Characteristics of main research directions investigated at the institute and the achievements 2010−2014

Institute	Institute of Animal Physiology and Genetics of the CAS, v. v. i.

Institute of Animal Physiology and Genetics is focused on animal reproduction, animal genetics, neuro-hormonal regulation and nutrition. In the past 20 years the activities of research teams have shifted in many topics from descriptive research (patterns) to the field of cutting edge knowledge (processes). The scientific activities of the Institute have differentiated into two principal themes:

Ontogenesis (animal and human) is focused on meiotic division, embryonal and selected tissues development, stem cells applications, cancer treatment and development of animal models for particular human diseases treatment.

The second theme **Biodiversity** (animal and microbial) is dealing with speciation, hybrid zone forming, occurrence of hybrids and hybrid zone effect on protein expression, climate change effects, fish/amphibian hybridization and asexual clonal reproduction all of them associated with increased ploidy levels (up 6n). Diversity of the gut microbiota is studied with the respect to effective and healthy digestion, as well as gut diseases and gut – brain axis.

Since the last evaluation of the Institute its performance, measured by bibliometric indicators, has increased significantly, furthermore also project ExAM financed from EU Structural Funds was awarded in 2012 to the Institute. During the last 5 years 3 laboratories (Laboratories of Endocrinology, Nutrition and Genomics) were closed down and 4 new (Cell Division Control, Molecular Ecology, DNA integrity, and Applied Proteomic Analysis) were established.

Structure of the Institute

Biomedical teams

Biomedical research in the institute is much more diversified. **The Developmental Biology** team's main research interest includes molecular mechanisms regulating resumption of meiosis in porcine and bovine oocytes, porcine cumulus cells – oocyte interactions, as well as gene expression during early embryonic bovine development in vivo or in vitro. **The Biochemistry and Molecular Biology of Germ Cells** team is focused predominantly on the study of regulation of meiotic division in mammalian female germ cells, oocytes. The understanding of mechanisms, which regulate meiotic maturation of oocytes, would have a high impact both on the field of reproductive biotechnology in farm animals (improvement of the conditions for in vitro culture of the oocytes, and in farther perspectives for obtaining in vitro produced preimplantation embryos of better quality), and on the field of human assisted reproductive technologies (improving ART techniques, identifying the factors affecting chromosome aneuploidy in oocytes). The **Animal Embryology** team aims to clarify the molecular and cellular mechanisms involved in the determination of the shape, number and final morphology of the teeth and a functional complex with the adjacent bone tissue. One of the research directions in this area relates to the current 3D view

morphogenesis crown and root of the tooth around the time of the eruption in its final anchoring the jaw. Simultaneously there are monitored candidate molecules and pathways participating in the definition of the space for the tooth and bone growth in the tooth, but also the bone remodeling.

Biodiversity teams

The activities of the **Evolutionary Biology** team address several issues of fish, rodent and also partially lower vertebrate genetics.

In **fishes**, the hybrid unisexual vertebrate systems of spiny loaches, genus Cobitis, Prussian carp, genus Carassius, partially also water frogs, genus Rana, are subjects of long-term interest. Hybrid diversity of asexual clones of European spiny loaches has no parallel among other unisexual vertebrates – they are widely distributed from Atlantic coast as far as Moscow region in Russia. Dealing with **rodent**, the research interests consists of the study of genetic variation, phylogeography and evolution of free-living house mouse species of the genus Mus (*M. macedonicus, M. spicilegus*) and their relationship to recently identified distinct subspecies and/or species (*M. m. spretoides, M. s. adriaticus, M. cypriacus*). The second research area consists of various aspects of hybridization in house mice (M. musculus complex) including introgression patterns for several autosomal and sex-linked molecular markers as well as morphometric characters across the *M. musculus/domesticus* hybrid zone, estimation of key evolutionary parameters (dispersal, selection, barrier to gene flow, fitness of hybrids), genomic and proteomic analysis of major urine proteins (MUPs) and digging for new suitable molecular markers of the SINE family. The adaptive phylogeography using NGS approach on rodent model demonstrated adaptation of single genes to climate changes.

The gut microbiota in human, ruminants, pigs, herbivores, reptiles and insects are the main research interest of the **Anaerobic Microbiology** team. Important are the inter-microbial (mainly bacterial, archaeal, fungal and protozoal) interactions, gut-microbiota interactions, stability and diversity of microbiota in healthy gut and inflamed intestine. Anaerobic fungi are producing unique fibrolytic complexes and genomes of several genera were not fully completed up to now.

PIGMOD Center

During the five recent years Center PIGMOD was established on the base of project Exam as an important regional centrum financed by EU Structural Funds. Advanced animal models are being developed for different biomedical purposes in the Centrum. This includes also the development of mini-pigs models for the **Huntington disease,** bearing N-terminal fragment of human mutated huntingtin. At the moment there are already 4 animal generations with the same gene expression. This model is starting to be used for testing of different HD treatments. One of the methods utilizes gene therapy approach in order to decrease huntingtin protein.

Another model has been developed for **spinal cord injury.** This model is using cell lines including human neural precursors or pluripotent stem cell lines, which are being analyzed for their therapeutic potential when grafted spinally in animals with ischemia-induced paraplegia. The goal is to identify differentiation potential, long term survival and functionality of grafted cells.

MeLiM strain of minipigs with hereditary melanoma is a unique model in which, after a period of tumor progression and metastasis, a spontaneous tumor regression occurs leading to complete cure. Tumor regression in MeLiM is associated with infiltration of

tumors with cells of the immune system, but their exact role in tumor regression is not yet known.

International status and relations

Institute reputation is built up on far-sighted individuals, significant results, novel techniques, scientific community activities, as well as on tradition of these activities in the organization. The significant persons in the Institute and their activities are summarized in the Personal paragraph. Participation in international projects is confirming the ability of our teams to contribute significantly to joint collaboration. Most of our teams are organizing international meetings and workshops to facilitate presentation and cooperation among interested partners.

International status is also documented by a number of foreign visitors and foreign PhD students. During the three months in the beginning of this year there were 9 visitors and there are 15 PhD students performing their studies in our Institute.

Within the scientific staff there are 18 scientists being appointed in international journal Editorial boards. Another 3 are in European Commission bodies.

Database LCI

Rumen bacteria sequencing data resulting from the FP7 Ruminomics project will be released at the end of 2015. Now they are available for project participants.

Collection of Anaerobes (Bacteria, fungi and methanogens)

The collection contains anaerobic bacteria from ruminants, mostly from the rumen from all over the world – Europe, Asia, Australia, America. Second part of the collection represents isolates from the human digesta. The biggest part a representing fibrolytic isolates of genera *Butyrivibrio, Pseudobutyrivibrio, Ruminococcus, Fibrobacter, Selenomonas, Eubacterium, Clostridium, Bacteroides, Bifidobacterium, Coprococcus, Roseburia, Prevotella, Megasphaera, Faecalibacterium, Escherichia, Treponema, Succinivibrio, Streptococcus, Wolinella, Veillonella,* and *Lactobacillus*. Anaerobic fungi are represented mainly by genera *Anaeromyces, Orpinomyces* and *Caecomyces*. Methanogens are represented by genus *Methanosarcina*. The collection contains more than 200 isolates which can be provided to other labs.

Fish Collection

Preserved fish samples in form of vouchers from Eurasia are stored for morphological, genetic and/or phylogenetic analyses. They are used not only by the Institute staff, but also in the international scale, some parts of collection were deposited in international collections in Paris and Berlin.

Research Report of the team in the period 2010–2014

Institute	Institute of Animal Physiology and Genetics of the CAS, v. v. i.

Scientific team	Team of Anaerobic Microbiology

Area of Research:

The most important results of the team:

Study of the human gut microbiome

Human gut microbiome harbors large number of microorganisms that are closely associated with human health and many diseases are reflected in microbiome imbalance. Plenty of bacteria are known for their beneficial or negative effect on host, yet even more bacteria are still waiting for their discovery. The description of gut microbiome is an important part in understanding of pathology in diseases such as inflammatory bowel diseases, gut cancer, liver diseases and celiac disease. In frame of gut – brain axis, it has been recently found out that Alzheimer and autistic patients also exhibit significant changes in gut microbiome. Alzheimer's disease is one of the major neurodegenerative diseases; the elderly population is affected (about 50% at age 85 years). According to some research on the emergence and development of the involved metabolites produced in the digestive tract and this gives rise to a hypothesis concerning the proportion of microbial activity in the etiology of this disease. Comparison of fecal microflora composition profiles in healthy persons and patients should help to comprehension of etiology and pathogenesis of the illness. Modifications of the overall bacterial composition of fecal samples were monitored by population fingerprinting using denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene amplicons and quantitative PCR method (Šimůnek et al., 2012).

Role of the intestinal microflora in the onset and development of autistic spectrum of disease (ADS) were repeatedly hypothesized as one of the potentially important factors. In particular, disturbances of the normal intestinal microflora by action of external factors can suppress potentially harmful bacteria and suppressed normal microflora can overgrowth and injure the host organism. The fecal samples of 11 subjects with various severities of autism and their family members living in the same conditions (dietary habits) were examined. The monitoring of differences in the composition of fecal bacterial populations was based on a culture-independent technique. Total bacterial DNA from feces was extracted and bacterial composition was assessed by DGGE analysis of 16S RNA. Special attention was paid to the microbial genus described as being involved in autism development and/or protection (*Desulfovibrio*, *Clostridium*, *Akkermansia, Sutterella, Bifidobacterium*) (Šimůnek et al., 2013).

IBD associated microbial changes are also studied on mice model. Commensal bacteria have been shown to modulate the host mucosal immune system. In following study we reported that oral treatment of BALB/c mice with components from the commensal, *Parabacteroides distasonis*, significantly reduces

the severity of intestinal inflammation in murine models of acute and chronic colitis induced by dextran sulphate sodium (DSS). The membranous fraction of P. distasonis (mPd) prevented DSS-induced increases in several proinflammatory cytokines, increased mPd-specific serum antibodies and stabilized the intestinal microbial ecology. The anti-colitic effect of oral mPd was not observed in severe combined immunodeficient mice and probably involved induction of specific antibody responses and stabilization of the intestinal microbiota. Our results suggest that specific bacterial components derived from the commensal bacterium, P. distasonis, may be useful in the development of new therapeutic strategies for chronic inflammatory disorders such as inflammatory bowel disease (Kverka et al., 2011). The study was carried under cooperation with Microbiological Institute of ASCR (Prague). Our department was responsible for microbial analyses by qPCR and PCR/DGGE approach including data analysis and discussion.

The research team was focused predominantly on the role of negative regulation of TLR signaling and gut microbiota in the development of colitis-associated cancer in mouse model was tested. Changes in microbiota composition during tumorigenesis were analyzed by bacteria specific qPCR and by pyrosequencing, and β-glucuronidase activity was measured in intestinal content by fluorescence assay. ATB treatment of wild-type mice reduced the incidence and severity of tumors. As compared with non-treated mice, ATB-treated mice had significantly lower numbers of regulatory T cells in colon, altered gut microbiota composition, and decreased β-glucuronidase activity. However, the β-glucuronidase activity was not as low as in germ-free mice. IRAK-M deficient mice not only developed invasive tumors, but ATB-induced decrease in β-glucuronidase activity did not rescue them from severe carcinogenesis phenotype. Furthermore, IRAK-M deficient mice had significantly increased levels of pro-inflammatory cytokines in the tumor tissue. We conclude that gut microbiota promotes tumorigenesis by increasing the exposure of gut epithelium to carcinogens and that IRAK-M negative regulation is essential for colon cancer resistance even in conditions of altered microbiota. Therefore, gut microbiota and its metabolic activity could be potential targets for colitis-associated cancer therapy (Klimešová et al., 2013). The study was carried under cooperation with Microbiological Institute of ASCR (Prague). Our department was responsible for microbial analyses by qPCR and PCR/DGGE approach including data analysis and discussion.

An important part of the scientific activities during evaluated period was physiology of a strictly anaerobic mesophilic chitinolytic bacterial strain *Clostridium paraputrificum* J4 was isolated in our laboratory from human feces. The bacterium produced an array of chitinolytic enzymes representing significant components of the J4 strain secretome. The active proteins we characterized by estimating the enzymatic activities of endochitinase, exochitinase, and N-acetylglucosaminidase. The spectrum of extracellularly excreted proteins was separated by SDS-PAGE and its variability was confirmed on zymograms of renatured SDS-PAGE. Protein components of the secretome were separated by 2D-PAGE analysis. Using 2D-E, MALDI-TOF/ TOFMS and zymography, significant spectrum of extracellular chitinases was identified. We were able to detect eight active proteins or isoenzymes using the zymography approach (Šimůnek et al., 2012). The Isolation and purification protocol was optimized (Dušková et al. 2011). Methods of separation of extracellular complex of chitinolytic enzymes of Clostridium paraputrificum by membrane ultrafiltration was optimalized (Tischenko et al., 2010, 2011). The components of extracellular chitinolytic complex containing at least six isoforms of

endochitinases and one β-N-acetylglucosaminidase (NAGase) were separated and subsequently concentrated by membrane ultrafiltration on a membrane with cut-off 30 kDa. The effect of pH and temperature on activity and stability of NAGase and endochitinases was evaluated. Our laboratory in this cooperation prepared bacterial culture and performed zymography analysis and we also participated on the manuscript preparation.

Study of the animal gastrointestinal microbiome

The members of our team were very focused on animal gut microflora which play an important role in animal food production affecting animal health and well-being as well as production costs. Understanding of gut microbiome is key to improve these areas. Following experiments were performed.

Methods of molecular biology revealed high numbers of uncultured bacteria. As these microorganisms interfere with diversity descriptions, following experiment was carried out to bring light into this area. 16S rRNA sequences of ruminal uncultured bacterial clones from public databases were phylogenetically examined. The sequences were found to form two unique clusters not affiliated with any known bacterial species: cluster of unidentified sequences of free floating rumen fluid uncultured bacteria (FUB) and cluster of unidentified sequences of bacteria associated with rumen epithelium (AUB). A set of PCR primers targeting 16S rRNA of ruminal free uncultured bacteria and rumen epithelium adhering uncultured bacteria was designed based on these sequences. FUB primers were used for relative quantification of uncultured bacteria in ovine rumen samples. The effort to increase the population size of FUB group has been successful in sulfate reducing broth and culture media supplied with cellulose (Rosero et al., 2012).

Special attention was paid to the exceptional micro-organisms, gut fungi inhabiting the gastrointestinal tract of ruminants and non-ruminant herbivores, only representatives of kingdom Fungi, which are obligately anaerobic, possessing hydrogenosomes instead of mitochondria. Gut fungi produce a broad range of excellent hydrolases, some of which are organized in cellulosomes. Anaerobic fungi are phylogenetically unique and comprise a separate clade, the *Neocallimastigomycetes*, among the basal fungi. Six genera have been described, namely the monocentric *Neocallimastix, Caecomyces* and *Piromyces* and the polycentric *Anaeromyces, Orpinomyces* and *Cyllamyces* (Fliegerova et al. 2012a, Gruninger et al. 2014). Diversity studies of LAM research group identified the new uncultured groups gut fungi from cow manure (Fliegerova et al. 2010) described later as cluster KF1 (GenBank acc. no.GQ850301) and cluster Piromyces 3 (GenBank acc. no. GQ850318) named in Index Fungorum as *Piromyces cryptodigmaticum* (Fliegerová et al. 2012). Molecular identification of axenic strains of anaerobic rumen fungi (Eckart et al. 2010) isolated by LAM research group contributed to introducing the nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi (Schoch et al. 2012). Axenic culture of *Anaeromyces mucronatus* JF1 (Novotná et al. 2010) isolated from faeces of deer was included as reference strain in RTL (RefSeq Targeted Loci) database of National Center of Biotechnology Information (NCBI, Schoch et al. 2014).

Pigs post-weaning are faced with new challenges like removal from the sow and change in diet and are vulnerable for gastro-intestinal disorders and infec-tions, often leading to diarrhoea and consequently economic losses. Therefore we have tried to assess the effects of intact dried *Ascophyllum nodosum* seaweed on piglet performances, gut bacteria and function and plasma oxidative status. It was found

that *A. nodosum* supplementation had no effect on daily weight gain, nor did it alter feed conversion ratio. Dendograms prepared using PCR-DGGE banding patterns did not indicate clustering of microbial profiles based on diet supplement. Plasma oxidative status and outcome of morphology and of electro-physiological measurements from gut tissues were similar for all treatments. Thus, the addition of A. nodosum seaweed to well digestible diets did not enhance performances of piglets nor some gut health parameters and plasma oxidative status (Michiels et al., 2012). The study was performed under cooperation with Institute of Life Sciences (Prague). Our department was involved in PCR-DGGE experiments with data analysis.

Bifidobacterial projects

Bifidobacteria are members of the numerous bacterial phylum Actinobacteria and represent a major component of the anaerobic microbiota of people, some mammals and economically important pollinators (honeybees and bumblebees). They are considered together with lactobacilli and other lactic acid bacteria as the main representatives of the so-called probiotic microorganisms which are defined as „live microorganisms-especially bacteria-which when administered in adequate amounts confer a health benefit on the host".

