important in modulating cellular events, such as cell proliferation for growth and apoptosis for fusion. Moreover, *Wnt11* siRNA results showed that apoptosis, induced by *Wnt11*, was necessary for palatal fusion.

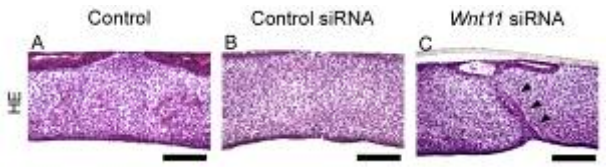

Fig. 1:H -E staining was examined after 500nM siRNA treatment.

DISCUSSION & CONCLUSIONS: *Fgfr1b* induced cell proliferation in the developing palate mesenchyme to grow and contact each palatal shelf; negative feedback of Fgfs, triggered by excessive cell proliferation, then inhibited the expression of *Fgfr1b* and activated the expression of *Wnt11* to fuse each palate via activating apoptosis.

REFERENCES: [1]Taniguchi, K., Sato, N., Uchiyama, Y. (1995) Arch. Histol. Cytol. 58:191-203. [2]Gritli-Linde, A. (2007) Dev. Biol. 301:309-326. [3]Martınez-Alvarez, C., Tudela, C., Perez-Miguelsanz, J., et al (2000) Dev. Biol. 220:343-357. [4]Majumdar, A., Vainio, S., Kispert, A., et al (2003) Development 130:3175-3185. [5]Shiomi, N., Cui, X.M., Yamamoto, et al (2006) Dev. Dyn. 235:1785-1793. [6] Nakajima, A., Ito, Y., Asano, M., (2007) Dev. Dyn. 236:791-801.

DENTIN BONDING: EXPRESSION AND ACTIVITY OF MMP-2 AND MMP-9 IN HUMAN ODONTOBLASTS CULTURED FROM TOOTH SLICES

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Studies revealed that host-derived proteinases to the collagen matrix breakdown could have potential implications in dentin bonding (1). Indeed, the results of these studies suggest that degradation of incompletely infiltrated zones within the hybridized dentin by host-derived dentin matrix metalloproteinases (MMPs) may proceed in the absence of bacterial enzymes. Thus, the objective of this study is to determine the origin of MMPs: dentin bonding systems either influence the expression of MMPs by odontoblasts or only active host-derived metalloproteinases within the dentin matrix. MMPs are zinc-dependent proteases that play a critical role in the normal turnover of extra cellular matrix. Few studies have shown that dentin pulp complex of healthy and carious teeth express and secrete also several type of MMPs including collagenases (MMP-1, -8, -13, -20), gelatinases (MMP-2, -9), membrane-type (MMP-14), enamelysin (MMP-20), (2,3). Interestingly, The MMP-2 and MMP-9 were expressed by both odontoblasts and pulp tissue (4).

Twenty fresh, non carious, human third molars were extracted from patients 15-18 years old for orthodontics reasons. An occlusal cavity was prepared on each tooth with a diamond bur (1.6 mm diameter) under water-spray cooling combined with culture medium. The size of these cavities was standardized, so that they did not extend over more than one half of the dentin thickness. Teeth were randomly assigned to two experimental groups (n=10). A dentin bonding system (Xeno III, Dentsply De Trey, Konstanz, Germany) was applied on each cavity of the first group. A flowable resin composite (Ceram X, Dentsply De Trey, Konstanz, Germany) was applied to all bonded specimens and light cured. The second group, without dentin bonding system was used as control. The teeth were carefully sectioned and thick slices were cultured as described previously (5). The slices were cultured up to 7 days.

The slices were treated for immunohistochemical detection of MMP-2 and MMP-9 and the culture medium was used for zymography analysis.

The results show that the dentin bonding systems influence directly the expression of metalloprotinases by odontoblasts. Indeed, after 7 days in culture, an intense immunoreactivity was observed for MMP-2 and MMP-9 respectively in the odontoblast layer and the pulp tissue in the group with dentin bonding system. The same results were shown with zymography analysis.

1. van Strijp A.J., Jansen D.C., De Groot J., Ten Cate J.M., Everts V. (2003) Caries Res 37 :58-65

2. Gusman H., Santana R.B., Zehnder M. (2002) Eur J Oral Sci 110 :353-357

3- Tjäderhane L., Larjava H., Sorsa T., Uitto V.J., Larmes M., Salo T. (1998) J Dent Res 77 :1622-1629

4- Palosaari H., Pennington C.J., Larmas M., Edwards D.R., Tjarderhane L., Salo T. (2003) Eur J Oral Sci 111:117-127 (2003)

5. Magloire H., Joffre A., Bleicher F. (1996) J Dent Res 75:1971-1978

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Bioinformatic and experimental analysis of the secondary structure of amelogenin

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INTRODUCTION: Amelogenin is a hydrophobic protein that is found during tooth enamel development. An enamel matrix derivative (Emdogain®), which consists mainly of amelogenin, is used as a therapeutic agent in periodontal regeneration. A secondary structure analysis can be valuable to elucidate the biological function of the protein. The aim of this study was to investigate whether experimental data could correlate the findings of the secondary structure of amelogenin obtained by computer based programs. We propose to elucidate its structural properties with two techniques; the Half-sphere exposure (HSE) program and Fourier Transform Infrared (FTIR). Neither has been used previously in structure determination of amelogenin.

METHODS: To examine the secondary structure, we developed a bioinformatic method, using HSE. And other bioinformatics tools such as JPRED, PSIPRED, disEMBL and COUDES. Experimental methods were carried out using FTIR and circular dichroism (CD).

RESULTS: Our HSE and secondary structure predictions of amelogenin confirmed previous structural studies as well as giving valuable insights into the structure of amelogenin. The results indicate that amelogenin is a protein where most of the amino acids are exposed to solvent, except for the N-terminal part that is slightly buried. The bioinformatic secondary structure analysis suggests that amelogenin lacks secondary structure elements such as alpha-helices and beta-sheets. The fact that the protein is rich in proline residues suggests the presence of beta-turn helices, rare structural elements. FTIR and CD findings strengthens the secondary structure theory further. The FTIR results indicate that amelogenin is mainly random coil, with a possibility of beta-turns. (See fig 1.) It does not contain other secondary structure elements, with the possible but unconfirmed exception of a 3_{10}-helix and a beta-sheet.

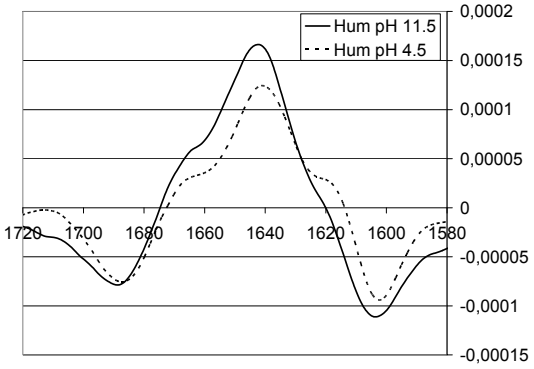

Fig 1. FTIR results of amelogenin at pH 4.5 (solid line) and 11.5 (dotted line). The peaks for both pH ranges suggest beta-turn structures at 1670 cm^{-1}. The peaks for both pH ranges at 1640 cm^{-1} suggest random coil. No other peaks (secondary structures) were found.

DISCUSSION & CONCLUSIONS: We report for the first time the application of two techniques for secondary structure analysis of amelogenin: HSE and FTIR. Both the bioinformatic and the experimental analysis suggest beta-turn as only secondary structures in amelogenin. This correlates with findings of other reported studies.

REFERENCES: Hamelryck. An amino acid has two sides: A new 2D measure provides a different view of solvent exposure. Proteins*: Structure, Function, and Genetics,* 59:38–48, 2005. Fincham et al. The structural biology of the developing dental enamel matrix. *Journal of Structural Biology*, 126:270–299, 1999.

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GENETIC CONTROL ON OSTEOBLAST DIFFERENTIATION: A GENE EXPRESSION ANALYSIS

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INTRODUCTION: Osteogenesis is an extremely complex process. At a molecular level, each factor can induce different responses activating multiple signalling and transcription factors with different biological effects.

We analyzed MSC (mesenchymal stromal cell) gene expression profile during osteogenic induction to investigate "critical" steps to osteoblast differentiation and mineralization.

METHODS: Bone marrow aspirates were collected from the femur during surgery in 4 male patients, without genetic or neoplastic diseases. A MSC culture schedule was defined to pinpoint relevant steps for genetic control of bone cell differentiation. mRNA was extracted before adding ascorbate and dexamethasone as differentiating agents (MD1), after 24h (MD2), at semiconfluence (MD3), at semiconfluent 'Colony Forming Units' (MD5), at full confluence before adding b-glycerophosphate mineralizing medium (MD4), 24 h after addition of mineralizing medium (MM1), 7 days later (MM2) and 14 days later (MM3), when mineral nodules were seen. Trascriptome analysis was performed on Human Genome U133 Plus 2.0 Array (Affymetrix GeneChip® Array). Differential expression of MD2/3/5 versus MD1, and MM1/2/3 versus MD4 were evaluated using bioinformatic analysis and 11,256 'probe sets' with paired T test p value < 0.05 were selected.

RESULTS: Our analysis focused on upregulated genes with a biological function relevant to osteogenesis, such as cell communication, morphological and skeletal development, Wnt signalling, TGF-beta signalling, angiogenesis, cell cycle and apoptosis.

We selected 217 genes: 69 acting in well recognized pathways, 103 reported on literature as relevant to skeletal development, and 45 with functions not described in bone cells.

In early stage (MD2) of differentiation we observed genes mostly belonging to cell cycle pathway, while in further stages (MD3-MD5) the expression of growth factor-signalling pathway, bone related genes and adhesion molecules, was gradually increased. Genes typical of angiogenesis and morphogenesis were upregulated in final steps of mineralization (MM2, MM3), suggesting a role of mature osteoblast in growth of other tissues involved in bone development.

DISCUSSION & CONCLUSIONS: Our results allow to design a gene expression profile of adult MSC during specific steps toward osteoblast differentiation, and to distinguish the genetic control of differentiation from mineralization. These informations can contribute to identify and clarify roles of new genes involved in osteogenesis regulation. Moreover, from a better understanding of molecular mechanisms of bone cells, molecular defects that hamper bone formation might be recognized.

ACKNOWLEDGEMENTS: This work is part of the NANOBIOCOM project supported by the 6th Framework European R&D Program (Project n° NMP3-CT-2005-516943).

SPROUTY GENES ARE REQUIRED FOR NORMAL MOUSE MAXILLARY INCISOR MORPHOGENESIS

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Tooth morphogenesis is classically divided into several phases, which are known as the placode, bud, cap, bell, and cytodifferentiation stages. Progression through these stages results from the interaction between two tissues, the ectodermally-derived epithelium and the neural crest-derived mesenchyme. The epithelium is of central importance throughout tooth development. It initiates tooth formation at the placode stage, and morphogenesis of the tooth at the cap stage is controlled by the epithelial enamel knot, which secretes a variety of growth factors. During the bell stage, the epithelium begins to differentiate into the enamel-secreting ameloblasts, as well as other tissues. The tooth serves as an excellent model for elucidation of the basic morphogenetic rules that govern epithelial bud development, because unlike the kidney, lung, and other organs that undergo budding of epithelium into mesenchyme, growth of the tooth bud is not complicated by multiple branching events. We have previously shown that Sprouty genes, which are inducible repressors of receptor-tyrosine kinase (RTK) signaling, are critical for maintaining normal tooth number in the molar region (Klein et al., 2006). In animals that are null for either *Spry2* or *Spry4*, supernumerary teeth develop in the molar region as a result of hypersensitivity to RTK signaling. In our current studies, we are examining incisor defects in Sprouty mutants, and we show here that mice heterozygous for *Spry2* and homozygous null for *Spry4* possess duplicated upper incisors. To analyze the morphogenetic mechanisms which underlie the incisor duplication, we have dissected epithelia from control and Sprouty mutant tooth germs and imaged the isolated epithelia using confocal microscopy. We show that the mutant tooth germs look grossly normal at the placode stage. However, at the early cap stage apparent duplications in the cervical loop precursors are detected. At the late cap stage, two enamel knots are detected in the mutant incisors, which promotes the continued development of the duplicated incisors. We propose that excess

RTK signaling causes incisor duplications by controlling cell death and proliferation, and that the role of Sprouty genes is to ensure normal tooth number in the incisor region by antagonizing RTK signaling between epithelium and mesenchyme. Study of the genetic regulation of tooth bud morphogenesis will lead to a broader understanding of the mechanisms that control epithelial bud size and shape in general.

REFERENCE: Klein, O. D., Minowada, G., Peterkova, R., Kangas, A., Yu, B. D., Lesot, H., Peterka, M., Jernvall, J., and Martin, G. R. (2006). Sprouty Genes Control Diastema Tooth Development via Bidirectional Antagonism of Epithelial-Mesenchymal FGF Signaling. Dev Cell *11*, 181-190.

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CRANIO-FACIAL BONE DYSMORPHOLOGIES IN RELATION WITH GENE MUTATIONS SCREENED BY CEPHALOMETRIC TOOL

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INTRODUCTION: Many mutations lead to craniofacial dysmorphologies which are difficult to screen. Whole Mount techniques have been used but they are not as accurate and time consuming. On the other hand, cephalometry is a technique of interpretation of cephalic extremity radiology. It illustrates the relations between bone structures, dento-alveolar processes and dental arches in the 3 directions of space. Therefore, we propose a new technique to screen cranio-facial bone dysmorphologies using a modified cephalometric analysis on profile micrographs to explore the sagittal and vertical directions.

METHODS: A microradiography is a radiography taken under conditions which permit subsequent microscopic examination. One of these conditions is called the X-Ray pin-hole source: the X-ray beam is collimated by a lead diaphragm with a pin-hole. This very small X-ray source allows to limit the half light phenomenon and so to optimize the image. We use high resolution films Kodak SO 343. The microradiographies were digitized with a scanner (EPSON Perfection 1640).
After determination specific points, measurements and shape analysis were done on four month Msx2 mutant animals.

Classical cephalometry analysis on profile microradiographies were used to analyze the craniofacial measurements. The following craniofacial distances and angles were calculated :(1) two craniofacial lengths and two craniofacial heights;(2)The thickness of the upper and lower incisors;(3)the thickness of their periodontal ligament;(4)the dental arch discrepancy which can be evaluated by the distance between the maximum of convexity of the mesial side of the first upper and lower molars to a vertical line lowered from the point of emergence of the mesial root of the first upper molar;(5)the antero-posterior bone basal discrepancy by the difference of those two angles; (6)the cochlear apparitus orientation evaluated by the angle between the highest point of the Internal Acousticus Meatus, the highest point of the External Acousticus Meatus and the infraorbital foramen

Shape analysis was done with the PROCUSTE superimpositions. This analysis allows to study and compare the shape of different geometrical figures with different size. Non parametric Wilcoxon test was used to statistical analysis.

RESULTS: Compared to the wild type and the Msx2 heterozygote's animal, vertical and sagittal cranio facial measurements showed that the msx2-/- mutant presents :(1) a smaller size ($p<0,05$), (2) a larger bone basis discrepancy ($p<0,05$), (3) a larger dental arches discrepancy ($p<0,05$), (4) a smaller thickness of the inferior incisor ($p<0,05$), (5) a larger thickness of the periodontal ligament ($p<0,05$) and (6) a clock direction rotation of the cochlear apparatus ($p<0,05$).
Compared to the wild type and the Msx2 heterozygote's animal the shape analysis showed (1) an overall craniofacial shape significantly different ($p<0,01$) and (2) a upper incisor shape with an inferior curvature ($p<0,01$)

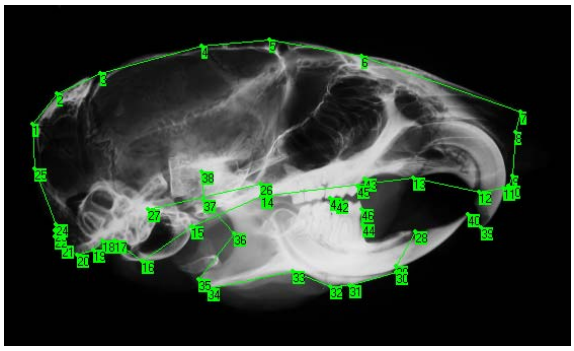

Fig. 1: 46 points have been chosen to characterize the dimensions and the shape of the head.

