

ABSTRACTS: ORAL PRESENTATIONS

General Tooth Development

O1

Early development of the lower incisor in mice

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Background: The multiple developmental origin of the upper incisor has been documented on serial histological sections and three-dimensional (3D) reconstructions during the period preceding a well-formed incisor bud (Peterkova et al., 1993). Using similar methods, the morphogenesis of the lower incisor from the bud stage to early bell stage has been systematically documented (Kieffer et al., 1999). However, there is still a lack of detailed information about the initial steps of lower incisor development, before this tooth enters the bud stage at embryonic day (ED) 13.5.

Aim of the study: Using serial histological sections and 3D reconstructions, we performed a detailed investigation of the initial development of the mouse lower incisor from the early epithelial thickening to the formation of the dental bud, and searched for

its developmental origin in wild type mice.

Methods: Contours of the dental and adjacent oral epithelium were drawn from the serial histological sections and superimposed with respect to the middle line and to the horizontal level for correct spatial positioning of the reconstructed structures. The drawings were digitalized and 3D reconstructions were performed to visualize the dental and adjacent vestibular epithelia. 3D analysis was undertaken in the lower jaw quadrants at ED11.5-13.5.

Results: At ED 11.75, an epithelial thickening was formed in the prospective incisor area. At ED 12.0, it was possible to observe formation of several epithelial buddings in the lower incisor region. At ED 12.5, the antero-laterally located budding corresponded to the anlage of the lower oral vestibule. Compared to the upper oral vestibule area, where only indistinct thickenings were apparent at ED 13.5, the epithelium of the oral vestibule in the mandible formed a well-marked ridge. More posteriorly, the incisor bud formed at ED 12.5 and became clearly distinct at ED 13.5. In contrast to the upper jaw, where the initial epithelial budding could be still detected on the mesenchymal surface of the incisor bud, the remains of the initial epithelial budding in the incisor region of mouse mandible were not detectable on the mesenchymal surface of the incisor bud. An epithelial connection between the incisor bud and the externally located ridge of the vestibular epithelium was apparent.

Conclusion: The detailed temporo-spatial description of normal mouse incisor development and the differences between incisors in the upper and lower jaws can inform our understanding of abnormal incisor development in various genetic disorders and provides a basis for the

investigation of molecular signalling pathways.

References: Kieffer S, Peterkova R, Vonesch JL, Ruch JV, Peterka M, Lesot H. (1999) Morphogenesis of the lower incisor in the mouse from the bud to early bell stage. *Int J Dev Biol* 43: 531-539. Peterkova R, Peterka M, Vonesch JL, Ruch JV. (1993) Multiple developmental origin of the upper incisor in mouse: histological and computer assisted 3-D-reconstruction studies. *Int J Dev Biol* 37: 581-588. **Acknowledgement:** This work was supported by the Grant Agency of the Czech Republic (grants 304/09/1579 and 304/07/0223).

O2

On the maintenance of the complex cusp pattern in continuously growing molars

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Background: All rodents have continuously growing incisors and the developmental aspects of this regenerating system have been reasonably well studied. Some rodent species such as the guinea pig and the sibling vole also have continuously growing molars. From one developmental perspective these open-rooted molars are vastly more interesting than the incisors: they have a complex cusp pattern. This pattern needs to be maintained over time because it is worn down constantly. We examined in this study how this pattern is maintained.

Methods: We used basic immunohistochemistry and radioactive in situ hybridization techniques to analyze the patterns of proliferation and the distribution of regulatory molecules, and we added an extra level of information by generating 3D reconstructions of whole teeth showing the entire pattern. For this analysis tissues were used of the mouse, guinea pig and sibling vole.

Results: We show that the most apical part of the tooth, where normally root formation occurs, remains proliferation free in continuously growing molars. This is unlike the situation in non-regenerating molar of the mouse, which does grow roots. Here the most apical zone is originally also proliferation free, but the proliferation zone shifts towards the apical mesenchyme at the moment when root formation is initiated. In the continuously growing molars the mesenchymal proliferation never shifts, keeping the roots dormant. We also show that different zones are associated with different regulatory molecules.

Conclusion: We speculate that both processes,

the maintenance of the complex cusp pattern and the postponement of root formation, are regulatory connected, and are possibly regulated by WNT signaling, through the spatial regulation of the proliferation in the mesenchymal component of the tooth.

O3

Evaluation of apoptosis and proliferation in the area of rudimentary premolar tooth primordia in the mouse embryonic mandible

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Background: Mouse dentition consists of one incisor and three molars separated by a toothless gap - diastema, at the place of missing canine and premolars. In mouse embryos, two rudimentary tooth primordia sequentially develop in the premolar region of the diastema at embryonic day 12.5 and 13.5, respectively. They later regress or become incorporated into the first molar. However, just in the presumed premolar position in the diastema, some genetically modified mice show a supernumerary tooth (ST), which is variable in its size and shape. The ST has been proposed to result from a revitalization of a rudimentary premolar anlage in mutant mice. However, it is not known whether only one or both rudimentary tooth primordia can play a role in the ST origin.

Aim: We aimed to characterize the proliferation and apoptosis during development of the two rudimentary tooth primordia (called MS and R2) in the embryonic mandible of WT mice and of the mutant mice that will go on to form a ST.

Methods: We studied the dental epithelium in the cheek region of the mandible in wild type (WT) and mutant (Spry2^{-/-}) mice at embryonic day (ED) 12.5 and 13.5. On series of frontal 7µm thick histological sections, we measured the area of the dental epithelium, and counted the

number of apoptotic elements and proliferating cells in the region of the rudimentary premolar primordia. The data were compared between WT and mutant embryos, and with the published data on the size, proliferation and apoptosis of the dental epithelium in the R2 bud and molar region at ED13.5 (Peterkova et al, 2009).

Results: First, we demarcated the region of the anterior rudimentary tooth primordium (MS) at ED 12.5. We performed shape and size analysis of the dental epithelium on histological sections. WT and mutant mice showed differences in the shape of dental epithelium on serial histological sections at ED 12.5. But it always remained possible to recognize the morphology characterizing the posterior end of the MS (peMS). We also analyzed the antero-posterior course of the size of the dental epithelium on graphical representations. The curves showed an ascent, peak and descent in the posterior direction. Although the antero-posterior position of the peMS was similar in all mice, the peMS was located at the peak of the *Spry2*^{-/-}-curves, but on the ascendant part of WT curves. The evaluation of the number of mitotic cells and apoptotic elements was made in the MS - on 5 sections located anteriorly to the peMS. The second evaluated region (corresponding to the R2) also comprised 5 sections, and was separated by a gap of 5 sections from the peMS. The number of apoptotic elements in MS and R2 was lower in *Spry2*^{-/-} than in WT embryos. Furthermore, the mitotic index was higher in both rudimentary tooth primordia in the *Spry2*^{-/-} mice than in WT at ED12.5. At ED13.5, the mitotic index in the mutant MS decreased and was similar to the MS and R2 of WT mandibles. However, the mitotic index in the mutant R2 stayed increased, being at a level similar to the molar region.

Conclusion: The increase of mitotic index and decrease of apoptosis in the mutant rudimentary tooth primordia suggested a sequential revitalization of the MS at ED12.5 and R2 at ED 13.5.

References: Peterkova et al. (2009). J Exp Zool B Mol Dev Evol 12B: 292-308.

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O4

Primary cilia in tooth development

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Primary cilia are surface organelles found on most cells in vertebrates that play critical roles in many aspects of biology, including development, and have recently been shown to be implicated in Shh signalling pathway that is also involved in tooth development. The aim of this study is to investigate the role of primary ciliary proteins in tooth development. Mice with mesenchymal conditional mutation of *Polaris* (*Polaris*^{fllox/fllox}/*Wnt1-Cre*) and *Kif3a* (*Kif3a*^{fllox/fllox}/*Wnt1-Cre*) and *Odf1* null mutant mice were used in this study. *Polaris*^{fllox/fllox}/*Wnt1-Cre* and *Kif3a*^{fllox/fllox}/*Wnt1-Cre* mice lacked incisors as a result of down regulation of Shh signalling. *Odf1* null mutants showed extra incisors. In the diastema and lingual region to the first molar, extra teeth were found in all three mutants, resulting both from ectopic Shh signalling. The action of primary cilia on Shh signalling is thus negative or positive, depending on the tissue context. In addition, different cilia proteins exert different functions on the mechanisms of cilia-directed regulation of Shh activity.

O5

Differential expression and functional significance of glucose transporters during murine tooth development

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Background: Glucose is an essential source of energy for mammalian cells and plays an important role for body metabolism, in which the transport of glucose across the cell membrane is accelerated by glucose transporters (GLUTs). To date, thirteen GLUTs have been identified and their tissue-specific expression patterns are suggestive to reflect the various

glucose requirements of different tissues. Class I transporter comprises the well-characterized GLUT1-4, and GLUT1 is known to be the major glucose transporter expressed in many tissues and be responsible for fundamental glucose uptakes. GLUT2 is a low affinity isoform with glucose, and GLUT3 and GLUT4 are high affinity isoforms, which are mainly expressed in brain. However, there has been no available data on the expression and the role of GLUTs during tooth development. This study aims to clarify the functional significance of GLUTs during murine tooth development using immunohistochemistry and organ culture experiment. **Methods:** ICR mice from embryonic day 13 (E13) to postnatal day 10 (P10) were used in this study. All mice were administered with an intraperitoneal injection of BrdU 2 hrs before the fixation and perfused with 4% paraformaldehyde. Following decalcification in 10% EDTA when necessary, the heads were embedded in paraffin and the frontal or sagittal sections of teeth were cut at 4 μ m. Immunohistochemistry was performed using antibodies against GLUT1-4, Ki67 and BrdU. For organ culture experiments, the mandibular molar germs were isolated from E13, E14 and E16 mice, and were cultured for 3, 7, 10 days with the inhibition of GLUTs by phloretin, a glucose transport inhibitor. **Results:** An intense GLUT1-immunoreaction was localized in the enamel organ of bud-stage tooth germ (E13) of mandibular first molars as well as the oral epithelium, where the active cell proliferation occurred. By the cap stage (E14), the expression of GLUT1 in the dental epithelial cells was dramatically decreased in intensity, and subsequently began to appear in the stratum intermedium at the bell stage (E19). On the contrary, GLUT2-immunoreactivity was observed in the stellate reticulum and dental papilla throughout all stages. Secretory ameloblasts and odontoblasts also showed immunopositive reaction for GLUT2. The expression of GLUT1 and GLUT2 was not overlapped in the dental epithelial and mesenchymal cells during tooth development. With regard to GLUT3 and GLUT4, their immunoreactions were not observed in the tooth germs. In vitro organ culture experiments, the inhibition of GLUT1/2 by phloretin in the bud-stage tooth germs (E13) induced the developmental arrest of explants at the bud stage and the squamous metaplasia of dental epithelial cells lacking the enamel organ. On the other hand, the development of tooth germs of E14 and E16 was not inhibited by phloretin treatment.

Three days after phloretin treatment of E13 tooth germs, cell proliferation was inhibited in the dental epithelial cells and the apoptotic features were observed in the primary enamel knot areas. These results suggest that the disturbance of primary enamel knot formation by lack of glucose uptake into the enamel organ cells is attributed to arrested tooth formation. **Conclusion:** The expression of GLUT1 and GLUT2 in the dental epithelial and mesenchymal cells seems to be precisely controlled spatiotemporally, and the glucose uptake mediated by GLUT1/2 plays a crucial role in the early tooth morphogenesis.

O6

The cadherin-catenin complex during zebrafish tooth development

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The development of teeth is the result of interactions between competent mesenchyme and epithelium, both of which undergo extensive morphogenesis. The importance of cell adhesion molecules in morphogenesis has long been acknowledged but remarkably few studies have focused on the distribution and function of these molecules during tooth development. We analyzed the expression pattern of an important epithelial cadherin, E-cadherin, during the formation of first-generation teeth as well as replacement teeth in the zebrafish, using *in situ* hybridization and whole mount immunostaining to reveal mRNA expression and protein distribution. The uninterrupted expression of E-cadherin in the epithelial-derived part of the tooth indicates that downregulation of E-cadherin is not required for formation of an epithelial tooth bud. In order to identify the intracellular partners in the adhesion complex, we are now embarking on a study of the expression pattern and distribution of several cadherin-associated molecules like p120catenin, β -catenin and plakoglobin and their role during the renewal of teeth.

O7

Advances in understanding abnormalities of tooth number

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The list of identified genes behind abnormal tooth number - either tooth agenesis or supernumerary teeth - is growing steadily. These genes involve all major signaling pathways or their downstream intracellular effectors. In humans, mutations have been identified mostly for syndromic tooth agenesis or supernumerary teeth and dominantly inherited isolated severe tooth agenesis. Recently apparently hypomorphic mutations in *EDA* and *WNT10A* have been associated with isolated tooth agenesis which is inherited in non-dominant manner. The identified mutations are often associated with strikingly characteristic phenotypes. Heterozygous mutations in *MSX1* and *PAX9* affect especially the posterior teeth, whereas anterior teeth are susceptible for impaired *EDA* signaling as shown in female *EDA* carriers and patients with hypomorphic *EDA* mutations. It is expected that hypomorphic mutations will also be found in other genes already associated with syndromic tooth agenesis. These may also shed light on the common types of tooth agenesis, incisor and premolar hypodontia and third molar agenesis. However, these candidate gene-based approaches must be complemented with whole genome searches which are able to reveal so far unknown connections to tooth development. We are currently performing both genome-wide and candidate gene-based approaches on tooth agenesis and supernumerary teeth. The results from this work including novel mutations will be presented and discussed.

O8

Eat it all: Tooth resorption in teleost fish Witten P.E.¹, Huysseune A.¹

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Teleost fish, like other non-mammalian vertebrates, replace lost teeth in a particular position by new teeth throughout life. Repeated tooth development requires bone and tooth resorption: (a) to remove all or part of the old tooth, (b) to move the successor tooth into the position of its predecessor, and (c) to prepare the bone surface to allow the new tooth to attach. Advanced teleosts must also resorb bone (d) for the downgrowth of the successional dental

lamina into the medullary cavity of the jawbone, and (e) to create space for the growing tooth as it works its way through the jaw bone. Remarkably, some teleosts, such as Atlantic salmon (*Salmo salar*), do not shed their teeth. Teeth are resorbed inside the oral mucosa likely to retain minerals (phosphorus), similar to mineral metabolism triggered scale resorption. Teleosts may also retain dentin and incorporate it into the jaw bone. Neither resorption nor preservation of dentin occurs in sharks (Chondrichthyan). Very different from teleosts, sharks shed entire tooth units (tooth + bony basal plate = odontode). Odontoclasts, osteoclasts or macrophages are not involved in this process. In teleosts the morphology of odontoclasts follows the osteoclast morphology. Basal teleosts with osteocyte-containing bone display mainly multinucleated odontoclasts, advanced teleosts with acellular bone display many mono- and oligonucleated odontoclasts. In Atlantic salmon, teeth are resorbed from inside the pulp cavity and from outside. Resorption from outside is clearly connected to - and likely mechanically triggered by - the growing replacement tooth. Indeed, studies on rodents have shown that the dental follicle, the dental epithelium, and the stellate reticulum release factors that regulate osteoclast activities such as CSF-1, RANK-L, OPG, TGF- β , and interleukin-1 α (Il-1 α) (reviewed by Witten & Huysseune 2009). Whether resorption from inside the pulp cavity is correlated to the outside-resorption, and how much the process depends on vascularization and innervation of the pulp cavity are questions that we address in our current studies. Witten, PE & Huysseune, A, 2009. *Biol Rev* 84: 315-346

Stem Cells And Signalling

O9

Purification and characterization of human dental epithelial stem cells

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Background: Mesenchymal stem cells, which give rise to dentin, bone, and cementum, have been isolated and are being studied extensively in the context of tissue regeneration. However, no studies exist on the regenerative potential of epithelial stem cells that could lead to enamel reconstruction. This missing gap is critical because dental enamel, in contrast to bone and dentin, cannot regenerate. Furthermore, without cells that can give rise to ameloblasts complete biological restoration of human dental crown can not be achieved. Our objective is to identify and characterize epithelial stem cells that can be differentiated to enamel producing cells. Our hypothesis is that human epithelial cell rests of Malassez (hERM), the only remaining dental-origin epithelial cells after tooth eruption, found in the periodontal ligament (PDL) of all teeth, have stem cell properties and can be isolated, expanded and induced to form enamel in vivo. **Methods:** hERM cells were clonally isolated from PDL using cell surface markers and flow cytometry and were expanded through inhibition of anoikis and manipulation of cell proliferation pathways. hERM clones were tested for self-renewal and karyotype stability, for the expression of stem-cell markers using immunofluorescence microscopy and PCR arrays and for their ability to express enamel specific proteins upon differentiation induction using ameloblast lineage molecular determinants and/or co-culture and in vivo co-seeding systems with dental pulp stem cells (DPSC). **Results:** We report a detailed protocol for the sorting and propagation of single live hERM expressing stem cell markers integrin- α 6, Notch-1 and CD34. We also show evidence that hERM clones express the epithelial stem cell super-marker LGR5 and share genetic programs with other stem and progenitor epithelial cells suggesting that hERM have stem cell like properties. We are also able to expand hERM in vitro and to induce differentiation of hERM into cells that express ameloblast phenotypic markers and produce enamel-like matrix. **Conclusion:** The isolation and characterization of hERM stem cells should help elucidate the molecular pathways that govern ameloblast lineage commitment and enamel tissue development. We are currently exploring combinations of epithelial and mesenchymal stem cells, biodegradable scaffolds and gene delivery systems to regenerate human enamel and dentin. These studies lay the foundation for innovative regenerative treatments in patients with defective or

missing dental tissues.