The most studied groups of bacteria by scientific community with the potential to positively affect the health of the hosts are bifidobacteria and lactobacilli. Characterization and description of a new species of prokaryotes is primarily based on genotypic analyses and then based on phenotypic analyses Until now, we have characterized and described three new, host-specific species of bifidobacteria (*B. actinocoloniiforme*, *B. bombi* and *B. bohemicum;*Killer et al., 2010a, Kopečný et al., 2010, Killer et al., 2011a, Bunešová et al., 2014, Killer et al., 2014) present in the digestive tract of bumblebees. It was the first report on presence of bifidobacteria in the digestive tract of these important pollinators. In addition, we have described based on results of modern phenotypic, chemotaxonomic and genotypic analyses a new genus within the family *Bifidobacteriaceae* (*Bombiscardovia*) occurring also in the digestive tract of bumblebees (Killer et al., 2010b). Representatives of the family *Bifidobacteriaceae* (genera *Bifidobacterium*, *Alloscardovia*, *Aeriscardovia Bombiscardovia*, *Scardovia*, *Parascardovia*, *Pseudoscardovia* and *Neoscardovia;* Killer et al., 2013a, 2013b) have specific pattern of glucose fermentation. They ferment glucose and other sugars through two active phosphoketolases (xylulose-5-phosphate/fructose-6-phosphate phosphoketolases) until fermentation end products in the form of acetic and lactic acids (molar ration 3:2). The modern taxonomic methods, primarily MLST (Multilocus Sequence Typing) have been used to describe another new genus *Pseudoscardovia* (species *P. suis* and *P. radai*) within the family *Bifidobacteriaceae*. Representatives of the new genus were isolated from the digestive tract of wild pigs and it was the first report on bifidobacteria and related bacteria in the specific ecological niche (Killer et al., 2013b).

Three new species of lactobacilli and a new species within the genus *Vagococcus* were characterized in our laboratory (Killer et al., 2014). Species *Lactobacillus apis* was the first new taxon observed and characterized within the digestive tract of honeybees (*Apis mellifera mellifera*). Because of inhibitory effect on causative agents of American and European Foulbrood, the new taxon is currently hopefully tested as probiotic candidate in beekeeping. Similarly, *Lactobacillus bombi* is the first documented taxon within the genus *Lactobacillus* occurring in the digestive tract of bumblebees and it is at the present time tested in cooperation with the Research Institute for Fodder Crops, Ltd. Troubsko (Czech Republic) as a probiotic candidate together with host-specific bifidobacteria in laboratory-reared bumblebees. Another new *Lactobacillus* taxon characterized and described by modern taxonomic methods in our laboratory is *L. rodentium* inhabiting the digestive tract of wild rodents (KIller et al., 2014b).
The LAM staff also participated in the research regarding the detailed identification of bifidobacterial isolates occuring in some food (ovine cheeses, Killer et al., 2014c) and digestive tract of unexplored mammals such as elephants (Killer et al., 2013c).

Methodology development

The study of microbial diversities always involves sample collection. The best way to preserve samples for following DNA analysis is freezing, but this step may not always be possible. We studied alternate methods for sample storage which involved RNA later and ethanol as liquid preservants. The samples were examined using culture-independent methods (PCR-DGGE, and Real-time PCR) to qualitatively and quantitatively assess fecal microbiota composition and to compare differences among the storage methods. Noticeably, freezing samples resulted in the highest recoveries of DNA. No significant differences in DNA recovery were found between freezings and using RNAlater; however, significantly lower DNA concentrations were recovered from samples stored in 96% ethanol. Using PCR-DGGE we found that either 96% ethanol, RNAlater or freezing were suitable for preserving bacterial DNA; however fingerprints obtained from RNAlater storage were more similar to those obtained from the frozen method; in comparison to the patterns resulting from storing samples in ethanol. Using qPCR, frozen samples yielded the highest values of bacterial counts, with the exception of Enterobacteriaceae, which showed the highest numbers using samples stored in ethanol. Sequences of amplicons obtained from PCR-DGGE belonged to the families Clostridiaceae, Lactobacillaceae, Staphylococcaceae, and Lachnospiraceae, phylum Firmicutes; however most amplicons showed sequence similarity to previously uncultured microorganisms. Bacteria belonging to the phylum Firmicutes were the most frequently identified species in the fecal bacterial communities of captive western gorillas. The study showed that RNAlater is an optimal storage method when freezing is not possible. We also later discovered that RNAlater did not stop all bacteria from growing, such as Leuconostoc sp. (Vlčková et al., 2012).

The effect of DNA extraction and sample preservation method on rumen bacterial population was also studied. The comparison of the bacterial profile of intracellular (iDNA) and extracellular DNA (eDNA) isolated from cow rumen content stored under different conditions was conducted. The influence of rumen fluid treatment (cheesecloth squeezed, centrifuged, filtered), storage temperature (RT, -80 °C) and cryoprotectants (PBS-glycerol, ethanol) on quality and quantity parameters of extracted DNA was evaluated by bacterial DGGE analysis, real-time PCR quantification and metabarcoding approach using high-throughput sequencing.

Samples clustered according to the type of extracted DNA due to considerable differences between iDNA and eDNA bacterial profiles, while storage temperature and cryoprotectants additives had little effect on sample clustering. The numbers of Firmicutes and Bacteroidetes were lower ($P < 0.01$) in eDNA samples. The qPCR indicated significantly higher amount of Firmicutes in iDNA sample frozen with glycerol ($P < 0.01$). Deep sequencing analysis of iDNA samples revealed the prevalence of Bacteroidetes and similarity of samples frozen with and without cryoprotectants, which differed from sample stored with ethanol at room temperature. Centrifugation and consequent filtration of rumen fluid subjected to the eDNA isolation procedure considerably changed the ratio of molecular operational taxonomic units (MOTUs) of Bacteroidetes and Firmicutes. Intracellular DNA extraction using bead-beating method from cheesecloth sieved rumen content mixed with PBS-glycerol and stored at -80 °C was found as the optimal method to study ruminal bacterial profile (Fliegerová et al., 2014). The team members participated in qPCR and PCR-DGGE data development and analyses and in writing the paper.

We describe a suitable procedure for preparation of IgY, which is based on freezing-thawing of the water diluted yolks followed by a filtration step to prepare a crude IgY water extract. Then, the specific sodium chloride precipitation of IgY at low pH, resulting in a highly purified fully immunoreactive IgY preparation, was used. Moreover, the purification protocol was optimized as regarded chemicals, instruments and sample handling (Hodek et al., 2013). The large chicken productivity makes the antigen-specific IgY an excellent tool for prophylactic and therapeutic treatment, where large amounts of antibodies are needed. Given the benefits of IgY production, it is rather surprising that a wider use of IgY for passive immunization and other human related applications is still rare. In our lab purification and isolation of antibodies was performed and also we take part in the manuscript preparation.

Modulation and stabilization of microbial ecosystems

The human gastrointestinal tract harbors a complex community of microorganisms that play a significant role in human health. The highest concentration of bacteria has been found in the large intestine and the relevance and effect of resident microflora on a host physiology has been well documented. However, the colon bacteria are often considered as a potentially harmful since they may initiate pathological disorders including intestinal obstruction, inflammatory bowel disease and even colon cancer. Critical role in the etiology of the inflammatory bowel diseases is played by intestinal microflora.

Probiotic bacteria can be used for the prevention and treatment of human inflammatory diseases including inflammatory bowel diseases (IBD). However, the nature of active components and exact mechanisms of these beneficial effects have not been fully elucidated. Our aim was to investigate if lysate of probiotic bacterium *L. casei* DN-114 001 (Lc) could decrease the severity of intestinal inflammation in a murine model of IBD. The preventive effect of oral administration of Lc significantly reduces the severity of acute dextran sulfate sodium (DSS) colitis in BALB/c but not in SCID mice. Our study provided evidence that even non-living probiotic bacteria can prevent the development of severe forms of intestinal inflammation by strengthening the integrity of intestinal barrier and modulation of gut microenvironment (Zákostelská et al., 2011). The study was carried under cooperation with Microbiological Institute of ASCR (Prague). Our department was responsible for microbial analyses by qPCR approach including data analysis and discussion.

Chitosan is natural biopolyaminosacccharide that is part of many commercial food supplements aiming to lower cholesterol and body fat, yet no one tested the influence of chitosane on gut microbiome. The changes in bacterial population can significantly alter condition in gastrointestinal tract. Chitosan (CS) and chitooligosaccharides (COS) can be with respect to its properties used as microbial modulating and mucus protective agent. Preparation and characterization of CS products with different molecular weight was essential for all chitosan projects. Primarily used enzymatic hydrolysis resulted in two fractions of low-molecular-weight (LMW) chitosans with Mw 34 kDa and 14.6 kDa. In the oxidative reaction at the hydrogen peroxide and acetic acid, two LMW chitosans with Mw 9 and 10 kDa were isolated with the total yield 86 wt%. In microwave irradiation, two chitosan fractions with Mw 80 and 120 kDa (total yield 70 wt%) were obtained (Tishchenko et al., 2011). The study was carried under cooperation with Institute of macromolecular chemistry of ASCR (Prague). Our department was responsible for chitosan preparation and purification, testing of bacterial sensitivity and experiments with rats and data analysis and discussion. The effect of chitosan and its splitting products on gut microbiome was studied in in vivo condition on rats and human supplemented by CS and COS and changes in the composition of microflora were monitored.

A feeding study was performed to monitor the effect of chitosan intake on the fecal microbiota of ten healthy human subjects. Diversity of microflora was monitored during 8 weeks including 4 weeks of chitosan supplementations. Using denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene amplicons and quantitative PCR method we revealed possible changes originating in the overall bacterial composition and also in the subpopulation of Bifidobacterium group. DGGE profiles displayed high complexity and individuality for each subject. Considerable variations in the composition of band patterns were observed among different persons. A raised level of fecal Bacteroides in response to chitosan intake was found in all samples. Bifidobacterium levels following chitosan intake increased or remain unchanged. Non-significant increase was, surprisingly, found in the numbers of butyrate-producing bacteria (Mrázek et al., 2010).

The concept of the impact of these biopolyamins with different degrees of polymerisation was completed by studies of the action on pure of human colonic bacteria in vitro. *Bifidobacteria* represent a significant part of the so-called beneficial microflora and play an important role in the eco-physiology of the colonic ecosystem. Our results support the concept that CS/COS represents one of the means for positive influencing the bifidobacterial population in the human colon (Šimůnek et al., 2010). Specific growth rate of seven selected nonpathogenic anaerobic bacterial strains *(Clostridium paraputrificum, Clostridium beijerinckii, Roseburia intestinalis,Bacteroides vulgatus, Bacteriodes thetaiotaomicron, Faecalibacterium prausnitzii and Blautia coccoides*) was determined in the presence of COS (2, 3, and 6 kDa), LMWC (10 and 16 kDa), and CS in vitro. The growth rate decreased in all strains in the presence of COS and LMWC in higher concentrations in comparison to control incubations. A relatively higher resistance to CS hydrolyzates was detected in *R. intestinalis* and *F. prausnitzii*, and more susceptible were bacteria belonging to *Bacteoides* sp. and *Clostridium* sp. The antimicrobial activity increased with t degree of polymerization (DP). We can conclude that CS and its oligomers possess the ability to modulate the composition of microbial ecosystem in human colon (Šimůnek et al., 2012).

In our study we used the rats with dextran sulfate sodium-induced colitis (DSS) for monitoring of changes in composition of microbial community in colon tissue and

fecal samples. The effect of the administration of CS and COS on rat fecal microbiota was analyzed in study of Koppova et al., 2012. The profile of total bacterial population was monitored during 3 weeks of CS or COS application using denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene amplicons. CS or COS per os administration changed the profile and structure of the microbial ecosystem of the gastrointestinal tract of healthy rats. COS have, in most cases, an opposite effect compared with CS.

Miscellaneous studies

Application studies of LAM research group investigated the influence of axenic cultures of rumen anaerobic fungi on biogas production from energy crops in laboratory conditions (Fliegerová et al. 2012b, Leis et al. 2014). Strains of anaerobic fungi isolated from feces or rumen fluid of cows were tested for their ability to integrate into anaerobic sludge bacterial ecosystem, to improve degradation of substrate polysaccharides and consequently to influence biogas production. Batch culture, semicontinuous, and continuous experiments have been performed using anaerobic microbial consortium based on fermented pig slurry with maize or grass silage, and different genera of anaerobic fungi (Prochazka et al. 2012). All experiments indicated positive effect of rumen anaerobic on biogas yield and quality. The biogas production was improved by anaerobic fungi by 9% up to 18 %; methane ratio in biogas was higher about 2.5% depending on the used substrate and species of rumen fungi. The best results were achieved during continuous experiments with mixture of fungal cultures Anaeromyces (KF8) and Piromyces (KF9), where increased biogas production and higher methane proportion was observed during the whole experimental period.

Genes encoding glycosyl hydrolase family 11 (GH11) xylanases and xylanases have been identified from Pseudobutyrivibrio xylanivorans. In contrast, little is known about the diversity and distribution of the GH10 xylanase in strains of P. xylanivorans. Xylanase and associated activities of P. xylanivorans have been characterized in detail in the type strain, Mz5. The aim of the present study was to identify GH10 xylanase genes in strains 2 and Mz5 of P. xylanivorans. In addition, we evaluated degradation and utilization of xylan by P. xylanivorans 2 isolated from rumen of Creole goats. After a 12-h culture, P. xylanivorans 2 was able to utilize up to 53% of the total pentose content present in birchwood xylan (BWX) and to utilize up to 62% of a ethanol-acetic acid-soluble fraction prepared from BWX. This is the first report describing the presence of GH10 xylanase-encoding genes in P. xylanivorans. Strain 2 and Mz5 contained xylanases which were related to GH10 xylanase of Butyrivibrio sp. Identifying xylanase-encoding genes and activity of these enzymes are a step toward understanding possible functional role of P. xylanivorans in the rumen ecosystem and contribute to providing an improved choice of enzymes for improving fiber digestion in ruminant animals, agricultural biomass utilization for biofuel production, and other industries (Grilli et al., 2014).

Instrumentation

Microbial diversity characterization is best achieved with modern tools of molecular genetics. A next-generation sequencing (NGS) approach was recently introduced as a novel tool allowing completely new insights into microbiome ecosystem: diversity description by PCR amplicon direct sequencing, bacterial characterization by whole genome sequencing, functional description by reverse transcription sequencing of mRNA etc. Ion Torrent Personal Genome Machine was

purchased in 2012 via Academy of Sciences of Czech Republic funding as NGS tool. Method of direct amplicon sequencing was later introduced in our laboratory and is currently being further developed and tested for new approaches.

Analysis of NGS data is a challenging task that requires quite high bioinformatic skills. Our laboratory group does not employ a bioinformatic, though we were able to learn this process through a Linux based software package QIIME. Obtained results are used for microbial alpha and beta diversity descriptions from human and animal digestive tract as well as from bio waste treatment facilities.

Geneious software package was also purchased to help analyze Sanger sequencing data (alignment, phylogeny, cloning and primer design).

Methods of anaerobic microbiology are dependent on generation of strictly anaerobic atmosphere in cultivating space. In our laboratory is routinely used for isolation and cultivation of strictly anaerobic microorganisms automatic glove box The Whitley Anaerobic Workstation.

Excellence

Last evaluation recommended LAM increased attention to the publication quality and to increase numbers of MS and PhD students. Both annual publication total impacts (from 14.3 in 2009 to 39.0 in 2014) and an impact per publication (from 1.3 in 2009 to 2.7 in 2014) has more than doubled in the last five years. Also number of MS and PhD students increased from 3 to 6 in the last five years.

Collection of Anaerobes (Bacteria, fungi and methanogens)

The collection contains anaerobic bacteria from ruminants, mostly from the rumen from all over the world – Europe, Asia, Australia, Amecica. Second part of the collection represents isolates from the human digesta. The biggest part are representing fibrolytic isolates for genera *Butyrivibrio, Pseudobutyrivibrio, Ruminococcus, Fibrobacter, Selenomonas, Eubacterium, Clostridium, Bacteroides, Bifidobacterium, Coprococcus, Roseburia, Prevotella, Megasphaera, Faecalibacterium, Escherichia, Treponema, Succinivibrio, Streptococcus, Wolinella, Veillonella,* and *Lactobacillus*. Anaerobic fungi are represented mainly by genera *Anaeromyces, Orpinomyces* and *Caecomyces*. Methanogens are represented by genus Methanosarcina. The collection contains more than 200 isolates which can be provided to other labs.

Cooperation

Academic cooperation is based on (1) students training as supervisor of their bachelor, master of sciences and doctoral theses. Following universities are involved: University of Chemistry and Technology (Prague), Charles University in Prague and Masaryk University (Brno); (2) lecture presentation at Czech University of Life Sciences, Prague.

Study of gut disorders involves research cooperation with Institute of Microbiology of the ASCR, Institute of Clinical and Experimental Medicine and Second Faculty of Medicine of Charles University, Prague.

Study of microbiome of animal digestive tract involves cooperation with Institute of Life Science (Prague), Faculty of Sciences of Charles University (Prague) and Mendoza University (Argentina).

International linkage of Laboratory of Anaerobic Microbiology:

The Rowett Research Institute, University of Aberdeen, Scotland (R.J. Wallace, H. Flint, K. Scott)

The University of New England, Armidale, Australia (Keith Gregg)
The University of Ljubljana, Slovenia (G. Avguštin, R. Marinšek-Logar)
The Hokkaido University, Japan (Y. Kobayashi)
The Mendoza University, Argentina (D. Grilli)
University of Innsbruck, Austria (H. Insam, S. Podmirseg)
University of Sassari, Italy (G. Moniello)
The Aberswyth University, Wales, UK (G. Griffith)
The Institute of Animal Physiology Košice (P. Javorský, P. Pristaš, V. Kmeť)
The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences (G. Belzecki, M. Czauderna)
University of Innsbruck (H. Insam, S. Podmirseg)
Zellkulturen GmbH, Germany (Dr. Peter Schumann, Dr. Rudiger Pükall)

Research Report of the team in the period 2010–2014

Institute	Institute of Animal Physiology and Genetics of the CAS, v. v. i.

