COST Action B23 ORAL FACIAL DEVELOPMENT AND REGENERATION
Joint Meeting of the Working Group 1–4 and the Management Committee
GROWTH FACTORS IN CRANIOFACIAL DEVELOPMENT AND REPAIR
4–7 May 2006

GROWTH FACTORS AS REGULATORS OF TOOTH SHAPE DURING EVOLUTION

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Whereas developmental biology studies of mice are uncovering details about molecular signaling and morphogenesis, present day knowledge about development remains often too crude to address question about evolutionary transitions. Small changes in spatial configuration of cusps is a frequently modified aspect of mammalian evolution. As intercellular signals regulating growth and placement of enamel knots, growth factors are likely to be the final mediators of evolutionary tinkering. Recent avenues to study how growth factors shape organs include 'modulatory' signaling molecules which seem to fine tune the overall effects of signaling molecule networks. Mice with dental phenotypes that lack functional copies of these modulatory molecules still have teeth but with altered cusp morphologies. These mice allow testing hypothesis how individual teeth and cusps are partitioned during morphogenesis. Both mathematical models and experimental evidence suggest that teeth lack a ‘hard-wired’ code for tooth number, cusp number or cusp positions. Rather, a dynamic balance between growth factors stimulating differentiation or growth regulate the final pattern.

Keywords: patterning, ectodin, ectodysplasin

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LOSS OF SPROUTY2 OR SPROUTY4 LEADS TO DEVELOPMENT OF SUPERNUMERARY TEETH BY MODULATING FGF SIGNALING

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Mammalian tooth development is controlled by signaling between the oral epithelium and the neural crest-derived ectomesenchyme. These interactions are mediated by several signaling pathways, including those activated by Fibroblast Growth Factors (FGFs) via binding to FGF receptor-tyrosine kinases (FGFRs). Members of the Sprouty family are intracellular proteins that modulate FGF signaling by antagonizing it downstream of the receptor. Our gene inactivation studies in mice show that two Sprouty family member, *Spry2* and *Spry4*, are essential for the formation of the normal number of teeth. In mice, a toothless gap known as a diastema is present between the incisor and molar regions. During embryogenesis, tooth primordia form in the diastema but cease developing and undergo apoptosis at the late bud stage. In both *Spry2* and *Spry4* null embryos, a diastema bud develops into a supernumerary tooth just anterior to the first molar on each side of the mandible. Thus, *Spry2* and Spry4 normally function to prevent the diastema bud from developing into a tooth. Remarkably, these two genes function in different tissues: *Spry2* is predominantly expressed in the epithelium and Spry4 in the mesenchyme. The formation of supernumerary teeth in *Spry2* null mice can be prevented by reducing the dosage of *Fgfr2*, suggesting that *Spry2* normally reduces the sensitivity of the diastema bud epithelium to FGF signaling, thus leading to its regression, and that elimination of Spry2 function results in hypersensitivity to FGF signaling and survival of the diastema bud. Based on gene expression studies, we propose that Sprouty genes modulate two discrete parts of an epithelial-mesenchymal FGF signaling loop: SPRY2 normally prevents maintenance of *Shh* by FGF3, and SPRY4 prevents induction of *Fgf3* expression by FGF4. Interestingly, in contrast to what has been observed in other mutants with supernumerary teeth, molar and incisor morphology is relatively normal in *Spry2* and *Spry4* null animals, indicating that regulation of FGF signaling may play a role in evolutionary control of tooth number independently of effects on cusp morphology.

NEW INSIGHTS IN THE FUNCTION OF BARX1 DURING MULTICUSPID TOOTH DEVELOPMENT

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In mice, the *Barx1* homeobox gene is strongly expressed during the development of three organs: molar teeth, salivary glands and the stomach. Using gain-of-function approaches we have shown that *Barx1* misexpression during incisor development results in a transformation of incisors into molars (1,2), and that *Barx1* misexpression in the developing digestive system results in a transformation of intestine into stomach (3). Using a loss-of-function approach, via generation of *Barx1* knockout mice using gene targeting, we show that *Barx1* is required for molar tooth development and salivary gland development. Moreover, in the absence of *Barx1*, the stomach is transformed into intestine.

In the developing stomach we have established that the molecular function of *Barx1* is to regulate the expression of the secreted Wnt inhibitors *Sfrp1* and *Sfrp2* in the mesenchyme. Since *Sfrp2* is locally expressed in presumptive molar mesenchyme and during salivary gland development, we are currently investigating if *Barx1* function is involved in the same genetic pathway in the development of these different organs.

To further explore the function of *Barx1* in multicuspid teeth of various sizes, we looked at *Barx1* expression during molar and premolar development in shrew, a small mammal exhibiting a dentition with premolar teeth. Significantly, the level of *Barx1* expression varies according to the number of cusps and size of shrew multicuspid teeth. Electroporations carried out in mouse suggest that *Barx1* levels may indeed regulate the final shape of multicuspid teeth.

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Keywords: *Barx1*, homeobox, stomach, teeth.