O10

MicroRNA regulated tooth epithelial stem cell differentiation: Specific MicroRNAs target BMP, Wnt and chromatin remodeling complexes

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Background: MicroRNAs are known to regulate gene function in many tissues and organs and in this study we investigate the functions of specific microRNAs in tooth development. **Methods:** *Pitx2*, *K-14* and *Wnt-1 Cre*'s were used to delete mature microRNAs in *Dicer1* floxed mouse models. Transgenic mice are used to understand the effect of specific microRNAs on tooth and craniofacial development. Molecular and biochemical assays reveal molecular mechanisms of specific microRNAs. **Results:** We have identified discrete sets of microRNAs expressed in molars compared to incisors as well as epithelium compared to mesenchyme at various stages of tooth development. Conditional knockout (cKO) of *Dicer1* (mature microRNAs) in the dental epithelium using the *Pitx2 Cre* mice results in multiple and branched enamel-free incisors and cusplless molars. Analyses of differentiating dental epithelial markers reveal a defect in ameloblast differentiation. Conversely, the cervical loop (stem cell niche) is expanded in *Dicer1* cKO. Epithelial microRNAs control dental stem cell differentiation demonstrating a unique role for microRNAs in regulating in vivo dental stem cell biology. Noggin, a potent BMP inhibitor, Lef-1 an essential transcription factor and HMG-17 a chromatin-associated remodeling factor are up-regulated in the *Pitx2 Cre-Dicer1* knockout mouse. We have identified specific microRNAs that target these genes in vitro and in vivo and are associated with the tooth/craniofacial anomalies and cell differentiation defects. **Conclusions:** These results demonstrate a critical role for microRNAs in regulating tooth and craniofacial development. Furthermore, we demonstrate microRNA regulation of Noggin/BMP signaling, Lef-1/Wnt signaling and chromatin remodeling during tooth/craniofacial development. Support for this research was provided from grant DE13941 from the National Institute of Dental and Craniofacial Research.

O11

Development of tooth cusps in the gecko *Paroedura picta*: Cusp generation without an enamel knot

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Background: The evolution of tooth cusps has played a significant role in the radiation of the vertebrates. The molecular basis of cusp development is relatively well known in mammals, where cusp development is directed by the enamel knots, transient signalling centre which direct the growth and shape of inner enamel epithelium (IEE). In contrast to mammals, reptiles do not show any evidence of enamel knots. This poses the question, what mechanism directs cusp formation in this amniotic group and does this mechanism represent a pre-adaptation for mammalian cusp formation? To address this question we have investigated cusp development in a gecko, *Paroedura picta*, which has bi-cuspid teeth. The expression pattern of growth factors that play a role in directing cusp development in the mouse was followed in this reptile, with particular emphasis on Sonic Hedgehog (Shh). The expression and role of Shh has previously been described in unicuspid snake teeth, but its role in formation of bicuspid teeth has not been analysed.

Methods: Embryos of *P. picta* at different stages were analysed. Shh was detected by *DIG in situ* hybridization. Cell proliferation and programmed cell death were detected by PCNA and TUNEL respectively on serial sections. Fibronectin, a component of the extracellular matrix of the stellate reticulum, was also assessed. Trichrome staining was used for the basic histological observations.

Results: At the bell stage, *Shh* expression was localized in the apical zone of the IEE, prior to the cells differentiating into ameloblasts. At this point in development the tooth has a unicuspid morphology. As the apical cells mature, they enlarge. Cell proliferation was not observed in this cluster of cells. At this stage the stellate reticulum (SR) is almost free of fibronectin in the apical area, where it has a relatively solid cellular character. In contrast, the lateral sides of the SR contain a high density of fibronectin. The mesenchyme of the dental papilla is also full of fibronectin. Enlargement of the IEE cells of the apical

complex results in the cells physically pushing into the dental papilla. The basic bicuspid shape is formed through this process. The shape is later fixed by the deposition of dentin. *Shh* expression in the apical ameloblasts disappears after the first signs of mineralization and the signal spreads along the differentiating cells of the IEE in the direction of the base of the tooth. The final shape of the cusps is re-enforced by the deposition of enamel. Proliferating cells, which direct the outgrowth of the tooth germ, were present in the non-differentiating area of the IEE and in the cervical loops from the bell stage until ankylosis. Apoptosis was detected in the apical zone of the SR during the mineralization stage, which may be connected with SR disintegration before tooth eruption. It would therefore appear that, unlike in the mouse, cell proliferation and apoptosis do not play significant roles in cusps formation.

Conclusions: Our studies indicate that tooth cusps develop in reptiles by a completely different from that reported in mammals. We saw no signs of any pre-adaptations that could have lead to the mechanism of mammalian cusp development. We plan to examine the expression patterns and functions of a number of other signalling factors (Fgfs, Bmps, Wnts) in the gecko in order to confirm this hypothesis. This research was supported by GA AV CR (grant KJB601110910).

O12

Ring1a/b polycomb proteins regulate the mesenchymal stem cell niche in continuously growing incisors

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Background: Rodent incisors are capable of growing continuously. The renewal of dental epithelium giving rise to enamel-forming ameloblasts and dental mesenchyme giving rise to dentin-forming odontoblasts and pulp cells is achieved by stem cells residing at their proximal ends. Although the dental epithelial stem cell niche (cervical loop) is well characterized, nothing is known about the dental mesenchymal stem cell niche. Ring1a/b are the core Polycomb repressive complex1 (PRC1) components that have recently also

been found in the protein complex with BCoR (Bcl-6 interacting corepressor) and Fbx10. **Methods:** Expression of PRC1 components was mapped using in situ hybridization and conditional postnatal gene activation using tamoxifen-inducible Cre was used to effects of loss of gene function on incisor growth. **Results:** During mouse incisor development, we found that genes encoding members of the BCoR/Ring1a/b complex are strongly expressed in the incisor apical mesenchyme. Analysis of *Ring1a*^{-/-};*Ring1b*^{fl/fl} cre+ mice showed that loss of Ring1a/b postnatally results in defective cervical loop and disturbances of enamel and dentin formation in continuously growing incisors. To further characterize the defect found in *Ring1a*^{-/-};*Ring1b*^{fl/fl} cre+ mice, we demonstrated that cell proliferation is dramatically reduced in mesenchyme and cervical loop epithelium of *Ring1a*^{-/-};*Ring1b*^{fl/fl} cre+ incisors in comparison to *Ring1a*^{-/-};*Ring1b*^{fl/fl} cre- incisors. Fgf signaling and downstream targets which have been previously shown to be important in the maintenance of the dental epithelial stem cell compartment in the cervical loop are downregulated in *Ring1a*^{-/-};*Ring1b*^{fl/fl} cre+ incisors. In addition, expression of other genes of the BCoR/Ring1a/b complex is also altered. **Conclusions:** These results suggest that the BCoR/Ring1a/b complex regulates the dental mesenchymal stem cell compartment and cell differentiation in developing mouse incisors. This is further supported by experiments showing that cells from this compartment are capable of directional migration in response to tooth damage and differentiation into odontoblast-like cells.

O13

Essential role of mesenchymal Notch-RBP-Jkappa signaling in controlling dental epithelial cell fate during mouse tooth development

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Background: The tooth is a well-established model of epithelial-mesenchymal interactions, which controls histogenesis, morphogenesis, and cell differentiation during tooth develop-

ment. Notch signaling is important in cell-cell communication and plays a key role in the control of cell fate, stem cell potential and differentiation. The best characterized “canonical” pathway of Notch activation involves translocation of NICD to the nucleus, where it associates with the DNA binding protein RBP-Jkappa (CBF-1 or CSL), converting it from a repressor into an activator of transcription. While attention have been already paid to the role of Notch signaling in the epithelial and mesenchymal compartments during tooth development, the role of this pathway in mediating mesenchymal-epithelial interactions has not been fully investigated. **Methods:** In this study, we applied Cre-loxP system for targeting disruption of RBP-Jkappa, the key effector of “canonical” Notch pathway, in the mesenchymal compartments of the developing teeth. Transgenic mice with Cre transgene expression driven by the promoter of the collagen type I (a2 chain) gene were crossed with mice with the RBP-Jk gene flanked by loxP sites. The Cre activity was evaluated by further crossing with mice of ROSA26R background. Histogenesis, morphogenesis as well as the differentiation of the teeth in the mice with RBP-Jkappa deletion were evaluated by using different approaches. The target genes, were analyzed by real time RT-PCR, and confirmed by western blotting and/or immunohistochemistry. To study whether the observed phenotype is intrinsic or secondary to systemic alterations resulting from the RBP-Jk deletion. To validate the *in vivo* gene regulation results, we applied adeno Cre virus in the cultured mouse dental mesenchymal cells to achieve acute deletion of RBP-Jkappa gene *in vitro*. To assess the functions of the relative signaling molecules, functional analysis was performed by applying recombinant proteins and/or chemical compound in the cultured tooth germs. Finally the direct down-stream targets of the RBP-Jkappa gene were confirmed by using chromatin IP. **Results and conclusion:** The deletion of RBP-Jkappa, in the mesenchyme, validated by different techniques, resulted in smaller tooth size, with no apparent changes of crown shape and cusp numbers. The reduced tooth crown size was due to a decrease of epithelial cell proliferation of stratum intermedium cells. In the incisor teeth, an enlarged apical end could also be identified, which suggested a possible expended stem cell pool/niche. Similar observations in dental epithelial cells were also achieved in the cultured tooth germs, suggesting an intrinsic effect of losing RBP-Jkappa in the dental mesen-

cymal cells. Molecular profiling indicated a common role of RBP-Jkappa, in regulating several key pathways involving in WNT, FGF and BMP signaling, in the mesenchymal cells including dental mesenchyme/pulp, dental sac, as well as surrounding bone tissues. Functional analysis suggests these signaling molecules could mediate the dental epithelial cell proliferation as well as differentiation. Thus our results suggest a novel role of Notch-RBP-Jkappa signaling is a key player in the mesenchyme compartment of a developing tooth in coordinating mesenchymal-epithelial interactions.

O14

The interaction between Wnt, Shh and Sostdc1 governs the spatial patterning of teeth

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Each vertebrate species displays specific tooth patterns in each quadrant of the jaw: the mouse has one incisor and three molars, which develop at precise locations and at different times. Sonic hedgehog (Shh) is involved in pattern formation of tooth and other ectodermal organs such as limb, hair, mammary gland, and feather by controlling proliferation and differentiation of cells. It has been reported that Shh in tooth development induces cell proliferation and increases cell polarity and cell differentiation. Here, by utilizing maternal transfer of 5E1 (anti-Shh antibody) through the placenta, we found the fusion between first and second molar or the fusion between second and third molar depending on the embryonic stages when 5E1 was injected. In addition to Shh, Wnt and Sostdc1 are also key signaling molecules involved in the spatial patterning of teeth and other ectodermal organs. In this study, we also found the close interactions between Wnt, Shh and Sostdc1 governing the spatial patterning in teeth and proposed a new reaction-diffusion model confirming the interactions do lead to patterning consistent with tooth patterning of wild type mouse.

O15

Hedgehog signaling directs generation of progeny from adult stem cells in the continu-

ously growing mouse incisor

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Background: The mouse incisor, unlike human teeth, grows continuously throughout the life of the animal. This constant growth requires the presence of both epithelial and mesenchymal stem cells that have the capacity to self-renew as well as to differentiate into enamel-producing ameloblasts, dentin-producing odontoblasts, and other cell types. Sonic Hedgehog (SHH) is expressed at multiple stages of tooth development, and Hedgehog (Hh) signaling has been proposed to regulate stem cells in several systems, including the adult brain. Here, we describe the role of Hh signaling in regulation of adult stem cells in the postnatal incisor. We show that SHH is a critical regulator of continuous incisor growth that directs progeny formation from stem cells.

Methods: K5tTA;H2B-GFP mice or BrdU-injected wildtype mice were used to visualize slowly cycling, label retaining cells (LRCs). Expression of Hh pathway members and cell type specific markers was detected using immunofluorescence, mRNA *in situ* hybridization, X-Gal staining or qRT-PCR. Genetic lineage tracing in Gli1CreER^{T2};R26R mice was used to identify Hh-responsive stem cells and to follow the lineage of their progeny *in vivo*. Effects of a Hh pathway inhibitor were studied using micro-CT, histological and marker analyses as well as cell proliferation assays and lineage tracing. **Results:** LRCs were present exclusively in the

posterior parts of both labial and lingual cervical loops and in the proximal incisor mesenchyme between the loops. *Shh* is expressed in pre-ameloblasts and ameloblasts, and expression analyses showed that it is the principal Hh ligand gene in the incisor. Using *Gli1^{lacZ}* and *Ptch1^{lacZ}* reporter mice, we found that high level Hh signaling is received specifically in those regions containing LRCs. The presence of double-labeled cells that were both BrdU label-retaining and *Gli1^{lacZ}* positive indicated that some of the putative incisor stem cells are Hh-responsive. Lineage tracing of *Gli1*-expressing cells confirmed that a subset of Hh-responsive cells are indeed stem cells in the mouse incisor that can give rise to progeny over extended periods of time. Furthermore, antagonizing Hh signaling using a small molecule inhibitor of Smoothed resulted in perturbed morphology and function of differentiated cell types, with ameloblasts being particularly affected. Interestingly, LRCs were not depleted after antagonizing Hh signaling for one month and the effects of the inhibitor were reversible. In addition, lineage tracing of Hh-responsive cells in inhibitor-treated mice indicated that antagonizing Hh signaling prevents generation of progeny that contribute to the ameloblast lineage. Significantly, after 14 days of antagonist treatment, proliferating cells in the transit-amplifying (T-A) region of the labial cervical loop were present only in one layer and stratum intermedium cells were still being generated from these T-A cells.

Conclusions: We showed that SHH signals to three stem cell niches in the incisor that contain Hh-responsive and Hh-non-responsive stem cells. Hh-responsive stem cells in the labial cervical loop are the progenitors of SHH secreting cells, thus, a positive feedback mechanism is in place between the stem cells and their progeny. Our data further suggest that Hh signaling is not required for stem cell survival but instead strongly support a role for Hh signaling in controlling generation of specific progeny- the ameloblasts- from epithelial stem cells.

O16

***Evc* regulates symmetric and asymmetric responses to Shh signalling in tooth development**

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Ellis-van Creveld (EvC) syndrome is an autosomal recessive disorder caused by mutations in *EVC* or *EVC2*. Both genes encode proteins that locate to the primary cilium, which is required for hedgehog signalling in mammals. Oral anomalies of EvC syndrome patients include multiple frenula, missing teeth and abnormal tooth morphogenesis. Consistent with a role for hedgehog signalling, we found an impaired response to Shh in the developing dentition of homozygous *Evc* mutant embryos. Remarkably, in the developing first molars of *Evc* mutants, the Shh pathway was transiently up-regulated at the lingual side but was subsequently inactive specifically at the buccal side, the latter associated with a severe, unilateral growth defect and a loss of the bucco-lingual symmetry. Unlike molars, the incisors of rodents deposit enamel only on the labial side and thus exhibit a highly asymmetric architecture along the oral-aboral axis. Paradoxically, in the lower incisors of *Evc* mutant mice this asymmetry is partly abrogated, a defect that is associated with an ectopic response to Shh signalling at the lingual cervical loop, while the response at the labial cervical loop is down-regulated. The results identified *Evc* as an important component of the spatio-temporal co-ordination of Shh pathway activities during tooth development. These modulations appear to contribute to the establishment of tooth-type specific symmetries and asymmetries along the oral-aboral axis of the mammalian dentition.

O17

Downstream targets of Ectodysplasin in developing teeth

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Ectodysplasin (Eda) is a signalling molecule which belongs to the Tumor Necrosis Factor family and is necessary for the normal development of teeth and other organs developing as appendages of the ectoderm. Mutations in the Eda pathway cause the hypohidrotic ectodermal dysplasia syndrome in humans and similar phenotypes in various animal models. Eda binds to its receptor Edar and the signalling is mediated via the NFκB transcription factor. In

the teeth, the Eda pathway regulates the function of the epithelial signalling centers including the dental placode, as well as the primary and secondary enamel knots. We have searched for downstream target genes of the Eda pathway by microarray analysis of embryonic Eda mutant epithelium exposed to Eda protein. The results indicate that Ectodysplasin signalling regulates the expression of multiple genes which encode components of the FGF, BMP, Wnt as well as Hedgehog pathways. We conclude that Ectodysplasin functions as a modulator of all major developmental signalling pathways.