Scientific team	Team of Biochemistry and Molecular Biology of Germ Cells

Area of research:

The research team is focused predominantly on the study of regulation of meiotic division in mammalian female germ cells, oocytes. The understanding of mechanisms, which regulate meiotic maturation of oocytes, would have a high impact both on the field of reproductive biotechnology in farm animals (improvement of the conditions for in vitro culture of the oocytes, and in farther perspectives for obtaining in vitro produced preimplantation embryos of better quality), and on the field of human assisted reproductive technologies (improving ART techniques, identifying the factors affecting chromosome aneuploidy in oocytes).

The most important results of the team

Study of the chromosomal aneuploidy in mammalian oocytes

Our research during evaluated period was mostly focused on identification of molecular mechanisms, which are in mammalian oocytes responsible for chromosome segregation errors and for creation of aneuploid eggs. The aneuploidy in eggs was linked to increased rate of abortions and severe mental and developmental disorders, such as Down syndrome. Moreover, the frequency of aneuploidy in oocytes significantly increases with maternal age. In search for appropriate animal model to study this phenomenon we have first analysed the frequency of aneuploidy related to maternal age in porcine oocytes. To our surprise we have discovered that porcine oocytes are not affected and therefore it seems that not all mammals are suffering from maternal age related aneuploidy **(Hornak at al., Plos One, 2011).** Then we have used mouse model, where the maternal age related aneuploidy is detected similarly to humans. Our research focused on a major cell cycle control mechanisms, which in somatic cells is able to prevent chromosome division in case when spindle apparatus is incorrectly assembled. Our results revealed that the mammalian oocyte is unable to prevent major segregation defects by postponing anaphase when chromosomes are not properly congressed **(Sebestova et al., Cell Cycle, 2012)**. This was an important discovery, which was also reported at the same time from other laboratories. The advantage of our study was however emphasized by the fact that we were able to obtain the data from living cells under physiological conditions. We have also used model system based on interspecific crosses between two mouse species, M. musculus and M. spretus, which allowed increasing the frequency of congression defects observed in oocytes.

Using similar set of techniques based on multichannel live cell confocal microscopy we participated on study focused on elucidation of post zygotic sterility in interspecific crosses in mammals **(Bhattacharyya et al., PNAS, 2013)**. This phenomenon is perhaps a major drawing force of speciation in mammals. Our contribution to the project was based on analysis of behaviour of chromosomes during meiosis I in intraspecific hybrids. Our data revealed that the increased aneuploidy levels seen in intraspecific hybrids are reflecting problems with spindle assembly and chromosome segregation in meiosis I and provided evidence about the molecular mechanisms behind such important phenomena with a major impact of speciation and evolution.

Recently, our laboratory made another important discovery relevant not only to the reproduction field. By comparison of the aneuploidy levels between various mouse strains we have revealed that although the overall aneuploidy is similar between analysed mouse strains, there were significant differences in precious segregation of sister chromatids (PSSC) in meiosis II **(Danylevska et al., Chromosome Res., 2014)**. Since PSSC is a main mechanism behind the aneuploidy in mammalian oocytes, our discovery is important for subsequent studies since it is possible to analyse in parallel strains with lower or higher frequency of PSSC and by their comparison study the molecular differences between those two strains.

Study of the condensin subunits in mammalian oocytes

The multi-protein complexes known as condensins (I and II) are major players in chromosome dynamics in mitotic and meiotic cells, especially in the process of chromosome condensation. Our team has studied these complexes in maturing porcine oocytes and documented for the first time the presence of different condensin subunits from both complexes in mammalian oocytes **(Liskova et al., Reprod.Fert.Dev., 2010)**. We have documented different localization of different condensin subunits and we have also proved that at least one of the subunits (CAP-D3) becomes phosphorylated during oocyte maturation, which is likely to have important consequences for the activation of the condensin complex.

Study of the role of protein degradation via ubiquitination during mammalian oocyte maturation and fertilization

Fertilization in mammals is characterized by formation of one male and one female pronucleus after incorporation of a single spermatozoon into an oocyte. Such a constellation of paternal and maternal chromatin leads to normal embryo development. However, fertilization by more than one spermatozoon, called polyspermy, causes aberrant development and death of the embryo at an early stage of development. Of particular interest for the study is the role of ubiquitin ligase-UCHL1 in the prevention of abnormal polyspermic fertilization in mammals (**Susor et al., 2010, Biol.Reprod.**). We have detected decreased levels in the monomeric ubiquitin and polyubiquitin pool after down-regulation of UCHL1. The down-regulation of UCHL1 in mammalian oocyte enhanced formation of presumptive UCHL1 oligomers and subsequently increased abundance of K63-linked polyubiquitin chains in oocytes. We have unveiled the alteration of dynamics of cortical granules (CG) by ubiquitin proteasome pathway. Our findings indicate that antipolyspermy defense in bovine oocytes relies on UCHL1-controlled functioning of CGs. These findings are important for understanding of biological processes which effects production of mammalian embryos in vitro for human medicine and agriculture.

In the study of **Mtango et al. (2012, J.Cell. Physiol.)** we have used mouse model with mutation in the UCHL1, which has shown an intriguing pattern of switched UCH localization, with UCHL3 replacing UCHL1 in the oocyte cortex. The embryos from homozygous Uchl1$^{-/-}$ mutant females fail to undergo morula compaction and do not form blastocysts in vivo, indicating a maternal effect related to UCHL1 deficiency. This suggests that the activity of oocyte UCHLs contributes to fertilization and embryogenesis by regulating the physiology of the oocyte and embryo.

Study of regulation of translation in mammalian oocytes and its specific effects on the major morphological changes during maturation

The members of our team have studied the regulation of translation initiation on the 3'-UTR of mRNAs in maturing porcine oocytes. The major regulatory event occurring in this part of mRNA in oocytes is cytoplasmic polyadenylation, which is believed to be essential for translational activation. This process is generally controlled by phosphorylation of cytoplasmic polyadenylation element binding protein 1 (CPEB1). We have proven that Aurora A protein kinase (which was previously reported as the main activator of CPEB-driven cytoplasmic polyadenylation of mRNAs in oocytes from various species) is essential neither for CPEB phosphorylation, nor for cytoplasmic polyadenylation of the mRNAs in maturing porcine oocytes **(Komrskova et al., Plos One, 2014)**. Our team is now elucidating the identity of the kinase involved in the activatory phosphorylation of CPEB.

Translational regulation at the 5'-UTR of mRNAs (the cap binding protein complex) has been also studied by our team recently. Specifically, we have concentrated on the regulation of the mTOR – eIF-4E pathway. It is known that the cap-binding protein eIF-4E is repressed by its binding protein, 4E-BP1. Phosphorylation of 4E-BP1 mediated by mTOR protein kinase releases eIF-4E from its repressor and in such a way enables initiation of the cap-dependent translation. We have found that during meiotic maturation of mouse oocytes, shortly after nuclear envelope breakdown, translational hotspots develop in the chromosomal area and in a region that was previously surrounded the nucleus. These hotspots are controlled by the activity of the mTOR–eIF4F pathway. Our results suggest the existence of the mechanism that—following the resumption of meiosis—controls the temporal and spatial translation of a specific set of transcripts required for normal spindle assembly, chromosome alignment and segregation **(Susor et al., Nature Commun., 2015)**. This article has been published in January, 2015, however, the majority of the experiments, as well as the reviewing process were realized in the 2013-2014. It was also available online in December 2014.

Most of the studies mentioned above (except for **Bhattacharyya et al., PNAS, 2013 and Mtango et al. 2012, J.Cell. Physiol.)** has been conducted entirely or mainly by the members of our team in our laboratory. Apart from these, the members of our team have participated also in the studies directed to the research of interactions between oocytes and surrounding cumulus cells (**Nagyova et al., Fertil.Steril., 2013, Nagyova et al., 2014, Domest.Animal Reprod., 2014**), study of the components of the follicular fluid (**Jarkovska et al., J.Proteome Res., 2010**), regulation of bovine preimplantation embryo development (**Toralova et al., Reproduction, 2012**), or polymorphism in porcine genes related to meat quality (**Cepica et al., Anim.Genet., 2010, Masopust et al., Mol.Biol.Rep., 2011, Cepica et al., Anim.Genet., 2012, Cepica et al., Meat Sci., 2013, Masopust et al., Mol.Biol.Rep., 2014**)

Awards granted to the members of the research team

In the years 2009-2013 EMBO Installation Grant a Purkynje Fellowship was awarded to Martin Anger by EMBO and Academy of Sciences of the Czech republis, respectively. Marie Curie Reintegration Grant was awarded to Martin Anger in the years 2009-2011.

Other factors or circumstances influencing the outcome of the Research team

During the years 2011-2012, Michal Kubelka was fully involved in the preparation and negotiation of the project Exam (Experimental Animal Models) for the European Operational Programme Research and Development for Innovations (OP RDI) – Regional Centers as the main coordinator. After this project was granted for Centre PIGMOD he was appointed as the temporary director in the years 2012-2013 (Feb) with the task to set up the management and launch the tenders and construction projects. These activities have significantly influenced his capacity to supervise his research projects as well as publication activity.

Research Report of the team in the period 2010–2014

Institute	Institute of Animal Physiology and Genetics of the CAS, v. v. i.

Scientific team	Team of Animal Embryology

a) Craniofacial development

The face is one of the three regions most frequently affected by congenital defects in humans. To understand the molecular mechanisms involved, it is necessary to have a more complete picture of gene expression in the embryo. We used microarrays to profile expression in chicken facial prominences, post neural crest migration and before differentiation of mesenchymal cells. Our study used a chip-wide analysis in which the whole genome is represented, therefore, differentially expressed genes, which were not well characterized or less well annotated, can be detected. Our study was the first to directly compare different regions of the face and has not only identified general trends in the expression profiles but also identified specific sets of genes that are differentially expressed in specific prominences.

The avian embryo was selected due to the fact that it is an amniote and, therefore, more related to humans and mice than fish or amphibians. We profiled the embryonic face at stage HH18, which is after neural crest cells have ceased migrating and before cell differentiation begins in the mesenchyme. Chip-wide analysis shows that out of the three regions of the face surveyed, the one with the most distinct profile is the frontonasal mass. This can be traced back to the source of the neural crest cells. The frontonasal mesenchyme is derived from the most anterior population of neural crest cells that originate from the prosencephalon and anterior mesencephalon, both other regions come from more caudal areas.

After analysis of variance (ANOVA) analysis, 3 094 genes were identified as significantly different between facial prominences at P value - 0.05. Tukey's post hoc testing determined there were 1 954 genes different between the frontonasal mass and mandibular arch, 1 985 differentially expressed genes between the frontonasal mass and maxillary prominence and only 839 genes differentially expressed between the maxillary and mandibular prominences.

We found sets of genes that can be used to identify the normal mandibular, maxillary, and frontonasal mass prominences. Genes characteristic for the frontonasal mass included ALX1, ASCL1, EYA2, FOXG1, SIX3, SOX10, SOX8, SP8 and TBX22, genes of maxillary prominence MAB21L2, MEIS3, ID1, TAGLN3 and mandibular genes DLX1, HAND2, LHX8, MSX2, PITX2, and TWIST2. These results are part of the paper „*Whole genome microarray analysis of chicken embryo facial prominences*".

Our expression profile study has identified many genes differentially expressed in the frontonasal mass and one of the most abundant was the T-box transcription factor (*Tbx22*). In situ hybridization studies in amniotes have documented expression of *Tbx22* in the frontonasal mass and maxillary prominences of chickens. We focused on the regulation and function of *Tbx22*, a repressor dynamically expressed in the frontonasal mass. Both FGF and Noggin (a BMP antagonist) strongly induce *Tbx22*, however, each has opposite effects on morphogenesis - Noggin inhibits whereas FGF stimulates growth. To determine whether *Tbx22* mediates these effects, we used retroviruses to locally increase expression levels. RCAS::hTbx22 decreased proliferation, reduced expression of *Msx2* and *Dlx5* and caused

cleft lip. Decreased levels of endogenous *Tbx22* were also observed but were not the primary cause of the phenotype as determined in rescue experiments. Our data suggest that genetic or environmental insults such as those affecting the BMP pathway could lead to a gain-of-function of *Tbx22* and predispose an individual to cleft lip. These results are part of the paper "*The Function and Regulation of TBX22 in Avian Frontonasal Morphogenesis*"

Other gene selected from microarray screen was Ecotropical viral integration site 1 (*Evi-1),* which is a transcription factor essential for vascularisation and cell proliferation during embryonic development. The chimeric transcription factor AML1-EVI-1 (MECOM) is activated in leukaemia where it plays a role as a differentiation block and stimulator of proliferation. We cloned chicken *Evi-1* and analyzed its expression during embryonic development. There was early expression in the pharyngeal arches, in the brain and intermediate mesoderm of chicken embryos at stage 15. Later at stage 20, *Evi-1* mesenchymal expression was concentrated in the second pharyngeal arch, and weaker expression was found in the mandibular and maxillary prominences. Facial expression decreased in intensity during development. *Evi-1* expression in the limb was also limited to the mesenchyme with the most prominent expression in the anterior margin. *Evi-1* was not detectable in the posterior limb bud. At later stages, *Evi-1* was expressed in the peripheral mesenchyme of the limb but not in the developing precartilage blastema. At stage 29, the expression became restricted to the perichondrium and interdigital areas; however, the cartilage condensations themselves were negative. These results are part of the publication "E*xpression, function and regulation of Evi-1 during embryonic avian development*".

To determine the effect of *Evi-1* on chondrogenesis, we established primary micromass cultures. As the expression was slightly asymmetrical at stage 24 limb buds, we cultivated the anterior and the posterior halves of the forelimb bud separately. After six days of cultivation, the level of chondrogenesis was evaluated in cultures by staining with Alcian blue. First, we evaluated if there are differences in the differentiation of cartilage between the two positions. We found more intense chondrogenesis in the posterior cells cultivated with medium in contrast to the anterior cells. Next, we applied siRNA and observed perinodular inhibition of chondrogenesis in both anterior as well as posterior cells with more intense inhibition in the posterior cultures. When Silencer select negative control no.1 siRNA was applied into cultures, no changes in extracellular matrix production were observed in comparison to medium with Fugene.

Since *Evi-1* was expressed adjacent to the apical ectodermal ridge and this structure is a source of FGFs, we tested whether endogenous FGF receptor signalling was necessary to maintain *Evi-1* expression. First, we used the most common inhibitor SU5402 (1mg/ml), which was soaked into beads and implanted into early limb buds (stages 20 and 24). Downregulation of *Evi-1*expression was observed 24h after implantation into the wing at both treated stages. As SU5402 is known to increase apoptosis in the surrounding cells, we also used another inhibitor PD161570, which was injected into the limb bud during stages 20–22. We observed a similar downregulation of *Evi-1* expression as in previous experiments. These results were confirmed by qPCR analysis of collected limb buds at three different time points after PD161570 injection. *Evi-1* expression was significantly downregulated both 6h and 16h after injection. At the latest collection time point 24h after treatment, there was no significant difference in the expression levels. Based on our results, *Evi-1* may be a novel gene mediating the effects of FGF during limb development. These results are part of the publication "*Expression, function and regulation of Evi-1 during embryonic avian development*".

Furthermore, the pig has recently become popular as a large animal experimental model in many fields of biomedical research. The aim of our study is to evaluate the basic

anatomical structures in the head region of the pig to lay the groundwork for its practical clinical usage or pre-clinical research in the future. We used three different diagnostic imaging methods: radiography, computed tomography (CT) and magnetic resonance imaging (MRI). The analysis showed that radiographic imaging is suitable only for general evaluation of the facial area of the pig skull. CT images showed excellent spatial definition of bony structures of the whole craniofacial area, and MRI images revealed fine soft tissue details. Radiography is preferentially suited to general assessment of bone structures of the facial skeleton; however, the thick layer of adipose tissue in the craniofacial region of the pig makes the imaging of some parts difficult or even impossible. CT is useful for revealing morphological details of mineralized tissues, whereas MRI is more suitable for soft tissue analysis and the detection of subtle pathologic changes in both bone and soft tissues. Therefore, before using pigs as an experimental model in craniofacial research, it is necessary to evaluate the suitability and disadvantages of potential imaging methods and how appropriate they are for accurate visualization of desired structures. These results are part of the publication "*Radiography, computed tomography and magnetic resonance imaging of craniofacial structures in pig*".

****

b) Odontogenesis

The first mouse molar (M1) is the most common model for odontogenesis, with research particularly focused on prenatal development. However, the functional dentition forms postnatally, when the histogenesis and morphogenesis of the tooth is completed, the roots form and the tooth physically anchors into the jaw. In our work, M1 was studied from birth to eruption, assessing morphogenesis, proliferation and apoptosis, and correlating these with remodeling of the surrounding bony tissue. The M1 completed crown formation between postnatal (P) days 0-2, and the development of the tooth root was initiated at P4. From P2 until P12, cell proliferation in the dental epithelium reduced and shifted downward to the apical region of the forming root. In contrast, proliferation was maintained or increased in the mesenchymal cells of the dental follicle. At later stages, before tooth eruption (P20), cell proliferation suddenly ceased. This withdrawal from the cell cycle correlated with tooth mineralization and mesenchymal differentiation. Apoptosis was observed during all stages of M1 postnatal morphogenesis, playing a role in the removal of cells such as osteoblasts in the mandibular region and working together with osteoclasts to remodel the bone around the developing tooth. At more advanced developmental stages, apoptotic cells and bodies accumulated in the cell layers above the tooth cusps, in the path of eruption. Three-dimensional reconstruction of the developing postnatal tooth and bone indicates that the alveolar crypts form by resorption underneath the primordia, whereas the ridges form by active bone growth between the teeth and roots to form a functional complex. These results are part of the publication "*Tooth-bone morphogenesis during postnatal stages of mouse first molar development*".