ECTODERMAL DYSPLASIA GENE (EDA) ANTAGONIZES BMP ACTIVITY AND UPREGULATES EXPRESSION OF SHH

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Ectodysplasin (Eda), a signalling molecule of the tumour necrosis factor (TNF) superfamily, and its receptor Edar are required for normal development of several ectodermal organs including teeth and hairs. Absence of *Eda* leads to an obvious molar cusp patterning defect associated with a smaller enamel knot, an epithelial signalling center regulating tooth shape whereas mice overexpressing *Eda* are characterized by supernumerary teeth. In addition, these mice lack of enamel in incisors which is associated with the absence of the epithelial cells producing the enamel matrix, the ameloblasts. We have recently discovered a similar enamel phenotype in mice overexpressing follistatin, and were able to demonstrate that this phenotype was due to inhibition the ameloblast-inducing activity of BMP4. These findings prompted us to test whether one of the functions of the Eda pathway would be to counteract BMP signalling. In this study, we show that recombinant Eda antagonizes the activity of BMP4 in developing incisors. Moreover, we provide evidence that suppression of BMP activity is compromised in *Eda* deficient embryonic skin. To search for endogenous targets of Edar signalling we analyzed the expression patterns of a number of known BMP inhibitors in developing tooth and hair. By using a novel approach we confirmed that the expression of connective tissue growth factor (ctgf, CNN2/Fisp12), a multifunctional secreted protein known to antagonize BMP4 activity is strongly induced by Eda. The expression pattern of ctgf correlated with that of Edar in developing teeth and hairs. In addition, follistatin was moderately upregulated by Eda. Finally, we show that *Shh* is also positively regulated by Edar suggesting an involvement of Shh in the pathogenesis of the Eda null tooth phenotype. To our knowledge, *ctgf* and *Shh* are first bona fide transcriptional targets of the Eda signalling pathway discovered.

Keywords: ectodysplasin, ectodermal appendages.

THE EXPRESSION OF RUNX2, MYF5 UND SOX9 DURING PRENATAL MORPHOGENESIS OF THE MANDIBLE OF THE QUAIL

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The morphological aspects during prenatal morphogenesis of the mandible have been described, at least in survey 3D reconstructions. Our knowledge, how tissue differentiation of e.g. Meckel's cartilage, bone, nerves, vessels, and muscles are controlled, is relatively sparse. Only recently an increasing number of signalling molecules has been identified, however, it is not clear how in detail they relate to creation of *gestalt*. In order to understand some of the processes, we investigated serial sections of Quails (coturnix japonica, stages HH 26 to HH 38). The expression of Runx2, Myf5 und SOx9 was identified by means of in-situ-hybridisation. The contours of the tissues and of the molecular activity have been digitised and, using the software Analysis (SIS, Münster), 3D reconstructions have been made. This way, for the first time, an interdependency between the spatiotemporal activity of Runx2, Myf5 und SOx9, and the formation of tissue could be shown in the mandible of the quail. However, the spatial extension of the Runx2 signalling did not exactly correspond with the extension of the bone formed, which needs to be discussed.

Acknowledgement: This study has been undertaken within the frame of the COST action B23

Keywords: Mandible, prenatal morphogenesis, signalling.

EXPRESSION OF THE *dlx* GENE FAMILY DURING FORMATION OF THE CRANIAL BONES IN THE ZEBRAFISH (*DANIO RERIO*): DIFFERENTIAL INVOLVEMENT IN THE VISCERAL SKELETON AND BRAINCASE

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We have used *dlx* genes to test the hypothesis of a separate developmental programme for dermal and cartilage bones within the neuro- and splanchnocranium by comparing expression patterns of all eight *dlx* genes during cranial bone formation in zebrafish from 1dPF to 15dPF. *dlx* genes are expressed in the visceral skeleton but not during the formation of dermal or cartilage bones of the braincase. The spatiotemporal expression pattern of all the members of the *dlx* gene family, support the view that *dlx* genes impart cellular identity to the different arches, required to make arch-specific dermal bones. Expression patterns seemingly associated with cartilage (perichondral) bones of the arches, in contrast, are probably related to ongoing differentiation of the underlying cartilage rather than with differentiation of perichondral bones themselves.

Whether *dlx* genes originally functioned in the visceral skeleton only, and whether their involvement in the formation of neurocranial bones (as in mammals) is secondary, awaits clarification.

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Key words: zebrafish, *dlx* genes, visceral skeleton

GENE EXPRESSION FOR TOOTH RENEWAL IN OSTEICHTHYAN FISH: DEVELOPMENTAL AND EVOLUTIONARY ORIGINS OF A DENTAL LAMINA

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The aim is to determine to what extent the spatio-temporal activation of those genes, required for primary tooth initiation, are instructing replacement tooth formation. Tooth renewal in the rainbow trout *Oncorhynchus mykiss* is an essential feature of the dentition at all locations, oral margins, palate, and fifth pharyngeal arch. *Shh*, *pitx2* and *bmp4* are expressed in the same spatio-temporal pattern for primary tooth initiation as reported from the mouse. This pattern of gene expression for the primary dentition in trout is first observed as a superficial epithelial and mesenchymal 'field' of co-incident expression, but that of replacement teeth is localised to restricted sites related to the predecessor tooth. Each site acts as a 'clonal' mechanism for tooth renewal able to independently regulate its own successor. It has been proposed that an epithelial stem cell niche exists to ensure tooth renewal, but how this is related to gene expression at secondary tooth initiation sites in teleosts is not clear.

We have identified these 'clonal' sites for induction of tooth renewal through expression of *pitx2* and *bmp4*, and note that shh is not expressed here. *Pitx2* is upregulated in the lingual, basal outer dental epithelium (ODE) of the dental organ in the appositional growth stage, prior to its attachment. We suggest these genes mark the sites of 'set-aside cells', the committed odontogenic cells of both the epithelium (*pitx2*) and ectomesenchyme (*bmp4*). Cichlids replace their teeth via an alternative mode to that of trout, from a deep dental lamina that buds from the ODE of the predecessor tooth and terminates deep within a bony crypt. Gene expression data is obtained from where the replacement bud will form in tooth renewal in both oral and pharyngeal jaws in cichlid species. We propose site-specific sequential tooth addition, across osteichthyans, and regard this mechanism as a transient dental lamina at one stage in evolution of the permanent successional lamina as observed in amphibians.