O18

Diverse roles of Lrp4 in craniofacial ectodermal organ development

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It has been known that ectodermal organs share common signaling pathways including Wnt, Shh, Bmp, and Fgf in their development and these pathways are functionally integrated in many biological processes. The precise mechanisms of the integration however are still unclear. *Lrp4* belongs to the low-density lipoprotein (LDL) receptor family which is a large evolutionarily conserved group of transmembrane proteins. Recent findings have shown that LDL receptor family members can also function as direct signal transducers or modulators for a broad range of cellular signaling pathways. *Lrp4* is expressed in the development of multiple craniofacial ectodermal organs including hair, tooth, palate, and tongue papillae. *Lrp4* mutant mice show fused molars and diastema teeth. Upregulation of Fgf, Bmp, and canonical Wnt signaling accompanied by changes in Shh activity are observed in the *Lrp4* mutant teeth. Molar fusion and/or diastema teeth are also found in mice with a mutation of *Wise* (a secreted BMP antagonist and Wnt modulator), *Sprouty2* (a negative feedback regulator of Fgf), and *Shh*⁽¹⁻³⁾. Palatal rugae, corrugated structures on the hard palate, are also believed to develop through reciprocal epithelial-mesenchymal interactions similar to tooth development. *Lrp4* mutants

present with disorganized palatal rugae. The palatal rugae of *Lrp4* mutants show upregulation of Fgf signaling and downregulation of Shh. Unlike tooth development, no changes in Bmp or canonical Wnt signaling are detected in developing palatal rugae. *Sprouty1/2* and *Shh* mutant mice also show similar palatal rugae deformities. The data identify different molecular changes in tooth and palatal rugae development in *Lrp4* mutants and indicate that *Lrp4* regulates diverse signaling networks depending on the tissue context. **References:** 1) Kassai et al. (2005) Regulation of mammalian tooth cusp patterning by ectodin. *Science* 309: 2067-2070. 2) Klein et al. (2006) Sprouty genes control diastema tooth development via bidirectional antagonism of epithelial-mesenchymal FGF signaling. *Dev Cell* 11: 181-190. 3) Ohazama et al. (2008) *Lrp4* modulates extracellular integration of cell signaling pathways in development. *PLoS ONE* 3: e4092.

O19

Amelogenin and RANK pathway in root and bone phenotype of the Msx2 null mutant mouse

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Background: The *Msx2* null mutant mouse (*Msx2*^{-/-}) presents a severe and complex phenotype. *Msx2*^{-/-} combines amelogenesis and dentinogenesis imperfecta and hypercementosis. In addition, regional RANKL down-regulation was suggested to be responsible for tooth eruption impairment associated to an alveolar osteopetrosis. Epithelial Malassez rests were hypothesized to be instrumental in this process. The aim of the present study was to analyse amelogenin and the impact of RANK pathway in this *Msx2*^{-/-} radicular and alveolar phenotype. **Methods:** Wild type, *Msx2*^{+/-} and *Msx2*^{-/-} mice were studied from birth until 3 months. Regarding RANK pathway implication, *Msx2*^{-/-} mouse line was mated with a transgenic mouse line over-expressing RANK in the monocyte precursors (RTg) in order to compensate the RANKL expression decrease. Their root and bone phenotype was compared to single *Msx2*^{-/-} mutant-ones. In situ hybridization, tartrate-

resistant acid phosphatase TRAP-histochemistry, immuno-histochemistry, histomorphometry and real-time PCR on RNA extracted from micro-dissected tissues were performed. **Results:** Amelogenin expression was increased in pathological root epithelial Malassez rests of *Msx2*^{-/-} mouse which continuously enlarged. RANK overexpression induces increased TRAP⁺ osteoclast number. In *Msx2* RTg mouse, osteopetrosis was rescued as well as tooth eruption. Root elongation timing was normalized but the final root length remained similar to single *Msx2*^{-/-} mouse. Interestingly, hypertrophic rests of Malassez seen in *Msx2*^{-/-} mouse were transformed into massive epithelial cysts located within wide resorption lacunae in *Msx2*^{-/-} RTg mouse. **Conclusions:** Amelogenin expression level was shown to be *Msx2*-dependent in epithelial Malassez rests. Increased amelogenin levels in *Msx2*^{-/-} would inhibit osteoclast RANK pathway, as reversely documented in amelogenin ^{-/-} mouse line. The *Msx2*^{-/-} RTg mouse analysis validates that RANKL expression decrease in *Msx2*^{-/-} mouse is instrumental in the regional osteopetrosis. In addition, the data outlines the fact that such increased osteoresorption would stimulate growth of odontogenic epithelial cysts.

O20

The role of *Pkd2* in tooth development

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Pkd2(encoding polycystin-2 or TRPP2) is a member of the transient receptor potential superfamily of cation channels. *Pkd2* has been implicated in various biological functions including cell proliferation, sperm fertilization, mechanoreception and asymmetric gene expression. The role of *Pkd2* in tooth development remains largely unknown. We carried out expression analysis of *Pkd2* in tooth development as a starting point. *Pkd2* is expressed ubiquitously at early stages of tooth development and shows expression in developing tooth pulp and periodontal ligament at post-natal stages. In order to investigate the role of *Pkd2* in tooth development, we examined mice with mesenchymal conditional *Pkd2* deletion (*Pkd2*^{flox/}

^{flox}/*Wnt1-Cre*). *Pkd2* mutant mice showed fractures of the middle portion of molar roots and thin roots at apical portion. Calcified structures were found in mutant molar pulp and blood vessels were abnormally expanded in mutant incisor pulp. *Pkd2* mutant mice also have multiple premature suture fusions, cleft palate and temporomandibular joint ankylosis. The results suggest that *Pkd2* is involved in molar root and pulp development.

O21

A genetic pathway involving *Msx1*, *Bmp4* and *Pax9* connects growth and morphogenesis during upper lip development

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Background: Cleft lip (CL) is among the most frequent craniofacial birth defects and may result from a disrupted regulation of growth and morphogenesis of the MNP (medial nasal process) and MxP (maxillary process), which ultimately prevents their fusion. The multifactorial nature of CL formation suggests the importance of gene-gene interactions that co-ordinate MNP and MxP development; however, these interactions are poorly defined at present. We previously found that unilateral or bilateral CL manifest in a significant proportion of newborn *Pax9*^{-/-};*Msx1*^{-/-} mutant mice, but not in single homozygous mutants [1]. We therefore aim to understand how *Pax9*- and *Msx1*-regulated developmental pathways interact with each other and how these interactions affect upper lip formation.

Methods: The generation of *Pax9* [2] and *Msx1* [3] mutant mouse lines has been reported. Scanning electron microscopy (SEM) and *in situ* hybridisation were carried out as previously described [1]. Facial primordia at E10.5 were dissected for organ culture (Trowell's standard method) and incubated with implanted beads soaked in human BMP4 protein. **Results:** During the early phase of upper lip development (E10.5 - E12.5) *Pax9* was mainly expressed in the mesenchyme of the MNP and LNP (lateral nasal process) surrounding the future nasal cavity, whereas expression of *Msx1*, *Msx2* and *Bmp4* was observed in overlapping patterns in the fusing regions of the MNP, LNP and MxP. SEM analyses at E11.5 demonstrated a hypoplastic MNP but also a clearly pronounced curvature of the MNP towards the MxP in *Msx1* mutants. In contrast, while MNP size was not af-

ected in *Pax9* mutants, the curvature of the MNP was measurably impaired. These results indicate that the combination of a growth defect (caused by *Msx1*-deficiency) with a morphogenesis defect (caused by *Pax9*-deficiency) is the major reason for CL formation in double homozygous mutants. Moreover, the *Pax9*-dependent, pronounced curvature of the MNP in *Msx1* mutants suggests that this phenomenon might involve changes of *Pax9* expression. Indeed, we not only found spatially expanded *Pax9* expression in the MNP of *Msx1*^{-/-} embryos but also down-regulated *Bmp4* expression in this region. Consistent with an important role of *Bmp4* to regulate *Pax9* expression patterns, BMP4 soaked beads were sufficient to suppress *Pax9* expression in the MNP in organ culture experiments.

Conclusions: Our results identified *Msx1*, *Bmp4* and *Pax9* as components of a genetic pathway that connects MNP growth with MNP morphogenesis. The *Pax9*-dependent, enhanced curvature of the MNP in *Msx1*^{-/-} embryos compensates for the MNP hypoplasia to allow sufficient contact between the MNP and MxP during a critical time-window of upper lip development. Impairment of this developmental robustness may underlie some sporadic CL cases that are occasionally seen in human oligodontia patients with *MSX1* mutations.

References: [1] Nakatomi *et al.* (2010) Genetic interactions between *Pax9* and *Msx1* regulate lip development and several stages of tooth morphogenesis. *Dev Biol.* in press [2] Peters *et al.* (1998) *Pax9*-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. *Genes Dev.* 12: 2735-2747. [3] Satokata and Maas (1994) *Msx1* deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. *Nat Genet.* 6: 348-356.

O22

Molecular regulations of supernumerary tooth formation

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Teeth are excellent models for studying organogenesis and regenerations. *Apc* is an inhibitor of canonical Wnt signaling. We showed that *Apc* inhibition of Wnt signaling regulates supernumerary tooth formation during embryogenesis and throughout adulthood. Even adult oral tissues are still responsive to loss of *Apc* or activation of canonical Wnt signaling, and are able to form supernumerary teeth. These supernumerary teeth can form from multiple regions of the jaw. Some more mature developed supernumerary teeth are properly mineralized, vascularized, innervated, and even start to form roots, thus may function as nature teeth. Moreover, only a small number of *Apc*-deficient cells are sufficient to induce surrounding wild type epithelial and mesenchymal cells to participate in the formation of new teeth. Strikingly, *Msx1*, which is necessary for endogenous tooth development, is dispensable for supernumerary tooth formation, highlighting the similarities and differences between endogenous and supernumerary tooth development. By comparison of differentially expressed genes in *Apc* loss-of-function and b-catenin gain-of-function mouse tooth germs, we identified a number of Wnt/b-catenin target genes that may be responsible for the initiation of new tooth formation. Knowledge of exactly how these genes fit within the gene regulatory network will contribute to our understanding of tooth development, and will assist in efforts aiming at building teeth or tooth components using adult tissues.

Ameloblasts and Amelogenesis

O23

Structural and cytochemical analyses of enamel and enamel forming cells in the teeth of amelogenin-deficient mice

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The amelogenin proteins comprise approximately 90% of the organic phase of the enamel matrix during early development. It has been reported that the *amelx*-null mice show thin chalky

white enamel with irregular surface and has disorganized crystals when viewed in the fractured planes, indicating a role of amelogenins for determination of enamel thickness and crystal arrangement. However, the previous phenotype analysis has been limited to macroscopic and SEM observations of the erupted teeth of the mutant mice and hence precise information of the intriguing process of amelogenesis without amelogenin gene and proteins has not been provided. **Methods:** In this study we examined growing molars and incisors of the 14-day and 50-day-old *amelx*-null mice by light and electron microscopy, and focused on the structural and cytochemical aspects of the ameloblasts and the growing enamel matrix. **Results:** In the incisors of *amelx*-null mice, the cells of the enamel organ showed normal arrangement and stage-related structural and functional features including the RA-SA modulation of maturation ameloblasts. The enamel matrix started to form on the mineralized dentin and the secretory ameloblasts developed Tomes' processes containing secretory granules in early development, although the thickness of the enamel was limited to 10 μm maximum. The immature enamel was immunopositive for both ameloblastin and enamelin although the enamelin reaction was limited to the "inter-rod" enamel in early development. Abnormal knob-like protrusions of enamel matrix up to 50 μm -long and 10 μm -thick were occasionally encountered on the growing enamel surface of the molars. Transmission electron microscopy of the undecalcified sections of the immature enamel revealed a halo of electron lucent layer along the individual ribbon-like crystals, highlighting the crystals from the surrounding amelogenin-free matrix that appeared relatively electron-dense. In some areas in immature enamel, the groups of enamel crystals were unusually thickened and some were apparently decorated with a bulk of irregular mineralized material. Such thickened crystals and deposits were lost by decalcification leaving a void space. **Conclusions:** These data indicate that a total absence of amelogenin gene and proteins does not interfere with the development of the tooth germs and formation of dental hard tissues other than enamel. Unusually thickened crystals and irregular mineral deposition in the amelogenins-free immature enamel support the regulatory role of amelogenins in crystal growth and arrangement. The causative factors of knob-like protrusions of enamel in the molars of amelogenin-null mice need to be explored.

O24

Induction of human keratinocytes into enamel-secreted ameloblasts

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Utilization of stem cells from a patient to develop bioengineered replacement teeth is the ultimate goal of regenerative dental medicine. Various postnatal mesenchymal stem cells have been shown capable of differentiating into odontoblasts and creating mineralized dentin. However, identification of stem cells that can be used as the epithelial component for tooth regeneration remains a challenge. We tested if keratinocytes could represent an applicable source for enamel production. Keratinocyte stem cells, isolated from circumcised human foreskins and confirmed by the expression of several molecular markers, were cultured to form confluent epithelial sheets. These epithelial sheets were recombined with E13.5 mouse molar mesenchyme that possesses an odontogenic potential. The recombinants developed into whole tooth crowns consisting of human and mouse tissues in 25% cases after 4-week culture under the mouse renal capsule. Immunohistochemical studies using specific antibodies against human or mouse MHC I antigens confirmed human origin of the epithelial component and mouse origin of the dental pulp, respectively. Histological examination revealed the presence of well-formed dentin, but a lack of enamel due to failed differentiation of the keratinocytederived epithelial cells into elongated ameloblasts. The human keratinocyte-derived epithelium thus supports mouse dental mesenchyme to form tooth structures but lacks ameloblastic differentiation capability under such condition. Next, we surveyed the potentials of several key growth factors to induce human keratinocytes into ameloblasts. Among them are SHH and BMP4 that are expressed in differentiating ameloblasts of mouse and/or human teeth, and FGF8 which is expressed in both odontoblasts and ameloblasts of developing human teeth. Growth factor-soaked beads were implanted in tissue recombinants of cultured human keratinocyte sheet and mouse molar mesenchyme, which were subsequently subjected to subrenal culture for 4-week. Nei-

ther SHH nor BMP4 induced ameloblast differentiation, while BMP4 rather caused bone formation instead of tooth in the recombinants. In contrast, inclusion of FGF8 beads, although did not enhance the success rate of tooth formation in the recombinants, indeed induced the elongation of keratinocyte-derived epithelial cells and the deposition of enamel in 6 cases of the 22 formed teeth. Immunostaining assays showed specific presence of ameloblastin and MMP-20, specific markers for differentiated ameloblasts, in the elongated epithelial cells and the enamel, confirming ameloblastic differentiation. It is interesting to note that in developing human embryo, the deciduous teeth begin to develop in the 6th week of gestation, but it is not until the 28th week the dental epithelium starts to differentiate into enamel-producing ameloblasts. However, in the human-mouse chimeric teeth, differentiated ameloblasts and deposited enamel are found within 4-week under the subrenal culture. This is likely due to a faster differentiation of the mouse odontoblasts, which in turn induces earlier ameloblastic differentiation of the human keratinocytes. These results demonstrate that human keratinocytes are capable of differentiating into ameloblasts and producing enamel, and identify the type of cell an appropriate source for generating bioengineered whole tooth crown for replacement therapy in the near future.

O25

Amelotin: Introducing a new player in the team of enamel matrix proteins

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Background: We have recently identified Amelotin (*Amtn*) as a unique gene that is highly and selectively transcribed in a transient fashion from birth to the time of tooth eruption in secretory stage ameloblasts of molars, and in late secretory to maturation stage ameloblasts of incisors. The AMTN protein is efficiently secreted *in vitro*. Studies in rat have located the protein to a basal lamina-like structure between ameloblasts and the enamel mineral surface, but the function of AMTN in amelogenesis remains unknown. The purpose of this project was to develop *in vivo* and *in vitro* models to provide insights into this functional role.

Methods: We have used immunohistochemical analyses to determine the developmental expression profile of AMTN in mouse molars

and incisors, and compared it with the expression patterns of other known enamel matrix proteins (EMPs). We have also conducted a series of biochemical analyses on bacterially expressed recombinant AMTN to characterize the protein in more detail and to investigate its effects on activities such as cell adhesion. A yeast-two-hybrid system was used to identify any potential AMTN-interacting proteins. We have created several transgenic mouse lines that overexpress the AMTN protein under control of the amelogenin (*Amel*) promoter, and mice in which the *Amtn* gene has been disrupted, and have used histological and various imaging methods to characterize their phenotype with a particular emphasis on enamel formation. Lastly, we have used luciferase assays on several *Amtn* promoter constructs to delineate the regions controlling *Amtn* transcription.