DISCUSSION & CONCLUSIONS: Classical cephalometry and PROCUSTE superimpositions applied to profile micrographs revealed to be an accurate and simple tool to screen cranio-facial dysmoprphlogies. A 3D cranio-facial bone dysmorphologies study is in process using a micro-computed tomography system (desktop Sky scan 1072) coupled to cephalometry analysis allowing 3D investigation of the whole mice head in relation with gene mutations.

CASPASES IN PRIMARY ENAMEL KNOT APOPTOSIS

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INTRODUCTION: Mammalian caspases, a family of intracellular cysteine proteases are initially produced as inactive zymogens (procaspases). Most of caspases play roles in apoptosis. Caspases may be divided into two functional subfamilies: initiator caspases which are involved in upstream regulatory events, and effector caspases which are directly responsible for cell disassembly steps. Targeted disruption of caspase genes in mice revealed different requirements for individual caspases during mammalian development.

The primary enamel knot (PEK) of developing molar teeth is an important signalling centre during transition from the cap stage to the bell, critical for forming the future tooth cusps. Cell population of PEK consists of non dividing cells expressing several signalling molecules, and is finally eliminated by massive apoptosis. PEK apoptosis may represent a passive process as well as act actively in tooth shape formation and size determination. Exact mechanisms of apoptosis in the PEK have not been known yet, however, dental apoptosis seems caspase dependent.

METHODS: Immunohistochemistry in serial frontal mandibular sections, pharmacological inhibition of tooth explant cultures and knock-out mice were exploited for investigation of intracellular caspase machinery in the mouse molar PEK. Apoptosis was detected using morphological criteria (apoptotic bodies) and TUNEL assay. Proliferation evaluation was based on mitotic figures and PCNA labelling.

RESULTS: Procaspase 3 was found in both epithelial and mesenchymal part of the tooth germ. Active caspase 3 was localized particularly in the primary enamel knot and apoptotic areas of the developing tooth germ. In caspase 3 knock-out mice/129X1/SvJ, the location of the first molar tooth germs was shifted posteriorly in the upper jaw. In contrast, in the caspase-3-/-/B57BL/6, altered morphology of the first molar tooth germs was found in both jaws. In particular, the concave region corresponding to former PEK appeared to be disorganised. No apoptotic bodies were found in the mutant tooth germs and TUNEL labelling showed negative or very weak staining. Knock-out mice (embryonic day 15.5 – bell shape of the first molar tooth germ) were exploited to investigate dental apoptosis in absence of Apaf-1, or caspase-9, crucial molecules of mitochondrial apoptosis pathways. In the Apaf-1 and caspase-9 deficient mice, the tooth shape corresponded to the wild type, however, no apoptosis was found in PEK, navel, stalk or surrounding tissues. All mutant mice showed the same PCNA staining pattern as the wild type mice.

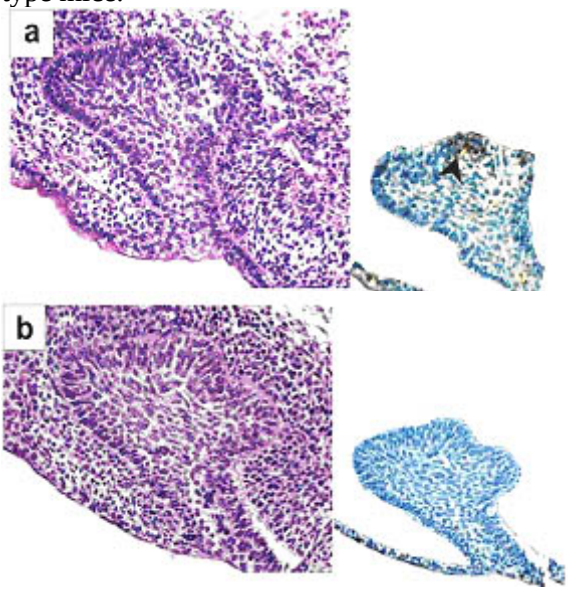

Fig. 1: Pharmaceutical inhibition of PEK apoptosis in explant culture (B), control (A). ED 13.5, 48h culture, TUNEL staining.

DISCUSSION&CONCLUSIONS: Mandibular explant cultures were used to apply pharmaceutical inhibition of specific caspases where mutants are not available or lethal before tooth formation (e. g. caspase-8), and to compare results with knock-out findings. Double-inhibitions will be applied to test any possible compensatory mechanisms among caspases as reported in systems *in vitro*. TEM is the method of choice to reveal any engagement of compensatory death (necrosis, autophagy). Compensatory pathways could explain disappearance of PEK cells despite apoptosis inhibition.

ACKNOWLEDGEMENTS: Supported by the Grant Agency of the Czech Academy of Sciences (B500450503). Research in Brno lab runs in the frame of IRP IAPG No. AVOZ 50450515 and COST Action B23.

MAP-1B EXPRESSION IN HUMAN ODONTOBLASTS

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Microtubule-associated protein 1B (Map-1B) belongs to a family of proteins that govern the dynamic state and organization of microtubules within cells. It is the first MAP to be expressed during development of the nervous system and particularly in neurons that are actively extending axons. It is essential to stabilize microtubules during the elongation of dendrites and neurites and its function is modulated by phosphorylation. We have previously identified Map-1B from a substractive cDNA library of fully differentiated cultured odontoblasts (1). It was tempting to suggest that Map-1B could be involved in the differentiation process of odontoblasts (polarization, cell process elongation, transport of molecules).

In the present study, we have identified the gene expression and localization of Map-1B in cultured human odontoblasts as well as in adult third molar and incisor germs from human embryos (20 weeks).

In situ hybridization and immunochemistry indicated that Map-1B was specifically expressed in human odontoblast *in vivo* and *in vitro*. Flow cytometry and real-time PCR enhanced these results showing that Map-1B expression is related to the differentiation state of odontoblasts. The two Map-1B phosphorylated forms clearly underlined the cellular architecture of odontoblasts (epitope 125) in vitro and in vivo and the cell processes only (SMI 31) of differentiated odontoblasts of human tooth germs.. GSK3 (glycogen synthase kinase 3) induces phosphorylation of Map-1B in growing axons or growth cones (probably for the regulation of microtubules dynamic instability), was also detected in odontoblasts.

On the basis of these data, Map-1B should be considered as a highly specific marker of terminal odontoblast differentiation and enhance the validity of our culture model (2)

REFERENCES: [1] R. Buchaille, ML. Couble, H. Magloire et al. (2000) Matrix Biol., 19: 421-430.

[2] ML. Couble, JC. Farges, F. Bleicher et al. (2000) Calcif. Tissue Int., 66: 129-138.

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PREVALENCE OF ENAMEL DENTAL DEFECTS IN THE DECIDUOUS TEETH OF PRESCHOOL CHILDREN IN BRASILIA, BRAZIL

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INTRODUCTION: Developmental defects of the enamel (DDE) are the result of alterations during amelogenesis due to hereditary, systemic or environmental factors. The aim of this study was to determine the prevalence and distribution of enamel defects in the deciduous dentition of pre-school children with low socio-economic status, in the Federal District, Brazil. **METHODS:** A cross sectional study was carried out in 20 public day care centers during 2006. The dental examination was done by two examiners and the inter-examiners reliability was 0.82 (Kappa). All buccal surfaces of the teeth were examined and the enamel defects were classified and recorded according to the modified DDE Index (FDI,1992). Descriptive statistics were performed and the difference in the prevalence was tested with the Chi-square test, at a level of significance of 5%. **RESULTS:** A total of 1755 children (836 girls and 919 boys) with ages comprised between 2 and 5 years old were clinically examined. The prevalence of DDE was 48.55%, with a significant difference between genders, showing that boys were more affected. The number of examined teeth was 34.672. The mean of affected teeth by children was 1,23 ± 0,86 and 42.5% of the children presented between 1 to 4 affected teeth. The most affected teeth were the lower canines (27.12%), followed by the upper canines (11.62%) and the upper molars (11.68%). The distribution of frequencies and percentages of the different types of enamel defects according to the DDE Index are shown in Table 1. The most prevalent enamel defect observed was the demarcated opacity. Small defects occupying 1/3 of buccal surface of the teeth were the most frequently observed defects

Table 1 – Distribution of enamel defects according to the DDE Index in Brazilian preschool children.

Types of Defects of Dental Enamel	n	Frequency (%)
Demarcated opacity	633	30.30
Diffuse opacity	616	29.47
Hypoplasia	468	22.39
Demarcated / Diffuse opacity	104	4.98
Demarcated opacity/ Hypoplasia	139	6.65
Diffuse opacity/ Hypoplasia	62	2.96
All three defects	68	3.25
Total	**2090**	**100**

CONCLUSIONS: The results indicate a high prevalence of enamel defects in the low socioeconomic sample studied as previously reported in other populations. Further studies are necessary in order to identify the etiology of these defects in this sample.

REFERENCES: FDI Commission on Oral health, Research and Epidemiology: A review of developmental defects on dental enamel index (DDE).Int Dent J 1992;42:411-26. Seow WK. Enamel hypoplasia in primary dentition; a review J. Dent Child 1991;58:441-452. Murray JJ,Shaw L. Classification and prevalence of enamel opacities in the human deciduous and permanent dentition. Arc Oral Biol 1979;24(1):7-. Chaves AM, Rosenblatt A, Oliveira OF. Enamel defects and its relation to life course events in primary dentition of Brazilian

children: a longitudinal study. Community Dent Health. 2007 Mar;24(1):31-6.

DIFFERENTIATION AND CHARACTERIZATION OF DENTAL FOLLICLE PRECURSOR CELLS (PCs)

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The dental follicle is an ectomesenchymal tissue surrounding the developing tooth germ. Previously our group reported the isolation of precursor cells (PCs) derived from the dental follicle of human third molar teeth, which are fibroblast-like, colony forming and plastic adherent cells [1]. PCs are unique undifferentiated cells residing in the periodontium prior or during tooth eruption. For PCs the differentiation capacity was demonstrated under in vivo and in vitro conditions [1,2].

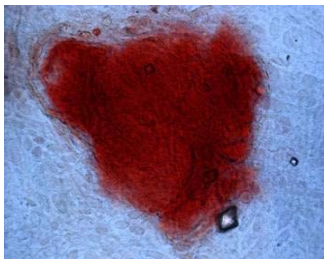


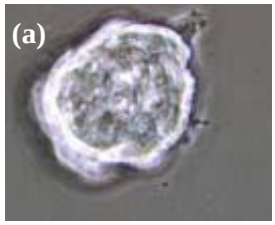
Fig. 1: Osteogenic differentiations of PCs. PCs were capable of differentiation into mineralizing cells. PCs were cultured (4 weeks) on cell culture dishes in Dulbecco's Modified Eagle Medium supplemented with 10 % fetal bovine serum and dexyamethasone (Alizarin staining).

Long-term cultures with PCs containing dexamethasone produced compact calcified nodules (Figure 1) or appeared as plain membrane structures of different dimensions consisting of a connective tissue like matrix encapsulated by a mesothelium-like cellular structure. Our work demonstrated also differentiation of PCs with an insulin-based protocol [2].

However, mechanisms for osteogenic differentiation are unknown in details. DLX-3, DLX-5, runx2, and MSX-2 are differentially expressed during osteogenic differentiation in bone marrow mesenchymal stem cells. In dental follicle cells, gene expression of *runx2*, *DLX-5*, and *MSX-2* was unaffected during osteogenic differentiation in vitro [3]. Like in bone marrow-derived stem cells, *DLX-3* gene expression was increased in dental follicle cells during osteogenic differentiation but similar to control cultures [3].

We suppose that molecular mechanisms in dental follicle precursor cells during osteogenic differentiation are different from those in bone marrow-derived mesenchymal stem cells.

Recently, we demonstrated differentiation of PCs in neural-like cells (Figure 2).



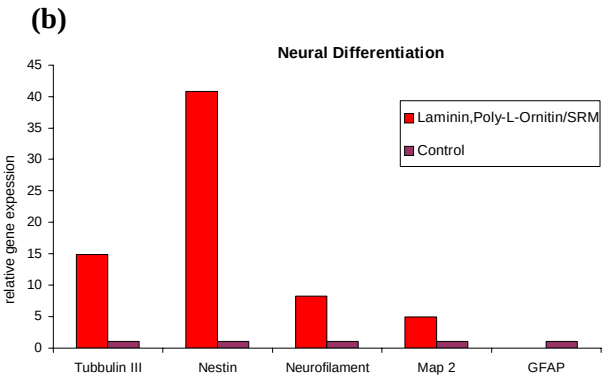


Fig. 2: Neural differentiations of PCs: (a) PCs generated neurosphere-like structures (NLS) after culturing on laminin/ornithin coated cell culture dishes and serum replacement medium (SRM); (b) quantitative reverse-transcription PCR (qRT-PCR) assay, NLS expressed differentially neural cell markers. Control: PCs in standard media (plastic).

REFERENCES: [1]C. Morsczeck, W. Götz, J. Schierholz, et al (2005) *Matrix Biol* **24**:155-165. [2]C. Morsczeck, C. Moehl, W. Götz et al (2005) *Cell Biol Int.* **29**:567-75. [3]C. Morsczeck (2006) *Calcif Tissue Int.* **78**:98-102

MOUSE MODEL COUPLED TO MICRO CT IMAGING ANALYSIS TO INVESTIGATE ALVEOLAR BONE INVOLUTION FOLLOWING TOOTH EXTRACTION

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INTRODUCTION: Both the mandible and maxillary jaw bones develop from the first branchial arch under the direction of homeobox genes that are expressed in a temporo-spatial manner and have a central role in skeletal pattern formation. However, alveolar bone contrarily to basal bone forms in relation to teeth and is dependent upon the presence of teeth for its preservation. Consequently, human alveolar bone ridge is progressively reduced following the extraction of teeth. Therefore, preventing or minimizing alveolar bone loss is a major clinical objective in dentistry.

The present study followed two main goals (1) to develop a new adult mice model system allowing investigation of human alveolar bone resorption specificity following teeth extraction surgery (2) to develop an automatic three dimensional software to obtain slices with a symmetry plane in order to measure and make left-right bone mass comparative analysis following teeth extraction. The study focused on the mice maxillary alveolar bone as an exemplary model system of this specific neural crest-derived skeletal unit.

METHODS: Twelve 3 months old animals were used in this study. Surgical procedure: Animals were anesthetized by an intraperitoneal injection of 0,20 ml of Avertine and ten minutes later, lied down on their back under the heat of a light with their oral cavity maintained open for extraction surgery. Only the second and the third right maxillary molar were extracted using a modified oral spatula type FP3 employed as a dental elevator and a haemostatic forcep to remove the teeth from the oral cavity when necessary. During their recovery and until their sacrifice, animals were maintained in individual cages. All the animals received ad libido 300 mg/kg of paracetamol diluted in water for 3 days. 10 animals were sacrified at one month after extraction surgery and 2 animals (surgery controls) were sacrified immediately after extraction surgery by intraperitoneal anesthesia of Avertine followed by cervical dislocation.

CT scan analysis: CT was used to measure the left and right vestibular plate height after the extraction procedure selected on 5 regions of interest exploring M2 and M3 alveolar ridge. Whole mice heads were scanned using a 1072 Skyscan® micro CT. 400 radiographic projections were acquired at 80 kV, 100 µA, 4 frames with a rotation step of 0.45° and a voxel size of 8µm. After scanning, slices were reconstructed with software based on Feldkamp algorithm. Secondly, the 3D dataset was reoriented using our automatic method. The determination of the symmetry plane relies on the calculation of moments of inertia and is additionally guided by robust least-square estimation of the rigid transformation. Rigid transformation was associated with a trilinear interpolation (A. Marchadier. et al 2006).