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Key words: gene expression, tooth renewal, osteichthyans

BONY FISH AS "MODELS" FOR BONE AND CARTILAGE DEVELOPMENT: CRIBS, CHALLENGES AND CHANCES

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Having been introduced as model organisms for the study of early development, nowadays bony fish (teleosts) such as zebrafish, medaka, or salmon are increasingly used as models for studying skeletal development. While the formation of the bauplan in early development (around to the phylotypic stage) can be reasonably good compared among different vertebrate lineages (e.g. fish, birds, mammals), skeletal development occurs late in ontogeny and depends on an increasing number of intrinsic and extrinsic factors that vary among vertebrate lineages. Among those factors that specifically impact teleost skeletal development are the phosphate driven mineral metabolism, factual weightlessness, infinite growth, and the capacity to regenerate parts of the skeleton. Furthermore, skeletal development generates a high number of unique skeletal tissues and, at the same time, leads to extraordinary complex anatomical structures. Examples for the latter are the teleost skull, with twice as much skeletal elements as the mammalian skull, the Weberian apparatus in zebrafish and the elements of the post-cranial dermal skeleton.

Using zebrafish, medaka or salmon as models offers great opportunities to obtain insights into the basic mechanisms of normal and pathological skeletal development, provided those traits and factors are taken into account that are unique to skeletal development in theses bony fish. On the other hand, regarding teleost as primitive or ignoring specific characters, bears the risks of a substantial misinterpretation of developmental data. The usefulness of any fish species as a model for skeletal development thus depends on considering its many unique characters and relies on discussing its phylogenetic position in order to understand which processes and tissues are conserved (comparable) and which are derived (unique).

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Keywords: teleost, bone and cartilage development.

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PHARYNGEAL DENTITION IN ASP: A NEW MODEL FOR EARLY STAGES OF PHARYNGEAL TEETH DEVELOPMENT?

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Odontogenetic mechanisms contributing to development of vertebrate pharyngeal dentitions received a considerable attention in recent years. Cyprinid fishes, one of few groups which lack teeth on the oral jaws, but actually possesess the large and entirelly functional pharyngeal dentition, presents a favoured model group for such studies. The dental development was investigated into great details in the zebrafish, nevertheless, the situation in other species of the group is largerly unknown. The present study concerns the asp (Aspius aspius), a carnivorous cyprinid fish, which exhibits a number of differences in comparison to the zebrafish. Among other it posseses 2-rowed teeth and the early stages of its dental and cranial development are much prolonged, what makes possible to study particular steps in early odontogenesis into great details.

I collected a large sample of embryonic, larval and juvenile stages scaled by 12 hrs from fertilization (0 dPF) to 64 dPF, when the r-phase of posthatching growth is terminated. Until now, 110 specimens were studied with double-staining technique, 30 specimens were serially sectioned and investigated by standard histological techniques.

The first tooth mineralized and attaches to ceratobranchiale 5 (cb5) at 17 dPF what preceeds the beginning of osification in cb5 (the first head element which ossifies). The larval dentition is the A-type. The shape of larval teeth succesive changes from simple conical to typical adult shape, i.e. the prolonged conical with small hook at its tip recurved in posterior-medially direction. The growth rate of the dentigerous surface of cb5 is much higher during early larval than during the juvenile period, by which the next generation teeth attach at a certain distance medially to the teeth of previous generation that do not shed (in contrast to the adult replacement system).

The hatching starts at 15 dPF with a peak at the end of 19 dPF (at 48 hPF in zebrafish). The second row appears (with two tooth germs) at 29 dPF.

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Keywords: cyprinid fishes, dental development, pharyngeal teeth, asp

WHY TRIBOSPHENIC?

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Monophyodont tribosphenic molars rank among the most eminent apomorphies of Mammalia. Tribosphenic teeth are ancestral to every other molar types and by that any molar structure in mammals can be potentialy homologized with that in any other molar via historical homology of both with the respectives structures in a tribosphenic tooth. Such a kind statements form essentials of the traditional method of phylogenetical reconstructions and the root of the central paradigm of comparative odontology. Despite of that almost any of such statements were subsequently invalidated, the idea on a key role of tribosphenic teeth in mammalian phylogeny never disappeared and, against expectancy, it received a very robust support from the recent fossil record of the earliest beginnings of mammals. The grade of tribosphenic dental organisation was achieven already in the early Jurassic and since then the mammalian divergences seem to proceed just in a good accord with the seemingly out-dated Cope-Osborn predictions. Under such a context, one question, which was never completely resolved as yet, comes again in light: why just the tribosphenic design became so essential for mammalian phylogeny, and why just that was retained almost unchanged until now, at least in a number of various extant clades which bear it? The question grows even a more intricated with the fact that vast majority of mammalian clades performed more or less succesfull attempts to abandon the tribosphenic dental organisation. Then, why it did not occured also at stage of the earliest mamalian divergences? Why the tribosphenic dentition has played a role of a bottle-neck in mammalian dental phylogeny? In search for answers we examined fine structural details of tribosphenic molars in several model taxa (mostly bats) and found some striking specificities in its embryogenetic and perieruptional developmental dynamics. We briefly report on some of them and discuss which of the factors contributing to the setting of mammalian adaptive strategies and their life-history traits might control them in essential way.

Acknowledgements: Study was supported by COST action B23

Keywords: tribosphenic molar, mammals, dental phylogeny

STRUCTURE OF DENTAL PHENOTYPE IN ARVICOLID RODENTS: ADAPTIVE VARIATION AND CONSTRAINTS

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Recent discoveries (Kassai et al.2005) demonstrating that scaling of expression of a single signal factor cause radical rearrangement of dental phenotype in muroid rodents turned in doubt the gradualistic schemes traditionally proposed (mostly by paleontologists) for dental evolution of that group. In respect to some results concerning arvicolids (a muroid group representing one of the peaks in mammalian dental specialisation), we critically reconsider such a possibility and discuss further factors possibly controlling the pathways of muroid dental divergences.