Results: In close correlation with the mRNA expression profile, we found the murine AMTN protein expressed transiently in ameloblasts of molars from postnatal day 2 (P2) to the time of eruption, and in late secretory to maturation stage ameloblasts in incisors. Comparison with other EMPs revealed a distinct expression profile for AMTN at relatively late stages of enamel formation. This profile was found to be highly similar to that of APin (ODAM), another recently identified enamel protein. The largest concentration of AMTN was found at the interface between the distal end of ameloblasts and the enamel mineral surface. The transcription of *Amtn* was mediated by a short, ca. 200 bp region upstream of the translation start site. Recombinant AMTN protein did not have any effect on cell adhesion in a variety of cell types, but formed large, homomultimeric aggregates in solution. This aggregation was confirmed in yeast-two-hybrid experiments, which revealed not only AMTN-AMTN, but also AMTN-APin interactions. APin was found to interact with ameloblastin (AMBN) and to a lesser extent with amelogenin (AMEL). Transgenic mice overexpressing AMTN showed a dose-dependent effect on the structure and mechanical properties of enamel, and AMTN deficiency resulted in hypomineralized enamel and premature enamel attrition. Comparisons of expression levels for other enamel proteins in AMTN-overexpressing and -deficient mice revealed alterations for APin and kallikrein 4 (KLK4). **Conclusion:** AMTN plays a critical role during amelogenesis, possibly forming co-polymeric aggregates with APin during the advanced stages of enamel mineralization. We hypothesize that AMTN and APin collectively provide the

specialized matrix that is involved in the transition from highly organized and structured bulk enamel to the compact surface layer of "final" enamel.

O26

The role of Amelotin on differentiation of ameloblasts

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Background: Amelotin has been shown to be a marker for maturation ameloblasts in developing rodent incisors. However the function of amelotin in tooth formation remains unclear.

Methods: In this study we immunolocalized amelotin in developing human incisors by immunofluorescent staining of cryosectioned bell stage primary tooth organs. Laser capture microdissected ameloblasts were used to quantify and compare the relative amounts of amelotin mRNA at different stages of ameloblast differentiation. Primary ameloblast lineage cells were used to determine the effects of amelotin on ameloblast lineage cell proliferation and differentiation, and a SV40 transformed ameloblast lineage cell line with a relatively high expression of amelotin was used to determine effects of serum growth factors and basement membrane proteins on amelotin expression.

Results: Immunohistochemistry revealed prominent amelotin staining in human presecretory and secretory ameloblasts, as well as in the developing enamel matrix. Quantitative PCR analysis showed that mRNA expression of amelotin could be detected in preameloblasts, and was dramatically up-regulated in the presecretory stage of ameloblast differentiation. *In vitro*, amelotin mRNA expression by immortalized ameloblast-lineage cells was significantly down-regulated when grown in 3D Matrigel (basement membrane proteins), while the addition of fetal bovine serum up-regulated amelotin. The addition of recombinant amelotin (rhAMTN) to primary culture ameloblast lineage cells showed that rhAMTN significantly up-regulated the mRNA expression of apin and KLK4. Taken together, these data suggest that amelotin is upregulated by serum related growth factors, and upregulation of amelotin directs amelob-

lasts to a more mature phenotype, as indicated by increased expression of apin and KLK-4.

Conclusions: These studies show that while amelotin is highly upregulated at the maturation stage of ameloblast differentiation, it is present at all stages of ameloblast differentiation. Amelotin may partially regulate ameloblast maturation.

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A high calcium supplement partially prevents fluoride toxicity on secretory amelogenesis in developing hamster tooth germs *in vitro*: Ultrastructural studies

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Background: Short-term exposure of fluoride (F⁻) to the developing hamster tooth germ *in vitro* induces a double response in secretory stage enamel: the mineralizing enamel deposited *in vivo* before the onset of F⁻ exposure hypermineralizes and the enamel matrix secreted during F⁻ exposure fails to mineralize. Fluoride administration during the secretory phase of amelogenesis *in vitro* also results in enhanced uptake of radio-labeled calcium and phosphate mainly due to hypermineralization of the pre-exposure enamel layer while newly secreted enamel matrix remained unmineralized. To test the validity of the hypothesis that the failure of the fluorotic enamel matrix to mineralize was due to a local shortage of calcium ions, medium calcium levels were increased concomitant with F⁻ exposure. The effects of elevated calcium concentration on the toxicity of fluoride to developing enamel were investigated at the ultrastructural level in hamster second molar tooth germs grown during the secretory phase of amelogenesis *in vitro*.

Methods: Mineralizing 3-4 day-old hamster second maxillary tooth germs were cultured in BGJ_b medium using the grid method. Most of the ameloblasts are in the secretory stage and continue to deposit enamel *in vitro*. The BGJ_b culture medium was prepared in our laboratory in order to be able to adjust the medium calcium concentration. The culture medium was supplemented with 15% FCS. The tooth germs were cultured for 48 hours in the presence of high (4.2 mM Ca²⁺) total calcium ions together with 1 mg/L F⁻. The contralateral control tooth germs were cultured in standard medium (2.1 mM calcium) also containing 1 mg/L F⁻. The medium containing standard calcium concentration used for the culture of the control germs support normal min-

eralization of enamel and dentine *in vitro*. Inorganic phosphate in the media was kept constant (1.6 mM). The culture media were changed every 24 hours. Some tooth germs were also cultured in standard medium but without fluoride but supplemented with an equimolar amount of Cl⁻ as control of the culture quality. At least six pairs of molars per group were examined. **Results:** The most profound effect of culturing the explants in calcium-enriched media on the toxicity of fluoride was the absence of unmineralized enamel matrix on top of the pre-exposure enamel layer. Instead, a relatively normal *in vitro* enamel layer was deposited on top of the fluoride-induced hypermineralized pre-exposure enamel layer. Another effect was that high calcium levels inhibited both the fluoride-induced disorganization of the Tomes' processes and improved the structural organization of secretory ameloblasts. However, the pre-exposure enamel deposited *in vivo* was still hypermineralized. **Conclusion:** Based on the findings that hypermineralization of secretory enamel during exposure to F⁻ could not be altered by increasing calcium levels, we conclude that the primary target of fluoride action during secretory amelogenesis is the pre-existing mineral crystals rather than the organic matrix being secreted by the ameloblasts.

O28

Ameloblastin in enamel formation: Rescue of amelogenesis imperfecta and processing by enamel proteinases

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Introduction: During enamel formation ameloblastin (Ambn) is expressed by secretory stage ameloblasts along with amelogenin and enamelin. Lack of Ambn or enamel proteinases results in amelogenesis imperfecta (AI). Mice with partial deletion of *Ambn* display hypoplastic AI. Disruption of proteolytic processing of enamel proteins causes hypomature enamel *in vivo*. **Objective:** The first goal was to determine if

Ambn is essential for enamel formation by rescuing the enamel defects in *Ambn* mutant mice using a transgenic mouse approach. The second goal was to identify the cleavage sites of Ambn recognized by enamel proteinases using recombinant Ambn and synthetic peptides. **Materials & methods:** A transgenic mouse model was developed to express full-length mouse Ambn (396 amino acids) under the control of the amelogenin (*Amel*) promoter in the *Ambn* mutant mice. The *Amel* promoter (4.6 kb) and its 3' UTR (1.1 kb) were cloned from mouse genomic DNA; the *Ambn* cDNA (1.7 kb) was cloned from mouse molar mRNA. pCR2.1-TOPO was used as cloning vector. The excised Ambn transgene (Tg) was injected into fertilized mouse oocytes. Ambn Tg expression levels of Ambn mutant mice were determined in the first molars of PN day 5. Proteins from molars were extracted with formic acid and analyzed by SDS-PAGE, CBB staining and Western blotting (WB). An anti-peptide antibody raised against a synthetic peptide from the deleted region was used in WB. The morphology of teeth was evaluated at 7 weeks. The thickness and organization of enamel were evaluated by scanning electron microscope (SEM) on cross-sectioned and fractured mandibular incisors. The enamel surface was analyzed on exposed labial surfaces. Recombinant pAmbn was expressed and secreted from a stable cell line (HEK293) and purified from medium. Ambn and synthetic peptides were incubated with rpMmp-20 and native Klk4. The digestion products were analyzed by SDS-PAGE, N-terminal sequencing and mass spectrometry. **Results:** From 17 Ambn Tg mouse lines, 5 lines were established and analyzed. Among lines Ambn Tg expression levels ranged compared to wild-type mice from no Ambn expression Tg(0), below normal Tg(+), above normal Tg(++), to much higher Tg(+++). The morphology of incisors and molars of Tg(+) mice appeared to be similar to wild-type mice. Analysis by SEM revealed that the enamel thickness at the central labial position was restored to normal in Tg(+), (++) and (+++) mice; however the distal enamel thickness was restored to normal only in Tg(++) animals. In Ambn Tg(+) and Tg(+++) mice, the distal thickness of the enamel was reduced and had a rough surface. In fractured enamel the decussating pattern was rescued in all Tg expressing mice but Tg (+++) mice had defined interrods. The enamel surface of the incisors of Tg(++) animals was similar to wild-type mice during all stages of enamel formation. rp-Mmp-20 cleaved rpAmbn and the synthetic pep-

tides at sites corresponding to those observed *in vivo*. Initial cleavages were made at the N-terminus, followed by cleavages at the C-terminus. In contrast, Klk4 cleaved rpAmbn and the synthetic peptides at sites not observed *in vivo*. **Conclusion:** The enamel defects of the Ambn mutant mice were at least partially restored to normal by expression of the full-length Ambn Tg. Our data demonstrate that Ambn is essential for enamel formation. Mmp-20 is capable of catalyzing cleavages of Ambn during the secretory stage, while Klk4 likely degrades Ambn during maturation stage.

O29

Immunolocalisation of NBCe1 in the mouse enamel organ

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Background: Enamel crystal growth generates protons that need to be neutralized to sustain enamel mineralization. The presence of carbonic anhydrase II (Car-II), the anion exchanger-2 (Ae2) and Cystic fibrosis transmembrane regulator (Cftr) in maturation ameloblasts supports the view that these cells are involved in pH regulation. Recently it was reported that mice deficient in electrogenic sodium bicarbonate cotransporter-1 (Nbce1) have enamel defects (Gawenis et al; J Biol Chem.282:9042-52, 2007); in addition, transcripts for Nbce1 have been detected in ameloblast-like cells (Paine et al.; J Dent Res 87, 391-395; 2008). This suggests that Nbce1 is operating in cells of the enamel organ during amelogenesis. The aim of this study was to examine a potential role of Nbce1 in pH regulation by ameloblasts by establishing its location during amelogenesis.

Methods: Mouse jaws were fixed in buffered paraformaldehyde, histologically processed in paraffin, serially sectioned and stained with rabbit anti-NBCe1 polyclonal antibodies.

Results: Staining for Nbce1 was located in the enamel organ during both secretion and maturation stage of amelogenesis. In early secretory ameloblasts, staining was located in membranes of stratum intermedium and cytoplasmically in secretory ameloblasts. In more advanced stages secretory ameloblast staining was very strong in the apical end of the amel-

oblast cell body near the proximal part of the Tomes' process. The distribution of staining for Nbce1 changed dramatically in maturation phase. The plasma membranes of the papillary layer adjacent to maturation ameloblasts became intensely stained whereas staining in maturation ameloblasts was reduced. The apical staining of the ameloblast membrane had disappeared with the onset of maturation stage but weak staining in the cytoplasm and in basolateral membranes was still apparent in these cells. **Conclusions:** The presence of electrogenic Nbce1 in both secretory ameloblasts and maturation ameloblasts suggests that this cotransporter functions in both stages of amelogenesis, likely involved in pH regulation during mineralization of enamel. In secretory stage the apical NBCe1 near the proximal ends of the Tomes' processes may be involved in bicarbonate extrusion into the enamel space in close vicinity of the plasma membranes of the Tomes processes where enamel crystals are forming. In maturation stage, as mineral accretion increases, proton production in enamel is intensified which requires more bicarbonate secretion. The joint activity of (cytosolic) Car II, basolateral Nbce1 and Ae2, and apical Cftr in maturation ameloblasts provides a basic mechanism to extrude bicarbonates into maturation stage enamel. The intense staining of the papillary layer cells for Nbce1 strongly suggests that the basal plasma membrane of the ameloblasts that is in contact with the papillary layer is a major route to import bicarbonates into the maturation ameloblast cell body. The present data further add to the concept that ameloblasts contain the machinery for local extracellular pH regulation in the enamel space. **Acknowledgments:** Part of this work was funded by NIH (grant DE13508-06; DL, THB, PdB and AB).

O30

The recombinant human amelogenin protein and regeneration of mouse non-union calvarial defect

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Background: The amelogenin protein constitutes 90% of the forming extra-cellular enamel matrix proteins and its function is to regulate the size, shape and direction of the mineral

crystal growth. We have previously reported that amelogenin is expressed in cells of alveolar bone, long bone, cartilage and bone marrow (Deutsch et al. 2006, Haze et al. 2007, 2009). Later we showed that the recombinant human amelogenin protein (rHAM⁺) alone, produced in eukaryotic system in our laboratory (Taylor et al. 2006), induces the regeneration of the periodontal tissues; cementum, periodontal ligament and alveolar bone, after induction of experimental periodontitis in the dog model, through recruitment of mesenchymal stem cells (Haze et al. 2009). We have also observed high amelogenin expression in active bone regions (growth and remodeling). The dynamic spatiotemporal pattern of expression of amelogenin in the developing mouse embryonic craniofacial complex strongly suggests that amelogenin has an important role during normal bone development (Gruenbaum-Cohen et al. 2009).

Objectives: To investigate the ability of amelogenin to repair / regenerate critical size calvarial defect (a membranous bone not associated with PDL and cementum).

Methods: (1) Creation of critical size defect (5mm, Aalamiet al. 2004) in the parietal bone of the mouse. (2) Application of collagen sponge into the defect. Suture soaked with rHAM⁺ (or its carrier PGA alone-control) was placed above the calvarial bone defect. (3) Characterization of the regenerated bone tissue 2-5 months after amelogenin application, using micro-CT. (4) Histological, immunological and molecular biology analyses of the regenerated bone (compared to control with PGA alone).

Results: Micro-CT analysis showed some calvarial bone regeneration 7 weeks after rHAM⁺ application, and the regeneration progressively increased at 3 to 5 months. In the experimental mice the regeneration process generally initiated at the sagittal line, just below the suture (soaked with rHAM⁺) and progressed laterally towards the defect boarder. 3-5 month after application of rHAM⁺, the regenerated bone was continuous at the sagittal line. Generally, in contrast, in control mice, no regenerated bone to very little bone regeneration was observed. In some defects sporadic islands of bone were observed, mostly close to the edges of the defect but not at the sagittal line. A direct correlation was apparent between rHAM⁺ concentration and the degree of regeneration at fixed 5 months period. Sagittal micro-CT section of the regenerated bone demonstrated full bone thickness as compared to non-operated bone. Histological

and immunological analyses of the regenerated bone showed new bone formation, characterized by expression of specific bone markers.

Conclusions: Amelogenin induces calvarial bone regeneration, most probably through recruitment of mesenchymal stem cells. Funded by the Israeli Ministry of Health, Grant number (D.D.): 3-4064

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Ameloblastoma characterization and *in vitro* stimuli response

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Background: Ameloblastomas are odontogenic neoplasms with a predilection for the posterior mandible although other jaw sites may be involved. They grow slowly, do not mineralize and are rarely metastatic. The usual treatment is surgical resection with the attendant disadvantages of significant morbidity and tendency for recurrence. Strategies for alternative therapies are therefore needed. Although still debated, it is generally accepted that these tumours originate from ameloblast precursors. Ameloblastomas have been reported to express genes important for enamel formation such as amelogenin and tuftelin and their histological appearance resembles pre-secretory ameloblasts. There is no established *in vitro* system developed to study these neoplasms. Attempts have been made to isolate stable ameloblastoma cell lines, but to date only one line transfected with a human papilloma-virus type-16 is available. The aim of this project was to establish an *in vitro* system to better understand how ameloblastomas interact with their local environment and maintain themselves in a tumorigenic state. The better understanding of tumour maintenance can lead to the development of new and effective therapies. Ameloblastomas grown in organ cultures can help us to understand them in 3-D context dissecting the behavior of the specific cell types within the tumour when challenged in a controlled environment. These tumours can be challenged with embryonic dental cells in co-cultures. These cells have been shown to produce factors capable of inducing adjacent tissues driving them towards their programmed fate.

Methods: We co-cultured ameloblas-

toma organs with murine embryonic dental epithelium or mesenchyme. **Results:** We established that the murine dental cells change the rate of proliferation and apoptosis on the cultured ameloblastomas. We also determined by *in situ* hybridization that the dental cells have an effect on the expression of genes important for tooth development such as BMP2, BMP4 and TGFb1 in the cultured ameloblastomas. In parallel experiments we used beads soaked with SHH, BMPs and FGFs proteins to determine the effects of these factors on the cultured ameloblastomas. These factors also have an effect on the rate of proliferation and apoptosis on the ameloblastomas. **Conclusion:** These results demonstrate the ability of these tumours to respond to stimuli derived from dental environment *in vitro*.

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New hypothesis of cross-striation formation mechanism

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In the enamel rods, periodic bands referred to as "cross-striation" are observed as incremental lines of circadian rhythm, and in some textbooks it has been written that human enamel is formed at a rate of approximately 4 μm per day and cross-striations appear at 4-μm intervals across enamel rods. However, the formation mechanism has not been elucidated clearly. The enamel formation proceeds through two-step process. In the first step the enamel protein has been deposited within the predicted enamel thickness and the second step involves mineral influx coincident with the removal of protein and water. Accordingly, cross-striation may not directly reflect the periodicity of mineralization in the enamel. Hematoxylin-eosin staining of non-calcified enamel matrix in canine tooth germ showed dark and light coloring pattern like cross-striation. And the pattern of fluorescent dynamic by amelogenin immunostaining is also very similar to cross-striation. Therefore, we consider that cross-striation reflects the contrasting density of amelogenin protein in the process of calcification. Firstly, to examine the diurnal fluctuation of

amelogenin expression in ameloblasts, we utilized the transcriptional assay of amelogenin promoter using luciferase. After transfecting the construct of amelogenin promoter into rat ameloblast cell line, the reporter activity was measured by luminometer and was observed by lumino-microscopy, time-dependently. The results showed that transcriptional activity of amelogenin promoter repeated the cycle of increase and decrease with a period of 20-26h.

