KLINIKUM DER UNIVERSITÄT REGENSBURG
Anstalt des öffentlichen Rechts

# Biomedical Symposium

## for Graduate Students

November 4-5th, 2011

BioMedIGS
Biomedical International Graduate School

Regensburg
International Graduate School
of Life Sciences

Biomedical Symposium for Graduate Students
November 4th and 5th, 2011

Tagungshaus Bayerischer Wald, Regen

Organisation: Johanna Canady
Benedikt Gröschl
Anke Rüdel

**PROGRAM** **BioMedIGS Symposium 2011**

**FRIDAY, 4th of November 2011**

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|---|---|---|
| 8.00-9.30 | DEPARTURE from the Klinikum Bus Stop | |
| 9.30-10.00 | CHECK-IN | |
| 10.00-10.30 | RECEPTION | |
| 10.30-11.30 | TALKS | GROUP 1 |

**Proteomic Analysis of the Cellular Response to Ionizing Radiation in *ATM*-Mutation Carriers**
Sophie Schirmer

**Functional Role of *DUSP4* in Colorectal Cancer**
Ben Gröschl

**Myocardial Infarction Family**
Ulrike Esslinger

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| 11.30-12.00 | POSTER | GROUP 1 |

**Gene Therapy for X-linked Juvenile Retinoschisis**
Sarah Hill

**Towards a Genetic Risk Model for Age-Related Macular Degeneration (AMD)**
Felix Grassmann

**First insight into the effect of LIM-homeodomain transcription factor LMX1B on its putative target genes**
Natalya Lukajczyk

**The role of repellent factors in the maintenance of joint compartment border integrity**
Anke Rüdel

**The peripheral nervous system and focal bony erosions in rheumatoid arthritis**
Dominique Muschter

**Generation of singlet oxygen by UVB-irradiation of endogenous molecules**
Alena Knak

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| 12.00-13.00 | LUNCH |
| 13.00-14.00 | MEET THE EXPERT<br>**Cristiano Ferlini, MD, PhD**<br>Director Medical Research,<br>Danbury Hospital Research Institute |

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| 14.00-15.00 | TALKS | GROUP 2 |
| | **Prediction of therapy response in short-term treated primary tumor-initiating cells in vitro**<br>Katharina Meyer | |
| | **The role of MIA/CD-RAP in chondrogenesis**<br>Rainer Schmid | |
| | **Adipocyte hypertrophy and non-alcoholic steatohepatitis in beta 2 syntrophin deficient mice on a high fat diet**<br>Sabrina Bauer | |
| | **KGF-induced OSM secretion by keratinocytes induces the active fibroblast phenotype in keloid and scleroderma**<br>Johanna Canady | |
| 15.00-15.30 | COFFEE BREAK | |
| 15.30-16.00 | INDUSTRIAL TUTORIAL<br>**"Life Technologies: Capillary Sequencer vs. NextGenSeq by Ion Torrent Personal Genome Machine and SOLiD 5500"**<br>Barbara Fitzky | |
| 16.00-17.00 | POSTER | GROUP 2 |
| | **Investigation of (new) photosensitizers for self-disinfection of surfaces**<br>Ariane Felgenträger | |
| | **Prediction of therapy response in short-term treated primary tumor-initiating cells *in vitro***<br>Sylvia Möckel | |
| | **Regulation of the transcription factor c- Jun in malignant melanoma**<br>Melanie Kappelmann | |
| | **Molecular Function and Transport of MIA**<br>Matthias Molnar | |
| | **The functional role of AP-2ε in chondrocytes and osteoarthritis**<br>Stephan Niebler | |
| | SHORT BREAK | |
| | **The role of sensory neuropeptides and catecholamines in callus differentiation**<br>Tanja Niedermair | |
| | **Investigation of proteomic changes in liver affected by non-alcoholic steatohepatitis**<br>Anja Thomas | |

**Biomarker Detection For Non-Alcoholic Fatty Liver Disease (NAFLD)**
Torsten Schön

**Utilizing Patient Specific Induced Pluripotent Stem Cell Derivatives to Functionally Characterize Cardiovascular Risk Alleles**
Maya Fürstenau-Sharp

**Alpha syntrophin deficienct mice are protected from high fat diet mediated obesity**
Kristina Eisinger

17.00-17.30 TUTORING for 1st year students (freshmen)