RESULTS: Micro-computed tomography analysis allows us to investigate the selected regions of interest. Vestibular cortical plate height measurements were analysed on forty slices centred on the ROI. Integrity of the vestibular alveolar wall during the extraction procedure was first verified by examination of the two animals sacrified immediately after extraction. Compared to the left side non extracted, all the animals after right molar teeth avulsion, display a bone height reduction of the right vestibular cortical plate ranging from 18 to 30% depending on the area selected on the M2-M3 bone ridge ($p < 0,005$).

DISCUSSION & CONCLUSIONS: These results demonstrate that (1) Mice alveolar bone follows a bone resorption comparable to what is observed in human being after teeth extraction (2) therefore, human alveolar bone involution may be studied in mice molar extraction models demonstrating the validity of such mice model to investigate the alveolar bone involution following teeth extraction procedure. (3) the automatic three dimensional software allowing to obtain CT scan slices with a symmetry plane used in the present study appear to be very accurate to measure morphological changes after molar extraction

REFERENCES : Marchadier, A. Basillais, M. Aioub, JR Nefussi, A. Berdal, CL Benhamou. Automatic three dimensional anatomic reorientation of micro CT imaging applied to mice molar dento-alveolar bone. 17th International Bone Densitometry Workshop, Kyoto (Japan) 5-7 November 2006

SALIVARY GLAND HYPOPLASIA AND APLASIA IS NOT SO UNCOMMON IN PERSONS WITH OLIGODONTIA AND ECTODERMAL DYSPLASIAS?

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INTRODUCTION: Previous reports by our group and others have demonstrated reduced salivary secretion in many persons with oligodontia as well as different forms of ectodermal dysplasias (ED) [1,2]. Also salivary gland hypoplasia and aplasia in a few persons with hypohidrotic ED [3,4] have been reported, but have been referred to as rare manifestations of the condition. However, with reference to the mentioned clinical studies, we postulate that disturbed salivary gland development is an underdiagnosed complication in many of these patients.

METHODS: Two cases will be presented.

RESULTS: Case I is a ten year old male with X-linked hypohidrotic ED, total anodontia in the mandible and aplasia of all but three teeth in maxilla. Computed tomography (CT) demonstrated that his parotid glands were hypoplastic, and his submandibular glands could not be visualised on the scans at all. Case II is a nine year old male with peg shaped incisors and canines, agenesis of five permanent teeth, taurodontia, delayed tooth development, and high caries activity. He also has delayed speech development and is diagnosed with ADHD. There are no findings indicating that he has an X-linked hypohidrotic ED. His mouth is clinically dry, and a salivary gland developmental disturbance was suspected. CT demonstrated that both his parotid and his submandibular glands were hypoplastic.

DISCUSSION & CONCLUSIONS: These findings emphasize the need to examine patients with both oligodontia and more general ectodermal developmental disturbances with regard to salivary gland function. In cases were both teeth and salivary glands are affected, the term oral ectodermal dysplasia may be used.

REFERENCES: [1] B. Bergendal, J. Norderyd, M. Bagesund et al (2006) *Int J Paediatr Dent*, **16**:320-6. [2] H. Nordgarden, J.L. Jensen, K. Storhaug (2001) *Oral Dis* **7**:226-232. [3] P. Singh, S. Warnakulasuriya (2004). *J Oral Pathol Med* **33**:634-636. [4] H. Nordgarden, S. Johannessen, K. Storhaug et al (1998). *Oral Dis* **4**:152-154.

Responses of dental pulp stem cells against exogenous stimuli

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INTRODUCTION: The dentin-pulp complex is capable of repair after tooth injuries such as dental caries, attrition, abrasion, and restorative procedures. This study focuses on the responses of dental pulp stem cells (DPSCs) against tooth injuries to clarify the pulpal healing mechanisms against exogenous stimuli.

MATERIALS & METHODS: Five to seven peritoneal injections of 5-bromo-2'-deoxyuridine (BrdU) into the pregnant Wistar rats labeled the adult stem cells in the matured tissues of born animals, 4 weeks and 100 days old, who were used in this study for cavity preparation and tooth replantation, respectively.

RESULTS & DISCUSSION: Four week-old animals contained numerous DPSCs in the center of the dental pulp except for the periphery of the pulp tissue including the odontoblast and subodontoblastic layer. Intense heal-shock protein (HSP)-25- and nestin-immunoreactivity (IR) was found in the cell bodies of coronal odontoblasts. Cavity preparation caused degeneration of the odontoblast layer resulting in the loss of HSP-25- and nestin-IR in the injured dental pulp at the early stages after tooth injury. Newly differentiated odontoblast-like cells with HSP-25- and nestin-IR were arranged at the pulp-dentin border during postoperative Days 2-3 after tooth injury. Interestingly, proliferative cells appeared in the dental pulp on Day 2 when the newly differentiating cells had already arranged along the pulp-dentin border, and increased in number in the wide range of the dental pulp during Days 2-5. The DPSCs were not committed into the newly differentiated odontoblast-like cells along the pulp-dentin border. These results indicate that progenitor cells equipped in the subodontoblastic layer firstly migrate and differentiate into new odontoblast-like cells to compensate for the loss of the odontoblast layer, and subsequently the reorganization of the dental pulp was completed by active proliferation of the DPSCs occurring in the wide range of the pulp tissue (Fig. 1). Tooth replantation also induced active cell dynamics in the dental pulp, although firstly the DPSCs proliferate during Days 3-5 after operation, and subsequently the transit amplifying cells were committed into the newly differentiated odontoblast-like cells to arrange along the pulp-dentin border during Days 5-7. These findings indicate that the DPSCs orchestrate the pulpal responses to the exogenous stimuli in cooperation with the progenitor cells by use of the different cell dynamics depending on the modes of tooth injuries.

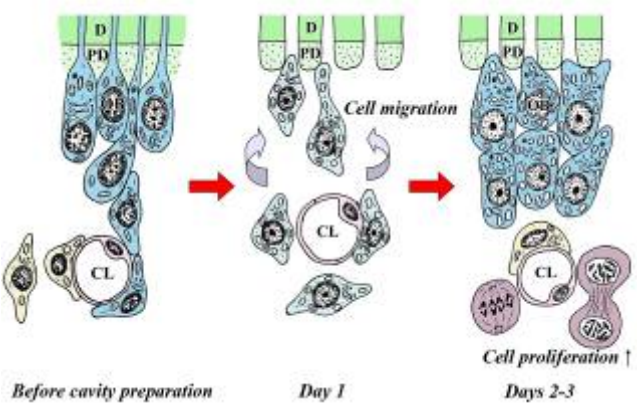

Figure 1. A schematic diagram summarizing cell migration and proliferation in the periphery of dental pulp following cavity preparation. Blue: HSP-25; CL: capillary lumen; D: dentin; OB: odontoblasts; PD: predentin.

ACKNOWLEDGEMENTS: This work was supported in part by grants from KAKENHI (B) (nos. 16390523 and 19390462 to H.O.) and KAKENHI (C) (no. 18592232 to K.N.-O.) from MEXT, Japan.

IMMUNOHISTOCHEMICAL LOCALIZATION OF GLYCOSAMINOGLYCANS AND PROTEOGLYCANS INVOLVED IN ENAMEL FORMATION

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Glycosaminoglycans (GAG) and proteoglycans (PG) are extracellular polyanionic macromolecules related with the biomineralization process in several biological systems. However, their participation in enamel formation during tooth morphogenesis is not clear. In order to probe the hypothesis that GAG and PG are involved in enamel mineralization, upper and lower molars of adult male White New Zealand rabbits were used. The animals were killed by overdose of sodium thiopental and the maxilar and mandibular segments were immediately fixed in paraformaldehyde-ethanol-acetic acid. The specimens were demineralized in 5% formic acid, dehydrated and embedded in paraffin. An indirect immunofluorescent technique or a streptavidin-biotin-peroxidase method was applied on serial sections of 5µm thickness. The following mouse monoclonal antibodies were used: anti-keratan sulfate (5D4), anti-chondroitin sulfate (clon CS-56) and anti-cartilage proteoglycan (MAB 2005 Chemicon. Temecula CA. USA). In order to determinate the relation between keratan sulfate and cartilage proteoglycans, confocal laser scanning microscopy was used. On the other hand, some specimens were partially demineralized in 0,1 M lactic acid and then processed for immunogold electron microscopy. As control, the primary antibodies were replaced by normal serum. As positive control, growth plates were used.

At light microscopy level, the immunolabeling was negative in the enamel matrix in the secretory stage of amelogenesis. However, in the transitional stage of molars, the immunolabeling, with all antibodies used, was observed at the enamel-dentin junction and in relation with ameloblast processes, leaving a wide central zone of unreactive organic matrix. In the maturation stage of enamel development, the antibodies stained progressively the matrix at the prism boundaries. A striking observation was that in the oldest enamel, near of dentogingival junction and occlusal tips, a stronger staining was detected in demineralized sections. The GAG studied colocalized with cartilage epitopes in the same optical section.

In mineralized specimens and at electron microscopic level, the gold particles were observed in relation with filamentous structures between hidroxyapatite crystals. Some labeling was detected in direct contact with the mineral phase of enamel.

Based on these results we propose that, in rabbit molars, GAG may play a regulatory role during enamel mineralization in the transitional and maturation stage of amelogenesis. Since cartilage PG epitopes were intensively stained in the maturing enamel, it is possible that these molecules could be related with the gel properties of enamel organic matrix. On the other hand, the molecules studied were not completely removed during enamel maturation remaining associated with hydroxyapatite crystals in fully mineralized enamel in rabbit molars.

EVOLUTION OF THE EDA PATHWAY IN VERTEBRATES: A STEP TOWARDS TOOTH "EVO-DEVO STUDIES"

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INTRODUCTION:

The development of teeth displays interesting similarities with the development of other vertebrate appendages such as scales, feathers, hair or glands. Indeed, the earliest stages involve similar tissue/cell behavior (epithelium-mesenchyme interactions, epithelial thickening and budding as well as mesenchyme condensation) and common signaling pathways[1]. Among them, the EDA pathway is one of the most interesting, since its disruption in fish and mammals still allows development of viable individuals, but with serious defects of ectodermal/dermal appendages[1,2]. The EDA pathway thus seems to be widely involved in vertebrate appendages development and, as a consequence, might have played roles in their acquisition and further evolution. Along these lines, the EDA pathway has been shown to be involved in armor plate reduction of fresh-water *versus* marine sticklebacks[3].

In order to provide a framework for further studies of the role of the EDA pathway in vertebrate appendage development and evolution, we studied the origin and evolution of EDA pathway genes. We focused our attention on the upstream part of the EDA pathway that is known to be specific for ectodermal/dermal appendages. We thus studied the molecular evolution of *eda*, *edar* and *edaradd* encoding the three classical components of the pathway: a TNF-like ligand, a TNF-R-like receptor and a death domain adaptor, respectively. We also included the genes encoding two related receptors, namely *xedar* and *troy*.

RESULTS & DISCUSSION:

Concerning the origin of the pathway, our observations suggest that a prototypical EDA pathway has been present in the chordate ancestor. However, many features have only been acquired during early vertebrate evolution. These features include new regulatory functions that are due to (i) receptor expansion (three receptors instead of one), (ii) alternative splicing of the *eda* gene (into A1 and A2 isoforms) (iii) the use of EDARADD.

Among vertebrates, *eda*, *edar* and *edaradd* are highly constrained genes and are thus likely to carry a function deeply anchored in vertebrate origins. Nevertheless, these genes experienced several modifications at different evolutionary levels (class, suborder, gender diversification), which may be responsible for subtle changes in pathway regulation or output. Moreover, our data suggest that *troy* and especially *xedar* underwent more drastic functional shifts. In particular, our analysis suggests that ligand-receptor relationships known for these genes in mammals are probably not conserved in other vertebrates.

CONCLUSION:

The evolution of EDA pathway genes reflects the evolution of vertebrate appendages. It combines strongly conserved features that might contribute to similarities found in the early development of vertebrate appendages with functional shifts that might have contributed to innovations and specializations. In the future, we will look for evolutionary modifications in these genes that can be correlated with tooth evolution in specific rodent lineages.

REFERENCES:

[1] Wu et al (2004) Int J Dev Biol 48:249-70.
[2] Sharpe, P (2001) Curr Biol 11:R751-752.
[3] Colosimo et al (2005) Science 307:1928-33.

ACKNOWLEDGEMENTS:

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VARIATION IN NUMBER AND MORPHOLOGY OF PRIMARY TEETH IN BRAZILIAN PRE-SCHOOL CHILDREN.

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INTRODUCTION: Dental anomalies are disturbances in tooth development determined by genetic and/or environmental factors. Number, size, shape alterations as well as defects of mineralized tissues are the most common dental anomalies reported. Epidemiological studies in primary dentition have shown a prevalence that ranges between 0.1% and 1% in the general population. The aim of the present study was to determine the prevalence and distribution of agenesis, size and shape anomalies of the primary dentition in Brazilian pre-school children.

METHODS: A cross sectional study was carried out in 20 public day care centers in the city of Brasilia during 2006. Of a total of 2065 children, 1755 children (836 girls and 919 boys) with ages comprised between 2 and 5 years old were clinically examined to detect teeth agenesis, peg-shaped teeth, double teeth, supernumerary teeth and dental shape alterations.

RESULTS: The percentage of children presenting at least one affected tooth was 5.75%. Only, six children presented two different types of dental anomalies simultaneously (tooth agenesis and double teeth; double teeth/shape alterations; supernumerary/shape alterations). The frequencies of dental anomalies of the preschool children sample are shown in Table 1. The most prevalent anomaly detected was dental shape alterations mainly supernumerary cuspids in upper canines. In The absent teeth detected were lower incisors (55%), upper incisors (36%) and lower canine (9%). No dermatological anomalies were observed in these patients. The double teeth were observed mainly in the lower jaw (88%) and in the incisor and canine region. Sixty five percent of these teeth were either fusion or germination of the lower central and lateral incisors, whereas fusion or germination of lower lateral incisors and canines were observed in 22 %. All the upper double teeth observed involved central and lateral incisors**.** Two cases of peg shaped teeth (0.11%) were observed with no association with tooth agenesis of deciduous teeth.

Table 1 *- Prevalence of preschool children with dental anomalies*

Type of dental anomalies	n	Frequency (%)
Shape alterations	63	3.59
Double teeth	19	1.08
Supernumerary	6	0.34
Tooth agenesis	5	0.28
Peg shaped	2	0.11
Double teeth/ Shape alterations	3	0.17
Double teeth/ Tooth agenesis	2	0.11
Supernumerary/ Shape alterations	1	0.06
No alterations	1654	94.25
Total	1755	100

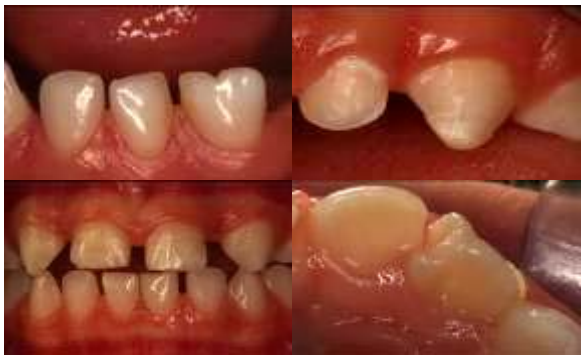

Fig.1 – Types of dental anomalies

CONCLUSIONS: The presents results are in accordance with studies in different geographic regions. Further family studies and radiographic analysis of the children are necessary in order to determine a familial aggregation and to verify the presence of agenesis in the permanent dentition of the affected individuals.