Next, to search the regulatory region controlling the transcriptional activity periodicity of amelogenin promoter, we designed the deletion mutant of amelogenin promoter and carried out the transcriptional assay in a same fashion. The rhythm was observed similarly in the deletion-mutant using -74bp promoter, the specific region controlling the periodicity could not be detected in the amelogenin promoter. Thirdly, because it has been reported that there are several CBP/α binding motifs in the promoter region and the activity is inhibited through Msx2 binding to CBP/α, we examined the effect of Msx2 on the periodicity of the amelogenin promoter activity. Overexpression of Msx2 led to the disappearance of the periodicity of amelogenin promoter activity. The results suggested that the binding motifs of CBP/α and Msx2 might be associated with the periodicity of transcriptional activity, however, further study is needed to clarify this. Taken together, we would like to hypothesize that cross-striation is the low-mineralization line which reflects the higher-density line of amelogenin at the stage of enamel matrix deposition.

Odontoblasts and Dentinogenesis

O33

How odontoblasts can sense enamel erosion

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Enamel erosion leads to dentine hypersensitivity, a painful disease that affects today a younger

population. The exposure of a dentinal surface results in pain arising in response to thermal, tactile, osmotic or chemical stimuli. The widely accepted hydrodynamic theory states that the consequent opening of dentinal tubules to the oral environment allows, under these stimuli, the movement of the dentinal fluid inside the tubules, indirectly stimulating the extremities of the pulp nerves, causing the pain. Nevertheless, growing evidence suggests that odontoblast could be a key cell in the detection of enamel erosion and the tooth pain transmission. We have demonstrated the presence of mechanosensitive ion channels at the surface of odontoblasts :BKCa, TREK-1 which is involved in polymodal pain perception, and $Ca_{v2.2}$ clustering at the base of the primary cilium of odontoblasts. Very recently, mechanosensitive TRP channels (transient receptor potential channels, a family of non-selective cation permeable channels) such as TRPV1-4 and TRPM3 were identified in odontoblasts at the gene expression and functional level (1). Moreover, we demonstrated the presence of TRPP1 and TRPP2, forming a mechanosensor / Ca^{2+} permeable channel heterodimeric complex, at the base of the primary cilium of odontoblasts. Therefore, odontoblasts are at least as well equipped as sensory nerves to sense enamel erosion. Moreover, we have demonstrated that these cells are able to generate action potentials and therefore are excitable cells. Taken together, all these data allow to devise a new schema to explain dentinal sensitivity pain where odontoblasts could sense the fluid displacement consecutive to stimuli, integrate this mechanical stress into a biological response that could be transduced to the sensory nerve endings. However, we need to precisely identify the proteins that mediate signals between odontoblasts and nerves although some candidate mediators are emerging. Therefore, the early step underlying dentin pain perception and transmission is a much more intricate mechanism than initially proposed by the hydrodynamic theory as odontoblasts are key cells involved in the pulp/dentin strain sensor complex. This explains the difficulty to develop more effective therapeutic strategies for the treatment of dentin sensitivity. References: (1) Son AR, Yang YM, Hong JH, Lee SI, Shibukawa Y, Shin DM. J Dent Res. 2009; 88:1014-1019. (2) Thivichon-Prince B, Couble ML, Giarmachi A, Delmas P, Franco B, Romio L, Struys T, Lambrichts I, Resnikoff D, Magloire H, Bleicher F. J Dent Res. 2009 ; 88 :910-915.

(3) Magloire H, Maurin JC, Couble ML, Shibukawa Y, Kimura-Tsumura M, Thivichon-Prince B, Bleicher F, J Pain, accepted

O34

Pathogen sensing by human odontoblasts

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Human odontoblasts are neural crest-derived, dentin-producing mesenchymal cells aligned at the periphery of the dental pulp. They become exposed to cariogenic oral bacteria as these progressively demineralise enamel then dentin to gain access to the pulp. Due to their situation at the dentin-pulp interface, odontoblasts are the first cells encountered by invading pathogens and/or their released components, and represent, in the tooth, the first line of defence for the host. Previous studies have shown that odontoblasts are able to sense pathogens and elicit innate immunity. In particular, they express several pathogen recognition receptors of the Toll-like receptor (TLR) and nucleotide-binding oligomerisation domain (NOD) families, which allow them to recognize specific bacterial and viral components. So far, most studies aiming at elucidating the role of odontoblasts in the dental pulp innate response have focused on Gram-positive bacteria, as these largely dominate the carious microflora in initial and moderate dentin caries lesions. In vitro, odontoblasts were found to be sensitive to Gram-positive bacteria-derived components, mainly lipoteichoic acid which is recognized through cell membrane TLR2. Our studies have shown that engagement of odontoblast TLR2 by LTA triggers TLR2 and NOD2 up-regulation, NF- κ B nuclear translocation, production of various chemokines including CCL2, CXCL1, CXCL2, CXCL8 and CXCL10, while promoting immature dendritic cell recruitment. Conversely, LTA down-regulates major dentin matrix components, including collagen type I and dentin sialophosphoprotein, as well as TGF- β 1, a known inducer of dentin formation. We provide

here additional data showing the fine localization of NOD2 in healthy dental pulps, as well as differential regulation of *TLR2*, *TLR4*, *NOD2*, *CCL2* and *CXCL8* genes by LTA and the synthetic TLR2 agonists Pam2CSK4 and Pam3CSK4. It appears from the aforementioned data that odontoblast-triggered immune events constitute potential targets for interrupting the signaling cascades which lead to excessive immune response and necrosis in the dental pulp tissue challenged with cariogenic bacteria. In particular, preventing Gram-positive bacteria recognition or signal transduction by pattern recognition receptors may represent a valuable strategy to achieve this goal. Future studies in the field will pave the way for designing novel therapeutic agents which modulate odontoblast behaviour to promote pulp healing and repair. This work was supported by University Lyon 1, CNRS, IFRO and Région Rhone-Alpes. TAK acknowledges support from the Deutsche Forschungsgemeinschaft (DFG), SFB670.

O35

Overexpression of the *Trps1* transcription factor in odontoblasts results in a dentinogenesis imperfecta-like phenotype

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Dentinogenesis imperfecta (DI) is a disorder of dentin formation. It can be manifested as a part of the osteogenesis imperfecta (OI) phenotype or the phenotype can be restricted to dental findings only. In the latter case, DI is caused by mutations of the *DSPP* gene encoding for dentin sialophosphoprotein (DSPP), a matrix protein expressed by odontoblasts. In dentin DSPP is cleaved to dentin sialoprotein (DSP) and dentin phosphoprotein (DPP), which together constitute the majority of non-collagenous dentin matrix proteins. Here we demonstrate that mice overexpressing *Trps1* in odontoblasts (*col1a1-Trps1* transgenic mice) present with a phenotype resembling DI. *Trps1* is a GATA-type transcription factor that has been shown to play role

in endochondral bone formation. Our previous studies suggested that *Trps1* may be a negative regulator of mineralization. To understand the function of the *Trps1* transcription factor in mineralizing tissues we generated transgenic mice overexpressing *Trps1* under the control of a 2.3 kb fragment of collagen 1a1 promoter. *Col1a1-Trps1* mice develop severe post-weaning growth retardation and lethality which are secondary to malnutrition. MicroCT and histological analyses revealed tooth fragility due to diminished dentin layer in the teeth of *col1a1-Trps1* mice. Biochemical analyses of SIBLING proteins extracted from dentin of wild type and *col1a1-Trps1* transgenic mice demonstrated decreased levels of both Dsp and Dpp proteins in transgenic animals, while there was no change in the level of dentin matrix protein 1 (Dmp1). Additionally, analyses of gene expression by RNA in situ hybridization demonstrated that overexpression of *Trps1* in odontoblasts results in inhibition of the *Dspp* expression in odontoblasts, but not in ameloblasts. Furthermore, by using chromatin immunoprecipitation (ChIP) assay in odontoblastic cell lines, we have shown that *Trps1* can bind GATA consensus sites in the *Dspp* promoter. Interestingly, during tooth development *Trps1* is expressed in preodontoblasts, but not in mature odontoblasts secreting dentin matrix. These data collectively demonstrate that *Trps1* is a negative regulator of dentin formation and serves this function, at least in part, through repression of the *Dspp* gene.

O36

The expression of GM-CSF and osteopontin in immunocompetent cells precedes the odontoblast differentiation following allogenic tooth transplantation in mice

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Background: Dental pulp elaborates both bone and dentin under pathological conditions such as tooth replantation/transplantation. Once osteoclast-lineage cells appear at the pulp-dentin border, bone-like matrix deposition can be induced, even beneath the pre-existing dentin, whereas the temporal appearance of dendritic cells there induces the tubular dentin forma-

tion (Figure 1). This study aims to clarify the expression of granulocyte macrophage colony-stimulating factor (GM-CSF) and osteopontin (OPN) in the process of reparative dentin formation by allogenic tooth transplantation using *in situ* hybridization for OPN and immunocytochemistry for GM-CSF and OPN at the both level of light and electron microscope. **Methods:** Crlj:CD1 (ICR) mice, 3 weeks old, were used in this study. The upper-right first molar was extracted under anesthesia, and the roots and pulp floor were resected. The coronal portion of the sample without the periodontal tissue was immediately transplanted into the sublingual region after cutting the ventral side of the tongue of the littermates. Materials were collected in groups of animals at intervals of 1, 3, 5, 7, and 14 days after allogenic tooth crown transplantation. The upper-left M1 of the same animal was used as control. **Results:** In the control group, GM-CSF-positive reactions were not observed in either the dental pulp or the periodontal ligament, whereas OPN-positive reactions were observed in the dentinal tubules at the pulp horn in addition to the osteoblasts, cementoblasts, and the matrix of cementum and bone. On the other hand, nestin-immunoreactivity was exclusively expressed in the coronal and root odontoblasts, and the other types of cells lacked nestin-positive reactions in the dental pulp. On Days 1-3, immunocompetent cells such as macrophages and dendritic cells expressed both GM-CSF and OPN, and some of them were arranged along the pulp-dentin border and extended their cellular processes into the dentinal tubules. On Days 5-7, tubular dentin formation commenced next to the pre-existing dentin at the pulp horn where nestin-positive odontoblast-like cells were arranged. Although the expression of GM-CSF and OPN disappeared in the pulp tissue except for the prolonged expressions of OPN and rarely GM-CSF in the lesion lacking the regenerated odontoblasts, the OPN-immunopositive matrices were recognized between the pre-existing and postoperative dentin. Until Day 14, bone-like tissue formation occurred in the pulp chamber, where OPN-positive osteoblasts surrounded the bone matrix. **Conclusion:** These results suggest that the secretion of GM-CSF and OPN by immunocompetent cells plays a role for the maturation of dendritic cells and the differentiation of odontoblasts, respectively, in the regenerated pulp tissue following tooth transplantation.

O37

Analysis of Col1a1-2.3GFP transgene during odontoblast differentiation

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Odontoblasts are exclusively dentin-producing cells that are morphologically and functionally distinct from osteoblasts secreting bone matrix. Although much has been learned about the cellular and molecular mechanisms that regulate the progression of osteoprogenitor cells into fully differentiated osteoblasts, the regulatory mechanisms involved in the differentiation program of odontoblasts are not fully understood. The overall goal of this study was to use pOBCol2.3GFP reporter transgenic mice as a novel experimental model to unravel the molecular mechanisms regulating the progression of neural crest-derived mesenchymal progenitor cells into odontoblasts. Our previous analysis of pOBCol2.3GFP transgenic mice showed that 2.3-GFP transgene is expressed at low levels in functional odontoblasts and at high levels in terminally differentiated odontoblasts. In the present study we have used the primary cell cultures derived from the coronal portion of dental pulp from P5-P7 pOBCol2.3GFP transgenic mice to 1) study the stage-specific activation of 2.3-GFP *in vitro* and 2) distinguish and identify sub-populations of cells at different stages of odontoblast differentiation *in vitro* by FACS sorting for further lineage analysis and expression profiling. Primary pulp cultures derived from P5-7 pOBCol2.3GFP transgenic animals were grown under mineralization inducing conditions and processed for various analyses, including FACS analysis and sorting, RT-PCR and immunocytochemistry. Epifluorescence analysis showed weak but detectable levels of 2.3-GFP expressions in scattered cells at day 3. At day 7, elevated levels of 2.3-GFP were expressed in clusters of cells and intensified in demarcated multilayered nodules during mineralization. There was a close correlation between the areas of the cultures expressing high levels 2.3-GFP and areas of mineralization identified by Xylenol Orange (XO) staining. FACS analysis showed increases in the number and intensity of cells expressing 2.3-GFP during *in vitro* mineralization of pulp cells. Confocal microscopy showed expression of DSP in the extracellular matrix and in the cytoplasm of the 2.3-GFP+ cells associated with mineralized nodules. PCR analysis showed that 2.3-GFP was activated at early stages of differentiation

and before the expression of markers of later stages of odontoblast differentiation (BSP, OC, DMP1, and DSPP). FACS sorting based on GFP expression on 7-days old cultures was used to separate GFP+ from GFP- sub-populations. Cultures established from the 2.3-GFP+ population expressed GFP in nearly all cells and the intensity of GFP gradually increased during the 14 days of culture. In cultures established from 2.3-GFP- population, a few 2.3-GFP+ cells appeared at day 7. There were increases in the numbers and the intensity of 2.3-GFP cells at days 10 and 14. The onset of mineralization in cultures obtained from 2.3-GFP+ population was around day 10, while in 2.3-GFP- population was around day 14. Despite increases in the amount and extent of mineralized tissue in both cultures, quantitative analysis of calcium content by Alizarin Red staining showed significantly lower calcium content in cultures derived from 2.3-GFP- as compared to 2.3-GFP+. These together indicated that 2.3-GFP provides a valuable experimental tool for the identification and isolation of cells in the early and late stages of odontoblast differentiation. Supported by Grant R01-DE016689.

Dental Pulp

O38

Neuronal differentiation of human dental pulp stem cells in vitro and in vivo - potential for tissue engineering
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Background: The plasticity of human dental pulp stem cells (DPSCs) has been demonstrated by several studies. The aim was to differentiate these cells to mature neuronal cells showing functional evidence of voltage gated ion channel activities. In addition, the fate of predifferentiated, implanted DPSC cells were studied in young rats following mild brain cortical lesion. **Methods:** Normal impacted human third molars were used following extraction. DPSCs were isolated and cultured as previously reported. For

neuronal differentiation we developed a three-set developed protocol. First, cultures were pre-treated with a medium containing the demethylating agent 5-azacytidine to dedifferentiate the cells. Then neural induction was performed by the simultaneous activation of protein kinase C and the cAMP pathways. Finally, maturation of the induced cells was achieved by a neurodifferentiation medium. Then Vybrant DiD labeled neuronally induced DPSCs were transplanted into the cerebrospinal fluid of 2-days-old male rats. Cortical lesion was induced by cold exposure applying metal stamp to the calvaria over the motor cortex. For visualization fluorescent and immunohistochemistry were applied. Voltage-dependent sodium and potassium channels were investigated by patch clamp recording. **Results:** Noninduced DPSCs cells already expressed vimentin, nestin, N-tubulin, neurogenin-2 and neurofilament-M. The in vitro neuroinductive treatment resulted in decreased vimentin, nestin, N-tubulin and increased neurogenin-2, neuron-specific enolase, neurofilament-M and glial fibrillary acidic protein expression. By the end of the maturation period all investigated genes were expressed at higher levels than in undifferentiated controls except vimentin and nestin. Patch clamp analysis revealed the functional activity of both voltage-dependent sodium and potassium channels in the differentiated cells in vitro. In non-injured brain implanted cells migrated into the progenitor zones of the brain. The cells displayed modest TTX sensitive voltage dependent sodium and TEA sensitive delayed rectifier potassium currents. In injured brain, four weeks after damage the DPSC cells were found the lesion, and expressed multiple neuronal marker proteins. Their sodium currents and potassium currents were about three times larger than those in uninjured brain. **Conclusion:** Our results demonstrate that DPSC cells can be efficiently differentiated into functional neurons in vitro. In addition, these differentiated cells are able to maintain their functional characteristics when implanted into injured brain. These data clearly indicate that the dental pulp contains a cell population that is capable of neural commitment, a valuable potential source for tissue engineering. Supported by the Hungarian National Scientific Research Fund (NI 69008 and CK 80928).