18.00-19.00 DINNER

20.00-open end GET TOGETHER

**SATURDAY, 5th of November 2011**

9.00-9.30 INTRODUCTION of BioMediGS

9.30-10.30 MEET THE EXPERT
**Christoph W. Turck, Prof. Dr. rer. nat**
Max-Planck-Institut für Psychiatrie, München

10.30-11.30 TALKS GROUP 3

**Strept. agal. infection in humanized mice**
Wolfgang Ernst

**Mouse ficolin B is produced by immature myeloid cells**
Katja Hunold

**9p21 CAD risk haplotype shows altered up-regulation of IL12B and IL1B in macrophages after inflammatory stimuli**
Christa Zollbrecht

**Functional characterization of mouse beta-defensin 14 (mBD14) in inflammation**
Nicola Barabas

11.30-12.00 POSTER GROUP 3

**Defining Immunological Parameters to be used as End- Point Measures in Clinical Transplant Studies**
Anja Kammler

**Cyclophilin A dependent HIV-1 replication of Cyclosporine A dependent viral variants can be interchangeably restored by structurally distinct in vivo and in vitro mutations**
Benedikt Grandel

**Basophils as regulators of CD4 T cell homeostasis**
Fabian Herman

**The homing of myeloid-derived suppressor cells and interaction with leucocytes differs between C57BL/6 and BALB/c after infection with *L. major***
Maximilian Schmid

**Characterization of the anti-inflammatory impact of molecular fragments derived from the adipokine CTRP-3 in adipocytes and monocytes**
Andreas Schmid

12.00-13.00 LUNCH

13.00-14.00 TALKS GROUP 4

**Functional role of natriuretic peptides in compensatory renal hypertrophy**
Andrea Schreiber

**Cellular Mechanisms of Renin secretion**
Dominik Steppan

**Localization and Regulation of the Angiotensin II Type 1 Receptor-associated Protein Arap1 in the Kidney**
Elisabeth Doblinger

**Fibrocyte development: role of monocytes recruited via the chemokine receptor CCR2**
Barbara Reich

14.00-14.30 POSTER GROUP 4 Part I

**MicroRNAs in the kidney and their relevance in renal diseases**
Susanne Baumgarten

**The impact of the basolateral potassium channel KCNJ10 for salt resorption in the distal tubule of the kidney**
Maria Ripper

**The role of TASK3 $K^+$ channels for the regulation of aldosterone secretion in neonatal mice**
Philipp Tauber

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| | **The physiological and pathophysiological relevance of the inwardly rectifying potassium channel Kir5.1 (KCNJ16) in the kidney**<br>Evelyn Humberg |
| | **Localization of Renin-producing cells in Cx40-deficient mice**<br>Christian Karger |
| | **Creation and Characterization of *Pkd2* Knock-in Mice**<br>Denise Schmied |
| 14.30-15.00 | COFFEE BREAK and PICTURE TIME |
| 15.00-15.30 | POSTER GROUP 4 Part II |
| | **Renal Function of cGKII**<br>Andrea Schramm |
| | **The role of Ecto-5'–nucleotidase (CD73) –mediated extracellular adenosine generation in renal fibrosis**<br>Isabel Carota |
| | **The role of WNK3-kinase in activation of NKCC2 in vivo**<br>Katharina Mederle |
| | **The impact of mutated Fanconi-associated protein on the mitochondrial proteome**<br>Nadine Aßmann |
| | **Chemokines and Their Receptors as Potential Regulators of Renal Development and Function**<br>Simone Wurm |
| 15.30-16.00 | ELECTIONS BEST POSTER / BEST TALK |
| 16.00-16.30 | AWARD PRESENTATION and FAREWELL |

**Abstract Band**

# 1. Group 1

TALKS

**Proteomic Analysis of the Cellular Response to Ionizing Radiation in *ATM*-Mutation Carriers**
Sophie Schirmer

**Functional Role of *DUSP4* in Colorectal Cancer**
Ben Gröschl

**Myocardial Infarction Family**
Ulrike Esslinger

POSTER

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**Towards a Genetic Risk Model for Age-Related Macular Degeneration (AMD)**
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Dominique Muschter

**Generation of singlet oxygen by UVB-irradiation of endogenous molecules**
Alena Knak

## 1.1 Proteomic Analysis of the Cellular Response to Ionizing Radiation in *ATM*-Mutation Carriers

**Sophie Schirmer**

Annually 57,000 women are diagnosed with breast cancer in Germany, 5-10% of the cases being familial. Beside mutations in *BRCA1/2*, *ATM* mutations confer an increased breast cancer risk. This holds in particular for missense mutations in the C-terminal FAT, kinase and FATC domains of the *ATM* gene, while heterozygous protein truncating and splice junction variants confer only a marginal risk.