REFERENCES: Brook AH, Winter GB. Double teeth. A retrospective study of 'geminated' and 'fused' teeth in children. Br Dent J. 1970 4;129(3):123-30. Carvalho JC, Vinker F, Declerck D. Malocclusion, dental injuries and dental anomalies in the primary dentition of Belgian children. Int J Paediatr Dent. 1998 Jun;8(2):137-41. Magnusson TE. An epidemiologic study of dental space anomalies in Icelandic schoolchildren. Community Dent Oral Epidemiol. 1977;5(6):292-300.

DYNAMICS OF CUSP PATTERN REGULATION

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INTRODUCTION: For most mammals, cusp patterning has direct consequences on fitness. After tooth eruption cusps can be modified only through wear, so tooth development must be strictly controlled to ensure the correct outcome on the first and only try. However, thus far not a single gene has been discovered to regulate a specific aspect of cusp patterning, such as cusp height, number, or location. On the contrary, cusp patterning seems to be a dynamic process regulated by the whole developmental history of the tooth.

In the mouse molar, cusp patterning begins around embryonic day sixteen when some of the epithelial cells differentiate into secondary enamel knot cells. These cells cease to proliferate whereas the cells of the surrounding epithelium continue proliferating. This uneven growth causes the folding of the occlusal surface, leaving the knot cells at the tips of the cusps. Factors that induce knot cell differentiation may include ActivinβA and Bone morphogenetic proteins (BMP), which induce the expression of the cyclin-dependent kinase inhibitor p21[1,2], one of the first differentiation markers of knot cells. This inductive potential might be regulated *in vivo* by inhibitors, such as Ectodin (a.k.a. SOSTCD1 or Wise), BMP3, and Follistatin.

METHODS: We have studied cusp pattern regulation by using a transgenic mouse that has Green fluorescent protein under a Sonic hedgehog promoter. Shh is expressed in the placode, the primary enamel knot, and the secondary enamel knots. By culturing these molars *in vitro* we have done time-lapse monitoring of how the cusp patterning responds to different protein treatments. We have also studied the expression patterns of *BMP3* and *Follistatin* in wild type mice by doing in situ hybridization on tissue sections.

RESULTS: BMP4 dose-dependently induced accelerated knot cell differentiation followed by rapid crown maturation. ActivinβA caused lingual supernumerary cusps in a dose- and time-dependent manner. *BMP3* and *Follistatin* were expressed in the dental mesenchyme, more strongly on the mesial and buccal sides.

DISCUSSION & CONCLUSIONS: Our results indicate that BMP4 affects cusp patterning through regulating the timing and rate of differentiation. Unlike BMP4, ActivinβA did not affect crown maturation, but the lingual supernumerary cusps might have resulted from ActivinβA's promoting of knot cell differentiation. However, the extra cusps could also be explained by ActivinβA's promoting of cell proliferation, leading to spontaneous cusp induction. ActivinβA is antagonized by BMP3 and Follistatin, both of which were expressed in the dental mesenchyme in mesial-distal and bucco-lingual gradients. Thus they were very weakly present in the distal lingual side, where the strongest response to excess ActivinβA occurred. Timewise, the response was strongest if ActivinβA was added to the culturing media at embryonic day fifteen, which coincides with primary knot apoptosis. Epithelial Follistatin is expressed only in the knot cells[2]. If its expression were to disappear along with the primary knot, reappearing only with the secondary knots, this would provide a narrow interval during which ActivinβA would be free to act on the epithelial cells. Further experiments should elucidate how BMP4 and ActivinβA function in regulating the cusp patterning.

REFERENCES: [1] J. Jernvall, T. Aberg, P. Kettunen, S. Keranen, and I. Thesleff (1998) *Development* **125**:161-169. [2] X-P. Wang, M. Suomalainen, C.J. Jorgez, M.M. Matzuk, M. Wankell, S. Werner, and I. Thesleff (2004) *Developmental Dynamics* **213**:98-108.

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SEASONAL INCIDENCE OF OROFACIAL CLEFTS IN THE CZECH REPUBLIC

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INTRODUCTION: Only a small part of inborn defects in man are explained either by genetic reasons (20% of cases) or by prenatal exposure to a harmful external factor (15% of cases), such as maternal diseases, acute infection accompanied by hyperthermia, specific drugs, occupation risks. Most developmental defects (65% of cases) are thought to result from prenatal exposure to the combined effect of several sub-threshold doses of external factors that act either simultaneously or sequentially; a genetic predisposition is presumed in some of these cases. Various harmful exogenous factors act seasonally during a year and can potentially be prevented. For example infections, extreme temperature, malnutrition, solar radiation, stressful state related to holiday or air-pollution can show seasonal variation. Therefore, we aimed to search for a seasonal variation in the incidence of newborns with an orofacial cleft in the Czech Republic.

METHODS: We analyzed data about seasonal variation of the mean month-number of newborn girls and boys with an orofacial cleft in a sample comprising more than 5000 patients treated at the Clinic of Plastic Surgery in Prague. The patients were born in the Czech Republic during 1964-2000. For this period, the mean numbers of newborns in each month in a year were calculated. The data were compared with the whole country population data by the Czech Statistical Office about the number of newborn boys and girls during the same period 1950-1999. The Fischer's exact test was used to test statistical significances.

RESULTS: In the Czech population, we found a seasonal variation in the mean month number of newborn living boys and girls with a significant maximum in March, April, and May, and with a significant minimum in October, November and December (Fig.1).
We found similar seasonal variation and significant maxima and minima for the number of newborn boys or girls with an orofacial cleft.

In comparison to whole country data about newborn boys, there were only two divergences in boys with an orofacial cleft: a significant decrease in April and a significant increase in May.

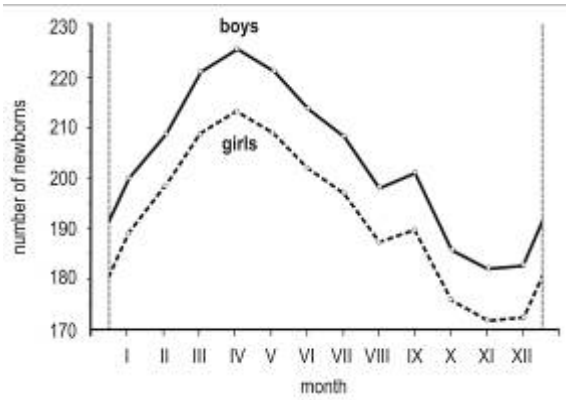

Fig. 1: A seasonal variation in the mean numbers of newborn living boys or girls in the Czech. The mean values were calculated for each month of a year from newborn data registered during 1950 -1999.

DISCUSSION & CONCLUSIONS: Seasonal variation in the birth rate has been related to the photoperiod or temperature (affecting hormonal concentrations, sperm quality or sexual activity), and may also be influenced by socio-demographic factors [1]. Therefore, seasonal differences in the number of newborns can be different and specific for each country (e.g. birth-rate in Australia [2] is significantly different from the Czech). It results that the evaluation of the incidence of a birth defect should always be correlated with appropriate data on natality at population level.

REFERENCES: [1] M. Bobak, A. Gjonca (2001) *Hum Reprod* **16**:1512-1517. [2] D.P. Weerasinghe, C.R. MacIntyre (2003) *Med Sci Monit* **9**:534-540.

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MORPHOGENESIS OF A SUPERNUMERARY CHEEK TOOTH IN *SPRY* NULL AND *TABBY/EDA* MICE

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INTRODUCTION: Mouse dentition consists of one incisor and three molars, separated by a toothless diastema. In mouse embryos, transient structures occur in the diastema that are thought to be vestiges of ancestral tooth primordia. Two large diastemal vestiges (buds) in front of the molars have been homologized to the premolars lost during mouse evolution [1]. The distal diastemal bud (R2) is involved in formation of the mesial part of the lower first molar (M1) [2], (Fig. 1A). A contribution from the mesial diastemal bud (MS) is assumed [3]. Either a mutation in the *Eda* gene or a loss of *Sprouty (Spry)* gene function can stimulate revival of the diastemal buds, leading to origin of a supernumerary cheek tooth (S) [3,4], which can be classified as an atavism [3].

Aim: To compare S morphogenesis and its developmental relationship to the diastemal premolar buds in the mandible of *Tabby/Eda* mutant mice and in *Spry 2* and *4* null mice.

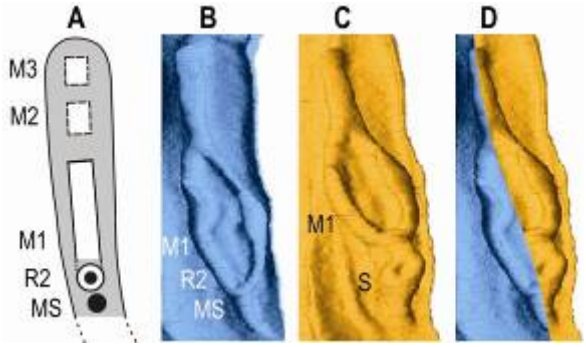

Fig. 1: Origin of the supernumerary tooth (S) in mouse mandible. ***(A)*** *Developmental relationship of premolar buds (MS, R2) and M1 in normal mice. The dental epithelium in 3D reconstruction at embryonic day 15.5: wild type* ***(B)*** *and Spry 4-/-* ***(C). (D)*** *A graphical chimera. The S forms at the presumable place of the mesial part of M1 in wild type mice.*

METHODS: Development of the lower cheek dentition was analyzed in *Tabby/Eda*, *Spry2* and *Spry4* null, and wild type (WT) mice at embryonic days 12.5-16.5. We used histological series of 7µm thick sections and computer-aided 3D reconstructions.

RESULTS: In *Spry* null and *Tabby/Eda* mice, abnormal segmentation of dental epithelium was observed, and the diastema bud was not incorporated into M1. The diastema bud R2 was either involved in formation of the S tooth primordium or failed to develop (in some *Tabby/Eda* jaws). The formation of S was accompanied by a reduction of M1 (Fig. 1C).

DISCUSSION & CONCLUSIONS: The loss of EDA signaling or *Spry* function enhances autonomous development of diastema primordia which are normally suppressed in WT mice. Reduction of the anterior part of M1 might result from failed integration of the diastema bud into M1. The size of the premolar bud is important in determining the number, shape and size of mouse cheek teeth. An improved understanding of the genetic and cellular pathways leading to the development of the supernumerary atavistic tooth may help to develop approaches of controlled tooth replacement [3], and to explain mechanisms driving tooth suppression during evolution.

REFERENCES:

[1]R. Peterkova, M. Peterka, L. Viriot, H. Lesot (2000) *J Craniofac Genet Dev Biol* **20**: 158-172. [2]L. Viriot, H. Lesot, J-L. Vonesch et al (2000) *Int J Dev Biol* **44**:233-240. [3]R. Peterkova, H. Lesot, L. Viriot, M. Peterka (2005) *Archs Oral Biol* **50**:219-225. [4]O.D. Klein, G. Minowada, R. Peterkova, et al (2006) *Dev Cell* **11**:181-90.

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REGULATORY LOOP OF MSX1 EXPRESSION BY ITS SENSE AND ANTISENSE TRANSCRIPTS

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INTRODUCTION: Msx genes play an important role in epithelial-mesenchymal interactions that lead to vertebrate organogenesis. Msx1 is particularly interesting as it plays a key role in the craniofacial morphogenesis as suggested by phenotypes of Msx1 mutations in human and Msx1 KO mice[1]. Msx1 is also involved in the development of other organs like hair follicles, limb buds, uterus and mammary glands. During adulthood, Msx1 is still expressed in skull, bones, uterus and mammary glands but its role during homeostasis is largely unknown. Msx1 is expressed in specific growth area of adult skeleton, thus suggesting Msx1 involvement in bone regeneration[2]. This is supported by in vitro data which revealed that Msx1 maintains cells in an undifferentiated state by stimulation of the cyclin D1 expression or inhibition of master genes like Runx2 in osteoblasts, MyoD in myoblasts[3].

Our previous studies on Msx1 expression lead us to map and characterize a natural antisense (AS) transcript generated from the same locus (cis-NAT) [4]. This Msx1 AS RNA may control Msx1 expression but its mechanism of action is still unknown. In vivo data showed that Msx1 protein and AS RNA expression patterns are complementary during tooth development.

The aim of the present study was to analyse the relations between Msx1 sense (S) and AS RNAs. For this purpose, *in vivo* and *in vitro* studies were carried out simultaneously.

RESULTS: In vitro part of the present study showed that transient overexpression of one Msx1 transcript modulated the complementary endogenous RNA level. Indeed, Msx1 AS RNA overexpression diminished endogenous S Msx1 mRNA level by a decrease of its half-life. Conversely, Msx1 overexpression led to an increase of endogenous AS RNA level probably because of an induction of AS transcription by Msx1 itself as suggested by RNA-FISH studies. These experiments showed a possible AS RNA nuclear localization when Msx1 was overexpressed probably resulting from the transcriptional activation.

In parallel, in vivo Msx1 expression studies in adult tissues showed that both Msx1 (S and AS) RNAs were detected in all tested tissues. Surprisingly, the level of expression was at least that measured in mouse embryos at E 12.5. These results rose the question of Msx1 function during adulthood. Moreover both RNA species exhibited similar patterns of expression further supporting a coordinated relation of their products. The focus on Msx1 expression in eyes (which is an organ able to regenerate in different species) showed that both RNAs and the protein were localized in the same areas.

DISCUSSION & CONCLUSIONS: The data showed that S and AS RNAs created a regulatory loop resulting in a definite Msx1 expression level. Msx1 expression is controlled by its AS RNA which is itself controlled in turn by Msx1. Moreover, during adulthood, both Msx1 transcripts were shown to be expressed in almost all tissues tested in this study and presented similar patterns of expression. The tight link between Msx1 S and AS RNAs evidenced in the present study would establish a fine tuned specific Msx1 expression level in vivo.

REFERENCES: [1] Satokata, I. and R. Maas. 1994. Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. *Nat Genet* **6**: 348-56. [2] Orestes-Cardoso et al. 2001. Postnatal Msx1 expression pattern in craniofacial, axial, and appendicular skeleton of transgenic mice from the first week until the second year. *Dev Dyn* **221**: 1-13. [3] Odelberg, S.J., A. Kollhoff, and M.T. Keating. 2000. Dedifferentiation of mammalian myotubes induced by msx1. *Cell* **103**: 1099-109. [4] Blin-Wakkach, C et al. 2001. Endogenous Msx1 antisense transcript: in vivo and in vitro evidences, structure, and potential involvement in skeleton development in mammals. *Proc Natl Acad Sci U S A* **98**: 7336-41.

DMP1 IS NOT INVOLVED IN DENTINOGENESIS IMPERFECTA OR DENTIN DYSPLASIA CASES.

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INTRODUCTION: Human dentinogenesis imperfecta (DGI) and dentin dysplasia (DD) are autosomal dominant disorders of the tooth that affect dentin biomineralisation. According to Shields, DGI is classified into 3 types (OMIM 166240, OMIM 125490) and DD into 2 types (OMIM 125420) based on clinical features. DGI and DD have been linked to mutations in the *DSPP* gene localized on the chromosome 4 (4q21.3). The *DSPP* gene encodes 2 major non collagenous dentin matrix proteins DSP and DPP. The role of this gene in the pathogenesis of DGI has been highlighted by *DSPP* null mice which developed tooth defects similar to those seen in human DGI-III. While different authors reported several mutations of the *DSPP* gene in the literature, direct sequencing of *DSPP* failed in some cases to explain the pathogenesis of dentin disorders. The variable expression of the phenotype and the low sensitivity of mutation detection might suggest the occurrence of other genetic events or a genetic heterogeneity. All the human dentin genetic diseases were demonstrated to be allelic and mapped on the same locus on chromosome 4q21. However, other important genes involved in biomineralization named the SIBLINGs map to the DGI loci on chromosome 4. In particular, the dentin matrix protein-1 (DMP1) plays a role in dentin extra cellular matrix formation and mineralization. The tooth phenotype of *DMP1* null mice is very similar to that of *DSPP* null mice. So *DMP1* has been proposed as a candidate gene in DGI and DD, even if, to our knowledge, no sequence variant in *DMP1* has been associated with these diseases yet. Here we describe the analysis of the *DMP1* gene mutations in families with no disease-associated sequence variation in *DSPP*.