In the late Cenozoic, arvicolids became the most abundant mammals of the Northern hemisphere and the fossils record of particular arvicolid clades is so dense that the history of dental adpatations can be traced into very fine details. It concerns even the pathways of dental rearrangement during a single glacial cycle. We investigated large samples of molar teeth of 5 different arvicolid clades (total n=6580) obtained from continuous sedimentary series covering the recent glacial cycle (Weichselian glacial and the Holocene).

Seven to 12 metric and 14 non-metric traits were examined at each tooth and the variation patterns of particular populations were analyzed with aid of several uni-, bi- and multivariate techniques. Besides significant common trends (such as gradual increase in size and total oclusal surface during the glacial), a number of clade specific adaptive rearrangements were demonstrated. The highest rate of such rearrangements was found in the most progressive taxa (such as Microtus arvalis, Microtus gregalis groups) which, at the same time, exhibits the lowest degree of mutual interdependence between particular components of the dental phenotype, i.e. those in which the control on dental variation by structural constraints is just the least pronounced. Our results suggest that a true link to the ultimate developmental factors of dental phylogeny, i.e. to the EVO-DEVO aspect of its real evolutionary dynamics, is to be searched for rather in the multi-factored mechanisms responsible for the above mentioned constraints than in a domain of seamingly more attractive single-factor relay dynamics.

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Keywords: arvicolids, muroid rodents, dental phylogeny, microevolutionary dynamics

SOREX ARANEUS AS A MODEL FOR TOOTH DEVELOPMENT

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Tooth development has been traditionally studied using mouse *Mus musculus* as a model animal. Mice, however, develop only one incisor and three molars in both sides of their upper and lower jaw. They also develop only the primary dentition. We wanted to find a model which is closer to humans and has addition to incisors and molars also canines and premolars, and the secondary dentition. According to old literature (Kindahl 1959), *Sorex araneus*, the common shrew, develops two dentitions during gestation. The first one disappears by apoptosis during the embryonic development and the second one erupts after birth. We started the project by catching shrews in eastern Finland by the lake Koitere in Ilomantsi. We got several different embryonic stages, 0,3 mm, 7,5mm, 12mm, and new born pups. From these we prepared serial paraffine sections and reconstructed the teeth by computer for 3D. We found that *Sorex araneus* has in the lower jaw one incisor (I), one canine (C), one premolar (P4) and three molars (M1, M2, M3) on both sides. We found that there was only one primary bud, lingually of P4. This could be seen in all 12mm embryos studied. In other stages or for other teeth no such buds could be seen. The P4 primary bud of 12mm embryos expressed Shh, a marker for tooth epithelia. We conclude that *Sorex araneus* develops only one secondary tooth. *Sorex araneus*, therefore, is not an optimal model for research on the development of the secondary dentition.

Keywords: *Sorex araneus*, tooth development

NF-KAPPAB – WNT INTERACTIONS IN TOOTH DEVELOPMENT

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The normal development of teeth requires epithelial/mesenchymal interactions and a cascade of signalling pathways. The Wnt and the NF-kappaB signalling pathways are both implicated in the early control of mammalian tooth development and there is evidence these two signalling pathways interact with each other.

The NF-kappaB pathway is involved in mediating cusp morphogenesis during tooth development. Wnt signalling is required in early tooth germ formation and many members of this pathway are involved throughout tooth development. Members of the Wnt family are expressed in the same regions as the NF-kappaB pathway in tooth development, which prompted us to establish how these two interact with each other.

In order to understand the interactions of Wnt and NF-kappaB, conductin-LacZ reporter mice were used to identify all cells with active canonical Wnt signalling in embryos through the different stages of tooth development. These mice were then crossed with mice containing mutations in genes in the NF-kappaB pathway, such as Ikkalpha to enable changes in Wnt signalling to be identified. The Wnt signalling pathway was also studied in explant cultures by using an inhibitor of GSK-3. The details of the results obtained are presented in this poster.

Key words: Wnt, NF-kappaB and tooth development.

THE IMPORTANCE OF ECTODIN IN DEVELOPMENT OF DENTITION

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The ectodin null mutant mouse has highly altered dentition including extra incisors and molars. The aim of this study was to determine why the removal of one gene, ectodin, leads to the formation of extra incisors in mouse. Are these structures new inventions or are they possibly some kind of rudiments that are normally eliminated during tooth development?

I investigated possible changes in apoptosis and in the expression of signaling molecules that are known to be active during tooth development. Ectodin is a BMP-induced BMP inhibitor. Ectodin is expressed around the enamel knot, which suggests that Ectodin might have a role in restricting the initiation and growth of these signaling centers in certain places during the tooth development.

The extra structures in the mutant mice can be at least partly explained by the lowered amount of apoptosis. In the regions giving rise to extra teeth, expression of Shh and p21 are upregulated more strongly in the mutants than in the wild type jaws. This is suggestive about the quantitative effect of Ectodin in inhibiting tooth placode formation. Tissue culture experiments show that extra incisors appear also in wild type samples in vitro culture conditions if the jaws are disssected before the developmental stage when apoptosis is strongest. Dissecting the placodes and culturing them in vitro would release them from the surrounding mesenchyme where inhibiting ectodin is expressed.

The relatively unrestricted effects of BMPs in ectodin knock out mice are shown in a culture experiment where BMP4 has been added in the culturing medium. Excess BMP accelerates crown differentiation in the ectodin -/- teeth but not in ectodin +/- teeth that have normal phenotypes.