On the other hand, the mouse third molar (M3) develops postnatally and is thus a unique model for studying the integration of a non-mineralized tooth with mineralized bone. Our study assessed the morphogenesis of the mouse M3, related to the alveolar bone, comparing M3 development with that of the first molar (M1), the most common model in odontogenesis. The mandibular M3 was evaluated from initiation to eruption by morphology and by assessing patterns of proliferation, apoptosis, osteoclast distribution, and gene expression. Three-dimensional reconstruction and explant cultures were also used. Initiation of M3 occurred perinatally, as an extension of the second molar (M2) which grew into a region of soft mesenchymal tissue above the M2, still far away from the alveolar bone. The

bone-free M3 bud gradually became encapsulated by bone at the cap stage at postnatal day 3. Osteoclasts were first visible at postnatal day 4 when the M3 came into close contact with the bone. The number of osteoclasts increased from postnatal day 8 to postnatal day 12 to form a space for the growing tooth. The M3 had erupted by postnatal day 26. The M3, although smaller than the M1, passed through the same developmental stages over a similar time span but showed differences in initiation and in the timing of bone encapsulation. These results are part of the publication "*Morphogenesis and bone integration of the mouse mandibular third molar"*.

Furthermore, we focused on comparative odontogenesis to solve selected developmental problems. The minipig provides an excellent experimental model for tooth morphogenesis because its diphyodont and heterodont dentition resemble that of humans. However, little information is available on the processes of tooth development in the pig. The purpose of our study was to classify the early stages of odontogenesis in minipigs from the initiation of deciduous dentition to the late bell stage when the successional dental lamina begins to develop. To analyze the initiation of teeth anlagens and the structural changes of dental lamina, a three-dimensional (3D) analysis was performed. At the earliest stage, 3D reconstruction revealed a continuous dental lamina along the length of the jaw. Later, the dental lamina exhibited remarkable differences in depth, and the interdental lamina was shorter. The dental lamina grew into the mesenchyme in the lingual direction, and its inclined growth was underlined by asymmetrical cell proliferation. After the primary tooth germ reached the late bell stage, the dental lamina began to disintegrate and fragmentize. Some cells disappeared during the process of lamina degradation, while others remained in small islands known as epithelial pearls. The minipig can therefore, inter alia, be used as a model organism to study the fate of epithelial pearls from their initiation to their contribution to pathological structures, primarily because of the clinical significance of these epithelial rests. These results are part of the publication "*Early morphogenesis of heterodont dentition in minipigs*".

Functional tooth germs in mammals, reptiles, and chondrichthyans are initiated from a dental lamina. The longevity of the lamina plays a role in governing the number of tooth generations. Monophyodont species have no replacement dental lamina, while polyphyodont species have a permanent continuous lamina. In diphyodont species, the dental lamina fragments and regresses after initiation of the second tooth generation. Regression of the lamina seems to be an important mechanism in preventing the further development of replacement teeth. Defects in the complete removal of the lamina lead to cyst formation and has been linked to ameloblastomas. In our study, we showed the previously unknown mechanisms behind the disappearance of the dental lamina, involving a combination of cell migration, cell-fate transformation, and apoptosis. Lamina regression starts with the loss of the basement membrane, allowing the epithelial cells to break away from the lamina and migrate into the surrounding mesenchyme. Cells deactivate epithelial markers (E-cadherin, cytokeratin), up-regulate Slug and MMP2, and activate mesenchymal markers (vimentin), while residual lamina cells are removed by apoptosis. The uncovering of the processes behind lamina degradation allows us to clarify the evolution of diphyodonty, and provides a mechanism for future manipulation of the number of tooth generations. These results are part of the publication "*Early regression of the dental lamina underlies the development of diphyodont dentitions*".

In monophyodont (single generation) species such as the mouse, no successional lamina develops. We have selected a reptilian monophyodont species – the Veiled Chame-

leon (Chamaeleo calyptratus) – to investigate whether this is a common characteristic of species that do not have replacement teeth. Furthermore, we focused on the sequence of tooth initiation along the jaw, and tooth attachment to the bones. Embryos of the Veiled Chameleon were collected during the first 6 months of incubation (from the 5th to 24th week) at 7-day intervals. After five weeks of incubation, an epithelial thickening was present as a shallow protrusion into the mesenchyme. A week later, the epithelium elongated more deeply into the mesenchyme to form the dental lamina. The formation of all tooth germs along the jaw was initiated from the tip of the dental lamina. Development of a successional dental lamina was initiated during the pre-hatching period but this structure became markedly reduced during juvenile stages. MicroCT analysis showed the presence of a heterodont dentition in young chameleons with multicuspid teeth in the caudal jaw area and simpler monocuspid teeth rostrally. Unlike the pleurodont teeth of most reptilian species, chameleon teeth are acrodontly ankylosed to the bones of the jaw. Odontoblasts produced a layer of predentine that connected the dentine to the support-ing bone, with both tooth and bone protruding out of the oral cavity and acting as a functional unit. Based on our observations, chameleons may provide new and useful information to study the molecular interaction at the tooth–bone interface in physiological as well as pathological conditions. These results are part of the publication "*Odontogenesis in the Veiled Chameleon (Chamaeleo calyptratus)*".

The incisors of the mammalian dental arch develop from tissues arising from separated facial prominences. These primordial craniofacial structures undergo complex morphogenetic processes as they merge and fuse in a time and space dependent fashion. However, local contributions of precursor facial prominences to the incisors that develop subsequently remain unknown. The purpose of our study was to characterize the development of all three deciduous upper rostral teeth in the pig (Sus scrofa f. domestica) for the identification of the likely facial prominence contributions to the incisors based on normal and pathological developmental relationships. We collected embryonic minipigs between gestational days 20-36 (E20-36) and processed them for histological analysis and subjected to computerized 3D modelling. The location and morphology of the incisors (i) in these specimens were characterized and compared between developmental stages. A second set of neonatal minipigs displaying cleft lip and/or cleft palate defects were also obtained and incisor locations and eruption patterns were morphologically examined. Palate formation begins during the third week of gestation (E20) in the minipig with ossification of the premaxilla initiating soon afterwards (E24). The third incisor (i3) develops caudally to the contact seam formed by the fusion of the primary and secondary palates in normal embryos. All cleft animals displayed normal i3 and canine, on other hand, development of i1 and i2 was often disrupted similar to human. In conclusion, our observations suggest a dual embryonic origin of the incisors in minipigs with the first and second incisors originating from the frontonasal prominence whilst the third incisor forms from tissues derived from the maxillary prominence. These results are part of the publication "*Comparative morphology of normal and cleft minipigs demonstrates dual origin of incisors*".

Molecular signaling of selected candidate genes was also analyzed during odontogenesis. The transcription factor c-Myb is involved in the control of cell proliferation, survival and differentiation. As these processes accompany the morphogenesis of developing teeth, our work investigated the possible role of c-Myb during odontogenesis. Analysis of the expression of c-Myb in the monophyodont mouse was followed by similar analysis in a diphyodont species, the pig, which has a dentition more closely resembling that of the human. The distribution of c-Myb was correlated with the pattern of proliferation and apoptosis and the tooth phenotype of c-Myb mutant mice was also assessed. In the mouse, c-Myb

expression was detected throughout prenatal development of the first molar tooth. Negative temporospatial correlation was found between c-Myb expression and apoptosis, while c-Myb expression positively correlated with proliferation. c-Myb-positive cells, however, were more abundant than the proliferating cell nuclear antigen positive cells, suggesting other roles of c-Myb in odontogenesis. In the minipig, in contrast to the mouse, there was an asymmetrical arrangement of c-Myb positive cells, with a higher presence on the labial side of the tooth germ and dental lamina. A cluster of negative cells was situated in the mesenchyme close to the tooth bud. At later stages, the number of positive cells decreased and these cells were situated in the upper part of the dental papilla in the areas of future cusp formation. The expression of c-Myb in both species was strong in the odontoblasts and ameloblasts at the stage of dentin and enamel production suggesting a possible novel role of c-Myb during tooth mineralization. These results are part of the publication "*Expression and characterization of c-Myb in prenatal odontogenesis*".

We also analyzed the expression pattern of c-MYB during postnatal stages of mouse molar odontogenesis using immunohistochemistry on serial sections. Along with an abundance of the c-MYB protein in proliferating zones, we confirmed the presence of this protein in differentiated ameloblasts, odontoblasts, and osteoblasts. In addition, c-MYB was also found in cementoblasts and alveolar fibroblasts. These findings suggest integration of c-MYB into regulatory networks during hard-tissue differentiation and mineralization. These results are part of the publication "*Localization of c-MYB in differentiated cells during postnatal molar and alveolar bone development*".

In mouse incisor, a population of somatic stem cells in the cervical loop is responsible for the incisor regeneration. Understanding of the specificities of these cells is therefore of an interest in basic research as well as regenerative therapies. The Myb transcription factors are involved in essential cellular processes. B-Myb is often linked to the stem cell phenotype, and c-Myb expression marks undifferentiated and proliferating cells such as the stem cells. In our study, temporo-spatial expression of B-Myb and c-Myb proteins was correlated with localisation of putative somatic stem cells in the mouse incisor cervical loop by immunohistochemistry. B-Myb expression was localised mostly in the zone of transit-amplifying cells, and c-Myb was found in the inner enamel epithelium, the surrounding mesenchyme and in differentiated cells. Taken together, neither B-Myb nor c-Myb was exclusively present or abundant in the area of the incisor stem cell niche. Their distribution, however, supports recently reported novel functions of c-Myb in differentiation of hard tissue cells. These results are part of the publication "*Mouse incisor stem cell niche and Myb transcription factors*".

Bone remodelling during tooth eruption was analyzed at molecular level with focus on BMP6. This bone morphogenetic protein with certain therapeutical potential was detected in tooth (odontoblasts, ameloblast, cementoblasts), bone (osteoblasts, osteoclasts) and periodontal tissues. Immunohistochemical approach was combined with 3D reconstructions of the bone remodeling in the interdental septa and underneath the dental primordia. Moreover, the work was supplemented by correlation analysis of BMP distribution with other morphogenetic events based on localization of specific markers in serial sections. These results are part of the publication "*Distribution of BMP6 in the alveolar bone during mouse molar eruption"*.

Next, we focused on the causes of dentinogenesis disruption. Enamel and dentin hypoplasia are the most common abnormalities of development and mineralization of human teeth. Several reports are available in the literature on the influence of dietary calcium on the

formation of human and rodent tooth; however, the information about the influence of dietary phosphorus on the tooth formation is scarce. The aim of the our investigation was to examine the chronic effect of high phosphorus diet and improper dietary calcium to phosphorus ratio on the mandibular incisor microstructure in a hystricomorph rodent - Octodon degu - using macroscopic observation, histopathological examination, transmission and scanning electron microscopy. Our study showed that enamel and dentin development is disturbed under high phosphorus diet and improper calcium to phosphorus ratio. Disturbed mineral metabolism resulted in enamel depigmentation, enamel hypoplasia, enamel pitting and altered dentin morphology. The results suggest that more attention should be focused on dietary phosphorus content when facing altered tooth structure in young patients with deciduous or permanent dentition. Furthermore, we showed that degus can be used as an experimental animal model for the study of the developmental teeth disturbances. These results are part of the publication "*Effect of high phosphorus diet on tooth microstructure of rodent incisors*".

****

c) Apoptotic signalling during odontogenesis and osteogenesis

The primary enamel knot (PEK) is a population of cells that shows spatio-temporal restricted apoptosis during tooth development. It has been shown that caspases are essential for apoptosis in the PEK as well as the central caspase-3. Caspase-7, as another executioner member in the caspase machinery, is considered to have caspase-3 like properties. The aim of our study was to detect caspase-7 activation during molar tooth development with a special focus on the cells of the PEK and to correlate the expression with the pattern of apoptosis and caspase-3 activation. Special attention was also paid to non-apoptotic functions of apoptotic molecules. Cleaved caspase-7 was found in the apoptotic region of the primary enamel knot (PEK), however, caspase-7-deficient mice still possessed apoptotic cells in the PEK in a similar distribution to the wild type. Caspase-7 is therefore not essential for apoptosis in the PEK. Notably, cleaved caspase-7-positive cells were found at later stages in odontoblasts and ameloblasts, but expression did not correlate with apoptosis in these tissues. The results indicate a non-essential apoptotic role of caspase-7 in the PEK apoptosis but suggest also possible non-apoptotic functions for caspase-7 in tooth development. These results are part of the publication "*Caspase-7 in molar tooth development*".

Cleaved caspase-7 was also analyzed during incisor development in mouse. At E17.5, the incisor tooth germ has reached the bell stage. At this stage the differentiation of functional odontoblasts proceeds as a gradient from the apex (cervical loops), while the ameloblasts are just starting to differentiate. Positive staining for cleaved caspase-7 was apparent particularly in the cytoplasm of the differentiating ameloblasts, cells of the stratum intermedium and also the polarized odontoblasts. Staining was detected on the inner portion of the epithelium on the labial part of the cervical loop. At P0, functional ameloblasts and enamel were covering the labial part of the tooth germ, although only in its anterior portion. Staining for cleaved caspase-7 was found in the odontoblasts and ameloblasts, whereas the neighbor cells of stratum intermedium were caspase-7 negative at this stage. In the labial part of the cervical loop, caspase-7 positive cells were detected on the inner portion of the epithelium, while the outer epithelium in contact with the peridental mesenchyme remained unstained. Later on, at P10, the active form of caspase-7 was detected in the cytoplasm of ameloblasts and odontoblasts while nuclei in both cell types were negative. A clear border was observed between the cleaved caspase-7 positive odontoblasts/ameloblasts and the cells of the peridental mesenchyme, next to these ameloblasts, which was negative. Caspase-7 positive odontoblasts were detected also in the root-analog of the incisor, whereas the stratum

intermedium cells remained caspase-7 negative. Few scattered, slightly positive cells were found in the marginal parts beneath the forming odontoblast layer.

Analysis of caspase-7-deficient mice revealed a significant thinner layer of hard tissue in the adult incisor. Micro CT scan confirmed this decrease in mineralized tissues. These data strongly suggest that caspase-7 might be directly involved in functional cell differentiation and regulation of the mineralisation of dental matrices. Our results are the first to report a non-apoptotic function of caspase-7 in osteogenesis and also demonstrates further specificities in endochondral vs. intramembranous ossification. These results are part of the publication "*Caspase-7 participates in differentiation of dental hard tissues*".

Next, we followed the localisation of the activated form of caspase-7 during intramembranous (alveolar and mandibular bones) and endochondral (long bones of limbs) ossification in mice. In both bone types, the activated form of caspase-7 was detected from the beginning of ossification during embryonic development and persisted postnatally. The bone status was investigated by microCT in both wild-type and caspase-7-deficient adult mice. Intramembranous bone in mutant mice displayed a statistically significant decrease in volume while the mineral density was not altered. Conversely, endochondral bone showed constant volume but a significant decrease in mineral density in caspase-7 knock-out mice. Cleaved caspase-7 was present in a number of cells that did not show signs of apoptosis. PCR array analysis of the mandibular bone of caspase-7-deficient versus wild-type mice pointed to a significant decrease in mRNA levels for Msx1 and Smad1 in early bone formation. These observations might explain the decrease in the alveolar bone volume of adult knock-out mice. In conclusion, our study is the first to report a non-apoptotic function of caspase-7 in osteogenesis and also demonstrates further specificities in endochondral versus intramembranous ossification. These results are part of the publication "*Non-apoptotic functions of caspase-7 during osteogenesis*"**.**

****

d) Novel methods were applied to investigate signalling centres of developing teeth in order to reveal cellular and molecular interactions

Laser capture microdissection (LCM) uniquely allows the selection of specific cell populations from histological sections. These selected cells are then catapulted into a test tube without any contamination from surrounding tissues. During the last ten years, many significant results have been achieved, particularly at the level of DNA and RNA where amplification techniques are available. However, where amplification procedures are difficult, the benefits of LCM diminish. To overcome such difficulties, a novel approach, combining laser capture microdissection and flow cytometry, has been tested here for detection of apoptosis and proliferation in tissue bound cell populations without any amplification steps. The mouse cap stage molar tooth germ was used as a model. At the centre of the inner enamel epithelium, the primary enamel knot is a clearly defined apoptotic population with minimal proliferation, flanked by the highly proliferative cervical loops on each side. Thus within the tooth germ epithelium at this stage, two distinct populations of cells are found side by side. These populations were selected by laser capture microdissection and then analysed by flow cytometry for apoptosis and proliferation. Flow cytometric results correlated well with immunohistochemical findings, demonstrating the success and sensitivity of this combined procedure. These results are part of the publication "*Cell proliferation and apoptosis in the primary enamel knot measured by flow cytometry of laser microdissected samples*".