METHODS: Seven pairs of primers were designed to amplify the coding seqence of *DMP1*. Genomic DNA from ten unrelated families negatively screened for mutations in *DSPP* was analysed. Sequencing was performed on both DNA strands using the ABI Prism BigDye® Terminator sequencing kit. Purified sequencing products were run on an ABI Prism 3100 Genetic Analyzer. Sequence analysis was performed using ABI Prism SeqScape software (PE Applied Biosystems).

RESULTS: The exon-by-exon screening for mutations in *DMP1* did not detect any deleterious sequence variant.

DISCUSSION & CONCLUSIONS: In many of our kindreds affected with DGI or DD, direct sequencing of *DSPP* and *DMP1* failed to explain the occurence of dentin disorders. Our perspective is to search for large rearrangements on these two genes by semi-quantitative multiplex genomic PCR, or explore the involvement of neighboring or related genes, in order to reach more comprehensive understanding of the inherited dentin disorders pathogenesis.

REFERENCES: M.L. Beattie, J.W. Kim, S.G. Gong, et al. (2006) *J Dent Res*, **85**(4):329-33. M. MacDougall, T.T. Gu, D. Simmons (1996) *Connect Tissue Res*, **35**(1-4):267-72. M. MacDougall, L.G. Jeffords, T.T. Gu, et al. (1999) *J Dent Res*, **78**(6):1277-82. S. Xiao, C. Yu, X. Chou, et al. (2001) *Nature Genet*, **27**:201-4. X. Zhang, J. Zhao, C. Li, et al. (2001) *Nature Genet*, **27**:151-2.

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FINE STRUCTURAL AND IMMUNOCYTOCHEMICAL OBSERVATIONS ON COLLAR ENAMEL AND GANOINE IN *POLYPTERUS*, AN ACTINOPTERYGIAN FISH

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INTRODUCTION: Collar enamel has not been reported in *Polypterus*, an actinopterygian fish, although the ganoine layer in the scales of polypterids corresponds to ectodermal enamel [1-3]. However, collar enamel was noted in *Lepisosteus*, which is also a primitive actinopterygian by electron microscopy and immunohistochemistry [4]. This is the first report of the collar enamel in *Polypterus*.

METHODS: Fine structure of the collar enamel and enamel organ during amelogenesis in *Polypterus senagals* was observed by transmission electron microscopy. To examine the collar enamel matrix, light and electron microscopic immunohistochemistry was performed using crude antiserum against 25 kDa porcine amelogenin, region-specific antibodies or antiserum against the C-terminus, central region and N-terminus of porcine amelogenin, respectively. Mature ganoine in the scales was examined with the same methods in order to compare with the collar enamel.

RESULTS: The enamel layer, 1 um thick, containing amorphous fine matrix that resembled the stippled material in mammals was found between the dentin and ameloblasts in the stage of tooth shaft formation. In the ameloblasts, there were developed Golgi apparatus, rough endoplasmic reticulum and secretory granules. The enamel and ganoine matrix showed intense immunoreactivity to crude antiserum against amelogenin and the C-terminal region-specific antibody, but not to the central region-specific antibody and the N-terminal region-specific antiserum.

DISCUSSION & CONCLUSIONS: The data suggests that both the enamel and ganoine matrices in *Polypterus* contain a domain that closely resembles the C-terminal region of porcine amelogenin.

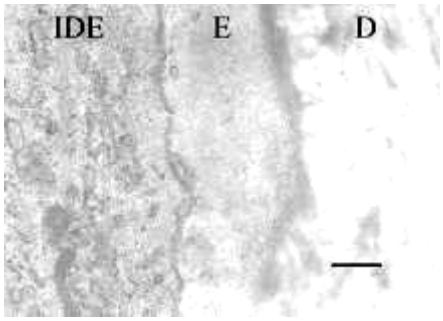

Fig. 1: Transmission electron micrograph showing the collar enamel layer (E) demineralized, bar = 200 nm

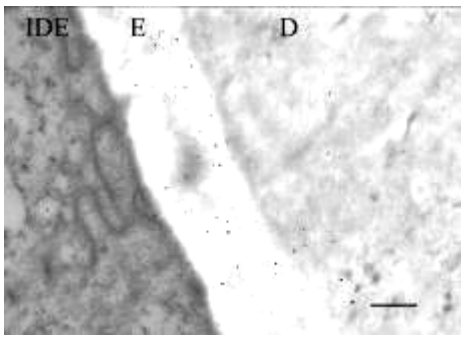

Fig. 2: Immunoreactivity against the anti-25kDa porcine amelogenin serum in collar enamel, bar = 500 nm, D; dentin, E; enamel, IDE; ameloblasts

REFERENCES:

[1] Sire J.-Y. (1995) Connect Tissue Res, 33. 535-544. [2] Zylberberg L., Sire J.-Y., Nanci A. (1997) Anat Rec, 249. 86-95. [3] Kogaya Y. (1997) Archives of Comparative Biology of Tooth Enamel, 5. 23-29. [4] Ishiyama M., Inage T., Shimokawa H. (1999) Arch Histol Cytol, 62. 191-197.

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CHANGES OBSERVED OVER TIME IN DENTIN DEVELOPMENT AFTER NEONATAL DESENSITISATION

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INTRODUCTION: The neonatal application of capsaicin has been known to produce a selective desensitisation of nociceptive fibers in a physiological system [1, 2]. Additionally, it has been observed that a lack of functional nociceptive fibers in the pulp has a detrimental effect on dentine development after 120 days [1]. The purpose of this study was to observe dentine development in the neonatally capsaicin-treated rat in a spectrum from 30 to 155 days of life.

METHODS: This study was carried out with the subcutaneous application of capsaicin at a dose of 50 mg/Kg body weight in a sterile vehicle in 48 Wistar rats on the 3rd day of life. This group served as the experimental group in which 12 rats lived to 30 days, 12 to 60 days, 12 to 90 days and 12 to 155 days. The control group was made up of 48 rats which were treated with the sterile vehicle of identical volume which did not contain capsaicin. Furthermore, in the control group 12 rats lived to 30 days, 12 to 60 days, 12 to 90 days and 12 to 155 days. All rats were deeply anaesthetized before being sacrificed with cardiac puncture and intravital perfusion and fixation. Jaws were then immediately dissected and further fixed for no more than 24 hours in 4% buffered paraformaldehyde. Jaws were opened sagitally from pulp horn to pulp apex and prepared for the scanning electron microscopy (SEM). Photo documentation was carried out at SEM magnifications of X200, X700, X1000, X1800, and X4000.

RESULTS: Our results demonstrate an extreme change in dentine development in a time spectrum from 30 days to 155 days in the experimental group, where changes in dentine development were not noted in the same time spectrum of the control group. More specifically, no changes were found between the 30 day experimental group and the 30 day control group. In the 60 day group, dentine defects such as irregular dentine tubuli diameter and voids in dentine structure are noted in the experimental group. These dentine defects are more extreme in the 90 day experimental group compared with the 60 day experimental group. Finally, while there is still dentine defects seen in the 155 day group compared with the 30 day experimental group, there is no progression of dentine deterioration when comparing the 90 day experimental group with the 155 day experimental group.

DISCUSSION & CONCLUSIONS: With this study we were able to show that the neonatal application of capsaicin produces changes in dentin development over time in the rat.

REFERENCES: [1] K. Chung, C.M. Klein, R.E. Coggeshall (1990) The receptive part of the primary afferent axon is most vulnerable to systemic capsaicin in adult rats. *Brain Res* **511**: 222-226. [2] T. Krage, I. Lambrichts, K. Zanger, W.H.-M Raab (2002) Capsaicin-treated animals vs. NO-3 knockout mice: a SEM and TEM comparison in *Proceedings to the International Conference on Dentin/Pulp Complex 2001* (ed Ishikawa et al.) Quintessence Publishing, Tokyo, pp 187-189.

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CERVICAL COLUMN MORPHOLOGY AND MANDIBULAR DEVELOPMENT

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INTRODUCTION: Malformations of the upper cervical vertebrae have been closely investigated in patients with cleft lip and/or palate. The association between the cervical vertebrae and the maxilla is caused by a developmental fault of the mesenchyme, as the areas are dependent on the same or similar paraaxial mesoderm. While the associations are well described for the maxilla, no studies have investigated deviations of the cervical vertebrae and the development of the mandible. So far three accepted studies have been carried out on the topic cervical column morphology and mandibular development and occlusion.

AIMS: The aims of studies were to describe the morphology of the cervical column in three groups: patients with mandibular condylar hypoplasia, patients with skeletal deep bite and patients with skeletal mandibular overjet and to compare the cervical column morphology in the three groups with the cervical column morphology in a control group with neutral occlusion and normal craniofacial morphology.

SUBJECTS AND METHODS: The mandibular condyler hypoplasia group consisted of 11 patients, the deep bite group of 41 patients, the mandibular overjet group of 57 patients and the control group consisted of 21 subjects. The subjects in all groups were between 18-45 years. A visual assessment of the cervical column and measurements of the craniofacial dimensions were performed on a profile radiograph of each individual.

RESULTS: Morphological cervical column deviations occurred in 72.7 percent in the condylar hypoplasia group, in 41.5 percent in the deep bite group, in 61.4 percent in the mandibular overjet group and in the control group 14.3 percent. Deviations occurred significantly more often in all three patient groups compared with the control group ($p<0.01$; $p<0.05$; $p<0.001$, respectively). Also the pattern of the deviations in the cervical column morphology was different in the groups. In the deep bite group as well as in the control group fusions of the cervical column always occurred between c2 and c3 (Figure 1), whereas in the condylar hypoplasia group the fusion also occurred between c4 and c5 (Figure 1). In the mandibular overjet group the fusions occurred between c2 and c3 but block fusions were also observed (Figure 1).

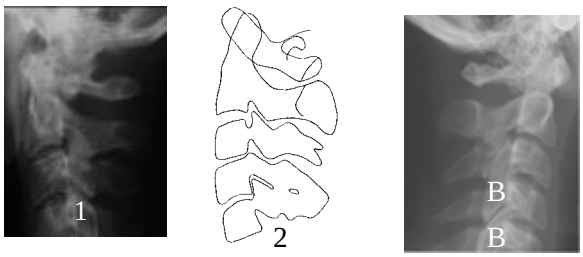

Fig. 1: Fusion of the cervical column vertebrae in the control group, deep bite group, condyler hypoplasia group and in the mandibular overjet group. 1: Fusion between c2 and c3, 2: Fusion between c4 and c5, B: Block fusion.

DISCUSSION & CONCLUSIONS: It is suggested that the key to understanding the associations between the apparently at random structures as the cervical column, the mandibular condyle, the deep bite and the mandibular overjet is the notochord and the common signalling from the notochord forming these craniofacial structures during the early embryogenesis.

METALLOTHIONEIN: A POSSIBLE NEW MARKER FOR HUMAN DENTAL PULP STEM CELLS

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INTRODUCTION: Human dental pulp stem cells (HDPSC's) is a term used for the remaining mesenchymal stem cell population in adult teeth. It has already been described that these cells exhibit odontogenic capacities when these cells are explanted into immunocompromised mice. They create a dentin-pulp like complex and in this way their use in tissue engineering and tooth regeneration seems very promising. However, there is still a lack of specific markers to identify this cell type. In this study, we investigated the immunoreactivity of HDPSC's for metallothionein (MT), a Cystein-rich, low molecular weight protein. MT has the capacity to bind both physiological (Zn, Cu, Se,...) and xenobiotic (Cd, Hg, Ag,...) heavy metals through the thiol group of its cysteine residues. Furthermore, the immunoreactivity fot MT was tested in human pulp tissue and human bone-marrow derived mesenchymal stem cells.

METHODS: All teeth used in the experiments were extracted for orthodontic or therapeutic reasons. After extraction, the apical part of the teeth was removed with a scalpel and the dental pulps were removed with a forceps. HDPSC's were isolated as described by Gronthos et al. (2000)[1]. Other dental pulps were prepared for immunohistochemistry using routine fixation (Unifix®) and embedding (paraffine sections) techniques. Mesenchymal stem cells were isolated from human adult bone marrow. Immunostaining for metallothionein was carried out using the DAB envision® kit.

RESULTS: HDPSC's isolated from human third molars were positive for MT (Fig.1a). When compared to mesenchymal stem cells derived from bone-marrow, only certain subsets of the latter were positive (Fig. 1b). These subsets were always showing mitotic figures or were located in an area surrounding these dividing cells. In normal human pulp tissue, only peri-vascular cell niches showed immunoreactivity for MT (Fig.2). This is in accordance with the current hypothesis of the localisation of HDPSC's in adult pulp tissue.

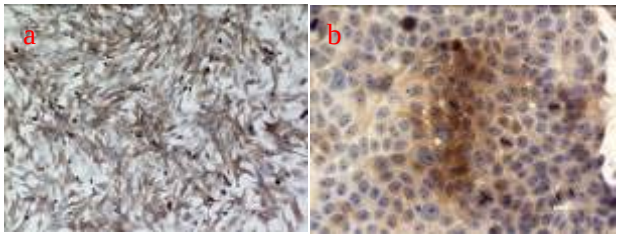

Fig. 1: (a) Human dental pulp stem cells showing immunoreactivity for metallothionein. (b) Bone-marrow derived mesenchymal stem cells are only found to be positive during mitosis or when neighbouring mitotic cells.

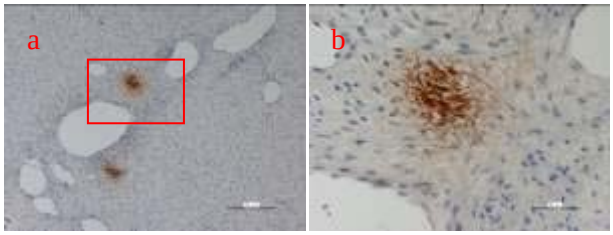

Fig. 2: (a) Presence of metallothionein-positive cell niches in healthy human dental pulp tissue. Note that these cell niches are only located in the peri-vascular area. (b) Higher magnification of boxed area in (a).

DISCUSSION & CONCLUSIONS: HDPSC's were found to be positive for MT. This suggests that MT can be used as a new marker that can distinguish HDPSC from other mesenchymal derived stem cells.

REFERENCES: [1] S. Gronthos, M. Mankani, J. Brahim et al (2000) Postnatal human dental pulp stem cells (DPSC'S) in *vitro* and in *vivo*, *Proc Natl Acad Sci USA. Dec 5;97(25):13625-30.*

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BALANCE BETWEEN BMP4 AND ACTIVIN SIGNALLING REGULATES FGF3 EXPRESSION AND EPITHELIAL STEM CELLS IN MOUSE INCISORS

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INTRODUCTION: In a continuously growing mouse incisor epithelial stem cells are located in the stellate reticulum in cervical loops [1]. Stem cells proliferate within stellate reticulum and the progeny invade the surrounding basal epithelial cell layer were they proliferate actively and differentiate. These cells differentiate into enamel forming ameloblasts on the labial side whereas on the lingual side differentiation does not occur. Labial side stellate reticulum tissue is large compared to the lingual side where only few cells can be found between inner and outer dental epithelium. Bone morphogenetic protein 4 (BMP4) is known to induce the differentiation of inner dental epithelium to enamel forming ameloblasts on the labial side of the incisor while the proliferation of the epithelium is stimulated by Fibroblast growth factor 10 (FGF10) [2, 3]. *Fgf3* and *Fgf10* have partially overlapping expression patterns in the mesenchymal cells underlying the epithelium in the cervical loop area. After E16 *Fgf3* expression becomes asymmetrical and restricted to the labial side while *Fgf10* is expressed in the mesenchyme on both the labial and lingual sides [4].