Since Ectodin appears to play such a central role in integrating signals in tooth induction it would be interesting to further resolve Ectodin's possible connections to molecules that are also known to affect tooth number and identity.

Keywords: Bmp-inhibitor, Incisors.

THE ROLE OF ECTODIN IN THE DEVELOPMENT OF ECTODERMAL ORGANS

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The recently discovered BMP antagonist, Ectodin, is known to been expressed in developing ectodermal organs includin teeth, vibrissae and hair follicles.(1) Ectodin knockout mice have extra teeth, enlarged enamel knots and highly altered cusp patterns. It is believed that Ectodin has a role as an inhibitor of induction during embryonic tooth patterning.(2)

In my studies I have analyzed the Ectodin knockout mice further in order to see if Ectodin has a significant role in other ectodermal organs besides teeth. No changes was seen in adult hair (tail, vibrissae and backskin hairs), sweat gland or nail phenotype. Instead, the hair placodes showed small irregularity in patterning.

The most visible change was seen in mammary gland phenotype. Whole mount in situ hybridization studies showed that Ectodin is expressed as a circle around the wild type mammary gland placode. The absence of Ectodin makes placodes larger compared to heterozygous Ectodin deficient mice. According to preliminary results no extra mammary placodes were seen along the milk line. Instead the adult homozygous females have extra nipples around and very close to the main nipples. The shape of the main nipple is bigger compared to heterozygous Ectodin knockout but the extra nipples are small in size. Moreover, preliminary results suggest that the spreading and the structure of the mammary tree during pregnancy is normal compared to heterozygous Ectodin knockout mice. The Ectodin deficient female mice can nurse their pups normally.

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Keywords: ectodermal organs, ectodin, mammary gland

CRANIAL MUSCLE MORPHOGENESIS IN LUNGFISHES AND AMPHIBIANS – A COMPARATIVE PERSPECTIVE

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Our goal is to describe the order of acquisition of innovations in selected phases of amphibian evolution. Both skull and cranial muscle development are in focus. I will present selected parts of this ongoing project including; cranial muscle differentiation and morphogenesis in the Australian lungfish and in the Mexican axolotl, and cranial muscle and skeleton development in Xenopus laevis and its relative Hymenochirus boettgeri. To determine the onset of differentiation we use antibodies against desmin and optical sectioning using confocal laser scanning microscopy on whole-mount immunostained embryos. Antibodies against acetylated tubulin are used to clarify muscle innervation patterns, and collagen II staining gives an overview of developing cartilage. This technique makes it possible to document head development in three dimensions while keeping the specimens intact. To obtain an appreciation of complicated three-dimensional structures in the head, we use reconstructions based on serial sections (two different methods will be shown). The project provides a morphological foundation for further studies of head skeleton as well as cranial muscle cell fate and early differentiation in a comparative approach. The focus is on understanding the developmental origins of morphological innovations.

Acknowledgements: Our research is supported by grant from the DFG the ARC, and by COST B23.

Keywords: head development, morphogenesis, cell migration, fate mapping

TIME-COURSE OF GENE EXPRESSION IN MURINE TOOTH GERMS AS MEASURED USING MICROARRAY ANALYSIS

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Current literature suggests that much remains to be learned regarding changes in gene expression occurring during maturation of the murine tooth germ. We have investigated gene expression in first mandibular molar tooth germs of CD1 mice embryos/pups, starting at 11.5dpc. Tooth germs were isolated at daily intervals up to 26.5dpc. Total RNA isolated from three separate tooth germs, isolated at an identical developmental stage, were pooled and used for cDNA synthesis. The expression patterns derived using this pooled RNA was analysed using triplicate 30K microarrays (NTNU Microarray Core Facility, Trondheim, Norway). The resulting cDNA was labelled with either Cy3 or Cy5 using the Genisphere 900 labelling kit. Microarray data were analysed using Spotfire software.

Data from microarrays covering the entire time-course were combined to facilitate analysis of gene expression throughout the time-course. The net median fluorescence values were normalized by subtraction of slide median value to facilitate comparison of data across all slides. The normalised values were clustered using self-organising maps (SOM). ANOVA analysis was used to select genes exhibiting consistent, and statistically significant ($P \leq 0.04$), patterns of expression throughout the time-course.

Characterization of expression profiles of the resulting 1292 known genes was facilitated by using the techniques of profile search to select genes following a particular expression profile, followed by principal component analysis of the resulting sub-populations of genes. Ontologies were established using David (1) or WebGestalt (2).

Initial validation of microarray data using real-time RT-PCR, or in situ hybridisation, appears positive.

(1) http://apps1.niaid.nih.gov/david/
(2) http://genereg.ornl.gov/webgestalt/

USING THREE-DIMENSIONAL RECONSTRUCTION TO STUDY TOOTH MORPHOLOGY AND GENE EXPRESSION PATTERNS

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Three-dimensional reconstructions provide a way to make reliable observations and measurements of small, complex shapes and patterns. Here I present two methods that use 3D reconstruction to allow detailed analysis of mouse tooth morphology and gene expression pattern.

A commonly used approach in developmental studies is to link differences in morphology with differences in developmental gene expression patterns. Usually this is done by in situ hybridisation on tissue sections. However, complex patterns are very difficult to reconstruct from two-dimensional slides. A 3D reconstruction of the slides can be made by first photographing each sample with light-field and dark-field illumination and then using National Institute of Health Image 1.63 software to convert the images into a three-dimensional projection. To make the histology more apparent, the bright-field pictures can be edited to show only the topography of the developing tooth. This method can also be used with any other sample that can be sectioned and digitally photographed.