The present proteomic study on the effect of compound heterozygous nonsense and heterozygous missense *ATM* mutations on the cellular response to ionizing radiation (IR) complements an earlier study that employed gene expression profiling. EBV-transformed lymphoblastoid cell lines (LCLs) of breast cancer patients heterozygous for 7271T>G, compound heterozygous A-T patients, and control patients were gamma-irradiated with 3 Gy and harvested after 6 hours of incubation. After nuclei separation the corresponding proteins were purified and purity was monitored by immunoblotting against several compartment-specific proteins.

The nuclear proteomes of the irradiated and non-irradiated cells were compared using differential in-gel electrophoresis (DIGE). The comparison showed 134 regulated spots with *P*-values ≤ 0.05 and a statistical power ≥ 0.8. These significantly regulated spots were picked, digested and analyzed by nano-LC-QTOF-MS/MS. We identified proteins such as SMC1A, Mre11 and Rad23B, which play a role in DNA repair and/or are known downstream targets of ATM. The SMC1A-containing spot was 1.6-fold down-regulated in controls ($P \leq 0.008$), while the Mre11-containing spot was about two-fold down-regulated in both controls and A-T patients ($P \leq 0.014$). These down-regulations occur presumably upon posttranslational modifications. In addition, the proteins RED, KHSRP and NONO were identified. Currently, regulated proteins are validated by immunoblotting.

## 1.2 Functional Role of *DUSP4* in Colorectal Cancer

**Ben Gröschl**

*DUSP4* (MKP-2), a member of the mitogen-activated protein kinase phosphatase (MKP) family and potential tumor suppressor, negatively regulates the MAPKs (Mitogen-activated protein kinases) ERK, p38 and JNK by dephsphorylation. MAPKs play a crucial role in cancer development and tumor progression.

We screened the expression of *DUSP4* mRNA in samples of colorectal cancer (CRC) and normal tissue in patients by qPCR. The majority of patients showed *DUSP4* overexpression in tumor. Furthermore, microsatellite unstable (MSI) samples showed *DUSP4* overexpression in tumor. Median expression levels in tumor samples of MSI patients were higher than in microsatellite stable (MSS) patients. Consistently MSI CRC cell lines showed higher *DUSP4* mRNA levels than MSS cell lines. To exclude a *DUSP4* mutation or methylation as an influence on mRNA overexpression, the gene was sequenced by Sanger sequencing in cell lines and promoter methylation was analyzed by Methy-QESD (Quantification of Endonuclease-Resistent DNA). No mutations were found in the protein-coding regions of the *DUSP4* gene in CRC cell lines and no methylation of the *DUSP4* promotor could be detected in CRC cell lines and patient samples. *DUSP4* overexpression by an expression vector in CRC cell lines resulted in changes of transcription levels of potential downstream targets. Proliferation of cell lines was affected after overexpression of *DUSP4*.

These findings suggest a potential correlation between microsatellite instability and *DUSP4* upregulation as well as a possible role of *DUSP4* in influencing tumorigenesis in colorectal cancer.

## 1.3 Myocardial Infarction Family

**Ulrike Esslinger**

In genome-wide association studies (GWAS) several novel genes were found to be associated with coronary artery disease (CAD) and myocardial infarction (MI). However, the common genetic variants underlying these genes explain only a modest fraction of heritability. The question came up whether rare genetic variants, so far not detected in GWAS, account for a significant fraction of unexplained heritability. For this reason, 25 extended MI families were collected. All of these families showed an autosomal dominant pattern of inheritance suggesting private mutations accumulated over the generations. To identify these rare genetic variants, the approach of exome sequencing was chosen.

From the largest MI family with more than 120 members and 23 affected individuals, three affected individuals were selected for exome sequencing. For enrichment of the exome part of the genome, the SureSelect Human All Exon Kit from Illumina was used. Captured DNA samples were sequenced on the Illumina Genome Analyser platform. The exome sequences were aligned to obtain single nucleotide variants (SNVs) shared between family members. Further filtering strategies were necessary to reduce the vast amounts of SNVs. Therefore, common genetic variants known by dbSNP, HapMap and control exome sequences (n=25) were excluded.