RESULTS: Mouse overexpressing *Follistatin* in the epithelium (*Keratin-14 Follistatin*) had small incisors with a hypoplastic cervical loop on the labial side. The stellate reticulum was missing and proliferation was diminished. The phenotype of the incisor resembled that of *Fgf10*$^{-/-}$ mouse [3]. In contrast, *Follistatin*$^{-/-}$ mouse had enlarged stellate reticulum on the lingual side which contained active proliferation. The expression of *Fgf3* was completely down regulated in the *Keratin-14 Follistatin* mouse but surprisingly in the *Follistatin*$^{-/-}$ incisor there was ectopic expression on the lingual side. Follistatin inhibits signalling of several Transforming growth factor (TGF) superfamily members such as BMP4 and Activin. Introduction of beads containing BMP4 or ActivinA to incisor explants revealed that BMP4 inhibits *Fgf3* expression while ActivinA induces ectopic expression of *Fgf3* on the lingual side and dramatically stimulates epithelial cell proliferation in the cervical loops. ActivinA was also able to induce proliferation in cervical loops and as expected the inhibition of Activin receptor-like kinase (ALK) receptors by SB431542 caused reduction in proliferation in both labial and lingual cervical loops. The enamel formation and growth of incisors were much more affected when there was less FGF signalling. In the *Fgf3*$^{-/-}$ the enamel appeared normal but in the *Fgf3*$^{-/-}$*; Fgf10*$^{+/-}$ mutant it was missing.

DISCUSSION & CONCLUSIONS: The epithelial stem cell niche in the incisor is redundantly regulated by FGF3 and FGF10, and BMP4 negatively controls the expression of *Fgf3* and epithelial cell proliferation. Activin counteracts the inhibition on the labial side and thus stimulates stem cell proliferation. Follistatin contributes to the formation of asymmetry by inhibiting the function of Activin on the lingual side.

REFERENCES: [1] H. Harada, P. Kettunen, H.S. Jung et al. (1999) *J. Cell Biol.* **4**:105-20. [2] X.P. Wang, , M. Suomalainen, C.J. Jorgez et al. (2004) *Dev. Cell* **7**:719-30. [3] H. Harada, T. Toyono, K. Toyoshima et al. (2002) *Development* **129**:1533-41. [4] P. Kettunen, J. Laurikkala, P. Itaranta et al. (2000) *Dev. Dyn.* **219**:322-32.

EXPRESSION AND LOCALIZATION OF ODONTOBLAST PRIMARY CILIUM PROTEINS

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Odontoblasts responsible for the dentine formation are organized as a single layer of highly polarized post-mitotic cells along the interface between the dental pulp and dentine. Each cell has an extension, the odontoblast process, running into a calcified dentinal tubule and deeped in a liquid phase, the dentinal fluid. This unique spatial situation of odontoblasts in the dentin/pulp complex strongly suggests that these cells could be subjected to dentine fluid movements resulting from a mechanical stimulation from external tooth stimuli (high pressure, osmotic, chemical or thermal shock). At the ultrastructural level, odontoblast cell body exhibits a primary cilium (9+0 formula) close to the centriole and in the vicinity of the Golgi apparatus. Antibodies directed against the ciliary marker detyrosinated □tubulin (ID5) identified the primary cilium in human odontoblasts both in vivo and in vitro (1). Its role remains unknown but this structure could constitute a critical link between external teeth stimuli and odontoblast responses. Indeed, fluid movements elicited in dentinal tubules by tooth stimuli may cause odontoblast cell membrane deformation thus initiating the signal transduction pathway. We suggest that odontoblast primary cilium deflexion by dentinal fluid flow could be involved in this process. The objective is to understand the role of the primary cilium in odontoblast using our unique in vitro human model (2, 3). Thus, the aim of this work was to analyze and characterize the primary cilium of human odontoblasts at the molecular level. We have thus successfully identified by RT-PCR and immunochemistry the expression and localization of acetylated □tubulin, OFD1 as well as inversin (PCR only), polycystin1 and 2, rootletin and KIF3A, proteins known to be essential for the cilium function in other cell types. Analyses of *OFD1* mutant mice molar tooth germs showed a dramatic impaired molars structure and the absence of differentiated odontoblasts. These results prompt us to analyse the functional role of cilium as a key organelle of signal transduction in odontoblasts during teeth stimuli.

REFERENCES: [1] H. Magloire, ML. Couble, A. Romeas et al.,(2004), *Cell Biol Int*, **28**: 93-99. [2] ML. Couble, JC. Farges, Bleicher F. et al.,(2000), *Calcif Tissue Int*, **66**: 129-138.
[3] R. Buchaille, ML. Couble, H. Magloire et al.,2000), *Matrix Biol*, **19**: 421-430.

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EGG TOOTH DEVELOPMENT IN THE SNAKE

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INTRODUCTION: The egg tooth is a vital structure allowing hatchlings to escape from the egg. In squamate (scaly) retiles the egg tooth is a real tooth that develops within the oral cavity at the top of the upper jaw. Primitive squamates, such as geckos, have two egg teeth, while more advanced squamates, including snakes, have a single medial egg tooth. We have investigated how the single egg tooth develops using two snakes, the python and corn snake, *Elaphe gluttata*, and investigated the relationship between the egg tooth and neighboring premaxillary teeth.

METHODS: Frontal and sagittal wax sections were made through the upper and lower jaw at a variety of embryonic ages. Slides were stained for histology or TUNEL staining. Snake Shh was cloned in the corn snake and African rock python, *Python sebae*, and *in situ* hybridisation carried out.

RESULTS: In the python and corn snake two closely positioned tooth germs are present in the midline of the upper jaw at early stages and these grow together and fuse during development to form a single large egg tooth associated with the premaxilla. The egg tooth is precocious in its development and much larger than the rest of the dentition. Teeth on the dentary (lower jaw) and maxilla, pterygoid and palantine (two tooth rows of upper jaw) develop deep within the jaw from an extended dental lamina[1]. In contrast, the egg tooth develops near to the oral surface. This superficial mode of development is also observed for the premaxillary teeth in the python. These teeth lie on either side of the egg tooth and share a shallow dental lamina. Unlike the python, the corn snake has no premaxillary teeth. In the embryo, however, it is clear that the premaxilllary tooth germs form and start to produce dentine but then regress during later stages of development. This regression appears not to involve apoptosis (programmed cell death), as is associated with the loss of vestigal tooth germs in the mouse diastema region.

DISCUSSION & CONCLUSIONS: The egg tooth in the snake forms by a fusion of two closely positioned tooth germs. This is in contrast to the situation observed in many lizards where two tooth germs initially form, but then one regresses, while the other moves into a central location[2]. The single egg tooth of a lizard, such as *Lacerta*, and that of a snake, therefore forms by a very different process.

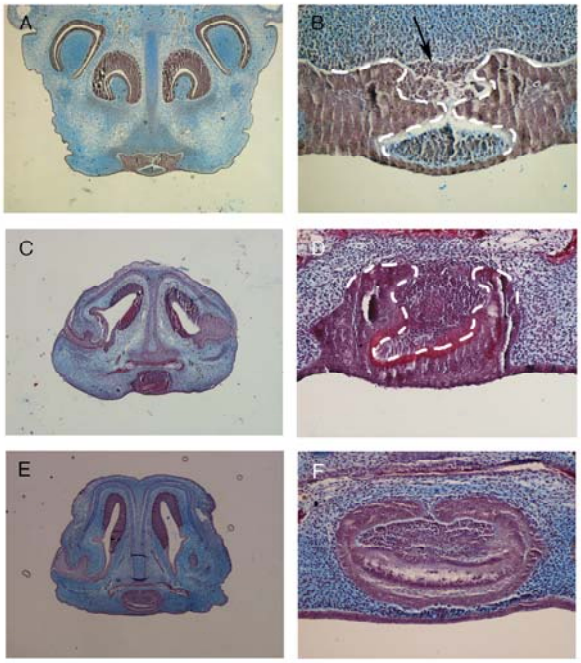

Fig. 1Fusion of two tooth germs into one in the corn snake.

Fusion of midline teeth in the upper jaw is also observed in humans with SMMCI syndrome. This syndrome has been related to a disruption in Shh signalling in the midline[3]. A reduction in Shh signalling may therefore have lead to the fusion of the egg tooth primordial during the evolution of the snakes. The close association of the egg tooth with the premaxillary dentition, implies that the egg tooth is not a novel structure but an adaptation of the premaxillary dentition.

REFERENCES: [1] M. Buctova, J.C. Boughner, K.Fu., V.M.Diewert, J.M.Richman (2007) Zoology **110**:231-251. [2] G.R. De Beer (1949) Proc. Linn Soc Lon. **161**:218-224. [3] L.Nanni, J.E.Ming, Y.Du, R.K.Hall, M.Aldred,A.Bankier, M.Muenke (2001) Am. J. Med.Genetics **102**:1-10.

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HISTOGENESIS AND PATTERN OF REPLACEMENT OF DENTARY TEETH IN WILD ATLANTIC SALMON (*SALMO SALAR* L., SALMONIDAE, TELEOSTEI)

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INTRODUCTION: The intriguing and complex life cycle of Atlantic salmon [1], the increasing use of Atlantic salmon as a model species in studies of skeletogenesis [2], and the continuous replacement of its teeth [3,4], have led us to study the development of the dentition in this species.

METHODS: We analysed the formation of first-generation and replacement teeth, and the pattern of tooth replacement on the dentary of wild Atlantic salmon throughout nearly all stages of its life cycle. We used serially sectioned heads and jaws, cleared and stained animals, and X-rays.

RESULTS: The dentary teeth are set in one row. First-generation teeth appear around hatching. They develop within the oral epithelium first in odd positions, followed by even positions. From position 8 further backwards, teeth are added sequentially.

The first replacement teeth appear in animals of about 30 mm fork length. The anlage of the replacement tooth is first seen as a placode-like thickening of the outer dental epithelium of the predecessor, at its lingual and caudal side. Ongoing development of the replacement tooth germ is characterized by the elaboration of multiple layers of epithelial cells apposed to the inner dental epithelium on the lingual side of the tooth germ, termed here the middle dental epithelium. Prior to the formation of the new successor, a single-layered outer dental epithelium segregates from the middle dental epithelium. The dental organ of predecessor and successor remain broadly interconnected.

The alternating pattern set up anteriorly in the dentary by the first-generation teeth changes in juveniles (parr) into a pattern whereby teeth are in a similar functional (for the erupted teeth) or developmental stage (for the replacement teeth) every three positions. This pattern is also observed in subsequent life stages, including the marine life phase and the freshwater migratory phase (grilse and salmon). In contrast to early life stages however, where each position holds both a functional and a mineralised replacement tooth, every position now holds either a functional tooth or a mineralised replacement tooth. This is likely due to the fact that replacement tooth germs have to grow to a larger size before mineralisation starts. The dentary tooth pattern of animals that have survived spawning (kelts) is highly variable. The abundance of functional teeth in post-spawning animals nevertheless indicates that teeth are not lost over winter.

DISCUSSION & CONCLUSIONS: The mode of tooth replacement in Atlantic salmon displays several ancient characters similar to those observed in chondrichthyans. Differences between Atlantic salmon and chondrichthyans can be explained by a heterochronic shift. The possibility that the middle dental epithelium functionally substitutes for a successional lamina, and could be a source of stem cells, whose descendants subsequently contribute to the placode of the new replacement tooth, needs to be explored.

REFERENCES: [1] P.E. Witten, B.K. Hall (2003) Seasonal changes in the lower jaw skeleton in male Atlantic salmon (*Salmo salar* L.): remodelling and regression of the kype after spawning. J Anat 203:435-450. [2] P.E. Witten, A. Obach, A. Huysseune, et al. (2006) Vertebrae fusion in Atlantic salmon (*Salmo salar*): Development, aggravation and pathways of containment. Aquaculture 258:164-172. [3] P.E. Witten, B.K. Hall, A. Huysseune (2005) Are breeding teeth in Atlantic salmon a component of the drastic alterations of the oral facial skeleton? Arch Oral Biol 50:213-217. [4] A. Huysseune, P.E. Witten (2006) Patterning of development in continuously replacing osteichthyan dentitions. J Exp Zool (Mol Dev Evol) 306B (3):204-215.

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THE EXON 6ABC REGION OF AMELOGENIN MRNA CONTRIBUTE TO INCREASED LEVELS OF AMELOGENIN MRNA THROUGH AMELOGENIN PROTEIN-ENHANCED MRNA STABILIZATION

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INTRODUCTION: Amelogenin is a major component of enamel matrix proteins and have an important role on enamel formation and mineralization. We have demonstrated that the reuptake of full-length amelogenin protein results in increased levels of amelogenin mRNA through enhanced mRNA stabilization. In present study, we examined the molecular mechanism of enhanced amelogenin mRNA stabilization.

METHODS: To identify the *cis*-regulatory region within amelogenin mRNA, we tested various reporter systems using a deletion series of reporter plasmids. The details are: HAT-7 cells were transiently transfected with each reporter plasmid separately, co-transfected with the *renilla* luciferase reporter plasmid (pRL). The levels of luciferase reporter mRNAs were determined by quantitative real-time RT-PCR and normalized against the pRL level. Northwestern blotting was performed to analyze *in vitro* RNA-protein binding. To know whether or not the amelogenin protein-mediated mRNA stabilization is via the exon 6ABC, the half-life analysis under amelogenin protein presence was performed.

RESULTS: A deletion at exon 6ABC of amelogenin mRNA resulted in a 2.5-fold increase in the amelogenin mRNA expression level, compared with that of full-length mRNA, indicating that a *cis*-element exists in exon 6ABC of amelogenin mRNA. Furthermore, northwestern analysis demonstrated that amelogenin protein binds directly to its mRNA *in vitro*, suggesting that amelogenin protein acts as a *trans*-acting protein that specifically binds to this *cis*-element. Moreover, recombinant mouse amelogenin protein extended the half-life of full-length amelogenin mRNA, but did not significantly alter the half-life of exon 6ABC-deletion mutant mRNA, suggesting that the amelogenin protein enhanced mRNA stabilization via exon 6ABC region, and the exon 6ABC is important for mRNA destability and amelogenin protein-mediated stabilization of amelogenin mRNA.

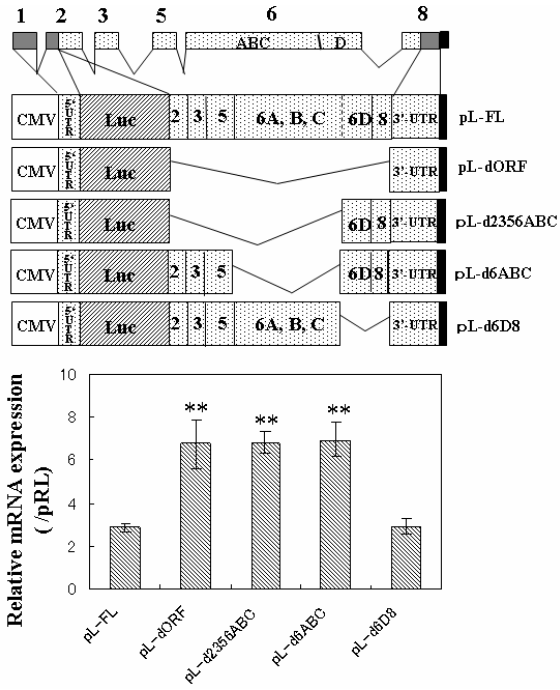

*Fig. 1: Schematic representation of reporter plasmids were shown in the upper panel and the constructure of rat amelogenin cDNA was showed in the top. Luc, partial luciferase sequence. Luciferase reporter mRNA levels in each reporter plasmid were shown in the lower panel. Error bars indicate mean± SD. **, p<0.001 versus pL-FL. (n=6)*

DISCUSSION & CONCLUSIONS: We have identified a *cis*-element, the exon 6ABC that is important for mRNA destability and amelogenin protein-mediated stabilization of amelogenin mRNA. The splice products produced by deletion of exon 6ABC are known as leucine-rich amelogenin peptides or LRAP, and have signaling effects on cells. Our findings also suggest that the regulation of full-length amelogenin protein expression differs from the regulation of LRAP expression.