Mineralized mouse teeth can be scanned with a laser confocal microscope and the pictures converted to elevation models with the National Institute of Health Image 1.63 software. They can be edited in Corel Photo Paint 12 and then converted to coordinate data with Scion Image. The morphological data can now be considered analogous to geographical data. The Surfer Mapping System can be used to make a "map" of the teeth and to measure features such as cusp heights and distances. The resolution of the scans (up to 8,28 μm) is maintained in the measurements. The Surfer can also be used to produce manoeuvrable 3D models of the teeth. This confocal scanning method is suited for any hard tissue sample smaller than 10 mm.

Three-dimensional reconstructions can be treated as two-dimensional pictures in any graphic software. They are easier to store than the original samples and can be made accessible to the scientific community through the Web.

Keywords: tooth, morphology, three-dimensional reconstruction

THE COMPLEX ORIGIN OF A TOOTH CAN CAUSE ITS DEVELOPMENTAL VULNERABILITY AND EXPLAIN THE FORMATION OF SUPERNUMERARIES

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In humans, one of the most frequent dental anomalies is an upper supernumerary lateral incisor, which is also often associated with orofacial clefting. In mutant mice, a supernumerary cheek tooth can occur mesially to the first molar. We searched for common aspects of the normal development of the upper lateral incisor (i2) in humans and the first lower molar (M1) in mice that could explain the origin of supernumeraries at these tooth positions in cleft patients and mutant tabby/EDA mice, respectively. Normally, the human i2 develops from two parts originating on the medial nasal and maxillary facial outgrowths, respectively. A failure of the two parts of i2 to fuse can explain the origin of a supernumerary i2 (i2 duplication). From a clinical aspect, a supernumerary i2 might represent a form of cleft caused by the non-fusion of the dental epithelia between the medial nasal and maxillary facial outgrowths (Hovorakova et al., 2006). This is in line with the high frequency of i2 duplication in cleft patients. The mouse M1 normally develops from two parts: the M1 proper and the vestigial premolar bud, which is incorporated into the mesial part of the M1 cap. Precisely at that location, the supernumerary tooth develops in the embryos of mutant tabby/EDA mice. This supernumerary tooth might result from a failure in the incorporation of the premolar bud into the M1 proper. The incorporation of the premolar vestige into the mesial part of the M1 can be regarded as a recapitulation of the disappearance of the remnant of the last premolar during evolution. Consequently, the supernumerary teeth in mutant mice can be interpreted as atavism (Peterkova et al., 2005). Conclusion: At positions where teeth arise by the fusion of two (several) developmental primordia, a failure of these primordia to fuse can lead to their separate development and the origin of tooth supernumeraries.

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Key words: supernumerary tooth, man, mouse

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3D MORPHOGENESIS OF THE SUPERNUMERARY CHEEK TOOTH IN *SPROUTY* EMBRYOS

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Fibroblast growth factor (FGF) signalling is thought to play a key role in epithelial-mesenchymal interactions during tooth development. Members of the Sprouty (Spry) family are intracellular proteins that modulate FGF signaling by antagonizing it downstream of the receptor. We investigated the morphogenetic effects of the elimination of *Spry2* and *Spry4* during odontogenesis. Development of the lower cheek dentition was analyzed in *Spry2* and *Spry4* null mice at embryonic days (ED) 12.5-16.5 using a series of histological sections and computer aided 3D reconstructions of dental epithelium. At ED 12.5, there was not a marked morphological difference between developing teeth in *Spry* null embryos and their wild type controls (WT) on sections. In 3D reconstructions, a single mound of dental epithelium was present in the cheek region of mandible. At ED 13.5, a wide bud was prominent in the mesial part of the dental epithelium in WT and *Spry2* null embryos, while a round shallow cap was seen in *Spry4* null samples. At ED 14.5, the first molar (M1) cap differentiated in WT as well as in *Spry* null jaws. A wide bud or a small cap was observed adjacent to the mesial end of M1 in *Spry2* or *Spry4* null embryos, respectively. At later stages in both *Spry2* and *Spry4* null fetuses, the supernumerary small cap progressively segregated from the M1, whose enamel organ appeared reduced mesially. However, the supernumerary cap and the M1 enamel organ remained connected by a wide mound of dental epithelium. Thus, as a consequence of the elimination of the Spry2 and Spry4, a supernumerary tooth develops at the mesial end of the lower M1. We hypothesize that the supernumerary tooth in *Spry* null animals is related to the premolar suppressed during mouse evolution.

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Key words: Sprouty, mouse, supernumerary tooth development

MORPHOLOGY OF TRIGEMINAL GANGLIONS (TG) AND DIFFERENT SALIVARY GLANDS AFTER UNILATERAL LONG-LASTING LIGATION OF *ARTERIA CAROTIS COMMUNIS*

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Blocking of blood supply of internal carotid artery may result in pathomorphological changes in salivary glands and TG. Aim of work was IMH investigation of qualitative and quantitative structural markers, growth factors of TG and salivary glands after long-lasting (3 months) ligation of *a. carotis communis sin.* in 4 experimental rabbits.

Results showed decrease or degradation of ganglionic cells with increase of nerves in TG and sclerotic blood vessels of ligation side. Numerous and/or abundance of NF- and M-containing neuronal structures were seen in both sides. GFAP was observed in neuronal structures from both sides. SP was not seen in any of TG. Buccal glands showed hypertrophy and degeneration of secretory parts, but submandibular and parotic glands demonstrated proliferation of excretory ducts and fibrosis. Apoptosis affected half from gangliocytes in TG, but apoptotic cell number in salivary glands was as following: in buccal - 40°æ 24.66, in submandibular - 51°æ15.01, in parotic - 43°æ 15.32. VEGF marked few to moderate endotheliocytes in salivary glands, NGFR - few nerve fibres in TG, but moderate nerves in salivary glands around the excretory ducts. EGFR1 stained some glial cells in TG and mainly was found in blood vessels of submandibular gland. FGFR1 richly stained glial cells and nuclea of neurons in TG, and excretory ducts in salivary glands.