Caspases are key enzymatic components of the intracellular apoptotic machinery, and their role in mammalian systems is often studied using fluoromethylketone (FMK) inhibitors. Despite many advantages of such approach, efficiency of the inhibitor and membrane permeability speed are often questioned. Our work therefore focused on an exact evaluation of caspase-3 FMK inhibition dynamics in camptothecin-induced mesenchymal micromasses. Two parameters of caspase-3 FMK inhibitor were investigated: first, the stability of the inhibitory potential in the time course of cultivation and, simultaneously, the dynamics of caspase-3 FMK inhibition after camptothecin-induced apoptosis peak. A photon-counting chemiluminescence approach was applied for quantification of active caspase-3. The sensitivity of the photon-counting method allowed for evaluation of active caspase-3 concentration in femtogram amounts per cell. The inhibitor penetrated the cells within the first minute after its application, and the peak of caspase-3 started to decline to the blank level after 30 min. The inhibitory effect of the FMK inhibitor was unchanged during the entire 48 h of cultivation. These results are part of the publication "*Dynamics of caspase-3 activation and inhibition in embryonic micromasses evaluated by a photon-counting chemiluminiscence approach*".

An investigation of apoptosis pathways on the level of individual cells is not only of biological but also medical importance. In our work, we focused on the development of a high-sensitivity instrumentation and protocol for detection of active caspase-3 in individual mammalian apoptotic cells. The technology is based on the specific cleavage of modified luciferin by caspase-3, an immediate bioluminescence reaction of free luciferin with luciferase followed by emissions of photons and their detection by photomultiplier tube working in the photon counting regime. Three different instrumental arrangements were compared for the determination of caspase-3 in free cells or tissue samples. Thus, in our best miniaturized system the mean amount as low as about 6.5 fg corresponding to 122 000 molecules of caspase-3 can be detected in individual apoptotic mouse leg cells. These results are part of the publication "*Bioluminescence determination of active caspase-3 in single apoptotic cells*".

Most of the methods available for the detection of apoptosis and caspases provide qualitative information only or quantification data as an average from cell populations or cell lysates. Several reports point to the importance of more accurate single-cell analyses in biomedical studies due to heterogeneity at tissue as well as cell level. To meet these requirements, we developed a miniaturized device enabling detection and quantification of active caspase-3/7 in individual cells at a femtogram level (10(-15) g). The active caspase-3/7 detection protocol is based on the bioluminescence chemistry commercially available as a Caspase-Glo™ 3/7 reagent developed by Promega. As a model, we used human stem cells treated by camptothecin to induce apoptosis. Individual apoptotic cells were captured from a culture medium under a microscope and transferred by a micromanipulation system into a detection capillary containing 2 µl of the reagent. Cells without activation by camptothecin served as negative controls. The detection limit of active caspase-3/7 achieved in the miniaturized system was determined as 0.20 and limit of quantification as 0.65 of the amount found in a single apoptotic human stem cell. Such a sensitive method could have a wide application potential in laboratory medicine and related clinically oriented research. These results are part of the publication "*A miniaturized device for bioluminescence analysis of caspase-3/7 activity in a single apoptotic cell*".

****

e) New topics were included into laboratory research plan

Neural crest cells (NCCs) derive early in vertebrate ontogenesis from neural tube as a population of migratory cells with exquisite differentiation potential. Abnormalities in NCC behaviour are cause of debilitating diseases including cancers and a spectrum of neurocristopathies. Thanks to their multilineage differentiation capacity NCCs offer a cell source for regenerative medicine. Both these aspects make NCC biology an important issue to study, which can currently be addressed using methodologies based on pluripotent stem cells. In our recent paper, we contributed to understanding the biology of human NCCs by refining the protocol for differentiation/propagation of NCC-like cells from human embryonic stem cells and by characterizing the molecular and functional phenotype of such cells. Most importantly, we improved formulation of media for NCC culture, we found that poly-L-ornithine combined with fibronectin provide good support for NCC growth, we unravelled the tendency of cultured NCCs to maintain heterogeneity of CD271 expression, and we showed that NCCs derived here possess the capacity to react to BMP4 signals by dramatically up-regulating MSX1, which is linked to odontogenesis. These results are part of the publication "*Properties of Neural Crest-Like Cells Differentiated from Human Embryonic Stem*".

Tooth absence and defects caused by various reasons are frequent events in humans. They are not life threatening but may bring about social consequences. Recent dentistry provides solutions in the form of prosthetics or dental implants; however, several complications and distinct limitations favour bioengineering of dental and periodontal structures. At least two types of cells (epithelial and mesenchymal) have to be recombined to produce a new functional tooth. Moreover, the tooth must be vascularized, innervated and properly anchored in the bone. To study these issues, different approaches have been established in both basic and applied research. In our review, recent strategies and techniques of tooth engineering are comprehensively summarized and discussed, particularly regarding manipulation using stem cells. These results are part of the publication "*Recent Approaches in Tooth Engineering Research*".

Research Report of the team in the period 2010–2014

Institute	Institute of Animal Physiology and Genetics of the CAS, v. v. i.

Scientific team	Experimental Animal Models

LBRP

In 2010-2014 scientific team of Laboratory of Cell Regeneration and Plasticity (LCRP) has bred and characterized the transgenic line of minipigs carrying the N-terminal part of human mutated huntingtin with 124 CAG/CAA glutamines (*http://www.ncbi.nlm.nih.gov/pubmed/25063429*; LCRP – ex vivo isolation of zygotes, microinjections with LVs into periviteline space of zygotes, laparoscopic transfer of microinjected zygotes into oviducts, genotypization of piglets, immunohistochemistry, biochemistry, Western Blots, manuscript preparation). During this period LCRP established the F1, F2, F3 and F4 generation of this unique transgenic line. This work was supported by the CHDI Foundation, the private initiative to transfer all new knowledge about Huntington's disease into clinics. Genotyping of offspring in each litter revealed that the wild-type and transgenic piglets are equal in all litters. This fact allows compare siblings in all phenotyping studies. These results formed background for preparation of Service Contract agreement between IAPG and CHDI Foundation (2010-2014) as well as grant of Technology Agency Czech Republic (TA01011466). The Service Contract has supported both, breeding and housing of the transgenic minipigs and also the experimental work on phenotyping of the large animal model of Huntington's disease. The new Service Contract supported by CHDI for breeding and housing of the transgenic minipigs was signed for the subsequent 6 years period (2014-2020). During period 2010-2014 LCRP with other IAPG laboratories (LAPA, LID, and LTB) have established a new biomedical Center PIGMOD (http://pigmod.avcr.cz/o-nas/centrum-pigmod/index.html) supported by the project EXAM (CZ.1.05./2.1.00/03.0124; http://www.isvav.cz/projectDetail.do?rowId=ED2.1.00%2F03.0124, http://www.iapg.cas.cz/en/oprojektu_exam).

In 2010-2014 the one of the crucial topic in LCRP as well as center PIGMOD was the study of spinal cord injury and its potential gene as well as cell therapy using minipig, as a large animal model. Firstly we characterized the optimal dosing regimen and safety profile of human spinal stem cells (HSSC) when grafted into the lumbar spinal cord (L2-L5) segments of naive immunosuppressed minipigs (*http://www.ncbi.nlm.nih.gov/pubmed/20412634*; LCRP – preparation of cells before transplantation, injection of cells into spine, neurological assessment after transplantation, immunosuppression, necropsy, partial immunohistochemistry, manuscript preparation). Based on the data of this study the safe total number of injected cells and volume of injections were determined to be 30,000 cells delivered in ≤6 µl of media. At the same time we also needed the standard and well quantifiable model of SCI in miniature pigs closely resembling the most situations after SCI in men which could be used for next stem cell therapy. Thus LCRP has characterized and optimized the spinal compression model of minipig´s spine at thoracic level (*http://www.ncbi.nlm.nih.gov/pubmed/22029501*; LCRP – creation of SCI in minipigs, neurological assessment after injury, necropsy, partial

immunohistochemistry, manuscript preparation). This preclinical large animal model will be rationally used for gene and cell therapy testing. Porcine human spinal stem cells (PSSC, HSSC) and other types of embryonic or iPSCs obtained from Neuralstem Inc. (http://www.neuralstem.com/) and/or UCSD have been tested and characterized at LCRP for safety and efficacy studies by minipig model of spinal trauma at different levels (*http://www.ncbi.nlm.nih.gov/pubmed/22889456, http://www.ncbi.nlm.nih.gov/pubmed/22291989*; LCRP – preparation of cells before transplantation, injection of cells into spine, application of LVs into spine, neurological assessment after transplantation, immunosuppression, necropsy, partial immunohistochemistry, manuscript preparation). For estimation of cell therapy effectiveness we used porcine neurological motor (PNM) score as well as pallet of sensory tests, *postmortem* MRI, immunohistology etc. These preclinical experiments were successfully used by Neuralstem, Inc. in obtaining of FDA approval to initiate two human clinical trials. These two trials are currently in progress in the USA.

In collaboration with UCSD LCRP also characterized effective long-term immunosuppression by different tacrolimus formulations (microspheres, pellets, liposomes) in rats with spinally grafted human neural precursors (*http://www.ncbi.nlm.nih.gov/pubmed/23748136*; LCRP – application of tacrolimus formulations to rats, blood collection from rats, partial immunohistochemistry, manuscript preparation). We also established the minipig model of brain ischemia (*not published yet*) whereas it was thought to be impossible in pigs due to *rete mirabile* (http://www.ncbi.nlm.nih.gov/pubmed/15646463).

LCRP also collaborate on research of gastrointestinal tract diseases with physicians from Military University Hospital Prague, Hořovice Hospital and Institute for Clinical and Experimental Medicine Prague. The aim of this collaboration is training of new and classical surgical techniques, the comparison their therapeutical effectiveness and introducing of new surgical approaches into clinical practice (NOTES – *Natural Orifice Transluminal Endoscopic Surgery*, POEM – *Peroral Endoscopic Myotomy*). The result of this collaboration was two projects supported by Ministry of Health (*http://www.isvav.cz/projectDetail.do?rowId=NS9994*; http://www.isvav.cz/projectDetail.do?rowId=NT13634), publications (*http://www.ncbi.nlm.nih.gov/pubmed/23073683, http://www.csgh.info/detail.php?stat=885, http://www.ncbi.nlm.nih.gov/pubmed/22826620, http://www.csgh.info/detail.php?stat=883, http://www.csgh.info/detail.php?stat=884, http://www.ncbi.nlm.nih.gov/pubmed/22509657, http://www.csgh.info/arch_detail.php?stat=524 http://www.csgh.info/arch_detail.php?stat=527, http://www.csgh.info/arch_detail.php?stat=543*; LCRP – POEM, endoscopy, laparoscopy, surgery, necropsy, partial immunohistochemistry, manuscript preparation) and introducing the POEM technique into the clinic in Czech Republic (Dr. Jan Martínek, IKEM). In 2010-2011 LCRP was also involved into the project of Ministry of Education, Youth and Sports (http://www.isvav.cz/projectDetail.do?rowId=2B06130) as a project participant and this collaboration resulted into publications (*http://www.ncbi.nlm.nih.gov/pubmed/21401296, http://www.ncbi.nlm.nih.gov/pubmed/22837133, http://www.ncbi.nlm.nih.gov/pubmed/21858919, http://www.ncbi.nlm.nih.gov/pubmed/19929138*; LCRP – isolation of bone blood for MSCs isolation /rabbit, pig/, FACS characterization of MSCs *in vitro*, seeding of

scaffolds with MSCs and their differentiation *in vitro*, immunohistochemistry, Biochemistry, Western Blots, manuscript preparation). LCRP was during period 2010- 2014 also involved into the study of iPSCs and neurodegenerative diseases and this effort was resulted into publication (http://www.ncbi.nlm.nih.gov/pubmed/21863007; LCRP – fibroblasts reprogramming, sample collection, manuscript preparation).

LAPA

A small proteomics-focused group evolved in 2002 as a part of the Laboratory of Biochemistry and Molecular Biology of Germ Cells at Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic (IAPG AS CR, v.v.i.) in Liběchov. Until 2011 the research focus was on reproductive biology mainly (http://www.ncbi.nlm.nih.gov/pubmed/20058866, http://www.ncbi.nlm.nih.gov/pubmed/21697218). Additionally, cancer research studies including the effects and biochemical mechanisms of anti-cancer drugs and drug resistance in collaboration with Faculty Hospital and Faculty of Medicine Palacky University in Olomouc and Microbiological Institute of AS CR, v.v.i. in Prague, facilitated the establishment of the Centre for Cancer Proteomics (http://www.ncbi.nlm.nih.gov/pubmed/23443080, http://www.ncbi.nlm.nih.gov/pubmed/19327356, http://www.ncbi.nlm.nih.gov/pubmed/23151231). Recently, collaboration with the group of Prof. Karel Smetana Jr. at the 1st Faculty of Medicine, Charles University in Prague, has added another cancer research aspect of epithelial-mesenchymal cell communication (http://www.ncbi.nlm.nih.gov/pubmed/23043537, http://www.ncbi.nlm.nih.gov/pubmed/24698078). A comprehensive proteome analyses of stem cells, namely differentiation of neural stem cells and neural precursor cell and their possible use in neurological or neurodegenerative disorders [http://www.ncbi.nlm.nih.gov/pubmed/21241017, http://www.ncbi.nlm.nih.gov/pubmed/23835433, http://www.ncbi.nlm.nih.gov/pubmed/25363140, http://www.ncbi.nlm.nih.gov/pubmed/25290828, http://www.ncbi.nlm.nih.gov/pubmed/25063502) represent the key interest of the Laboratory of Applied Proteome Analyses (LAPA) at IAPG AS CR, v.v.i. which was officially established in January 2014. The members of the group always had crucial (higher than 50%) contribution and Proteomics part was exclusively performed by this group

LTB

Laboratory of Tumor Biology (LTB) focused research activities mainly on mechanisms of tumour progression and spontaneous regression (SR) in two own animal models: the MeLiM (*Me*lanoma-bearing *Li*bechov *M*inipig) strain of miniature pigs with hereditary melanoma and the Lewis rat with inoculated sarcoma. The role of various subpopulations of immune cells, cytokines and extracellular matrix proteins and changes of basic haematological parameters was studied during these processes. New procedures for induction of anti-tumour immune reaction were also tested using these animal models.

In the MeLiM model, long-term (since 3 weeks till 8 months of age) immunohistochemical monitoring of two important extracellular matrix (ECM) proteins, collagen IV and laminin, showed that SR of porcine melanoma is a highly dynamic process. The expression of collagen IV and laminin correlated with changes in population of melanoma cells. Tumours of 3-week-old animals consisted primarily of melanoma cells with a granular expression of collagen IV and laminin around

them. Thereafter, melanoma cells were gradually destroyed and tumour tissue was rebuilt into the connective tissue. Collagen IV expression slightly increased in tumours of 10-week-old pigs showing extracellular fibrous appearance. In tumours of older animals, areas lacking melanoma cells demonstrated a low expression and areas containing melanoma cells a high expression of both proteins. We considered the age of 10 weeks as a turning point in the transition between tumour growth and SR of the MeLiM melanoma. These results were published by Planska et al. (http://www.ncbi.nlm.nih.gov/pubmed/25861134). Moreover, the dynamics of another two ECM proteins (tenascin C and fibronectin), matrix metalloproteinase 2 (MMP-2) and changes of basic haematological parameters and of subpopulations of immune cells (in peripheral blood and skin tumours) were also studied. All these results were already summarized and now they are being prepared for publication.
The melanoma-bearing MeLiM minipigs, especially those with progressing melanoma, showed often a significant decline of number of red blood cells and other associated haematological parameters (haematocrit, haemoglobin concentration). This erythropenia was studied in collaboration with the Institute of Chemistry and Biochemistry, the Mendel University in Brno. New fully automated electrochemical measuring system was tested for quantification of iron ions in blood samples of MeLiM minipigs. Some experimental conditions such as composition and pH of the supporting electrolyte, value of the deposition potential and preparation of erythrocytes were optimized. It was found that the iron concentration in erythrocytes of the healthy (control) minipigs was significantly higher (10 µg Fe ions/ml) than in the group of melanoma-bearing animals (3.5 µg Fe ions/ml) (*Kreplova, M., Krejcova, L., Hynek, D., Barath, P., Majzlik, P., Horak, V., Adam, V., Sochor, J., Cernei, N., Hubalek, J., Vrba, R., Kizek, R.: Automated electrochemical detection of iron ions in erythrocytes from MeLiM minipigs suffering from melanoma; Int. J. Electrochem. Sci. 7: 5893-5909, 2012*). The LTB (H.V. with technical assistants) selected for this study suitable experimental minipigs, monitored their health status (including extent of skin tumours), carried out collection of peripheral blood samples, measured basic haematological parameters and participated in preparation of the article.