REFERENCES: [1] Xu. L, Harada. H, Tamaki. TY, Matsumoto. S, Tanaka. J, and Taniguchi. A. (2006) J *Biol Chem* **281**, 2257–2262. [2] Xu. L, Harada. H, and Taniguchi. A. (2006) *J Biol Chem* **281**, 32439-32444.

GAP JUNCTIONAL COMMUNICATION REGULATES AMELOBLAST DIFFERENTIATION

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INTRODUCTION: Tooth development is regulated by their specific gene expression and has an interesting features, especially ameloblasts differentiation. Therefore, we thought that ameloblasts must be regulated by tooth specific genes. But only a few molecules have been reported their specific function in ameloblast differentiation. To identify the molecules that express stage specifically in tooth germ, we performed the digital differential display (DDD) method using pooled molar UniGene library. Using this methods, we identified Gja1 as gene specifically expressed in tooth germ. Gap junctional intercellular communication (GJIC) is important for the tissue organization, especially in epithelium and cardiac muscle. Gap junction membrane channel protein alpha 1 (Gja1) is one of the GJIC proteins and highly expressed in tooth germ as previously reported (Fukumoto E. *et al* IADR 2005). Gja1-null mice showed congenital heart disease and malformation of lung. However, the role of GJIC in tooth development has never clearly understood. Here we focused on the function of Gja1 during amelogenesis.

METHODS: Expression of Gja1 in mouse incisor was determined by immunofluorescence.To determine the in vivo function of Gja1, We analyzed the Gja1 null-mice histologically. To analyze the regulatory mechanism of ameloblastin expression by Gja1, we used gap junction inhibitor, oleamide.

RESULTS: The expression of Gja1 in mouse incisor was stage specific in amelogenesis, and in odontoblasts. Expression of Gja1 started at presecretory stage of ameloblasts and strongly in secretory stage. This expression was diminished in maturation stage. Further, Gja1 null-mice showed the inhibition of the morphogenesis of tooth germ, salivary gland, and lung, similar to occulodentodigital dysplasia (ODDD) . In mutant mice, expression of ameloblastin in ameloblasts was dramatically reduced comparing with wild type and heterozygote. Mutant ameloblasts lose cell polarity as similar to ameloblastin knockout mice[1)] (Fig.1). TGF-b1 induced ameloblastin expression transiently in dental epithelial cells. Ameloblastin expression induced by TGF-b1 was inhibited in presence of oleamide. Simillar results were observed in over-expression of mutant Gja1, R76S and R202H, which were gene mutations observed in ODDD patients. Cells over-expressing R76S and R202H Gja1 showed enhanced proliferation and down regulation of ameloblastin expression. On the other hand, over-expression of wild type Gja1 dramatically inhibited proliferation of dental epithelial cells.

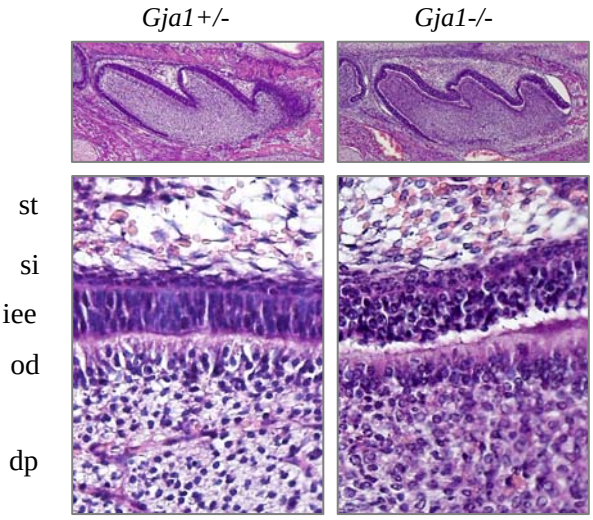

Fig. 1: Ameloblast polarization was disturbed in Gja1 null mice:heterozygote(left),Gja1-null mice(right). si:stratum intermedium, iee:inner enamel epithelium

CONCLUSIONS: Gja1 may regulate proliferation and ameloblastin expression in dental epithelium and associated with ODDD phenotype in tooth, especially anodontia, microdontia and amelogenesis imperfecta.

REFERENCES: [1] S.Fukumoto, et al (2004) *J Cell Biol* **167(5)**:973-983.

ROLE OF P38 KINASE AND JNK IN TOOTH DEVELOPMENT

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INTRODUCTION: Patients who manifest Hypohidrotic ectodermal dysplasia (HED) show ectodermal abnormalities, including sweat glands defects, impaired hair formation, anodontia/ologodontia, and amelogenesis imperfecta. Evidence is accumulating that Ectodysplasin (Eda), Edar, the receptor for Eda, and EDARADD, an adaptor protein of Edar, are responsible for HED. Mice possessing spontaneous mutation in Eda, Edar, or EDARADD gene, known as *tabby*, *downless*, or *crinkled* respectively show similar phenotype to HED. It is also shown that EDARADD binds to TRAF6 relays Eda/Edar signaling into the cytoplasm and that mice deficient in TRAF6 gene showed more severe ectodermal abnormalities than *tabby*, *downless*, or *crinkled* mice, suggesting that there is another pathways that plays an important role in tooth development via TRAF6. TRAF6 is known to transduce signals to NF-κB, p38 kinase, and JNK. Although importance of NF-κB in tooth development has been already reported, the role of p38 and JNK pathway in tooth development is unknown. Thus we investigated the role of p38 kinase and JNK pathways in tooth development.

RESULTS: To examine the role of Eda/Edar in tooth development, we generated soluble Edar (sEdar), which consists of only an extracellular domain of Edar, and thus is expected to function as a decoy receptor for Eda, and examined the effect of sEdar on the development of the mouse tooth germs in organ culture. sEdar clearly impaired the growth of tooth germs. Furthermore, sEdar markedly inhibited the expression of amlogenin and ameloblastin in tooth germs. These results indicate that Eda/Edar signaling has important roles in ameloblast differentiation.

We next examined whether p38 pathway and/or JNK pathway are involved in tooth development. To address this, we determined the effect of p38 inhibitor (SB203580) and JNK inhibitor (SP600125) on development of tooth germ. Both agents dramatically inhibited tooth germ development and reduced the expression of ameloblastin mRNA. Of note addition of SP600125 showed reduction in cusp formation.

DISCUSSION & CONCLUSIONS: Collectively these data suggest that p38 and JNK may play a role in tooth development..

REFERENCES: [1] M.L.Mikkola, I. Thesleff (2003); *Cytokine Growth Factor Rev* **14**; 211-224

[2]A.Naito, H. Yoshida, E. Nishioka, M. Satoh, S. Azuma, T. Yamamoto, S. Nishikawa, J. Inoue (2002) *Proc Natl Acad Sci U S A* **99**; 8766-8771

CLINICAL HETEROGENEITY OF THE ORAL MANIFESTATIONS IN NEPHROCALCINOSIS PATIENTS

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INTRODUCTION: Nephrocalcinosis (NC) is a rare condition characterized by calcium deposition in the renal parenchyma[1]. Several inherited conditions that affect the pH and/or mineral balance may be involved in the pathogenesis of NC. Studies have also reported a rare syndrome associating NC and Amelogenesis Imperfecta (AI) (OMIM 204690). The aim of the present study was to describe the oral manifestations of six patients with different renal pathologies and NC.

METHODS: Informed consent approval was obtained from all patients. Patients treated in the Pediatric Nephrology Service of two general hospitals were submitted to biochemical tests and ultrasonography. When NC was diagnosed, they were referred to the Dental Anomalies Clinic at the University Hospital of Brasilia for oral examination. All patients were submitted to complete dental and radiographic examination and intra and extra-oral photography.

RESULTS: Six NC patients were referred to be examined (4 males and 2 females). Patients` age ranged between 2 and 16 years old. The associated diseases included renal congenital malformation, Henle's limb tubulopathy, distal renal tubular acidosis (dRTA) and NC/AI syndrome. The 16yr male diagnosed with NC/AI syndrome, as previously reported[2], presented hypoplastic enamel with yellow discoloration of all teeth, intrapulpal calcifications, tooth eruption delay, dental follicular hamartoma and enlargement of the gingival tissue. The other patients presented only dental enamel defects which included diffuse and demarcated opacities, hypoplasias in the form of grooves and pits and teeth discoloration. Associations of defects were observed in different degrees of severity. In these patients, there were no alterations in tooth eruption (fig1).

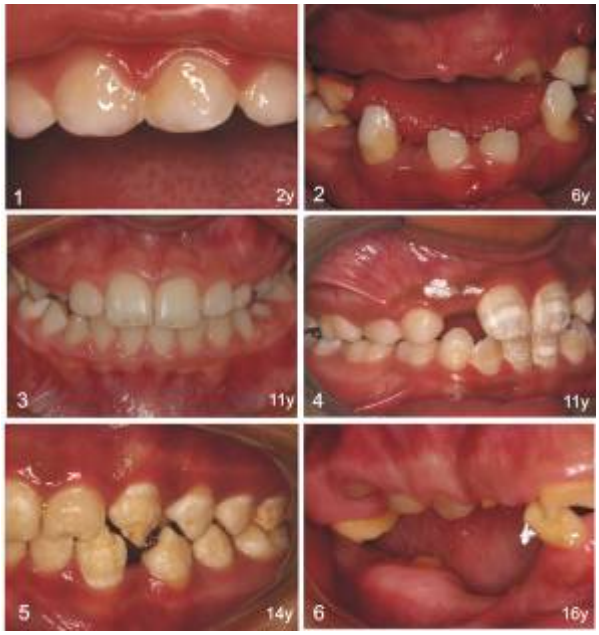

Fig. 1: Different dental phenotypes observed in NC patients induced by different renal pathologies. 1 and 2: renal congenital malformation;3: and 5: dRTA; 4: Henle´s limb tubulopathy; and 6: NC/AI syndrome.

CONCLUSIONS: Enamel defects were observed in all NC patients examined. However, a clinical heterogeneity was visualized between patients. Patients with NC due to different renal pathologies presented dental enamel defects in varied degrees of severity and the clinical features were different from those found in the NC/AI syndrome patient.

REFERENCES: [1]Sayer JA, Carr G, Simmons NL. *Nephrocalcinosis: molecular insights into calcium precipitation within the kidney*. Clin. Sci. 2004 Jun;106(6):549-61. [2]Paula LM; Melo NS; Silva Guerra, EN; Mestrinho DH; Acevedo AC (2004). *Case Report of a Rare Syndrome Associating Amelogenesis Imperfecta and Nephrocalcinosis in a Consanguineous Family*. Arch Oral Biol 50, 237-242.

WNT10A REGULATES DENTIN SIALOPHOSPHOPROTEIN MRNA EXPRESSION AND POSSIBLY LINKS ODONTOBLAST DIFFERENTIATION AND TOOTH MORPHOGENESIS

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Abstract: We have explored the role of Wnt signaling in dentinogenesis of mouse molar teeth. We found that Wnt10a was specifically associated with the differentiation of odontoblasts and that it showed striking colocalization with dentin sialophosphoprotein (Dspp) expression in secretory odontoblasts. Dspp is a tooth specific non-collagenous matrix protein and regulates dentin mineralization. Transient overexpression of Wnt10 in C3H10T1/2, a pluripotent fibroblast cell line induced Dspp mRNA. Interestingly, this induction occurred only when transfected cells were cultured on Matrigel basement membrane extracts. These findings indicated that Wnt10a is an upstream regulatory molecule for Dspp expression, and that cell–matrix interaction is essential for induction of Dspp expression. Furthermore, Wnt10a was specifically expressed in the epithelial signaling centers regulating tooth development, the primary and secondary enamel knots. The spatial and temporal distribution of Wnt10a mRNA demonstrated that the expression shifts from the secondary enamel knots, to the underlying preodontoblasts in the tips of future cusps. The expression patterns and overexpression studies together indicate that Wnt10a is a key molecule for dentinogenesis and that it is associated with the cell–matrix interactions regulating odontoblast differentiation. We conclude that Wnt10a may link the differentiation of odontoblasts and cusp morphogenesis.

Fig. 1: Odontoblasts (od) are columnar cells lining the pulpal surface of dentin (de), and preodontoblasts (pod) are the odontoblast precursors in the apical end of the root. At the apical end of the growing root, Wnt10a *transcripts were present in the preodontoblasts (pod). (**D**) The expression was continuous and maintained in differentiating and secretory odontoblasts (od).*

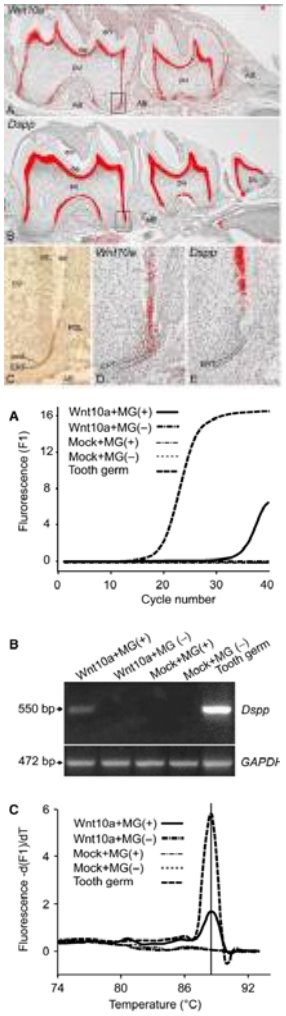

***Fig. 2** (**A**) C310T1/2 cells were transiently transfected with pCMV-Wnt10a (Wnt10a) or its vehicle pCMV (Mock). Amplification of Dspp was observed in C310T1/2 cells transfected with pCMV-Wnt10a and cultured on Matrigel (Wnt10a+Mg (+)), and positive control (tooth germ). (**B**) Forced-expression of Wnt10a induced Dspp expression when the transfected cells were cultured on Matrigel dishes (Wnt10a+Mg (+), 40 cycles). Wnt10a transfected cells cultured on normal culture dishes (Wnt10a+Mg (−), 40 cycles) and Mock transfected cells cultured on Matrigel dishes (Mock+Mg (+) or normal culture dishes (Mock+Mg (−)) did not show Dspp expression. Positive control (Tooth germ) showed intense Dspp expression. (**C**) PCR products were subjected to melting peak analyses to determine the specificity of the products.*

ACKNOWLEDGEMENTS: Parts of the data has been published in Differentiation journal (2006).

FGF-9 PLAY A ROLE FOR THE MAINTENANCE OF STEM CELL NICHE VIA FGF-10 EXPRESSION IN THE MOUSE INCISORS

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INTRODUCTION: Mouse incisors are regenerative tissues, which grow continuously throughout life. In the teeth, Fgf-10 being continuously expressed in the apical end of mouse incisors, plays a role of maintenance of dental epithelial stem cell compartment as referred to apical bud [1, 2]. However, little is known about the epithelial signaling inducing/maintaining Fgf-10 expression in the apical end mesenchyme of incisors. We investigated the expression of growth factors thoroughly in the apical bud, and found that the candidate is Fgf9.

METHODS: Mandibular incisors of neonatal mice were used. For RT-PCR analysis, we extracted total RNA from apical bud, after cDNA synthesis, PCR was performed using the sets of primers. For immunohistochemical detection, rabbit anti-mouse Fgf-9 antibody (0.5 μg/ml, CHEMICON, USA) was used. For cell proliferation assay, primary dental mesenchymal cells were dissected from incisor germs. $0.5x10^4$ cells were inoculated onto 96 well plates containing 100 μl medium (MEM, 10%FBS) and recombinant Fgf-9 protein (CHEMICON, USA) at various concentration, and counted cell number time-dependently. For investigate the effect of Fgf-9 to mesenchyme, dental mesenchymal tissues were separated form the epithelium were cultured with Fgf-9 releasing beads. After 24-36 hr culture in vitro, tissues proceed for whole-mount in situ hybridization analysis and Annexin V staining (1μg/ml, Molecular Probes, USA).