Conclusions. Dystrophy raised by carotid artery ligation in TG occur in changed cell morphology, cytoskeleton degradation and partial apoptosis, but in salivary glands – in changed structure of excretory ducts and hypertrophy of secretory parts. Compensation after long-lasting regional ischemy are sclerotization of blood vessels, proliferation of nerves and fibrosis in salivary glands. EGFR1 and FGFR1 are main growth factors found in ischemic TG and salivary glands. Absence of tachykinins in TG seems to be a response of neurons to disordered blood supply and raises disordered pain reflex pathway in both sides.

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Key words: trigeminal ganglion, salivary glands, ischemy, morphology, rabbits

THE ROLE OF *GAS1* DURING EARLY CRANIOFACIAL DEVELOPMENT

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The growth arrest-specific gene (*Gas1*) encodes a GPI-linked membrane glycoprotein, originally identified by its ability to induce cell cycle arrest in cultured mouse fibroblasts. However, *Gas1* -/- mice provide evidence of a cell context-dependent role during embryonic development; Gas1 can also induce proliferation and cell differentiation in regions as diverse as the cerebellum, limb and eye. Moreover in the dorsal somite, Gas1 is a Wnt-inducible marker able to inhibit Shh-induced growth via a direct physical interaction. The expression domains of *Shh*, *Ptc1* and *Gas1* in several regions of the mouse embryo support the notion that Gas1 may act as an inhibitor of Shh signalling. *Gas1* expression is complementary to Ptc1, being upregulated in cell populations that are unresponsive to the Shh signal. Interestingly, *Gas1* -/- mice display multiple craniofacial defects, which include anomalies of midline facial development. In particular, cleft palate and fused maxillary incisors occur with variable penetrance. These defects of midline patterning are characteristic of a mild form of holoprosencephaly; a condition associated with perturbations in Shh signalling in both mouse and humans. Thus, an apparent contradiction exists; evidence of Gas1 negatively regulating Shh function *in vitro* but a craniofacial phenotype in *Gas1* -/- mice suggestive of reduced, not increased Shh signalling. In an attempt to further define any putative relationship between these molecules in the craniofacial regions we have analysed Shh transduction in Gas1 -/- embryos. Our preliminary results suggest that Shh signalling remains normal in these mutant embryos and that the midline defect is not secondary to perturbations in Shh signal.

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Keywords: Gas1, Shh

MICRODISSECTION AS A TOOL FOR CELL SIGNALLING ANALYSIS IN TEETH

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Detailed molecular analysis of tissue samples is very complicated due to their heterogeneous structure with different cell types locked in specific morphologic units. Heterogeneity of studied tissues can be overcome by microdissection method that allows separating and collecting particular cells of the tissue and subsequent DNA, RNA, or protein analysis. Analysis of nucleic acids after microdissection has been well established. Moreover, cDNA micro arrays allow parallel investigation of global gene expression in a specific cell type or tissue compartment. On the other hand, protein analysis of microdissected tissues is far more complicated. Western blotting or 2D electrophoresis are usual methods of choice. However, the sensitivity of both assays is relatively low and microdissection may not yield sufficient number of cells. This is a limiting factor in analyses of specific signalling molecules that are low abundant but with high impact on crucial cellular events.

During tooth development, reciprocal interactions between small numbers of dental epithelial and mesenchymal cells drive the tooth germ morphogenesis. Primary enamel knot, the signalling centre that is believed to control the bud to cap stage transition, consists of only several hundreds of cells. For such tiny structures, conventional western blot analysis after microdissection is not sensitive enough. However, these few enamel knot cells are of a great importance and thus a suitable modification of after-microdissection analysis of this cell population becomes challenging. We performed a novel method enabling laser microdissection of enamel knot cells from cryopreserved sections and dissociation of these cells followed by high sensitive flow cytometry after specific antibody labelling. Moreover, this method is open for many other applications in detailed protein analysis of limited cell populations.

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Keywords: microdissection, protein analysis

BALANCE BETWEEN ACTIVIN, FOLLISTATIN AND BMP4 REGULATES THE FUNCTION OF FGF3 AND PROLIFERATION OF STEM CELLS IN MOUSE INCISORS

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Mouse incisor is a continuously growing tooth. On the labial side epithelial stem cells have been located in the center of cervical loop. Stem cells give rise to the progenitor cells which move to the enamel epithelium. Bone morphogenetic protein 4 (BPM4) is known to induce the differentiation of enamel epithelium to enamel forming ameloblasts on the labial side of the incisor while the proliferation of the epithelium is stimulated by fibroblast growth factor 10 (FGF10). Fgf3 and Fgf10 have partially overlapping expression patterns in the mesenchymal cells underlying the epithelium in the cervical loop area. After E16 Fgf3 expression becomes asymmetrical and restricted to the labial side while Fgf10 is expressed in the mesenchyme on both the labial and lingual sides. We discovered that the incisor phenotype of K14-Follistatin overexpressing mouse resembles the phenotype of Fgf10-/- mouse at new born stage. In K14-Follistatin mouse the labial cervical loop is small and the proliferation is diminished. In contrast to this phenotype in Follistatin-/- mouse both the labial and lingual cervical loop is large and contains active proliferation. When we did tissue culture experiments with E16 incisors cultured with a bead containing BMP4 the expression of Fgf3 was missing. BMP inhibitor Noggin protein and surprisingly also ActivinA protein were able to induce upregulation of Fgf3 expression on the lingual side. This suggests that BMP4 inhibits Fgf3 expression on the lingual side of the incisor. We then compared the structure of incisors in adult Fgf3 knockout and Fgf3-/-; Fgf10+/- compound mutant mice. In the Fgf3 knockout the enamel appeared normal but in the Fgf3-/-; Fgf10+/- mutant it was missing. These results indicate that Fgf3 signaling is negatively regulated by BMP4 and the inhibition can be counteracted by Activin. The redundant functions of FGF3 and FGF10 regulate the formation of cervical loop and proliferation of stem cells.