O39

Establishment of *in vitro* culture system for evaluation of the dentin-pulp complex regen-

eration with special reference to differentiation capacity of the BrdU-label-retaining dental pulp cells

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Background: Our recent study has demonstrated that a pulse of the thymidine analog BrdU given to the prenatal animals revealed the existence of slow-cycling long-term label-retaining cells (LRCs), putative adult stem cells, reside in the pulp tissue. Furthermore, we have proposed the new hypothesis that both progenitors and dental pulp stem cells (DPSCs) are equipped in the dental pulp and that the DPSCs with proliferative capacity play crucial roles in the pulpal healing process following the exogenous stimuli in cooperation with the progenitors (Figure: modified from Niigata Dent J 39: 171-176, 2009). This study aims to establish *in vitro* culture system for the evaluation of the dentin-pulp complex regeneration with special reference to differentiation capacity of the LRCs using immunocytochemistry for BrdU, Ki67, nestin, and α -smooth muscle actin (SMA), and to compare the results obtained from *in vitro* system with those obtained from *in vivo* animal models such as tooth replantation/transplantation for the verification of our hypothesis. **Methods:** Three peritoneal injections of BrdU were given to pregnant CrIj:CD1(ICR) mice to map dense LRCs in the mature tissues of born animals. The labeled born animals or GFP-transgenic mice were used for *in vitro* and *in vivo* experiments. The upper-right first molars (M1) of BrdU-labeled or GFP-transgenic mice (3 weeks old) were extracted under anesthesia. For *in vitro* culture, the extracted teeth were divided into the two pieces and cultured for 0, 1, 3, and 7 days using the Trowel's method. For *in vivo* experiments, the extracted teeth were replanted in the original socket or the crown portion without roots were allogenic transplanted in the sublingual region of non-labeled animals. **Results:** We succeeded to establish the *in vitro* culture system for the evaluation of the dentin-pulp complex regeneration, where most odontoblasts were occasionally degenerated and lost nestin-immunoreactivity because of the separation of cell bodies from cellular processes in

the dentin matrix until the beginning of *in vitro* culture. Numerous dense LRCs were mainly resided in the center of the dental pulp associating with blood vessels throughout the experimental periods. On postoperative Days 1-3, the periphery of pulp tissue including the odontoblast layer showed the degenerative features, although some odontoblasts survived throughout the experimental periods. Until Day 7, nestin-positive odontoblast-like cells were arranged along the pulp-dentin border and dense LRCs were committed in the odontoblast-like cells. These chronological changes in the pulp-dentin border *in vitro* organ culture were similar to the changes in the *in vivo* experimental models. **Conclusions:** These results suggest that dense LRCs in the center of the dental pulp associating with blood vessels were supposed to be dental pulp stem cells possessing regenerative capacity for forming newly differentiated odontoblast-like cells.

O40

Responses of BrdU-label-retaining dental pulp cells to allogenic tooth transplantation into mouse maxilla

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Background: Allogenic tooth transplantation is now a common procedure in dentistry for replacing a missing tooth. However, there are many difficulties in clinical application of allogenic tooth transplantation because of immunological rejection. Recently, we have established the successful experimental animal model using mice for allogenic tooth transplantation into the maxilla (Anat Rec 292: 570-579, 2009). Furthermore, our recent study has demonstrated that BrdU given to the prenatal animals revealed the existence of slow-cycling long-term label-retaining cells (LRCs), putative adult stem cells, reside in the pulp tissue. This study aims to clarify responses of BrdU-label-retaining dental pulp cells to allogenic tooth transplantation into mouse maxilla using *in situ*

hybridization for osteopontin (OPN) and periostin and immunocytochemistry for BrdU, nestin, OPN, and periostin. Furthermore, the relationship between donor and host cells in the healing process has been analyzed using GFP mice. **Methods:** Two to 3 peritoneal injections of BrdU were given to pregnant Crlj:CD1(ICR) mice to map dense LRCs in the mature tissues of born animals. The labeled born animals at 2 weeks after birth were used for tooth transplantation. The upper-right first molars (M1) of BrdU-labeled and non-labeled mice (2 weeks old) were extracted under anesthesia, and the extracted teeth were allografted in the original socket in the non-labeled and BrdU-labeled mice, respectively, after the extraction of M1. Materials were collected in groups of animals at intervals of 1, 3, 5, 7 days, 2, 4, and 8 weeks after allogenic tooth transplantation. Furthermore, the allogenic tooth transplantation was performed between GFP and non-GFP mice. The upper-left M1 of the same animal was used as control. **Results:** In the control group, nestin-immunoreactivity was exclusively expressed in the odontoblasts, and numerous dense LRCs were mainly resided in the center of the dental pulp of BrdU-labeled animals, associating with blood vessels. Tooth transplantation caused degeneration of the odontoblast layer, resulting in the disappearance of nestin-positive reactions in the dental pulp. On postoperative Days 1-3, the pulp chamber was mainly occupied by inflammatory lesions including numerous neutrophils, fibrin networks, and a hemorrhage. On postoperative Days 5-7, tertiary dentin formation commenced next to the preexisting dentin where nestin-positive odontoblast-like cells were arranged in the successful cases. Three types of healing patterns were recognized until Day 14: tertiary dentin, the mixed form of dentin and bone-like tissue formation, and immunological rejection. In the case of BrdU-labeled transplanted teeth, dense LRCs were maintained in the center of the dental pulp beneath the newly differentiated odontoblast-like cells, whereas LRCs disappeared in the area beneath the bone-like tissue. On the other hand, LRCs were not recognized in the pulp chamber of non-labeled transplants through the experimental period. Interestingly, the periodontal tissue recovered even in the case of immunological rejection in which the pulp chamber was replaced by sparse connective tissue. In such cases, the donor periodontal tissue was replaced by the host tissue. **Conclusions:** These results suggest that the maintenance of BrdU-label-retaining dental pulp

cells is the decisive factor for the regeneration of odontoblast-like cells in the process of pulpal healing following tooth transplantation.

Tooth Bone Interface

O41

The role of Wnt signaling for patterning of molar tooth roots in mammals

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Background: The diet is one of the essential factors for survival. The tooth crown structure is adapted for diet, and thought to contribute to the diversification and adaptation of extant mammals. Importantly, the structure of tooth roots also varies to effectively support the applied force. Tooth morphogenesis is regulated by reciprocal interactions between the dental epithelium and odontogenic mesenchyme. Following tooth crown formation, the dental epithelium forms a double-layered Hertwig's epithelial root sheath (HERS) derived from inner and outer enamel epithelium. Previous studies have reported that HERS plays an important role in tooth root development. The morphology of tooth roots is considered to be determined by genetic and environmental factors, but basic information on the morphology of tooth root patterning and the molecular mechanism of root morphogenesis is largely unavailable in mammals. **Methods:** The jaws of extant mammals were collected to examine correlation between the number of major tooth cusps and tooth roots in molar teeth. The jaws of Tabby, Downless, Crinkle, and Nfkb1 mutants indicating microdontia, and of Sostdc1 null, Lrp4 null mutants indicating macrodontia were collected and analysed by microCT. The tooth raw were dissected from Top-GAL and Axin2-LacZ mice at postnatal

day 5, 6, 7, 8, 9, and 10 and performed whole-mount LacZ staining and histological analysis. **Results:** Firstly, we studied about the correlation between the number of major cusps of the tooth crown and number of tooth roots of molars and last premolar teeth in several extant mammals. The number of molar tooth roots have strong coorelation in maxilla but weak in mandible. Next, similar analysis was performed with several mouse mutants indicating abnormal molar morphology, similar coorelation was identified. The dynamic change of LacZ-staining was observed on early development of molar root in Axin2-LacZ and TOP-GAL mice, and LacZ-positive tissues were identified as developing HERS. **Conclusion:** The number of tooth roots was correlated to the major tooth cusps in extant mammals strongly in maxilla but weakly in mandible. The number of molar tooth roots are determined by the elongation pattern of epithelial diagram of HERS in early development of tooth roots, and the staining pattern of HERS of Axin2-LacZ suggested that Wnt-signaling play important role for patterning of molar tooth root

O42

Label-retaining epithelial cells in mouse epithelial cell rests of Malassez

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Slow-cycling somatic stem cells have been identified in some tissues by their ability to retain bromodeoxyuridine (BrdU) label for long periods of time. Using these BrdU pulse-chase experiments, lineage-specific stem cells have been identified in epithelium such as skin, intestine, and cornea. However, epithelial stem cells have not yet been identified in ERM. Epithelial cell rests of Malassez (ERM) are located in the periodontal ligament (PDL). ERM are clusters of epithelial cells that are derived from fragments of Hertwig's epithelial root sheath (HERS). Epithelial cell rests of Malassez in the PDL are thought to be completely quiescent. On the other hand, ERM actively proliferate when they are expanded *in vitro*, suggesting that some ERM are mitotically active. This study was designed to identify putative epithelial stem cells in ERM, based on their retention of BrdU label *in vivo*. C57BL6 mice were injected subcutaneously with BrdU daily for

three days. Tissues were obtained at postnatal day 5, 10, 32, and 90 (P5, P10, P32, and P90) to identify and characterize the label-retaining epithelial cells (LRECs) in the ERM. At P5, several BrdU-stained cells were detected in both inner and outer enamel epithelia in HERS. BrdU and cytokeratin double-stained cells were seen in the clusters of ovoid cells in the PDL at P10, P32, and P90. Furthermore, cytokeratin and Ki67 double-staining of cells with a proliferative activity was apparent in the clusters of ovoid cells in the PDL at P32. This study suggested that populations of LRECs that were quiescent, slow-cycling, or undergoing asymmetric division were identified in the ERM using BrdU pulse-chase experiments.

O43

The biology of periodontally accelerated tooth movement

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It is apparent that tooth movement is enhanced by procedures that elevate the remodeling of alveolar bone, and of periodontal and gingival fibrous tissues. The periodontally accelerated osteogenic orthodontics (PAOO) involves full-thickness labial and lingual alveolar flaps accompanied with limited selective labial and lingual surgical scarring of cortical bone (corticotomy). Most of the authors suggest that the RAP is the major stimulus for alveolar bone remodeling, enabling the PAOO. We have shown that detachment of the bulk of dento-gingival and interdental fibers from coronal part of root surfaces by itself suffice to stimulate alveolar bone resorption mainly on its PDL surfaces which largely attributes to PAOO. Our results propose that unstrained gingival fibroblasts secrete ATP which activates P2X7 purinoreceptors in human gingival fibroblasts. Also, stanniocalcin-1 is up-regulated which in turn stimulates Pi transport. It seems that extracellular ATP is modulating the pathway leading to alveolar bone remodeling. Moreover, marginal gingival fibroblast closely control the remodeling of alveolar bone. Therefore, by fiberotomy the physiological strain of marginal gingiva cells is reduced, thus activating alveolar bone resorption, this way increasing the efficiency of orthodontic tooth movement. Also, normal tension integrity of the marginal gingiva is important for normal function of the periodontium.

O44

Fibrillin-1 is indispensable for normal collagen fiber architecture and gene expression in periodontal ligament

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Background: Periodontal ligaments (PDLs) are a soft connective tissue between the cementum covering the tooth root surface and alveolar bone. PDLs are composed of collagen and elastic system fibers, blood vessels, nerves, and various types of cells. Elastic system fibers are distributed among various types of connective tissue where elasticity is required. Depending on the relative amount of elastin, they are classified into elastic, elaunin and oxytalan fibers. Oxytalan fibers, lacking elastin, are made up of bundles of 10-12-nm microfibrils which are predominantly composed of glycoproteins, fibrillin-1 and -2. In PDLs, the main elastic system fibers are oxytalan fibers oriented in an occluso-apical direction. Compared to collagen fibers, there is only limited information on microfibrils in PDLs. In order to clarify the unknown role of microfibrils in PDLs, mice underexpressing fibrillin-1 (MgR mice) were examined.

Materials and methods: Light and electron microscopic analysis was performed on PDLs of 2 to 8 week-old MgR and wild-type (WT) mice. *In situ* hybridization was carried out to examine the expression of PDL-related genes.

Results: In PDLs, homozygous mgR mice had only one quarter of the elastic system fibers compared to WT mice. Electron microscopic observation showed that microfibrils were seen adjacent to each PDL-cell, suggesting a close relationship between the cell and the microfibril in both WT and MgR mice. An association between the elastic system fibers and capillaries was noted in wild-type (WT), homozygous, and heterozygous mgR mice, and capillaries in PDLs of homozygous mice were dilated or

enlarged compared to those of WT mice. Interestingly, there were degenerative PDL-cells exclusively around blood vessels in homozygous mice but not in other genotypes. Next, structure of collagen fibers in mgR mice was examined. *In situ* hybridization showed that a comparable level of type I collagen, which is the most major collagen in PDLs, was expressed in PDL-cells of mice with three genotypes. Sections were stained with Picrosirius red solution and observed three dimensionally using a polarizing light microscope to examine the collagen fiber architecture in PDLs. The observation showed that well-organized definite collagen fiber bundles were seen in WT mice. In contrast, multi-oriented collagen fiber bundles with a thinner appearance were noted in homozygous mice. Moreover, there was a marked decrease in periostin expression in homozygous mice.

Conclusion: PDLs are well vascularized tissue, which reflects the high levels of cellular and extracellular constituents. The present study indicates the fibrillin-1 is required to maintain the normal vascularization. The exact reason for the abnormal collagen architecture in homozygous mice is unknown; however, it is interesting to speculate that the architecture is related to the suppressed expression of periostin, which is known to regulate collagen crosslinking and fibrillogenesis. The present study opens a new insight that the microfibrillar protein, fibrillin-1, is indispensable for the normal tissue architecture and gene expression in PDLs.

O45

Regulation of osteoblast differentiation and ECM remodeling by BMP2/4 in vitro

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Both of bone morphogenetic proteins 2 and 4 (Bmp2 and Bmp4) are two closely related members of the transforming growth factor beta superfamily and play diverse roles in normal and

pathological processes. However, detail understandings of mechanisms through which Bmp2 and Bmp4 exert their effects remain elusive due to their functional compensations each other. **Objective:** To study roles of Bmp2/Bmp4 in osteoblast differentiation and extracellular matrix (ECM) remodeling. **Methods:** Bmp2/4 conditional mice with Cre recombinase recognition site (loxP) were isolated from calvarial osteoblasts and transfected with simian virus 40 large antigen to generate immortalized BMP2^{C/C}4^{C/C} (iBMP2^{C/C}4^{C/C}) osteoblast lines. The BMP2/4 genes in the iBMP2^{C/C}4^{C/C} cells were double knocked out by Cre recombinase. Expression of bone-related genes in iBMP2^{C/C}4^{C/C} knock-out (KO) cells were detected by immunohistochemistry, western blot and real time quantitative PCR analyses. ECM remodeling was also observed in fluorescent microscope. **Results:** Expression of Runx2, Osx, AFT4, DMP1, OC and other genes were dramatically decreased in the iBMP2^{C/C}4^{C/C} KO cells compared to that of the iBMP2^{C/C}4^{C/C} osteoblasts. Cell differentiation and mineralization were also reduced in these KO cells by alkaline phosphatase and alizarin red S staining. Furthermore, Bmp2/4 double knock-out cells have major defects in remodeling the ECM as reflected by changes in collagen type I, IV and gelatin processing. **Conclusions:** We for the first time demonstrate the establishment of immortalized floxed Bmp2^{C/C}4^{C/C} cells and Bmp2/4 double null iBmp2^{C/C}4^{C/C} cells. Expression of bone-related genes and cell differentiation in the iBmp2^{C/C}4^{C/C} KO cells were decreased. Furthermore, ECM processing in these KO cells was impaired. This indicates the BMP2/4 play important roles in osteoblast differentiation and ECM remodeling.

O46

Non redundant role of Msx1 homeogene on neurectodermal osteogenesis

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Background: Anodontia and cranio-facial dysmorphology characterise Msx1 -/- null mutant mice. Long bone defects require additional Msx2 gene invalidation. The proper Msx1 impact on

osteogenic differentiation was studied here. The divergent cell origin (mesoderm/neurectoderm) or site (limb/maxilla) were taken into account. **Methods:** Histology, Von Kossa staining and μ CT were realised at 18 days of gestation and birth in Msx1 -/- (versus Msx1 +/- and Msx1 +/+ controls). Osteogenic and chondrogenic markers were screened in microdissected oral (maxilla and mandible without teeth) and tibial bones by RT-qPCR and in situ. In vitro, cell-cycle phases were studied under transient Msx1 overexpression. **Results:** Msx1 was shown to alter cell differentiation program: in vitro, Msx1 overexpression induced G0/G1 phase increase. In the reverse in vivo situation (Msx1 -/- mice), late bone differentiation markers were up-regulated and mineralization, enhanced. These defects were significant in oral osteoblasts where Msx2 expression was interestingly found Msx1-dependent. **Discussion:** Our results showed a non-redundant role of Msx1 on oral osteoformation. This indicates that a proper regulation of homeoprotein expression levels is independently critical for tooth and bone morphogenesis. Such a proposal was revisited by the analysis of a Utrecht/Paris cohort. Oral phenotype variability (oligodontia/clefts) were related to specific Msx1 RNA expression levels and gene mutations.

Craniofacial Biology And Tissue Engineering

O47

The planar cell polarity effector gene, *Fuz* is essential for craniofacial and tooth development

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Background: *Fuz* has been identified as a planar cell polarity effector gene and shown to be involved in ciliogenesis. We asked if *Fuz* expression was critical for craniofacial and tooth development.