The Lewis rat sarcoma model has been extended and improved isolating seven different cell clones from the original R5-28 sarcoma cell line. Three of these clones – C4, C7 and D6 – were characterized *in vitro* and *in vivo* (http://www.ncbi.nlm.nih.gov/pubmed/23150138). Cells of all three clones were morphologically distinct, showed significant proliferative capacity and expressed vimentin, fibronectin, laminin, collagen IV, MMP-2 and MMP-9. They varied in secretion of five cytokines (MCP-1, TIMP-1, VEGF, CINC-2, LIX) into culture medium. Their karyotype greatly differed from diploid chromosome number of normal rat somatic cells. Subcutaneous inoculation of all three clones into the Lewis rats give rise the development of tumours. Spontaneous tumour regression (ascertained also in the R5-28 cells) was observed in the C4 clone only. It was associated with significant changes in lymphocyte subpopulations. These three clones represent new rat cancer models. They can be used for detailed study of sarcoma progression (the C7 and D6 clones) and/or spontaneous regression (the C4 clone) and for evaluation of the role of expressed cytokines on tumour growth. Moreover, they can be suitable for testing sensitivity to various drugs *in vitro* and for development of new therapeutic approaches *in vivo*.
Another study in the Lewis rat model utilized inoculation of D6 cells and dealt with analysis of changes in haematological parameters, immune cell subpopulations and

cytokine profiling during tumour growth, after tumour excision and after second inoculation of D6 cells. Tumour progression was found to be associated with an increased number of leukocytes in peripheral blood. This leukocytosis was caused by the increased proportion of CD11b+ cells (neutrophil granulocytes). Serum concentration of chemokine (c-c motif) ligand 2 (CCL2; monocyte chemotactic protein 1 – MCP-1), L-selectin and intracellular adhesion molecule-1 (ICAM-1) also increased with growing tumour. On the contrary, the proportion ofCD4+, CD8+ and MHC II+ cells decreased with growth of tumours probably due to immunosuppressive effect of increased population of neutrophil granulocytes. After tumour excision, all these parameters returned to pre-inoculation levels and did not change even after a second inoculation of D6 cells. Absence of subcutaneous tumours after the second D6 cell inoculation gives suggests development of anti-tumour immunity stimulated by primary tumour (http://www.ncbi.nlm.nih.gov/pubmed/25667449).

New strategies for a possible cancer therapy were also tested in the animal models. Effects of the field of pulse vector magnetic potential (PVMP) was solved in collaboration with the Enjoy Ltd., Brno (MEYS CR – NRP II – No. 2B08063; the LTB – co-investigating workplace). Several types of applicators creating PVMP field of different intensity (developed in the Enjoy Ltd.) were used *in vivo* (on porcine skin melanoma and rat subcutaneous sarcoma) as well as *in vitro* (on various cancer and normal cell types). Various application scheme of the PVMP field (various exposure times, single or repeated application) were tested and extent of tissue or cell damage, level of apoptosis, heat shock protein expression, proliferation of cells and their metabolic activity were analysed. Unfortunately, no significant evidence of the inhibitory or destructive effect of the PVMP field on tumour (or normal) cells *in vitro* and on tumour tissue *in vivo* was revealed (*Holubová M., Palko L., Leba M., Kovalská J., Mishra R., Rampl I., Horák V.: Influence of pulsed electromagnetic field and field of pulse vector magnetic potential on sarcoma cells and skin fibroblasts of the Lewis rat; Abstracts – XX. Biol. Days, 25.-27.10. 2011, Plzeň, p.70-71*). All these *in vivo* and *in vitro* experiments were prepared, realized and evaluated in the LTB.
Shock waves (primarily used in medicine to disintegrate urolithiasis) could bring a putative therapeutic effect in cancer diseases. Various types of cell damage are known from *in vitro* studies but knowledge from *in vivo* are limited. In collaboration with the Institute of Plasma Physics, AS CR, v.v.i. in Prague, effects of focused shock waves on subcutaneous tumours in our Lewis rat model were monitored. Shock waves were produced by a multichannel pulsed-electrohydraulic discharge generator with a cylindrical ceramic-coated electrode (developed in the Institute of Plasma Physics). In tumours treated with shock waves, a large area of damaged tissue was detected which was clearly differentiated from intact tissue. Localization and a cone-shaped region of tissue damage visualized by TUNEL reaction apparently correlated with the conical shape and direction of shock wave propagation determined by high-speed shadowgraphy. A strong TUNEL reaction of nuclei and nucleus fragments in tissue exposed to shock waves suggested apoptosis in this destroyed tumour area. However, specificity of the TUNEL technique to apoptotic cells is ambiguous and other apoptotic markers (caspase-3) that we used in our study did not confirmed this observation. Thus, the generated fragments of nuclei gave rise to a false TUNEL reaction not associated with apoptosis. Mechanical stress from high overpressure shock wave was likely the dominant pathway of tumour necrotic damage (http://www.ncbi.nlm.nih.gov/pubmed/25200989). The cancer model (the Lewis rats with subcutaneous sarcomas), immunohistochemical and histological staining as well

as evaluation, interpretation and photo-documentation of ascertained results and writing of the corresponding parts of the article were done in the LTB.
Under a contract with the Swiss Bellefontaine Medilab SA (Henniez, Switzerland), effects of ISATID (Implantable Securing Autologous Tissue Immunization Device) *in vivo* were studied using the MeLiM model. The aim of the evaluation is to provide the biological basis necessary for obtaining official clearance for the clinical testing of the patented device (US Patent No 7 160 716), which is to be used in clinical practice for cancer immunotherapy. ISATID with autologous porcine melanoma was implanted at different places of the body (subcutaneously, into the abdominal cavity) for various period and response of the organism (body temperature, reaction around the ISATID, haematological parameters) and changes of melanoma tissue inside the ISATID were monitored. Neither any significant changes in the monitored parameters nor any inflammation around the ISATID were ascertained. The ISATID was usually covered with a thin layer of fibrous tissue which did not restrict the migration of immune cells. The melanoma persisted inside the ISATID up to two weeks showing varying degree of tissue disintegration. This first phase of the study clearly demonstrated safety of the ISATID *in vivo*. Anti-tumour immune reaction induced by the melanoma inside the ISATID is now under study. All the mentioned experiments, tissue and blood analyses and evaluation of results were done in the LTB.
Under a contract with Areko s.r.o. (Prague, Czech Republic), basic haematological parameters and proportion of lymphocyte subpopulations in the peripheral blood of cancer patients taking Ovosan for a long period were determined. Ovosan, a food supplement containing a mixture of biologically active phospholipid on natural base, is recommended for general health improvement, acceleration of regeneration, support of immunity and suppression of undesirable side effects of chemotherapy and radiotherapy. Basic haematological parameters and proportion of lymphocyte subpopulations (helper and cytotoxic T lymphocytes, NK and NKT cells, T regulatory cells, B lymphocytes) has been detected at regular intervals in the peripheral blood of patients affected by different cancers. Stabilization of monitored parameters without significant fluctuations caused by chemo- or radiotherapy was observed in a number of long-term monitored patients. These results demonstrate a supportive effect of Ovosan on the stabilization of haematological and immunological parameters in cancer patients treated by standard therapy (*Horák V., Holubová M., Pokorná E.: Effect of long-term administration of Ovosan on haematological parameters and proportion of immune cells in cancer patients; Areko workshop „Research of BAF 2010“, 3.5.2011, Prague, Lecture Proceedings, pp.22-26; Horák V., Pokorná E.: Haematological and immunological parameters in cancer patients receiving Ovosan; Abstracts of Areko workshop „Research BAF 2011-2012“, 20.11.2012, Prague, p. 7; both contribution in Czech*). All blood analyses, evaluation of results and preparation of the contributions were realized in the LTB.

LID

In the field of reproductive a developmental biology Laboratory of DNA Integrity (LID) focused mainly on the role of mitotic kinases Aurora kinase A (AURKA) and Polo-like kinase 1 (PLK1) in the oocyte meiosis and the first mitosis in the zygote.

We produced mice with overexpression of wild-type and kinase-dead AURKA specifically in the oocyte to study its function in vivo. We have shown that AURKA activation is biphasic with the initial activation not requiring CDC25B-CDK1 activity, whereas full activation, which is essential for the increase in the amount of microtubule organizing centers (MTOCs), depends on CDK1 activity. AURKA activity

also influences spindle length and regulates, independent of its protein kinase activity, the amount of MTOC associated gamma-tubulin (Solc et al 2012, PMID: 22837479). This work was done under our collaboration with Richard Schultz (University of Pennsylvania) supported by our two collaborative grants. This collaboration also resulted in the work where we highlighted similarities of meiotic resumption with G2-checkopint recovery in somatic cells (Solc et al 2010, PMID: 20453035).

We invested main effort to establishment quantitative confocal live cell imaging to study chromosome dynamics in oocytes and embryos. It was done under great collaboration with Jan Ellenberg (EMBL, Heidelberg). It was also supported by our common bilateral grant. This collaboration resulted in common paper published recently. We showed that PLK1 becomes activated at meiotic resumption on MTOCs and later at kinetochores. PLK1 is required for efficient meiotic resumption by promoting nuclear envelope breakdown. PLK1 is also needed to recruit centrosomal proteins to acentriolar MTOCs to promote normal spindle formation, as well as for stable kinetochore-microtubule attachment. Consequently, PLK1 inhibition leads to metaphase I arrest with misaligned chromosomes activating the spindle assembly checkpoint (SAC). Unlike in mitosis, the metaphase I arrest is not bypassed by the inactivation of the SAC. We show that PLK1 is required for the full activation of the anaphase promoting complex/cyclosome (APC/C) by promoting the degradation of the APC/C inhibitor EMI1 and is therefore essential for entry into anaphase I. Moreover, our data suggest that PLK1 is required for proper chromosome segregation and the maintenance of chromosome condensation during the meiosis I-II transition, independently of the APC/C. Thus, our results define the meiotic roles of PLK1 in oocytes and reveal interesting differential requirements of PLK1 between mitosis and oocyte meiosis in mammals (Solc et al 2015, PMID: 25658810). Importantly this collaboration is continuing and also it resulted in the establishment of new collaboration with Tomoya Kitajima (RIKEN Centre for Developmental Biology, Kobe). We also showed that function of PLK1 in mitotic zygotes is more similar to PLK1 meiotic function in contrast to known function of PLK1 in somatic cells (Baran et al 2013, PMID: 23649868).

Our experiences with oocytes mRNA microinjection and advanced live imaging produced also collaborative work with Petr Svoboda (Inst. of Molecular Genetics, ASCR). In this work we characterized role of ELAVL2 that acts a translation repressor. Our group conducted experiments characterizing a meiotic phenotype of Elavl2 knock down and gain of function experiments (ELAVL2 overexpression). We have done also analysis of histological samples (Chalupnikova et al 2014, PMID: 24553115).

We also started project focusing on the role of small G-protein Ran in the spindle formation in mouse oocytes. This work is done under collaboration with Petr Kalab (National Cancer Institute, NIH, Bethesda). Until know we published review article in this fied (Kalab et al 2011, PMID: 21630149) and continuing in the Ran research using FRET sensors in live oocytes.

In summary, in the last period our research in reproductive biology focused on several signalling pathways regulating spindle formation and chromosome dynamics in oocytes and early embryos. We used advanced techniques such micromanipulation, transgenic Cre/lox system and live cell imaging. We have done these activities under broad range of international collaboration (University of Pennsylvania, EMBL, RIKEN) and published several papers.

Research Report of the team in the period 2010–2014

Institute	Institute of Animal Physiology and Genetics of the CAS, v. v. i.

Scientific team	Team of Evolutionary Biology

Before the evaluated period (2007, 2008) we were first to prove, through molecular analysis of a huge sample and rigorous statistical treatment of the data, that introgression of X chromosome across the hybrid zone between two European house mouse subspecies (*Mus musculus musculus* and *M. m. domesticus*) is impeded relative to movement of autosomes. However, in 2011 we revealed, on a larger number of X-linked markers, that the introgression pattern is much more complex: in two-dimensional space, 14 loci showed linear contact, in four we found introgression islands to the east of the zone suggesting past westward zone movement, and two showed westward salients coinciding with Y chromosome invasion reported by our team in 2008. These results were confirmed and refined by one-dimensional analysis where the geographic axis of introgression was replaced with the hybrid index. The follow-up study employing 1401 markers (single nucleotide polymorphisms – SNPs) and using measures of linkage disequilibrium and haplotype structure among neighbouring markers in two geographically distinct transects confirmed recent zone movement because of either geography-dependent asymmetrical dispersal or selection favouring one subspecies over the other. These studies were accompanied by a comparative analysis based on resampling of localities across two independent transects. We explored the transition of 13 X-linked markers asking whether such a comparison can distinguish the effects of selection from random factors. A heuristic search in the likelihood landscape revealed more complex likelihood profiles for data sampled in two-dimensional space relative to data sampled along a linear transect. Randomized resampling of localities analyzed for individual loci showed that deletion of sites away from the zone centre can decrease cline width estimates whereas deletion of sites close to the centre can significantly increase the width estimates. Deleting localities for all loci resulted in wider clines if the number of samples from the centre was limited. The results suggest that, given the great variation in width estimates resulting from inclusion/exclusion of sampling sites, the geographic sampling design is important in hybrid zone studies and that our inferences should take into account measures of uncertainty such as support intervals. The comparison of the two transects suggests that genetic incompatibilities may have at least partly common architecture in the house mouse hybrid zone (HMHZ). [Members of the team participated in designing the studies, in molecular analyses (scoring a part of X-linked markers, SNP development), in data analyses, and writing the papers.]

Our studies of the HMHZ showed that whereas this is characterized by frequency discontinuities for some sex chromosome markers, there is an unexpected large-scale regional introgression of a Y chromosome across the barrier, in defiance of Haldane's rule. Since it was suggested that a major force maintaining the species barrier acts through sperm traits, we tested whether the Y chromosome penetration of the species barrier acts through sperm traits by assessing sperm characteristics of wild-caught males and comparing the data with the hybrid index of each male using

1401 diagnostic SNPs. We found that both sperm count and sperm velocity were significantly reduced across the natural spectrum of hybrids. However, sperm count was more than rescued in the presence of the invading *musculus* Y. Our results imply an asymmetric advantage for Y chromosome introgression consistent with the observed large-scale introgression. We suggest that selection on sperm-related traits probably explains a large component of patterns observed in the natural hybrid zone, including the Y chromosome penetration. [The team members participated in SNP data development and analysis, and in writing the paper.]

In a 2011 study we analyzed two mate-recognition signal systems, based on urinary and salivary proteins, across a Central European portion of the HMHZ. Introgression of the genomic regions responsible for these signals, the major urinary proteins (MUPs) and androgen binding proteins (ABPs), respectively, was compared to introgression at loci assumed to be nearly neutral and those under selection against hybridization. The preference of individuals taken from across the zone regarding these signals was measured in Y mazes, and we developed a new mathematical model for the analysis of the transition of such traits under reinforcement selection. The strongest assortative preferences were found in males for urine and females for ABP. Clinal analyses confirmed nearly neutral introgression of an *Abp* locus and two loci closely linked to the *Abp* gene cluster, whereas two markers flanking the *Mup* gene region revealed unexpected introgression. Geographic change in the preference traits matched our reinforcement selection model significantly better than standard cline models. Our study thus confirmed that behavioural barriers are important components of reproductive isolation between the house mouse subspecies. [The team members performed all behavioural experiments and most of molecular analyses, carried out a part of statistical analyses, participated in modelling reinforcement, and wrote the paper.]

While most studies have dealt with selection against hybrids little attention has been paid to an opposing mechanism maintaining the HMHZ: dispersal. In a series of studies we focused on various aspects connected with mouse dispersion. First, using open field experiments we tested whether males of the two subspecies differ in their propensity to disperse and in their character of exploration. We revealed that *M. m. musculus* males were less hesitant to enter the experimental arena than were *M. m. domesticus* males, but once inside the arena their movements were more timid. Interestingly F1 hybrids differed from both parental strains, with longer latencies to enter the arena, but explored the arena in a similar fashion as the *M. m. domesticus* males, thus displaying transgressive behavioural phenotypes. One of obstacles to mouse dispersion can be water barriers. House mice are believed to be, unlike rats, only reluctant swimmers. If water is a barrier to mouse dispersal water bodies and streams can have a substantial impact on the genetic structure of populations. In our study we therefore used a simple motivation experiment to test the disposition of both wild and inbred male mice representing the subspecies to overcome a water barrier. Contrary to a common belief tested animals entered water rather easily, often even engaging in repeated swimming. We found significant differences in scored behavioural parameters between the subspecies. Under the 20 °C regime, both wild and inbred *domesticus* males entered and crossed the water earlier and more often swam even when satiated. Strikingly, under the 10 °C regime, the results were rather equivocal but with the opposite tendencies, with *musculus* males being more willing to swim. A final experiment employed Morris water maze to investigate how the two

subspecies, known to differ in aggressiveness, cope with stressful situations. We found that less aggressive *musculus* males performed significantly better in solving the MW task than more aggressive *domesticus* males. This suggests that *M. m. musculus* is more flexible and could be more successful under stressful and/or dynamic situations typical of dispersal bouts. It seems plausible that this difference may have had an influence on the secondary contact between *musculus* and *domesticus* populations in the past and perhaps still can affect the dynamics of the European hybrid zone between the subspecies. [All these studied have been carried out by members of the team.]

Our research over the past five years has largely revolved around the response of temperate organisms to climate change using the Eurasian bank vole as the model. We have been funded by two major grants, one focused on the role of haemoglobin function in adaptive response of the bank vole to the global warming at the end of the last glaciation, and second on the patterns and processes responsible for the divergence of the genome between bank vole populations that survived in northern versus southern glacial refugia.

Specifically, we have investigated the functional consequences of structural differences between two bank vole haemoglobins deriving from different glacial refugia, one of which partially replaced the other in Britain during the end-glacial climate warming. This allowed us to examine their adaptive divergence and hence a possible role of selection in the replacement. We determined a single amino acid substitution in the bank vole beta-globin gene as the allelic difference and showed that possessing cysteine at the site beta52 increased the resistance of bank vole erythrocytes to oxidative stress. We thus found striking evidence for physiological differences between products of genic variants that spread at the expense of one another during colonization of an area from different glacial refugia.