DIVERGENCE OF HEDGEHOG SIGNAL TRANSDUCTION MECHANISM BETWEEN DROSOPHILA AND MAMMALS

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The Hedgehog (Hh) signaling pathway has conserved roles in development of species ranging from Drosophila to humans. Responses to Hh are mediated by the transcription factor Cubitus interruptus (Ci; GLIs 1-3 in mammals), and constitutive activation of Hh target gene expression has been linked to several types of human cancer. In Drosophila, the kinesin-like protein Costal2 (Cos2), which associates directly with the Hh receptor component Smoothened (Smo) is essential for suppression of the transcriptional activity of Ci in the absence of ligand. Another protein, Suppressor of Fused (Su(Fu)) exerts a weak negative influence on Ci activity. Based on analysis of functional and sequence conservation of Cos2 orthologs, Su(Fu), Smo and Ci/GLI proteins, we find here that Drosophila and mammalian Hh signaling mechanisms have diverged, and that in mouse cells, major Cos2-like activities are absent, and the inhibition of the Hh pathway in the absence of ligand critically depends on Su(Fu).

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Keywords: Signalling, pathway, evolution, Hedgehog, Su(Fu) and Cos2

GEKKOS TEETH: AN ALTERNATIVE MODEL OF AMNIOTE TOOTH-CUSPS FORMATION?

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Origin of multicuspidate crown pattern (e.g. in mammals) from the conical reptiles teeth ranks among the paradigmatic predictions of comparative odontology. The morphogenetic pathways mediating that transition are mostly unknown. We report here the data on development of bicuspidous fissident teeth in a non-mammalian amniote, Paroedura pictus, using a fine scaled embryonal series (n=45 ind.) examined with aid of different histological techniques. The teeth of the first generation are monocuspid, do not attach to the jaw bones and are all subsequently dislocated or resorbed. The second generation teeth are bicuspidate, they develop in between first generation teeth via separate budding from dental lamina and are formed by much larger cell population. At the late cup stage a clear divergence in histogenetic programme among cells of the inner enamel epithelium (IEE) is seen: whereas central cells remain undifferentiated and interestingly, undergo fast population growth until late bell stage, the laterally situated IEE cells rapidly attain the shape and function of mature ameloblasts. In other words, the bicuspidous phenotype of adult teeth is here apparently produced by heterotopic histogenesis of IEE: its axial cells remain less differentiated, whereas the growth and prolongation of the lateral cells create the effect of epithelial bifolding. As in other lepidosaurian reptiles, the enamel knot of the mammalian type was not identified. Nevertheless, the above-described pattern of heterotopic differentiation of the IEE seems not only to produce quite a similar effect as the enamel knot do but, possibly, it may represent its basal phylogenetic stage. Analysis of its signaling activity is the subject of further study.

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TBX1 IS EXPRESSED AT MULTIPLE SITES OF EPITHELIAL-MESENCHYMAL INTERACTION DURING EARLY DEVELOPMENT OF THE FACIAL COMPLEX

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Tbx1 encodes a T-box-containing transcription factor, which is thought to be a key player in the aetiology of the DiGeorge and Velocardiofacial syndromes (DGS/VCFS). In addition to defects affecting structures derived from the pharyngeal pouches, these subjects exhibit varying degrees of facial dysmorphology and cleft palate. We have analysed the expression of murine *Tbx1* during early facial development and find transcripts at sites of known epithelial-mesenchymal interaction. In particular, *Tbx1* was expressed in epithelium of the early facial processes, including the fronto-nasal, medial and lateral nasal, and palatine. Transcripts were also localised to the epithelium of developing tooth germs and hair follicles at several stages during their early development. Together, these expression domains suggest a role for *Tbx1* in mediating epithelial-mesenchymal signalling in regions of the developing face. A finding consistent with the spectrum of facial deformity encountered amongst subjects affected by DGS/VCFS.

LOCALIZATION OF EPITHELIAL STEM CELLS IN THE MOUSE INCISOR

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Rodent incisors are continuously growing organs, suggesting that stem cells are present in these teeth. A highly proliferative area, which is located in the apical part of the incisor's epithelium – the cervical loop – has been proposed as a stem cell niche. The epithelial tissue of the incisor apex consists of a core of stellate reticulum cells surrounded by basal epithelial cells contacting the dental papilla and the dental follicle mesenchyme. Cells from the cervical loop of the labial side of the incisor proliferate and differentiate into four dental cell populations, including the ameloblasts. The precise location and identity of the putative stem cells, nevertheless, remain elusive. To get more information about the exact location of the stem cell niche, we have applied DiI labelling to follow the cell movements in various locations of the cervical loop during incisor development. The tooth germs have been sliced in vivo in 250um thick slices using a tissue chopper, and thereafter have been cultured on a filter. Cell populations within the cervical loop that contribute to the different incisor epithelial layers, (i.e. ameloblasts, stratum intermedium, stellate reticulum, outer enamel epithelium), have been ascertained, thereby indicating the exact location of the putative stem cell rich region.

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Key Words: Stem Cells, Incisor, DiI