Methods: *Fuz*^{-/-} mutant mice and mouse embryo fibroblasts were used to analyze the effect of *Fuz* expression on tooth development and gene expression pathways, respectively. Immunohistochemical, molecular and biochemical assays were used to understand the defects and molecular mechanisms underlying the phenotype of the *Fuz*^{-/-} mice.

Results: The *Fuz*^{-/-} mutant mice exhibit massive craniofacial deformities such as a hypoplastic mandible, a complete lack of upper and lower

incisor development, malformed molars, hyperplastic Meckel's cartilage and missing eyes and tongue. *Fuz* has an essential role in ciliogenesis, and the primary cilium has been shown to repress canonical Wnt signaling. We demonstrate that canonical Wnt signaling is up regulated in *Fuz* mutant mice, shown by increased β -catenin expression. Given the critical function of Wnt signaling in craniofacial development, we believe that this up-regulation of Wnt signaling results in hyperplastic cartilage formation and disruption of incisor and tongue development as well. Over-expression of *Fuz* represses the Topflash reporter demonstrating a role for *Fuz* in canonical Wnt signaling. The *Fuz* 2.4kb promoter contains eleven Wnt response elements, and LiCL treatment can activate both *Fuz* 2.4kb and 1.1kb promoter constructs and endogenous *Fuz* in cell cultures. We have identified several transcription factors that are regulated indirectly by *Fuz* through Wnt and Shh signaling mechanisms, which are associated with specific craniofacial developmental processes. **Conclusions:** These data suggest that *Fuz* is involved in a negative feedback loop of canonical Wnt signaling regulation, and this feedback loop is essential for precise regulation of craniofacial and tooth development. Support for this research was provided from grant DE13941 from the National Institute of Dental and Craniofacial Research.

O48

Interactions between *Gas1*, *Cdo* and *Boc* during early development of the craniofacial midline and dentition

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Background: Sonic hedgehog (Shh) is a secreted signaling molecule essential for normal craniofacial development. Disruptions within the Shh signalling pathway can result in Holoprosencephaly (HPE), a developmental disorder characterized by failure of the forebrain to divide and varying degrees of midline facial anomalies. *Gas1*, *Cdo* and *Boc* are membrane proteins able to bind to Shh and regulate transduction of the signal. Both *Gas1*^{-/-} and *Cdo*^{-/-} mice exhibit disrupted craniofacial midline patterning and microform HPE as a result of reduced Shh signalling activity; however, *Boc*^{-/-} mice have normal craniofacial structures. Interestingly, in the absence of *Gas1* function, supernumer-

ary premolar teeth also form in the diastema region of both jaws with complete penetrance.

Methods: We have investigated the relationship between *Gas1*, *Cdo* and *Boc* during early craniofacial development using phenotypic analysis of varying combinations of *Gas1/Cdo* and *Boc* mutant mice.

Results: We find combinatorial expression of these genes within overlapping domains in the embryonic head. Loss of a single allele of either *Gas1* or *Cdo* on a *Gas1*^{-/-} or *Cdo*^{-/-} background respectively, results in exacerbation or increased penetrance of the midline craniofacial phenotype. Moreover, *Gas1*^{-/-/Cdo}^{-/-} mice display a severely dysmorphic craniofacial midline, with many structures either synostotic or absent. In contrast, the loss of *Boc* does not affect gross craniofacial development in combination with either *Gas1* or *Cdo*. However, whilst an important individual role and strong interaction was demonstrated between *Gas1* and *Cdo*, but not *Boc* during midline craniofacial patterning, different requirements amongst all these genes were clear during tooth development. Whereas *Gas1*^{-/-} mice display both maxillary and mandibular supernumerary premolar teeth, tooth morphology and number in both *Cdo*^{-/-} and *Boc*^{-/-} mice was normal.

Conclusions: This phenotypic analysis has demonstrated a strong interaction between *Gas1* and *Cdo* that influences Shh signaling activity during early craniofacial and tooth development. However, these requirements appear to vary between different regions of the craniofacial complex during development.

O49

Variation of tooth root lengths in human X chromosome aneuploids

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Background: Human tooth development is a continuous process from primary to permanent teeth, the permanent molars developing as clones from the second primary molars. Teeth are single organs and develop from the tip of the crown to the root, and their size is final once the root apex has closed. Murine studies have shown that separate homeobox-containing genes and signalling molecules are expressed in the anterior and posterior fields during den-

tal development. Mutations in tooth-specific genes usually affect all teeth of the same type in mice, but in humans the teeth in the clone (ICM shape series) that develop later are the first to be absent. Tooth patterning in humans seems to be more complex, indicating that each clone is separately controlled. More than 300 genes are now known to be involved in tooth development. Human sex chromosomes also influence human tooth root lengths, men having longer roots than women. The aim of this study was to compare and describe the variation of the X chromosome influence on tooth root lengths in human X chromosome aneuploids.

Methods: Permanent tooth root lengths in 56 normal women were compared with those in seven 47,XXX, three 48,XXXX and fifteen 45,X/46,XX females studied in the Kvantti research project headed by Professor Alvesalo. Measurements were made on panoramic radiographs of the permanent teeth (except third molars) on both sides of the jaws available from three 48,XXXX females and three female relatives using a digital calliper and following established procedures. The other root lengths, measured in the same manner, have been published earlier.

Results: The influence of the X chromosome on tooth root lengths differed between the maxilla and mandible in all the groups studied. Its additive effect of the X chromosome was obvious in the mandible and was larger anteriorly. In the maxilla the root lengths with one extra X chromosome were similar to those in normal women but those with two extra X chromosomes were posteriorly longer than in any other group, and while root development was inhibited anteriorly. A lack of sex chromosome material had an overall inhibiting effect on root length development.

Conclusions: The results showed variations between the jaws and between the anterior and posterior regions of the permanent maxillary dentition in the tooth root length differences between X chromosome aneuploids and normal women. It is suggested that the genetic influence of the X chromosome on permanent tooth root development is mediated by a number of inductive and inhibiting agents.

Keywords: Aneuploidy, chromosomes human X, dental field, growth and development, tooth root

O50

Characterization of two novel genes expressed in tooth and associated epithelia

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Background: Our group previously conducted a functional genomic screen for cDNAs encoding secreted and membrane proteins in rat enamel organs (EOs). This allowed us to identify and characterize Amelotin and Apin (Odam) as novel secretory products of maturation stage ameloblasts and junctional epithelial (JE) cells. We present here two additional novel genes expressed in the tooth referred to as clones *EO063* and *EO463*.

Methods and results: Northern blotting on a panel of RNAs extracted from rat tissues reveals that the expression of *EO063* and *EO463* is restricted to tooth. In microdissected strips of rat EOs, transcripts of *EO063* are more abundant in the secretory stage, whereas those of *EO463* are highly enriched during the maturation stage. In silico data mining indicates that *EO063* matches a mouse Riken clone (5430401F13). The gene is present in 3 copies in the mouse (chr. 6F3) and in a single copy in the rat (chr. 4q42), but it is apparently absent in human. *EO063* encodes for a secreted protein having 145 residues and a C-terminal enriched in glycine, glutamine, and lysine. To understand the function of *EO063* in vivo, we generated a transgenic mouse model overexpressing Flag-tagged-*EO063* under the ameloblastin promoter. No obvious alterations on molars and incisors were observed. This was probably due to the fact that *EO063*-Flag protein expression was below immunodetectable levels despite obvious transgenic expression at the mRNA level. Hence, this model did not allow us to determine the role of *EO063* in tooth formation and mineralization. The *EO463* clone does not correlate to any known sequences available in various databases. The gene encoding *EO463* is also not annotated in existing genomes and is considered totally novel. Cloning of the cDNA for *EO463* allowed us to precisely map the gene and to determine its chromosomal architecture. The *EO463* gene resides within the so-called 'secretory calcium binding phosphoprotein' (SCPP) cluster. In several mammal genomes *EO463* contains 10 exons transcribed in the same orientation as the adjacent *SPARCL1* gene. Very recently, Kawasaki (Dev. Genes Evol. (2009) 219:147-157) identified *EO463* gene by in silico cloning and named it *SCPPPQ1*. *EO463*

is a highly conserved short 75 residue long secreted protein rich in proline, leucine, glutamine and phenylalanine. Using an antibody generated against the bacterial recombinant protein, EO463 was strongly immunodetected along the apical surfaces of maturation stage ameloblasts, in line with its gene expression profile. No or very little signal was found over the enamel layer suggesting it does not accumulate to any significant extent within the calcified matrix. The only other site immunoreactive for EO463 was the JE which attaches to erupted tooth surfaces. The immunolabeling pattern for EO463 in the EO and JE is reminiscent of Amelotin and Apin. **Conclusion:** Together with their respective sequence features, clustering and phylogenetic evolution within the SCPP locus, their expression at a developmental time when the enamel layer becomes almost fully mineralized is consistent with the intriguing possibility that Amelotin, Apin and EO463 may have evolved as “hypermineralizing” molecules. Their presence at the cell-matrix interface where an atypical basal lamina is present further suggests that these unique molecules may be part of the multimolecular complex involved mediating attachment of epithelia to mineralized tooth surfaces.

O51

Genomic copy number alteration analysis of sporadic and Gorlin-syndrome associated keratocystic odontogenic tumour (odontogenic keratocyst)

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Background: Apart from reported aberrations in the PTCH1 gene (9q22-31), very little is known about the genomic structural rearrangements in sporadic and Gorlin syndrome (basal cell nevus syndrome, BCNS, OMIM #109400) associated keratocystic odontogenic tumour (KCOT). The aim of the present study was to find out deletions and duplications in the genome, collectively referred to as copy number alterations (CNA), in KCOT. Further aim was to find out po-

tential differences in the CNA regions between the sporadic and syndrome associated forms of the tumour to aid their differential diagnosis.

Methods: DNA originating from 20 sporadic and seven Gorlin-syndrome associated KCOT samples were included in this study. All samples were hybridized on Genome-Wide Human SNP arrays 6.0 (Affymetrix®) and the chromosomal regions with copy number changes (gains and deletions) were analyzed using Genotyping Console 3.0 (Affymetrix®) software. Additional analyses included loss of heterozygosity (LOH). Principal component analysis (PCA), hierarchical clustering and Pearson correlation coefficient were adopted for bioinformatics analyses.

Results: Gorlin-associated KCOT samples exhibited more CNA and LOH regions than the sporadic tumours. PCA and hierarchical clustering mapped most of the sporadic tumours and three Gorlin-associated tumours together indicating similarity in their molecular pathogenesis, whereas the remaining four Gorlin-associated tumours formed its own cluster.

Conclusions: Gorlin-associated KCOT shows more genomic aberrations than sporadic KCOT, which is in accordance with its more aggressive behaviour. Furthermore, neither sporadic nor Gorlin-associated KCOT appears to share common CNA regions, which could have offered an option for future discovery of a diagnostic biomarker.

Keywords: DNA segments, genome-wide studies, molecular biology, intermediate-scale, structural abnormalities, odontogenic tumours

O52

Tooth engineering: Organization of dental matrices in implanted dental cell-cell reassociations

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Background: Embryonic dental cells have been used to set up an experimental approach for tooth engineering (1) and check a set of criteria to be achieved for this purpose. We have successively answered a series of constraints to be respected in order to obtain crown morphogenesis, epithelial histogenesis, organ vasculariza-

tion, as well as root and periodontium development (1,2,3). The purposes of the present work were to investigate the organization of the different dental matrices (predentin/dentin, enamel and cementum), which form and mineralize after implantation of cultured cell-cell reassociations, and compare these matrices to those forming after implantation of cultured tooth germs. **Methods:** First lower molars from ED14 mouse embryos were dissected and dissociated in epithelium and mesenchyme using trypsin as previously published (2). For each tissue, cells were further dissociated as single cells. Pellets of epithelial cells were cultured in contact with mesenchymal cells for 8 days on a semi-solid medium and implanted for two weeks under the skin of adult ICR mice (1,3). These implants were processed for histology and TEM. For comparison, molar tooth germs were cultured for 6 days before implantation. **Results:** After two weeks of implantation, the cultured cell reassociations were fully re-vascularized as cultured tooth germs (3). In the reassociations, gradients of odontoblasts differentiated. These cells were in close contact with capillaries. They were ciliated, polarized and extended cell process in predentin and dentin. This context allowed the induction of ameloblast functional differentiation. These cells accumulated enamel, which also mineralized. Enamel crystals organized in implanted cell-cell reassociations as in tooth germs. Finally in the developing root, odontoblast differentiation proceeded, cementogenesis occurred, and periodontal ligament fibers could form and interact with the root surface and newly formed bone. This was observed in both types of implants. **Conclusion:** This work shows that the implantation of cultured dental cell-cell re-associations allows reproducing a correct functional differentiation of odontoblasts, ameloblasts and cementoblasts. The same will have to be searched for, in reassociations where one dental compartment is replaced by non-dental cells. **References:** 1) Hu B, Nadiri A, Kuchler-Bopp S, Perrin-Schmitt F, Peters H, and Lesot H. Tissue engineering of tooth crown, root and periodontium. *Tissue Engineering*, 2006, 12, 2069-2075. 2) Hu B, Nadiri A, Bopp-Kuchler S, Gendault JL and Lesot H. Dental epithelial histo-morphogenesis in vitro. *J. Dent. Res.*, 2005, 84, 521-525. 3) Nait Lechguer A, Kuchler-Bopp S, Hu B, Haïkel Y, and Lesot H. Vascularization of engineered teeth. *J Dent Res*. 2008, 87, 1138-1143.

Evolution And Development

O53

Of teeth, pouches and pores: the ectoderm/endoderm connection in development and evolution

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In basal osteichthyans, teeth are distributed throughout the oropharynx, including the roof of the oral cavity, and the surface of the hyoid and five more posterior pharyngeal arches. In actinopterygians and sarcopterygians, teeth became restricted to the mandibular and to the last pharyngeal arch (constituting the oral and pharyngeal dentition, respectively). We postulate that the presence of oral and pharyngeal teeth in the various osteichthyan lineages is linked to the potential of competent tooth-forming ectoderm to penetrate into the oropharyngeal cavity via regular openings, such as the mouth, the spiraculum, the opening of the nasopharyngeal duct or of the pharyngeal pouches (gill slits), and we propose that the ectoderm acts instructively or permissively in tooth formation. The fossil record presents many examples that support this view. Zebrafish (*Danio rerio*), an increasingly popular model to study non-mammalian teeth, possesses pharyngeal teeth only. We use zebrafish to monitor the sequence of events and the cellular changes that characterise the formation of the endodermal pharyngeal pouches and their contact with the ectoderm (skin). Contacts are established already at 24 hrs post-fertilisation as an extremely flattened epithelial sheet connecting the two body sides, followed by substantial proliferation and shape changes within this epithelium. Pharyngeal tooth formation precedes cavitation within this solid epithelial strand. Zebrafish *van gogh* mutants, defective in the transcription factor *tbx1*, display impaired pouch formation (Piotrowski et al., 2003). Interestingly, these mutants show defects in their pharyngeal dentition, coinciding with the level of impairment of ectodermal-endodermal contacts. These data are in support of our hypothesis that the loss of ectodermal-endodermal contacts, and thus of (open) gill slits, could be responsible for the evolutionary loss of pharyngeal teeth in tetrapods (Huysseune et al. 2009). Huysseune et al. 2009. *J. Anat.* 214: 465-476. Piotrowski et al. 2003. *Development* 130: 5043-5052

Rudimentary structures and dental anomalies

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Background: Rudimentary structures (rudiments) are abortive primordia of developmental features of ancestors that are only found in embryos. Under specific conditions, rudiments can express a retained old developmental potential and give rise to an extinct phenotypical feature (atavism or reversal), (Hall, 2003). Rudimentary structures occur during odontogenesis in various animal species (Peterkova et al, 2006), and represent an integral component of the developing dentition in the laboratory mouse (Peterkova et al, 2002). For example, although premolars are absent in muroids, the rudimentary anlage of premolar teeth transiently develops in mouse embryos and can give rise to a supernumerary tooth in mice with gene alterations. Such a supernumerary tooth is, at least partially, homologous with a premolar lost during evolution, and so represents an evolutionary recovery (atavism) (Peterkova, 1983; Peterkova et al, 2005).

Aim: The goal of this study was to search for and to document further examples of pathologies that can be correlated with rudimentary structures in the oral cavity.

Methods: Examination of the oral cavity, histological techniques, analysis of serial histological sections and 3D reconstructions were used.

Results: Prenatally, rudimentary structures transiently develop during the formation of the dentition and oral vestibule in mice and humans. These observations were completed by published data on rudimentary structures in the oral cavities of several species (for review see, e.g., Peterkova et al, 2006) and compared with current knowledge on developmental anomalies of the dentition (e.g., recent reviews by D'Souza and Klein, 2007; Fleischmannova et al, 2008; Townsend et al, 2008; Cobourne and Sharpe,

2010) and with clinical reports on oral pathologies in humans. The comparison showed that some types of tooth developmental anomalies (supernumerary teeth, supernumerary cusps) and dentigerous cysts occur at loci where rudimentary structures transiently develop prenatally.

Conclusion: Rudimentary structures can be implicated in the origin of supernumerary formations in the oral cavity under pathological or even physiological conditions.