This approach resulted in seven SNVs shared between the three individuals of this family. To test which SNV is associated with MI, the regions of SNVs were sequenced to consider co-segregation. No complete co-segregation (50–70%) could be established, however, this might be in agreement with a complex rather than Mendelian disease (limited penetrance and phenotypical diversity within the family). After analysing the conservation of the mutation site as well as Polyphen prediction, a frameshift mutation leading to protein truncation in a plausible candidate gene, was considered to be the causal variant. In subsequent mutation scanning of the gene using hetero-duplex analysis in 483 young MI cases, 562 controls, as well as in 81 members of extended MI families, additional six rare (private) missense mutations were identified. Two of these mutations were found in members of two unrelated extended MI families. These results confirm that this gene plays an important role in the pathogenesis of myocardial infarction. Functional studies are ongoing to decipher the precise biological consequences of the mutations identified in this study.

## 1.4 Gene Therapy for X-linked Juvenile Retinoschisis-Proof-of-concept in a Knockout Mouse Model

**Sarah Hill**

The *RS1* gene encodes an extracellular protein, retinoschisin, which is secreted from photoreceptors and bipolar cells of the retina and has a predicted function as a cell surface protein implicated in cell-cell interactions. Mutations in *RS1* cause X-linked juvenile retinoschisis (XLRS), which is a common cause of juvenile macular degeneration in males. Our group and others have generated knockout mice deficient in *Rs1h* (the murine ortholog of *RS1*).[1-3] Gene therapy studies for RS1 have been pursued involving of various vectors into RS1 knockout mice.[3-8] While previous studies have shown the effectiveness of gene transfer on the structure and function of *Rs1h* deficient murine retina, it remains challenging to express the protein at physiological levels. In order to move toward a clinical trial in humans, a well-regulated RS1 expression vector is required.

Recently, our group showed that the cone-rod homeobox (CRX) controls RS1 expression.[9] Evaluation of two murine genome-wide data sets revealed two highly conserved regions near *RS1* that bind CRX, named CRX-bound region 1 (CBR 1; immediately upstream from the TSS) and CBR 2 (within the first 700bp of intron 1). Additionally, our group screened 5Kb surrounding the *RS1* transcription site for CpG islands (CGI), and located one CGI of greater than 500bp upstream of *RS1* (3kb upstream from the TSS; bp -2878 to -3386). Using luciferase assay and *in vitro* electroporation of mouse retinal explants, expression effects were evaluated for vectors containing various combinations of CBR 1, CBR 2, and the CGI. Based on these experiments, Krause et al. suggested a vector containing all three elements for future XLRS gene therapy studies.

Three constructs containing the recommended promoter elements from Krause et al. were cloned into a pTR vector (used for packaging into adeno-associated viral vectors). The first construct includes RS1 cDNA under the regulation of the recommended promoter elements, the second construct additionally contains an N-terminal MYC tag, and the third construct is a GFP fusion protein also regulated by the recommended promoter elements. Initial testing of the GFP fusion construct in 661W cells (mouse cone photoreceptor cell line) did not show GFP expression when transfected alone and when co-transfected with CRX. Currently, the constructs are being electroporated *in vitro* into wild type and RS1 knock out mouse retinal explants. The electroporated retina will be tested for RS1 mRNA and protein expression via RT-PCR, immunohistochemical staining of retinal cryo sections, and Western blot.

Validated vector constructs will be packaged in to adeno-associated viral vectors injected into RS1 knockout mice at various ages. The novel vector construct will be compared to the previously validated AAV5-mOP500-hRS1 vector. Electroretinography will be performed at

various post-injection time points, and analysis of the expression and localization of RS1 will be performed post-mortem.

## 1.5 Towards a Genetic Risk Model for Age-Related Macular Degeneration (AMD)

**Felix Graßmann**

Purpose: Age-related macular degeneration (AMD) is the leading cause of blindness in the Western World and affects an estimated 50 million people worldwide. AMD is a multifactorial trait involving both genetic and environmental effects although the precise aetiology still remains elusive. Age, smoking, and to a lesser extent diet and sunlight exposure are among the most commonly reported risk factors for disease onset. A genetic contribution to AMD is well established by familial aggregation analyses and twin studies. Recently, variations in the complement pathway as well as other loci have been found to be associated with AMD. Testing single susceptibility variants is of limited value for prediction of complex diseases. Genotyping and evaluating several disease associated variations (genetic profiling) however, could be the key to accurately predict disease risk.