In our original study of the replacement involving bank voles in Britain (published prior to the evaluated period; Searle, Kotlík et al. 2009 Proc R Soc B 2009) it was not possible to determine whether the replacement involved a selective sweep of mtDNA (the marker screened) or a complete population replacement. Therefore, in our recent study we tested whether the phylogeographic pattern in Britain, with one peripheral mtDNA phylogroup (originating from a northern refugium) and one central mtDNA phylogroup (from a southern refugium), is reflected by population structure at a large number of single nucleotide polymorphism (SNP) loci derived from the bank vole transcriptome by RNA-Seq. The analysis of over 10 thousand SNPs revealed a clear north-south genomic gradient in the proportion of admixture by the ‘genes’ (SNP alleles) from the second colonizing population, with only the northernmost locality (Aberdeen) retaining the genome without admixture, while at the two southernmost localities (Cornwall and Devon) the genes of the first colonists were completely replaced with genes of the second colonists. This rejects the possibility that the phylogeographic pattern in Britain was the result of an mtDNA selective sweep and shows that it is possible by using large SNP datasets to reveal true population replacements and distinguish them from selective sweeps at one of a few loci.

The fact that the second colonizing (replacing) population carried the haemoglobin variant conferring the enhanced antioxidative capacity to red blood cells (beta-

cysteine allele) provides strong support for the role of selection in the population replacement involving bank voles in Britain. Given the various life-history correlates of ROS production (e.g. muscular activity, increased growth rate or reproduction, or thermal stress), the members of the second colonizing population could be at a selective advantage under a multitude of ecological conditions. Therefore, if selection may have had a place in determining distributions of current genetic lineages, there is the possibility for a genetic lineage emanating from a particular glacial refugium to have a wide current distribution not because of geographical factors (e.g. because the refugium is closer to colonized areas than other refugia) but rather because conditions in the refugium selected for characteristics advantageous during colonization. It is possible that conditions in different refugia selected for ecological and/or physiological characteristics providing advantage at different stages of climate change. For example, populations that survived in more northerly refugia (under more harsh conditions) could have been more cold tolerant and thus had advantage during earlier stages of the end-glacial warming, but they could have later been outperformed by populations originating from more southerly refugia. The endeavour to understand whether adaptive differentiation and selection do have influences like that is what we mean by 'adaptive phylogeography' (Kotlík et al., 2014 Proc R Soc B). If different populations were involved in populating species ranges at different stages of the end-glacial warming, such a finding would be relevant to the response to the current global warming as it would mean that some populations may be more important for survival of a species under warmer climate than other populations within the same species.

The main topics we focused in evaluated period are naturally occurring clonality and asexuality in lower vertebrates, specifically in our model (s) – spiny loaches of the genus *Cobitis*, partly also cyprinid genus *Carassius* and water frogs of the genus *Pelophylax.* The topic of assexuality is inherently associated with evolutionary polyploidy and microspeciation issues that we addressed in model of sturgeons and salmonids, groups that experienced their group specific whole genome duplication (WGD) events.

Considering the ubiquity of sexual reproduction, we aimed to test whether stable coexistence of sexual and asexual spiny loaches is mediated by parasite load in hosts, because clonal asexual individuals suffer more from parasitic infections due to their lower genetic variability or partial niche shift between sexually and asexually reproducing strains. However, we did not reveal a clear correlation between infection load by helminth parasites and proportion of sexuals as well as we found similar infection rate among sexual and asexual females. These findings suggest that the mechanism of Red Queen Hypothesis are directly involved in stabilization of asexual complex. Contrary, the temporally stable gradient of sexuals/asexuals along the river and correlation with gradients of environmental parameters suggests that sexual and asexual forms prefer different habitats.

We studied a complex of hybridizing species, which are known to produce clonal hybrids, to discover how one of the species, *Cobitis tanaitica*, has uniquely achieved a pattern of mito-nuclear mosaic genome over the whole geographic range. We applied three distinct methods, including the method using solely the information on gene tree topologies, and found that the contrasting mito-nuclear signal might not

have resulted from the retention of ancestral polymorphism. Instead, we found two signs of hybridization events related to *C. tanaitica*; one concerning nuclear gene flow and the other suggested mitochondrial capture. Interestingly, clonal inheritance (gynogenesis) of contemporary hybrids prevents genomic introgressions and non-clonal hybrids are either absent or too rare to be detected among European *Cobitis*. Our analyses therefore suggested that introgressive hybridizations are rather old episodes, mediated by previously existing hybrids whose inheritance was not entirely clonal. *Cobitis* complex thus supports the view that the type of resulting hybrids depends on a level of genomic divergence between sexual species. The problem is, by now addressed and further developer by NGS approach.

We were also interested theoretically in problem whether lower evolutionary ages of clones, relative to their sexual ancestors demonstrated by phylogenetic studies may be attributed to heightened extinction risk of asexual organisms. We previously criticized such interpretations and demonstrated that the life span of clones is ultimately limited by neutral drift depending on the rate at which new clones are spawned into an asexual community of a finite size. Therefore, it is important to investigate whether the natural rates of such influxes are sufficiently high to account for the relative ephemerality of clones without assuming their increased extinction rate. We also applied the neutral clonal turnover model to phylogenies of polyploid asexual ferns and simulated the coalescent trees over a wide range of demographic structures and sampling schemes. On parameterizing the model with biologically relevant estimates of population sizes and plant polyploidization rates, simulated clonal assemblages appeared younger than their sexual counterparts even in the absence of selection against clones. Therefore, differences observed between the ages of sexual and clonal lineages may be explained by the neutral clonal turnover and the possibility that natural clones may get lost by neutral drift before their fate could eventually be affected by any long-term constraints of asexuality should be seriously considered.

As other teams studying asexual vertebrates we tried to artificially synthetize asexual clones of our model spiny loaches. Because most known clonal vertebrates have hybrid genomic constitutions, tight linkages are assumed among hybridization, clonality, and polyploidy. However, predictions about how these processes mechanistically relate during the switch from sexual to clonal reproduction have not been fully understood. Therefore, we performed a crossing experiments to test the hypothesis that interspecific hybridization *per se* initiated clonal diploid and triploid spined loaches (*resulting* in their gynogenetic reproduction. We reared two F1 families resulting from the crossing of 14 pairs of two sexual species, and found their diploid hybrid constitution and a 1:1 sex ratio. While males were infertile, females produced unreduced non recombinant eggs (100%). Synthetic triploid females and males (96.3%) resulted in each of nine backcrossed families from eggs of synthesized diploid F1s fertilized by haploid sperm from sexual males. Five individuals (3.7%) from one backcross family were genetically identical to the somatic cells of the mother and originated via gynogenesis; the sperm of the sexual male only triggered clonal development of the egg. Our reconstruction of the evolutionary route from sexuality to clonality and polyploidy in these fish shows that clonality and gynogenesis may have been directly triggered by interspecific hybridization and that polyploidy is a consequence, not a cause, of clonality. We thus succeeded experimentally to synthetize clonal and polyploid individuals by simple interspecific

hybridization as the first group after long 80 years since description of the first clonal vertebrate.
Collection of studies dealing with all these interrelated topics received Main Award of the Czech Academy of Sciences in 2012 entitled: Clonal vertebrates: discovery, mechanisms, biodiversity and reconstruction in model cobitoid loaches

Due to the hybrid genomic constitutions and increased ploidy of asexual vertebrates, we tried to identify processes related to the origin and maintenance of clonal diversity which can provide information about the evolutionary consequences of interspecific hybridization, asexuality and polyploidy. We performed fine-scale genetic analysis of Central European hybrid zone between two sexual species using microsatellite genotyping and mtDNA sequencing to understand the processes driving observed diversity of biotypes and clones in the *Cobitis taenia* hybrid complex. We found that the hybrid zone is populated by an assemblage of gynogenetically reproducing di-, tri- and tetraploid hybrid lineages and that successful clones, which are able of spatial expansion, recruit from two ploidy levels, i. e. diploid and triploid. We further compared the distribution of observed estimates of clonal ages to theoretical distributions simulated under various assumptions and showed that new clones are most likely continuously recruited from ancestral populations. This suggested that the clonal diversity is maintained by dynamic equilibrium between origination and extinction of clonal lineages. On the other hand, an interclonal selection is implied by nonrandom spatial distribution of individual clones with respect to the coexisting sexual species. Importantly, there was no evidence for sexually reproducing hybrids or clonally reproducing non-hybrid forms. Together with previous successful laboratory synthesis of clonal *Cobitis* hybrids, our data thus provide the most compelling evidence that 1) the origin of asexuality is causally linked to interspecific hybridization; 2) successful establishment of clones is not restricted to one specific ploidy level and 3) the initiation of clonality and polyploidy may be dynamic and continuous in asexual complexes.

Studies in biodiversity of cobitoid loaches was and is intimately related with long term interest of laboratory with asexual clonal reproduction in vertebrates in model of cobitid and cobitoid fishes. In previous period we have essentially uncovered until now unknown actual biodiversity of this fish group describing at molecular level their major phylogenetic lineages and groupings (new families and genera), one of the largest components of Palearctic freshwater fish fauna. In result we had understood finally the correct phylogenetic assignment of our model group – spiny loaches of the genus *Cobitis*. As example published in evaluated period, is another speciose cobitid genus *Pangio*, discovering at least 30 intrageneric phylogenetic lineages. This study provides an example from a number of molecular phylogenetic studies in our laboratory. In evaluated period, the studies into biodiversity of this group continued by two large still ongoing projects – actual biodiversity of the most speciose family Nemacheilidae (3 new genera and 8 new species already described and fundamental phylogenetic organization published soon) and the problem of evolutionary origin of diploid-tetraploid relationships in the family Botiidae, in both using molecular markers, in the latter also NGS data-set (discussed in section about polyploidy and related issues).

Evolution of sturgeons and paddlefishes (order Acipenseriformes) is inherently associated with group specific polyploidization events which resulted in differentiation

of ploidy levels of extant acipenseriform species. Moreover, allopolyploidization as well as autopolyploidization is apparently an ongoing process in these fishes and individuals with abnormal ploidy levels were occasionally observed within sturgeon populations.

We have participated using our molecular cytogenetic methodology in long lasting study that firstly described occurrence of hexaploid individual of Siberian sturgeon (*A. baerii)* and confirmed its autopolyploid origin. In addition to that, the first detailed evidence about fertility of spontaneous hexaploid sturgeon was provided. If 1.5 fold increment in number of chromosome sets occurring in diploids, resulted triploids possess odd number of chromosome sets causing their sterility or subfertility due to interference of gametogenesis. In contrast, 1.5 fold increment in number of chromosome sets in naturally tetraploid *A. baerii* resulted in even number of chromosome sets and therefore in fertility of the hexaploid specimen under study. We also tested the fertility of this individual in comparison with normal individuals of sterlet (*A. ruthenus*), Russian sturgeon (*A. gueldenstaedtii*) and Siberian sturgeon (*A. baerii*). *Acipenser ruthenus* possessed 120 chromosomes, exhibiting paleodiploidy (2n), *A. gueldenstaedtii* and *A. baerii* had ~245 chromosomes representing paleotetraploidy (4n), and *A. baerii* male with abnormal ploidy level had ~ 368 chromosomes, indicating paleohexaploidy (6n). Genealogy assessed from the *mtDNA control region* did not reveal genome markers of other sturgeon species and this individual was supposed to originate from spontaneous 1.5 fold increment in number of chromosome sets with respect to the number most frequently found in nature for this species. Following hormone stimulation, the spontaneous hexaploid male produced normal sperm with ability for fertilization. Fertilization of *A. baerii* and *A. gueldenstaedtii* ova from normal 4n level females with sperm of the hexaploid male produced viable, non-malformed pentaploid (5n) progeny with a ploidy level intermediate to those of the parents. This result showing full fertility of such individual has enormous significance for sturgeon aquaculture and stocking pure individuals with not altered ploidy level.

To distinguish individual genomes of artificially produced sturgeon hybrids between sterlet, *Acipenser ruthenus* female (~120 chromosomes) or Russian sturgeon, *A. gueldenstaedtii* female (~240 chromosomes) and a spontaneous triploid Siberian sturgeon *A. baerii* male (~360 chromosomes), respectively, we applied comparative genomic hybridization (CGH) and genomic in situ hybridization (GISH). We found that the species-specific regions were surprisingly identifiable only on some micro- and small(er) macrochromosomes in hybrid metaphases. We hypothesized that these distinguishable regions are represented by species-specific repetitive sequences driven by more dynamic molecular evolutionary mechanisms. On larger chromosomes, GISH faintly visualized only blocks of pericentromeric and telomeric repetitive sequences, remaining regions were equally shared by both parental species. We concluded that the interspecies hybridization producing viable and even fertile progeny is enabled by the fact that genomes of the species involved are likely divergent at the level of the repetitive sequences only and probably highly conserved in the coding sequences. These small differences of coding sequences are in concordance with previous estimations of relatedness of examined species producing artificial as well as natural hybrids. CGH and GISH represent a challenge in sturgeon cytogenetics as a valuable though technically not simple tool to discriminate chromosomes of parental species in hybrids.

We have employed molecular cytogenetic tools to identify the genomic differences between the two lacustrine sympatric species at the sub-chromosomal level of resolution. Sympatric species pairs are particularly common in freshwater fishes associated with postglacial lakes in northern temperate environments. The nature of divergences between co-occurring sympatric species, factors contributing to reproductive isolation and modes of genome evolution is a much debated topic in evolutionary biology addressed by various experimental tools but not, to the best of our knowledge, nobody approached this field using molecular cytogenetics. We examined chromosomes and genomes of sympatric European winter-spawning *Coregonus albula* and the local endemic dwarf-sized spring-spawning *C*. *fontanae*, occurring in Lake Stechlin. FISH experiments consistently revealed a distinct variation in the copy number of loci of the major ribosomal DNA (the 45S unit) between *C*. *albula* and *C*. *fontanae* genomes. In *C*. *fontanae*, up to 40 chromosomes were identified to bear a part of the major ribosomal DNA, while in *C*. *albula* only 8–10 chromosomes possessed these genes. To determine mechanisms how such extensive genome alternation might have arisen, a PCR screening for retrotransposons from genomic DNA of both species was performed. The amplified retrotransposon *Rex1* was used as a probe for FISH mapping onto chromosomes of both species. These experiments showed a clear co-localization of the ribosomal DNA and the retrotransposon*Rex1* in a pericentromeric region of one or two acrocentric chromosomes in both species. We demonstrated genomic consequences of a rapid ecological speciation on the level undetectable by neither sequence nor karyotype analysis. We provide indirect evidence that ribosomal DNA probably utilized the spreading mechanism of retrotransposons subsequently affecting recombination rates in both genomes, thus, leading to a rapid genome divergence. We attribute these extensive genome re-arrangements associated with speciation event to stress-induced retrotransposons (re)activation. Such causal interplay between genome differentiation, retrotransposons (re)activation and environmental conditions may become a topic to be explored in a broader genomic context in future evolutionary studies.

Research Report of the team in the period 2010–2014

Institute	Institute of Animal Physiology and Genetics of the CAS, v. v. i.

Scientific team	Team of Developmental Biology

At present, our knowledge of mammalian oogenesis and early embryogenesis is mainly based on studies performed in mouse model. However, in important aspects, e.g. molecular mechanisms of meiosis resumption, embryonic genome activation, cleaving timing, demands on composition of culture media, porcine and bovine oocyte and bovine preimplantation embryo reflects for more closely the situation in human and other non – rodent mammals. Thus our, main research interest includes molecular mechanisms regulating resumption of meiosis porcine cumulus – oocyte complex, porcine and bovine oocytes maturation and gene expression during early embryonic bovine development in vivo or in vitro.

The first part of our studies oriented on elucidations of molecular mechanisms regulating resumption of meiosis in preovulatory mammalian follicles or in intact cumulus-oocyte complexes (COCs) cultured in vitro. We are especially interested in intercellular communication of somatic follicular cells with germ cell – the oocyte. In the antral follicles, the somatic cells differentiate into two phenotypically distinct populations: the mural granulosa cells, lining the follicle wall and the cumulus cells, surrounding the oocyte in several layers. Both populations of granulosa cells fulfil different roles during follicle development: the mural granulosa cells are predominantly involved in the perception of signals from outside follicle, the production of steroid hormones and follicular rupture; the cumulus cells provide nutrients and regulatory molecules for oocyte growth, final maturation and ovulation. In turn, the range of function of cumulus cells, including steroidogenesis, gene expression, extracellular matrix formation and metabolism, are modified by factors secreted by the oocyte. During the growth phase, the oocyte is arrested in the first meiotic prophase, but it gains full meiotic competence in several steps. However, these meiotically competent oocytes still remain in the dictyate stage if they are retained within follicles. They only resume meiosis following the preovulatory surge of luteinizing hormone (LH).

Preovulatory surge of LH results in the resumption of meiosis in oocytes, expansion of surrounding cumulus cells and ovulation of matured COCs into oviduct. The mechanism by which LH triggers the meiotic resumption is not completely known yet. LH-receptors are highly expressed in mural granulosa cells but their expression in cumulus cells is suppressed by factors produced by the oocyte. We have raised a hypothesis that epidermal growth factor (EGF) may have a physiological role in regulation of pig oocyte maturation and cumulus expansion in preovulatory follicles, presumably as a mediator of signals elicited by the LH surge. Recent data indicate that these LH-induced events are mediated in preovulatory follicles by several members of EGF-like protein family. Experiments on mice revealed that LH binds to its receptor on mural granulosa cells and induces expression of amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC). These peptides then bind to EGF receptor (EGFR) on cumulus cells and in the autocrine manner, via MAPK 3/1 (also known as ERK 1/2) stimulate transcription of genes involved in regulation of meiotic resumption and cumulus expansion. The production of EGF-like peptides can also be induced by FSH in isolated COCs.