References: Hall BK. Biol Rev Camb Philos Soc. 2003;78:409-33.

Peterkova R, Lesot H, Peterka M. J Exp Zool B Mol Dev Evol. 2006;306:234-50.

Peterkova R, Peterka M, Viriot L, Lesot H. Connect Tissue Res. 2002;43:120-8.

Peterkova R. J Craniofac Genet Dev Biol. 1983;3:133-42.

Peterkova R, Lesot H, Viriot L, Peterka M. Arch Oral Biol. 2005;50:219-25.

D'Souza RN, Klein OD. Cells Tissues Organs. 2007;186:60-9.

Fleischmannova J, Matalova E, Tucker AS, Sharpe PT. Eur J Oral Sci. 2008;116:1-10.

Townsend G, Harris EF, Lesot H, Clauss F, Brook A. Arch Oral Biol. 2009;54 Suppl 1:S34-44.

Cobourne MT, Sharpe PT. Semin Cell Dev Biol. 2010. Jan 18. [Epub ahead of print]

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O55

Signaling centers in mouse embryonic mandible reflect an ancestral tooth pattern

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Background: Mouse molar development has long been an important experimental model. Although mice have only three molars and no premolar, two large rudimentary (vestigial, diastemal) buds transiently develop in both maxilla and mandible of mouse embryos in front of molars, and have been correlated with premolars lost during mouse evolution (Peterkova et al, 2000). Since the transient development of these rudimentary buds has been claimed for years only on the basis of morphological criteria, we used an experimental approach to further support their existence and their dental origin in the mouse embryonic mandible, where they are called MS and R2.

Methods: We used Shh reporter mice, whole mount in situ hybridization, histology and 3D reconstructions in mouse embryonic mandible. The mouse embryos were precisely staged on the basis of day of embryonic development (ED) combined with the wet body weight of embryos (Peterka et al., 2002).

Results: The Shh signal was co-localized with the rudimentary buds on frontal sections and 3D reconstructions made from the whole mount in situ hybridized mandibles. These data showed that each rudimentary diastemal bud has its own signaling centre with a transient Shh activity. These Shh-signaling centres appeared sequentially at embryonic day (ED) 12.7 and 13.3, and the duration of the Shh expression could be determined on the basis of a range of embryonic body weight: MS at ED12.7, range 65 - 100 mg, R2 at ED13.3, range 125 - 160mg. The Shh expression in the primary enamel knot of the first molar (M1 pEK) started at ED 14.3, in embryos heavier than 210mg. Both MS and R2 signaling centers also expressed other markers that have been reported in the signaling center M1 pEK (Jernvall et al, 1994).

Conclusion: These data document that the two rudimentary diastemal buds are tooth primordia which exhibit their own transient signaling centres, develop in the mouse embryonic mandible anterior to and before the forming M1, and thus reflect the tooth pattern in the ancestors of muroids.

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References: Peterková R, Peterka M, Viriot L, Lesot H (2000). Dentition development and budding morphogenesis. *J Craniofac Genet Dev Biol* 20:158-72. Peterka M, Lesot H, Peterkova R (2002). Body weight in mouse embryos specifies staging of tooth development. *Connect Tissue Res* 43:186-190.

Jernvall J, Kettunen P, Karavanova I, Martin LB, Thesleff I (1994). Evidence for the role of the enamel knot as a control center in mammalian tooth cusp formation: non-dividing cells express growth stimulating Fgf-4 gene. *Int J Dev Biol* 38:463-469.

O56

Evolutionary analysis of enamelin in mammals, sauropsids and amphibians provides new insights on its function

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Enamelin (ENAM) plays an important role in the mineralization of the forming enamel matrix but the functions of its different regions and residues are still poorly understood. Here, we have compiled the results of an evolutionary analysis of mammalian ENAM and the characterization, for the first time, of ENAM in non mammalian vertebrates. The main objectives of these studies were to (i) identify highly conserved residues or regions that could have important function (selective pressure), (ii) predict mutations that could be associated with *amelogenesis imperfecta* in humans, (iii) reveal possible adaptive evolution of ENAM during 200 millions years (Ma) of mammalian evolution, and (iv) improve our understanding of ENAM origin and evolution.

Indeed, although ENAM was known only in mammals, previous molecular analyses strongly suggested that this protein was present early in vertebrates.

This finding was supported both by the ENAM relationships with the other enamel matrix proteins (amelogenin and ameloblastin) and the presence of enamel/enameloid tissues in early jawless vertebrates, 450 Mya. We have obtained the sequences of (i) ENAM mRNA in two reptiles, a lizard and a crocodile, and in a frog, and (ii) the gDNA of frog and lizard ENAM. Using these sequences we have been able to find the putative sequence of ENAM pseudogene in the chicken genome. Taken together our results show a remarkable conservation pattern in the region of the 32 kDa fragment of ENAM, especially its phosphoryla-

tion, glycosylation and proteolytic sites. In primates and rodents, we have also identified several sites under positive selection, which could indicate recent evolutionary changes in ENAM function. Twenty five amino acids have been conserved unchanged during 350 Ma of tetrapod evolution. These data provide new directions for further investigation of ENAM functions and reveal several positions that are predicted to lead to *amelogenesis imperfecta* when changed. Furthermore, our studies indicate (i) the recruitment, in mammals, of an unusual signal peptide that provides new insights on the possible regulation of ENAM secretion, (ii) the presence of an additional coding exon 8b in the common amniote ancestor, and (iii) many sequence variations in the large exons compared to conserved regions in the small exons encoding the N-ter of the protein. Furthermore, lizard *ENAM* was identified in a region that was not expected when considering gene synteny in mammals. This discovery allowed to find the *ENAM* pseudogene in an homologous region of the chicken genome. In contrast in the frog the enamel gene cluster is conserved in a similar synteny as in mammals. This result indicates that the enamel gene cluster was probably translocated to another chromosome in an ancestor of the sauropsid lineage. Our studies indicate that *ENAM* origins have to be found earlier in vertebrate evolution and *ENAM* invalidation in chicken confirms that this gene encodes a tooth specific protein.

O57

The evolution and development of the mammalian dentition: Insights from the marsupial *Monodelphis domestica*

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To understand developmental mechanisms of evolutionary change, we must first know how different morphologies are formed. The vast majority of our knowledge on the developmental genetics of tooth formation derives from studies in mice, which have only molars and incisors and only one tooth generation. In contrast, the marsupial *Monodelphis domestica* has a heterodont dentition with incisors, canines, premolars, and molars on both the upper and the lower

jaws. The complexity of the *M. domestica* dentition ranges from simple, unicusped incisors to conical, sharp canines to multicusped molars. Here, we present data on the development of the teeth in *M. domestica* that reveals the normal program of development of these teeth as compared to mouse wild-type and mutant dentitions. We show that the tooth germs of *M. domestica* express fibroblast growth factor (FGF) genes and Sprouty genes in a manner similar to wild-type mouse molar germs, but with a few key differences. We find differences in the expression of *Fgf10* and *Sproutys 2* and *4* along the tooth row of *M. domestica* as compared to mouse and hypothesize that these differences are attributable to differences in tooth shape.

O58

Developmental mechanisms in the evolution of morphological key innovation in small mammals

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Since mammalian appearance in the Triassic period, different morphological key innovations in mammalian tooth evolution were involved in their adaptive radiation 65 My ago (for example, strengthen of the enamel structure, cusps appearance, hysodonty...). Despite the more recent evolutionary origins of voles (5-6 My), they show all the features of an explosive radiation leading to the appearance of modern voles 2 My ago. In temperate climates, voles supplanted mice species by a rapid ecological and morphological diversification. We have already demonstrated that, during this radiation event, vole tooth evolution is characterized by a sudden and stable elongation of the first lower molar (m1). Moreover, this radiation event is contemporaneous with the onset of the Pliocene glacial/interglacial cycles. There is an ongoing debate about the factors affecting phenotypic evolution between developmental constraints and environmental pressure. Our aim is to understand the developmental mechanisms involved in the m1 elongation of voles.

Two first objectives are developed in this direction: 1) which specific genes can be expressed in the m1 anterior part of voles? and 2) which growth factors are involved in the elongation of the m1 anterior part of voles? The first objective is studied through gene expression experiments with *in situ* hybridization of the molars at early embryonic developmental stages (E13 and E14). Results of this first objective are used to perform *in vitro* experiments to functionally target the main growth factors responsible in the m1 elongation of voles. These experiments will help to uncover developmental mechanisms that allow rapid evolutionary change in small mammals.

Teeth at the margins in lungfish fill a phylogenetic gap

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Bilateral symmetrical addition of teeth sequentially along the jaws is normal for development of any vertebrate dentition. One of the striking aspects of the Australian lungfish is that only the lower jaw marginal teeth form in this way, as what differentiates lungfish from other osteichthyan fish is the palatal tooth plate. The characteristic tooth plate phenotype is in marked contrast to the typical osteichthyan dentition with marginal rows of teeth. In lungfish a radial pattern of sequential tooth addition in dental development is unique for lungfish, present in all three living genera and retained from 400 MYBP. Genetic pattern information, for evolution of the dipnoan phenotypic dentition into the disparate types occurred early in the Devonian record. The balance of two processes, odontogenesis and odontoclasia is towards complete tooth loss in the marginal tooth rows, and this happens early in development. One phylogenetic position of lungfish within the Osteichthyes is as the immediate living sister group of the tetrapods and provides the closest link between them. To understand how the dipnoan dentition was derived through developmental evolution from the generalised osteichthyan one it replaced, we have studied development of the marginal dentition on the lower jaw in *Neoceratodus forsteri*. Crucial data is from the sequence and timing of tooth initiation in the dentary row and the activation of genes associated with this developmental process. We can now claim that the early

and initial patterning of the marginal dentition is one that is stereotypic for other osteichthyans. The early pattern of tooth loci in *Neoceratodus* is observed to be a single pioneer tooth at position two, then sequential teeth added in adjacent tooth positions, first three and then one, on the lower jaw. *Shh* is one gene in the molecular cassette for the developmental mechanism that is essential to iteratively create teeth in three crown group gnathostome species. Novel probes were made by extracting the cDNA from a staged series of embryos for these key events in development of the dentition. From *in situ* hybridization studies *shh* expression in *Neoceratodus* is shown to be intensely focused on tooth positions at different times corresponding with their initiation order. Cyclopamine inhibition of *shh* signalling prevented tooth formation in relation to stage specific initiation and retarded development of teeth initiated prior to experimental treatment. Moreover, different levels of *Nfshh* expression reveal cryptic timing differences of tooth initiation at selected developmental stages. This observation was checked in stained and cleared skeletal preparations, in all toothed bones in 6 stages of the hatchling dentition. A previously unknown bilateral asymmetry of timing was apparent at all stages of development with the left side in advance of the right in all new tooth positions. Variation in symmetry of tooth initiation is a novel example of regulation of development in a bilaterally symmetrical system.

O60

How bats reach perfect occlusion? Late odontogenesis of tribosphenic molar

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Background: The vast majority of odontogenetical studies are concerned with the early stages of molars and incisors of mouse. Little attention was paid to developmental dynamics of the tribosphenic molar, i.e. the tooth type ancestral to all diverse types of mammalian molar teeth. The complicated system of crests and cusps and perfect pattern of interlocking between the occluding upper and lower teeth are perhaps the most prominent characteristics of the tribosphenic dentition. **Methods:** To understand the way the inter-

locking pattern is established, we studied postnatal dental development (including premature enamel coat) in a bat, *Myotis myotis*, from newborns where molars were already in bell stage, enamel maturation was not finished and tooth eruption was about to start, to the stage of completely established adult dentition pattern. The complete series covered 98 juvenile individuals (mostly of known age). We studied them with the aid of detailed craniometry and scanning electron microscopy. **Results & conclusion:** We found that (i) molars exhibit a considerable expansion in size during their eruption, (ii) mutual position of particular molar elements (cusps, crests) is not entirely fixed until the moment of eruption what together with (iii) delayed enamel maturation make teeth flexible and capable to refine the crest interlocking pattern during first occlusion efforts. (iv) The eruption of lower molars (especially trigonids) is faster and their posteruptional dimensions are more constant, what suggests they can serve as the template for the upper molars.

O61

Tooth replacement in *Xenopus tropicalis*: A WNT-WNT situation

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The most popular model to study tooth development, the mouse, has an evolutionary very derived dentition in which teeth are never replaced. To understand the process of continuous tooth replacement which characterizes all non-mammalian vertebrates, an experimental model is required for which a large and diverse set of genetic and molecular tools is readily available. The frog *Xenopus tropicalis* has recently emerged as a popular model to address developmental questions. For instance, the WNT pathway, suggested to play a crucial role during continuous tooth replacement (Huysseune & Thesleff 2004), can be visualized using a transgenic approach and studied functionally by gain- and loss-of-function strategies. Although the dentition of *X. tropicalis* in many ways resembles that of its close relative *X. laevis*, aspects relevant to the tooth replacement process have not been well documented. Such knowledge is nevertheless required to correctly interpret data from functional studies. Here we describe tooth formation and replace-

ment in *Xenopus tropicalis* using histological sections, focusing on the identification of the dental lamina, and mapping the epithelial connectivity. A multi-layered dental lamina, running parallel with the oral epithelium, links all teeth (whether developmental stages or functional teeth) to each other and to the oral epithelium. Also, another cell layer links the outer dental epithelia of a functional tooth and its successor within a tooth family. We discuss the role of these different cell layers during tooth replacement. In addition, we have analyzed the activity of the WNT pathway using a new transgenic reporter line. Preliminary results suggest activation of the pathway in early cytodifferentiation, but not in initiation stages of tooth germs. We discuss the potential role of the WNT pathway during continuous tooth replacement in this species and elaborate on the similarities and differences to what is known on tooth development in other model organisms. Huysseune, A. and I. Thesleff. 2004. Continuous tooth replacement: Possible involvement of epithelial stem cells. *BioEssays* 26: 665-671.

Workshop

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***In vitro* investigations of the Pax9/Msx1/Bmp4 interrelationship in tooth bud mesenchyme**

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In vivo and *in vitro* evidence points to an interaction between Pax9, Msx1 and Bmp4 during early tooth development. *In vivo* experimentation showed that Bmp4 from dental epithelium (DE) induces Msx1 in dental mesenchyme (DM) which consequently with the help of unknown cofactor(s) leads to the expression of Bmp4 in DM. Pax9 was found to be expressed in DM independently from Msx1 and Bmp4 but to be necessary for the expression of both, Msx1 and Bmp4 in DM. Our earlier *in vitro* investigations seemed to provide a straight forward explanation for the *in vivo* observations: We found that wild type Pax9, but neither Msx1 nor hypodontia-causing mutants of Pax9, can activate gene transcription from proximal *Bmp4* and *Msx1* promoter con-

structs. Pax9 and Msx1 co-immunoprecipitate and low (but not high) concentrations of co-transfected Msx1 potentiate the Pax9-induced activation of *Bmp4* and *Msx1* promoters. This synergism between Msx1 and Pax9 has so far been the only mechanism that could explain the *in vivo* observed dependence of Bmp4 expression on Msx1 in tooth bud mesenchyme. Our more recent *in vitro* studies show that

1. Pax9 can only be co-immunoprecipitated (Co-IP) with Msx1 not with Msx2, Dlx1, Dlx2 and Dlx5. The physical interaction during Co-IP is not mediated by the homeodomain of Msx1 and is not related to the functional interaction with Pax9 during promoter activation.
2. Potentiation of the Pax9-induced Bmp4 activation is achieved by both, Msx1 and Msx2 but not by Dlx proteins. It must be mediated either by the homeodomains of Msx1 and Msx2 or by the conserved C-terminal amino acid sequence common to both, Msx1 and Msx2.
3. Potentiation of the Pax9-induced promoter transactivation by Msx2 is consistently stronger with the human *Bmp4* proximal promoter than with an equivalent of the mouse *Bmp4* promoter.
4. Mutant forms of Pax9 that cause human tooth agenesis lead to a loss of DNA binding ability which correlates with the severity

of the patient phenotype.

5. Most mutant forms of Msx1 that cause human tooth agenesis do not affect the transcriptional cooperation with Pax9 in the activation of proximal *Bmp4* or *Msx1* promoters.
6. The transcriptional activity of more than 70kb of 5'extended *Bmp4* gene sequence is repressed by Msx1 except for one short area which shows slight activation. We will test if this area contains the enhancer which controls Bmp4 expression in tooth bud mesenchyme (Chandler et al. 2009)

These data indicate that:

The protein interactions between Msx1 and Pax9 seen in Co-IP can explain the decreased efficiency of the Msx1/Pax9 synergism which is observed with higher Msx1 concentrations: Large amounts of Msx1 are likely to sequester Pax9 by forming inactive complexes. Msx2 may play a more important role in human odontogenesis as compared to mouse. Pax9's involvement in human tooth agenesis appears to be primarily a consequence of the loss of DNA-binding capacity. Msx1's ability to cause human tooth agenesis is largely independent from its potentiation of Pax9 induced Bmp4 activation suggesting that there must be other mechanisms for Msx1 to activate Bmp4 such as *Bmp4* enhancer engagement or a different pathway for Msx1 to cause hypodontia.