Methods: Our study included 979 AMD patients and 793 matched controls recruited from the Lower Frankonian area at the University Eye Clinic of Würzburg (Dr. Claudia N. Keilhauer). Genotyping of patients was performed by various methods including i-plexing, Taqman assays, and direct sequencing. Conditional and unconditional logistic regression was carried out with R, a scripting language used in statistics and epidemiology.

Results: We genotyped previously reported variations that were found to be associated with AMD in European samples and found the most parsimonious set of variations by conditional logistic regression analysis. Ten SNPs showed significant and independent association with late stage AMD. These SNPs were used to build a multivariate logistic regression model. With the log-odds ratios obtained from this model, a genetic risk score for each individual study member was calculated and a hypothetical population assuming different prevalences was modeled. By adjusting the prevalence in this population, we can allow our population to age and thus can predict disease risk at any given age.

Conclusion: Here we suggest a model to predict disease risk for late stage AMD from a well characterized case control study by modeling a hypothetical population and linking the prevalence in this population with age strata obtained from previous publications. This enables us to predict a person’s risk to develop late stage AMD assuming the genotype at the ten SNPs used in the model is known.

## 1.6 First insight into the effect of LIM-homeodomain transcription factor LMX1B on its putative target genes

**Natalya Lukajczyk**

**Introduction:** LMX1B is a transcription factor that belongs to the family of LIM-homeodomain proteins and is crucial for the development of several organs. Mutations in the *LMX1B* gene are associated with a rare autosomal-dominant disorder called nail-patella syndrome which affects nails, kneecaps and kidneys. The binding of LMX1B to its target genes is mediated by the central homeodomain which specifically recognizes so-called FLAT (FAR linked AT-rich) elements. Microarray studies of glomeruli isolated from inducible podocyte-specific *Lmx1b* knock-out mice have shown a significant increase of the mRNA levels of several genes in comparison to control mice. Due to their time-course of induction and their common physiological function four promising LMX1B target genes were chosen for further investigation. Three of them encode proteins associated with the actin cytoskeleton, they are Abra, Arl4c and Transgelin. The fourth candidate of the studies is CRCT1 (Cysteine-rich C-terminal 1). Currently there is no biological information available regarding this gene, making it attractive as a research object.

**Research aims and methods:** The main goal of the project is to characterize putative LMX1B target genes. To start with, 6 kbp long promoter fragments of the putative LMX1B target genes were studied using a set of bioinformatical research tools and databases, such as UCSC genome browser and NCBI. The bioinformatical studies were focused on the human and murine promoter regions because the *in vitro* experiments will be performed in a stably transfected HeLa cell line synthesizing the human LMX1B in an inducible manner. Then the studies will be continued in the podocyte-specific *Lmx1b* knock-out mouse model. Afterwards promoter fragments of putative target genes were isolated and cloned in pGL4.10 Luciferase Reporter Vector. Obtained constructs were studied using dual-luciferase reporter assay.

**Results:** The dual-luciferase reporter assay performance was impeded by unexpected effects of LMX1B on the *Renilla* vector which is used for normalization. Therefore it was necessary to optimize the normalization method. The achieved results provide a first attempt in understanding the mechanism by which LMX1B regulates the maintenance of the glomerular filtration barrier.

**Perspectives:** In order to characterize precisely these genes it is planned to continue studies with gene expression analysis, site-directed mutagenesis and RNA interference.

## 1.7 The role of repellent factors in the maintenance of joint compartment border integrity

**Anke Rüdel**

The mechanical functions of joints depend on the strict separation of different compartments. In inflammatory and degenerative joint diseases like rheumatoid arthritis (RA) or osteoarthritis (OA) this separation cannot be maintained. Synovial fibroblasts (SF) cross the compartment boundaries between the hardstand and the cartilage compartment and destroy cartilage by the release of proteases such as matrix metalloproteinases (MMPs). Isolated rheumatoid arthritis synovial fibroblasts (RASF) have been demonstrated to be able to migrate and destroy cartilage in early passages by means of Boyden chamber assays and in cartilage destruction assays. They also show differences in the expression pattern compared to normal SF and late stage RASF, e.g. Robo3 and Unc5C are strongly overexpressed in early stage RASF. By Slit3 and Netrin1 treatment respectively the migratory activity of these cells is inhibited. Therefore the aggressiveness of the RASF is reduced which provides a new possible target for RA treatment in patients. We now aim to identify the signaling pathways responsible for the Robo3/Slit3 and Unc5C/Netrin1 signal transduction.