The aim of this work was to answer the question of whether EGF-like peptides can substitute for gonadotropins in stimulation of cumulus expansion, oocyte maturation and acquisition of developmental competence in vitro or whether additional gonadotropin-induced signaling, besides the induction of the EGF-like peptides, is essential for occurrence of these events in pig COCs. For this reason, we studied expression of EGF-like peptides in FSH-stimulated COCs and compared the effect of the peptides and FSH on expression of the expansion related genes, activation of signaling pathways and progesterone production in the COCs. In addition, we assessed the efficiency of EGF-like peptides and FSH in inducing maturation of oocytes, expansion of cumulus cells and acquisition of the oocyte developmental competence. We have shown that FSH promptly stimulated expression of *AREG* and *EREG*, but not *BTC* in the cultured COCs. Expression of *AREG* and *EREG* reached maximum at 2 or 4 h after FSH addition, respectively. FSH also significantly stimulated expression of expansion related genes (*PTGS2*, *TNFAIP6* and *HAS2*) in the COCs at 4 and 8 h of culture, with a significant decrease at 20 h of culture. Both AREG and EREG also increased expression of the expansion related genes, however, the relative abundance of mRNA for each gene was much lower than in the FSH-stimulated COCs. In contrast to FSH, AREG and EREG neither stimulated expression of *CYP11A1* in the COCs nor an increase in progesterone production by cumulus cells. AREG and EREG stimulated maturation of oocytes and expansion of cumulus cells, although the percentage of oocytes that had reached metaphase II was significantly lower when compared to FSH-induced maturation. Nevertheless, significantly more oocytes stimulated with AREG and/or EREG developed to blastocyst stage after parthenogenetic activation when compared to oocytes stimulated with FSH alone or combinations of FSH/LH or PMSG/hCG. We conclude that EGF-like peptides do not mimic all effects of FSH on the cultured COCs, nevertheless they yield oocytes with superior developmental competence. The EGF-like factors thus appear to be convenient stimulators of meiotic maturation of pig COCs under in vitro conditions. The results were published in Reproduction (2011; 141:425-435).

The above described results raised a question about specific roles of gonadotropin- and EGF-like factor-stimulated signaling pathways in cumulus cells and about their significance for resumption of meiosis and cumulus expansion. FSH/LH binding to their membrane receptors leads to a prompt activation of several signaling pathways. A well-characterized signaling cascade is G-protein mediated activation of adenylate cyclase and subsequent production of cAMP. Cyclic AMP in turn activates cAMP dependent protein kinase (PKA) that phosphorylates many substrates within the cell including transcription factor CREB that facilitates in granulosa cells transcription of specific genes such as aromatase and inhibin. However, recent studies have shown that FSH signaling in granulosa and also in cumulus cells is more complex and some molecular events occur independently of PKA activation. We have recently demonstrated that FSH activates in granulosa/cumulus cells MAP kinase and PI3 kinase/PKB signaling pathways. Moreover, stimulation of granulosa/cumulus cells by FSH leads to a transactivation of EGF-receptor via expression of EGF-like factors. These peptides bind to a classical EGF-receptor, activate its intrinsic tyrosine kinase and downstream signaling cascades, mainly MAPK, protein kinase C (PKC) and phosphoinositiol 3 kinase (PI3K). It is obvious that gonadotropins activate in the cultured COCs a broad signaling network, which results in essential changes in transcription profiling in the cumulus cells and consequently in series of post-transcriptional molecular events in oocytes leading to resumption of meiosis. The purpose of our work was to characterize the FSH/LH and AREG/EREG signaling in pig COCs during the period of meiotic resumption and to define signals driving meiotic resumption and cumulus cell expansion.

The specific aim of this work was to define specific roles of **signaling pathways** activated in cumulus cells by the FSH and EGF-like peptides in major events accompanying

meiotic resumption in the pig. For this purpose, specific inhibitors of PKA, MAPK14, MAPK3/1, PI3K/AKT and EGFR tyrosine kinase were employed and their effects on maturation of oocytes, expansion of cumulus cells and expression of the expansion related genes, namely hayluronan synthase 2 (*HAS2*), prostaglandin-endoperoxidase synthase 2 (*PTGS2*) and tumor necrosis factor α-induced protein 6 (*TNFAIP6*) were studied. To define signaling pathways that drive FSH- and EGF-like peptide-induced cumulus expansion and oocyte meiotic resumption, in vitro cultured pig cumulus-oocyte complexes were treated with specific protein kinase inhibitors. We found that FSH-induced maturation of oocytes was blocked in germinal vesicle (GV) stage by PKA, MAPK14, MAPK3/1 and EGF receptor tyrosine kinase inhibitors (H89, SB203580, U0126 and AG1478, respectively) whereas PI3K/AKT inhibitor (LY294002) blocked maturation of oocytes in metaphase I (MI). Amphiregulin (AREG)-induced maturation of oocytes was efficiently blocked in GV by U0126, AG1478; H89 and SB203580 allowed the oocytes to undergo breakdown of GV and blocked maturation in MI. Both FSH- and AREG-induced cumulus expansion was incompletely inhibited by H89 and completely inhibited by SB203580, U0126, AG1478 and LY294002. The inhibitors partially or completely inhibited expression of expansion related genes (*HAS2*, *PTGS2* and *TNFAIP6*) with two exceptions: H89 inhibited only *TNFAIP6* expression and LY294002 increased expression of *PTGS2*. The results of this study are consistent with the idea that PKA and MAPK14 pathways are essential for FSH-induced transactivation of the EGF-receptor and synthesis of EGF-like peptides in cumulus cells and MAPK3/1 is involved in regulation of transcriptional and post-transcriptional events in cumulus cells required for meiotic resumption and cumulus expansion. PI3K/AKT signaling is important for regulation of cumulus expansion, AREG-induced meiotic resumption and oocyte MI/MII transition. The present data also indicate existence of an FSH-activated and PKA-independent pathway involved in regulation of *HAS2* and *PTGS2* expression in cumulus cells. The results of this study were published in Reproduction (2012, 144:535-546).

In addition, we studied an effect of **lapatinib**, a small molecule tyrosine kinase inhibitor that is used for anti-cancer treatment, on meiotic maturation of porcine cumulus-oocyte complexes. Lapatinib competes with ATP-binding site in the kinase domain of the EGFR and inhibits downstream signaling inclusive of the MAPK3/1 and PI3K pathways resulting in a growth arrest or apoptosis. In this study, we asked a question of whether lapatinib through EGFR tyrosin kinase inhibition is able to affect the oocyte meiotic maturation, the expression of cumulus expansion-related genes, hyaluronic acid synthesis and progesterone secretion by in vitro cultured pig COCs. The methods used in this study included real time RT-PCR, immunofluorescence, immunoblotting and radioimmunoassay. We found that lapatinib (10 μM) inhibited FSH/LH induced maturation of oocytes and cumulus cell expansion. The expression expansion-related genes (*PTGS2, TNFAIP6*) was significantly decreased when compared to control FSH/LH stimulated complexes, and so was activation of MAPK3/1, production of hyaluronic acid and progesterone. The results were published in Fertility and Sterility (2013; 99:1739-1748). This study was undertaken in collaboration with the Laboratory of Cell Regeneration and Plasticity. Lucie Němcová from the Laboratory of Developmental Biology was responsible for assessment of an effect of EGFR inhibitor on expression of expansion-related transcripts (*PTGS2 and TNFAIP6*), evaluation of data and manuscript preparation.

Next, we studied **SMAD 2/3** and EGFR signaling pathways in cumulus cells regulating expansion of cumulus cells and maturation of pig oocytes. The aims of this study were: 1/ to determine the requirements for EGFR and SMAD2/3 signaling for porcine cumulus expansion 2/ to investigate involvement of these signaling pathways in steroidogenesis by granulosa

cells and 3/ in expression of cumulus expansion-related transcripts (*HAS2, TNFAIP6, pentraxin3-PTX3*). Porcine oocyte-cumulus complexes (OCCs) were cultured 24 h either with FSH or EGF in the absence/ presence of the following inhibitors: AG1478 (an EGFR tyrosine kinase inhibitor), SB431542 (a specific inhibitor of SMAD2 and SMAD3 activation) and SIS3 (a specific inhibitor of SMAD3 activation). Progesterone and hyaluronic acid (HA) synthesis was measured by radioimmunoassay. The transcripts were quantified using real-time RT-PCR. AG1478 blocked FSH-/EGF-induced germinal vesicle breakdown (GVBD), cumulus expansion by preventing synthesis of HA and significantly decreased FSH-stimulated progesterone production. SIS3 significantly inhibited FSH-stimulated synthesis of HA and progesterone. Although SB431542 only partially inhibited the FSH-induced cumulus expansion, it significantly decreased both total production and matrix incorporation of HA. In addition, SB431542 significantly increased FSH-stimulated progesterone production and expression of pentraxin3 mRNA. In summary, the activation of EGFR pathways by gonadotropin is essential for GVBD, the organization of the expanded cumulus extra-cellular matrix (ECM) and steroidogenesis. The activation of SMAD2 and SMAD3 is required for HA synthesis and production of proteins involved in the ECM organization. Moreover, while the activation of specific SMAD2 signaling pathway is indispensable to prevent luteinization, the activation of SMAD3 signaling pathway is involved in the progesterone synthesis. The results were published in Molecular Reproduction and Development (2011; 78:391-402). This study was undertaken in collaboration with the Laboratory of Cell Regeneration and Plasticity. Members of our laboratory Lucie Němcová and Radek Procházka were responsible for assessment of an effect of SMAD inhibitors on expression of expansion-related transcripts (*HAS2, TNFAIP6, PTX3*), evaluation of data and manuscript preparation.

In the next study, we have elucidated a role of **cyclic guanosin monophosphate (cGMP)** in regulation of meiotic resumption and cumulus expansion in the pig. Recently, a crucial role of cGMP in maintenance of oocyte meiotic arrest has been described on the mouse model of follicular culture. Cyclic GMP is produced by granulosa and cumulus cells by a quanylyl cyclase NPR2 and passes through gap junction into the oocyte, where it prevents hydrolysis of cAMP by inhibition of phosphodiesterase 3A. This inhibition maintains a high concentration of intra-oocyte cAMP and blocks meiotic progression. LH reverses the inhibitory signal by lowering production of cGMP in somatic follicular cells and by closing gap junctions between the cells, thereby causing a decrease in oocyte cAMP, leading to resumption of meiosis. The aim of our study was to verify hypothesis that resumption of oocyte meiosis and expansion of cumulus cells in isolated pig cumulus-oocyte complexes (COCs) can be blocked by high levels of cGMP and that this effect is mediated by cGMP-dependent inhibition of mitogen-activated protein kinase 3/1 (MAPK3/1), as proposed in other laboratory. For this purpose, we used a NPR2 ligand – the natriuretic peptide type C (CNP) and two synthetic cGMP-analogues (8-bromo-cGMP and 8-chlorophenylthio-cGMP) to manipulate with cGMP concentrations in COCs and to assess their effect on FSH-induced maturation of oocytes and expansion of cumulus cell. In addition, we assessed effect of the analogues on activation of MAPK3/1 and on expression of cumulus expansion related genes (*HAS2, PTGS2, TNFAIP6*). We found that expression of *NPR2* decreased in the FSH-stimulated COCs whereas expression of the natriuretic peptide type C precursor (*NPPC*) remained stable. CNP and both cGMP analogues significantly increased concentration of cGMP in COCs and inhibited spontaneous maturation of cumulus enclosed and denuded oocytes but they did not affect expansion of cumulus cells. Neither expression of the expansion-related genes nor activation of MAPK3/1 was affected by cGMP analogues in FSH-stimulated COCs. In conclusion, these data indicate that high cGMP concentration blocks maturation of pig oocytes in vitro but not through inhibition of MAPK3/1 signaling in

cumulus cells. The study has been completed in 2014 and the results published in Reproductive Biology and Endocrinology (2015; 13:1)

In vitro produced (IVP) bovine embryos represent a valuable resource for embryology research and recently also for routine embryo transfer. Nevertheless, IVP process still suffers from low efficiency (<40% IVP embryos reaching blastocyst stage). This is caused not only by low oocyte quality but also embryo culture conditions. To address these problems, several groups have concentrated on embryonic preimplantation development period where maternal-to-embryonic transition (MET) takes place. This shift from utilization of maternally produced and stored transcripts to mRNAs produced by newly activated embryonic genome occurs at a species specific time-point. In the bovine embryos, a minor genome activation was reported to start between 1-4-cell stage, while major genome activation takes place at 8-16-cell stage.

In order to characterize **changes in gene expression profiles** caused by *in vitro* culture environment, we employed microarray constructed from bovine oocyte and preimplantation embryo-specific cDNAs. The analysis revealed changes in the level of 134 transcripts between *in vitro* derived (cultured in COOK BVC/BVB media) and *in vivo* derived 4-cell stage embryos and 97 transcripts were differentially expressed between 8-cell stage *in vitro* and *in vivo* embryos. The expression profiles of selected transcripts (*BUB3, CUL1, FBL, NOLC1, PCAF, GABPA* and *CNOT4*) were studied in detail. We have identified a switch from Cullin 1-like transcript variant 1 to Cullin 1 transcript variant 3 expressions around the time of bovine major gene activation. New fibrillarin protein was detected by immunofluorescence already in early 8-cell stage and this detection correlated with increased level of fibrillarin mRNA. The qRT-PCR analysis revealed significant differences in the level of *BUB3, NOLC1, PCAF, GABPA* and *CNOT4* gene transcripts between *in vivo* derived and *in vitro* produced embryos in late 8-cell stage. The combination of these genes represents a suitable tool for addressing questions concerning normal embryo development *in vivo* and can be potentially useful as a marker of embryo quality in future attempts to optimize *in vitro* culture conditions. This study was undertaken in collaboration with Veterinary and Pharmaceutical University in Brno, University of Veterinary Medicine in Wien and Constantine the Philosopher University in Nitra. These coworkers were responsible for hormonal treatment of donor animals and non-surgical recovery of *in vivo* bovine embryos in different cell stages. On the other hand, all molecular biology methods (suppression subtractive hybridization, array hybridization, scanning, data analysis, quantitative RT-PCR) were performed in Institute of Animal Physiology and Genetics, Libechov. The results of this study were published in Theriogenology (2011, 75:1582-95).

Our further studies have dealt with detailed characterization of such potentially crucially important genes. In the reporting time, we published a paper characterizing the expression and role of nucleophosmin during bovine preimplantation development. **Nucleophosmin** was for our study chosen based on our previous unpublished results. We have found that embryonic transcription of nucleophosmin starts during the major wave of embryonic genome activation which indicates its necessity for bovine preimplantation development. The mRNA expression level of nucleophosmin was found to decrease from MII stage to early 8-cell stage and started to increase at late 8-cell stage. This increase was α-amanitin sensitive and thus represents the start of transcription from embryonic genome. Consequently, we monitored the influence of nucleophosmin mRNA silencing on the early embryogenesis. We showed that the embryos with silenced nucleophosmin mRNA were able to develop until the blastocyst stage. There was only a slight decrease in number of embryos injected with nucleophosmin dsRNA reaching blastocyst stage. Moreover, the embryos showed normal nuclear shape and regular tubulin polymerization. This was likely caused by the fact that a great deal of maternal protein was stored even after embryonic genome activation. The localization of nucleophosmin

protein reflects the formation of embryonic nucleoli. In pre-EGA embryos, we detected diffuse staining in nucleoplasm, during mitosis the protein was dispersed throughout whole blastomere. In early 8-cell stage, nucleophosmin formed shell-like structures that corresponded to the conformation of the forming nucleoli. From the late 8-cell stage, nucleophosmin presented typical nucleolar staining. In late blastocyst stage, the nucleophosmin staining predominated in the trophectoderm. This was evident especially in the hatched blastocysts. The decrease in protein level was verified using western blot analysis. However, there was still enough protein to monitor its localization. The difference in localization distinction between groups with silenced and non-silenced nucleophosmin mRNA was minimal. We only detected a slight delay in nucleophosmin relocalization from nucleoplasm to nucleoli. In non-treated embryos, the protein was localized to nucleoli already at 8-cell stage, whilst in embryos with silenced nucleophosmin mRNA the relocalization occurred at 16-cell stage. In summary our data have shown that a small amount of nucleophosmin protein is preserved throughout whole preimplantation development and enables the development of embryos until the blastocyst stage. The results of this study were published in Reproduction (2012, 144:349-359).

Currently, our most important research issue concerns ubiquitination during bovine preimplantation development. We concentrate on **Skp1-Cullin1-Fbox (SCF) complex**, the expression of its invariant components (Cullin 1, Skp1, Rbx1) and its contribution to degradation of maternal proteins. Our up to now results have shown that embryonic expression of all these genes starts in initial stages of development. Especially Cullin 1 is activated very early, already at 4-cell stage. Genes participating in ubiquitination are usually activated at 8-cell stage, and the early activation of Cullin 1 suggests its necessity for embryonic genome activation. Protein localization analysis showed interesting results especially at the blastocyst stage. There was clear concentration of protein expression and SCF complex activation to trophectoderm. These results allowed us to initiate the experiments dealing with the application of SCF complex during preimplantation development and especially in maternal protein degradation.