RESULTS: Immunostaining showed that Fgf-9 was expressed in the basal epithelium (BA), stellate reticulum (SR) and inner enamel epithelium (IEE) in the apical bud (Fig.1), and the expression area underlay the mesenchyme expressing Fgf-10. Next, to study the effect on proliferation in primary cultured mesenchymal cells of apical end, cell proliferation assay was carried out. Recombinant Fgf-9 protein stimulated the increase of number of mesenchymal cells in a concentration-dependent manner. Annexin V staining and whole mount in situ hybridyzation using organ culture showed that recombinant Fgf-9 protein inhibited the apoptosis of mesenchymal cells of apical end and furthermore maintained the expression of Fgf-10 (Fig.2).

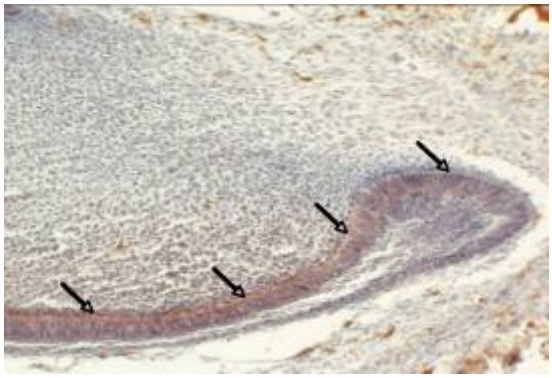

Fig. 1: Localization of Fgf-9 in the apical bud. Fgf-9 is recognized at BA, IEE and SR in the apical bud, but it is absent from OEE and ameloblasts.

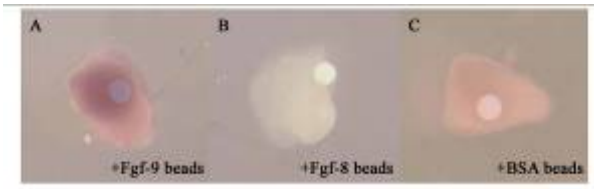

Fig. 2: Expression of Fgf-10 in organ cultural dental mesenchyme. Fgf-10 expression is seen around the Fgf-9 bead. No expression could be seen around Fgf-8 and BSA bead.

DISCUSSION & CONCLUSIONS: The results suggest that Fgf-9 plays a role of maintenance of mesenchymal cells expressing *Fgf-10* in the mice incisors. and that the stem cell niche of incisors are formed by the epithelial - mesenchymal interaction via the signaling of Fgf-9 and Fgf-10.

REFERENCES: [1]H. Harada, et al (1999) *J. Cell Biol.* **147**, 105-120 [2]H.Harada, et al (2002) *Development* **129**, 1533-1541

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ASSOCIATION OF TIMP-2 WITH EXTRACELLULAR MATRIX EXPOSED TO MECHANICAL STRESS AND ITS CO-DISTRIBUTION WITH PERIOSTIN DURING MOUSE TOOTH DEVELOPMENT

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INTRODUCTION: Matrix remodeling is regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). Of the 4 known TIMPs, TIMP-2 has been thought to be soluble and does not bind to ECM. However, our previous data indicated that TIMP-2 bound to ECM in a peculiar distribution pattern during mouse tooth development [1, 2]. Periostin, originally identified from a mouse osteoblastic library, plays a role in cell adhesion and migration, and in mechanical stress-induced matrix remodeling. Although localization of periostin has not been fully analyzed during molar and incisor tooth morphogenesis, the reported localization of periostin is intriguingly similar to that of TIMP-2 [3]. In order to test the hypothesis that TIMP-2 would associate with ECM of specific areas and co-distribute with periostin, we analyzed tissue distribution patterns of TIMP-2 and periostin in mouse mandible development using immunohistochemistry. Furthermore, dissected mandible was cultured in the presence of anti-TIMP-2 antibody, and localization of periostin was evaluated.

METHODS: Immunohistochemical stainings for TIMP-2 and periostin were carried out on serial cryosections obtained from mice at embryonic day (E)13-E16, postnatal day (P)2, P35, and 12 weeks. Dissected gingival tissue of P2 was cultured in serum-free DMEM for 4 days. For antibody inoculation, anti-TIMP-2 antibody was added to the culture medium, and antibodies against TIMP-1 and TIMP-3 were used as controls at the same concentration.

RESULTS & DISCUSSION: TIMP-2 and periostin exhibited strikingly similar protein distribution, and their patterns were asymmetrically and spatiotemporally regulated during tooth development. From bud to early bell stages of molars, TIMP-2 and periostin were highly expressed on the lingual and anterior sides of the basement membrane, and on the adjacent jaw mesenchyme (Fig.1). In pre- and postnatal incisors, basement membrane of the apical loop and dental follicle were stained with TIMP-2 and periostin. At postnatal stages, TIMP-2 and periostin were prominently confined to the extracellular matrix (ECM) of gingival tissues, periodontal ligaments, and tendons, which are all receiving mechanical strain. However, periostin was solely detected in the lower portion of the inner root sheath of hair follicles. Gingiva of P2 cultured in anti-TIMP-2 antibody-conditioned medium showed markedly reduced staining of periostin, however, neutralization of TIMP-1 or TIMP-3 did not alter the localization of periostin. We suggest that TIMP-2 and periostin are co-distributed on the ECM exposed to mechanical forces, and coordinately function as ECM modulators.

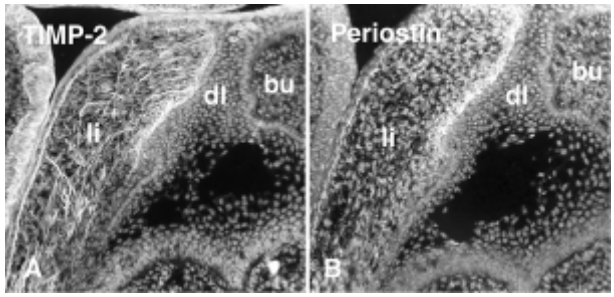

Fig. 1:Immunohistochemical detection of TIMP-2 (A) and periostin (B) at E16. On serial frontal sections both proteins are detectable along the dental lamina (dl) and in the jaw mesenchyme on the lingual side (li). In contrast, the buccal side (bu) is devoid of TIMP-2 and periostin.

REFERENCES: [1] N. Yoshiba, K. Yoshiba, C. Stoetzel et al. (2003) *Dev Dyn* 228:105-112. [2] N. Yoshiba, K. Yoshiba, C. Stoetzel et al. (2006) *Cell Tissue Res* 324:97-104. [3] A. Kruzynska-Frejtag, J. Wang, M. Maeda et al. (2004) Dev Dyn 229:857-868.

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NEUROTROPHIC FACTOR NT-4 REGULATES AMELOBLASTIN EXPRESSION

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INTRODUCTION: Tooth development is regulated by epithelial-mesenchymal interaction, and various growth factors mediate this event. Neurotrophic factors (NTs) are expressed in neuronal cells and important for neurite extension, synapus formation and their survival. These factors are also found in tooth germ. Neurotrophin-4 (NT-4) is one of the NTs, and expressed in dental epithelium, but not in mesenchyme. NT-4 binds to their receptor TrkB expressing in presecretory ameloblasts. However, the role of NT-4 in tooth development has been clearly understood. Here, we focused on the role of NT-4 in ameloblast differentiation, which is detected at high level in ameloblasts at early stage of amelogenesis.

METHODS: To determine the effect of growth factors including NTs on cell proliferation, BrdU incorporation method was performed. To analyze the expression of enamel matrix in dental epithelial cell line (HAT-7), we performed RT-PCR after stimulated with or without NT-4. ERK1/2 phosphorylation by exogenous administration of NT-4 was analyzed by western immunoblotting. To analyze the function of NT-4 signaling in amelogenesis, TrkB-FL and TrkB-T1 expression vectors were constructed. Those expression vectors were transfected into HAT-7 cells using lipofectamine 2000 to create stable transfectant cells. Further, to examine the in vivo function of NT-4, NT-4 disrupted mice were used for histological analysis of tooth development.

RESULTS: First, we tried to determine the effect of growth factors including NTs on cell proliferation using BrdU incorporation method. BrdU positive cells were decreased after treatment with NT-4 or TGF-b1. To determine the effect of dental epithelial differentiation, we performed RT-PCR to analyze the expression of ameloblast markers. NT-4 dramatically induced the Ameloblastin expression in dental epithelial cell line (HAT-7). In order to assess the biological function of full-length isoform of TrkB (TrkB-FL) and truncated form TrkB (TrkB-T1), we constructed V5-His-tagged both receptors, and then obtained stable transfectants using these vectors. Ameloblastin expression was enhanced in stable transfectant TrkB-FL, but not in TrkB-T1. ERK1/2 is one of the down stream of TrkB signaling. TrkB-FL transfectants enhanced the phosphorylation of ERK1/2 after stimulation of NT-4. On the other hand, TrkB-T1 transfectants decreased the phosphorylaton of ERK1/2. Further, Trk signaling inhibitor, K-252a, inhibited Ameloblastin expression induced by NT-4. These results indicated that NT-4 regulates the Ameloblastin expression via TrkB-MAPK pathway. To analyze the in vivo function of NT-4 in dental epithelium differentiation, we used NT-4 null mutant mice. Ameloblastin expression was transiently decreased in early secretary stage of ameloblast of NT-4 null mutants. These results suggest that expression of Ameloblastin is regulated by NT-4 in vitro and in vivo.

DISCUSSION & CONCLUSIONS: Our results demonstrated that NT-4 regulates dental epithelial cell differentiation and enamel matrix gene expression via TrkB-FL, but not truncated TrkB forms. NT-4 inhibited cell proliferation and also induced enamel matrix genes such as Ambn in dental epithelial cells. NT-4 deficienct teeth resulted in a thin enamel layer during the initial stage of amelogenesis. Our findings are the first time to demonstrate that a neurotrophic factor plays an important role in tooth development.

SEMI-QUANTITATIVE RT-PCR ANALYSIS OF LIM MINERALIZATION PROTEIN 1 AND ITS ASSOCIATED MOLECULES IN CULTURED HUMAN DENTAL PULP CELLS

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INTRODUCTION: LIM mineralization protein 1 (LMP-1), an intracellular signaling molecule, regulates osteoblast differentiation and maturation, as well as bone formation. However, the role of LMP-1 in the differentiation of human dental pulp cells and formation of dentin has not been determined. The study was to investigate the expression of LMP-1, the related proteins, such as bone morphogenetic proteins 2, 6, and 7 (BMP-2, BMP-6, and BMP-7), and core binding factor alpha 1 (Cbfa1) during the differentiation of cultured human dental pulp cells and the formation of mineralized nodules.

MATERIALS & METHODS: Dental pulp cells were cultured by tissue explant. Differentiation of human dental pulp cells was induced by dexamethasone, asorbic acid and β-glycerophosphate. The formation of mineralized nodules, was determined by Von Kossa staining and immunocytochemistry detection of dentin sialoprotein. Expression of LMP-1, the related proteins, and the differentiation marker alkaline phosphatase (ALP) was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR).

RESULTS: After 14 days' induction by differentiation medium, ALP expression was enhanced, mineralized nodules was formed, and DSP was expressed. The above evidence suggests that dental pulp cells are characteristic of odontoblasts upon differentiation induction. The expression of LMP-1, BMP-2, BMP-6, BMP-7 and Cbfa1 was significantly increased in the process of dental pulp cells differentiation and the formation of mineralized nodules, while the pattern of the expression was distinct. We found a 1.8-fold increase in LMP-1 expression on day 2 after induction and a maximum three-fold increase on day 9. LMP-1 expression then started to decline and returned to baseline on day 28. BMP-2 expression started to increase on day 3, reaching the peak on day 11, 2 days later than LMP-1. Similarly, BMP-7 expression increased on day 3 and reached the peak on day 11 with almost a two-fold increase. However, the expression of BMP-6, which was different from BMP-2 and BMP-7, increased on day 5 and reached the peak on day 21, 2 weeks later than LMP-1. In addition, the expression of Cbfa1, was increased on day 2 and peaked on day 11 with a significant increase of about three-fold. The correlation analysis revealed significant correlations between expression of BMP-2 and BMP-7, BMP-2 and Cbfa1, and the expression of BMP-7 and Cbfa1, whereas the correlation of other molecules expression was not found.

DISCUSSION & CONCLUSIONS: We found that, for the first time, LMP-1 expression was increased during the differentiation of human dental pulp cells. Furthermore, expression of LMP-1, BMP-2, BMP-6, BMP-7, and Cbfa1 was elevated in different stages of differentiation. Our data suggest that LMP-1, together with BMPs and Cbfa1, might play an important role in the differentiation of human dental pulp cells and the formation of mineralized nodules. The peak of LMP-1 expression in induced dental pulp cells appeared 5 days earlier than ALP, suggesting that LMP-1 might play a role in the early stage of the differentiation of human dental pulp cells. Further direct evidence was needed to explore the role of LMP-1 and the mechanism of LMP-1 signaling pathway.

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AN EXPRESSION SURVEY OF GENES CRITICAL FOR TOOTH DEVELOPMENT IN HUMAN EMBRYONIC TOOTH GERM

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INTRODUCTION: The recent development of new molecular technology allows fast proliferation of studies on molecular mechanisms underlying tooth morphogenesis in mice. The tooth phenotype seen in mouse mutants is often found in humans carrying mutations in the counterpart gene. It indicates that human and mouse tooth development does not only share many similarities in morphological processes but may also utilize similar molecular mechanisms. Understanding the molecular mechanisms underlying the human odontogenesis is a prerequisite to the realization of human tooth regeneration in the future. Here we report the expression of several regulatory genes in the human embryonic tooth germ and a comparison of the expression patterns between the mouse and human.

METHODS: Human embryos of eighth to fourteenth week gestation were collected and fixed in 4% PFA at 4ºC overnight and then processed for paraffin sectioning at 10 m. To obtain human tooth germ at late differentiation stage, premolar tooth germs were dissected out from 14-week old human embryos and grafted under the kidney capsule of adult male nude mice. All the probes except *BMP4* that were used for examining human gene expression in this study were amplified from exon of each gene using human genomic DNA. Section in situ hybridization was performed as described previously [1Zhang et al., 1999].

RESULTS: In the developing human tooth germ, *BMP4* expression was detected in the dental pappila mesenchyme as well as the dental epithelium at the cap stage in both incisor and premolar. The expression in the inner enamel epithelium became significant in both incisor and premolar at the bell stage, while the expression in the dental pappila was maintained at a relatively lower level. *BMP4* expression was also detected in the odontoblasts and ameloblasts of the grafted teeth. *MSX1* expression was restrictedly detected in the dental pappila mesenchyme of tooth germ at the cap stages of both incisor and premolar. *MSX1* transcripts were also detected in the odontoblasts and ameloblasts of the tooth graft after long term culture. *PITX2* expression was detected only in the dental epithelium of tooth germs at the late bud stage, the cap stage and the bell stage. The expression of *PITX2* was found in ameloblasts of the graft. *FGF8* transcripts were strongly detected in the dental epithelium but also slightly in the dental mesenchyme. In addition, *FGF8* expression in the dental epithelium appears to be restricted to the central portion where the enamel knot will form. *PAX9* was found to be expressed in both dental mesenchyme and dental epithelium of incisor and premolar at the cap stage. *SHOX2* expression was indeed mainly localized in the dental epithelium.

DISCUSSION & CONCLUSIONS: Our results show that these genes exhibit basically similar expression patterns in the human tooth germ as compared to that in the mouse. However, slightly different expression patterns were also observed for some of the genes. Our results indicate that the human and mouse teeth do not only share considerable homology in odontogenesis[2] but also utilize similar underlying molecular networks.

REFERENCES: [1] Y.D. Zhang, X. Zhao, Y.P. Hu, St. Amand, et al (1999). *Msx1* is required for the induction of *Patched* by *Sonic hedgehog* in the mammalian tooth germ. *Dev. Dyn.* **215**, 45-53. [2] Y.D. Zhang, Z. Chen,, Y. Song, et al (2005). Making a tooth: growth factors, transcription factors, and stem cells. *Cell Res*. 15, 301-316.

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