Electromobility shift assays (EMSAs) showed that AP-1 binding to its binding site is reduced in RASF after Slit3 treatment and is therefore a very interesting candidate for Robo3/Slit3 signaling. For Netrin1 the most interesting candidate to this date seems to be CREB. In EMSAs stronger binding of this factor could be detected after Netrin1 treatment. However, also a high variation in the intensity was observed in RASF from different donors. We therefore search for another receptor contributing to the Netrin1 effect. In ongoing experiments we try to confirm the results and to understand the signaling pathway more in detail.

In second part of the project we aim to analyze the role of single nucleotide polymorphisms (SNPs) in the CDH2 promoter on the severity of OA and to understand the mode of action. Two of the analyzed SNPs were associated with a positive outcome/less severity of OA. We now try to find the transcription factors responsible for a different expression of CDH2 and therefore leading to the positive association.

## 1.8 The peripheral nervous system and focal bony erosions in rheumatoid arthritis

**Dominique Muschter**

In recent years the interaction between bone tissue and the peripheral nervous system has been studied extensively. Numerous factors have been identified which contribute to the regulation of bone homeostasis. Catecholamines like norepinephrine favor bone degradation by stimulating osteoclastogenesis and inhibiting osteoblast actions via β2-receptors. On the contrary the neuropeptides vasoactive intestinal peptide (VIP) and calcitonin-gene related peptide (CGRP) enhance bone formation by inhibition of osteoclast differentiation and activation of osteoblasts. Neuropeptide Substance P (SP) activates osteoclasts and therefore stimulates degradation of bone. Various neurotransmitters and neuropeptides can affect bone metabolism either positively or negatively. The mode of action depends on the local transmitter concentration and composition as well as on the stage of differentiation of osteoblasts and osteoclasts and their respective precursors.

Rheumatoid Arthritis (RA) is a systemic chronic inflammatory condition predominantly affecting the joints and leading to destruction of articular cartilage and focal bony erosions. In the course of arthritis a loss of catecholaminergic nerve fibers and the sprouting of sensory nerve fibers into synovial tissue has been demonstrated leading to a change of neurotransmitter concentrations in the microenvironment of the tissue. It is not clear whether the sympathetic nerves are lost or if a sympathetic transition took place inducing a VIP/cholinergic phenotype instead of catecholaminergic fibers.

This research project will focus on the focal bony erosions in the context of a possibly altered local neurotransmitter milieu and especially on the cells responsible for bone matrix degradation, the osteoclasts. We will use an animal model of collagen-induced arthritis (CIA) where we want to compare osteoclastogenesis at various stages of disease progression to healthy controls by gene expression analysis of typical osteoclastic markers like calcitonin receptor, cathepsin K and tartrat-resistent acid phosphatase as well as various neurotransmitter receptors for acetylcholine, VIP and norepinephrine. The influence of acetylcholine, VIP and norepinephrine on differentiation and activity of osteoclasts will be examined and compared between untreated animals and CIA animals.

The results should lead to a better understanding of the communication of bone tissue and the peripheral nervous system in the context of chronic inflammatory diseases like rheumatoid arthritis.

## 1.9 Generation of singlet oxygen by UVB-irradiation of endogenous molecules

**Alena Knak**

Introduction

It is generally known that UVA (320 – 400 nm) and UVB radiation (280 – 320 nm) can cause damage in cells or bacteria. Usually, the mechanisms of cell damage are classified to either direct DNA damage for UVB radiation or oxidative damage for UVA-radiation. This oxidative damage is predominantly mediated by singlet oxygen, which is generated after absorption of UVA radiation in various endogenous photosensitizers. It has already been suggested that UVB radiation can also generate singlet oxygen and therefore cause oxidative damage in cells and bacteria.

Results and Methods

Part of my PHD work is to investigate whether and to which extent UVB radiation is also able to generate singlet oxygen via endogenous photosensitizers. Therefore potential endogenous photosensitizers such as different vitamin molecules and unsaturated fatty acids are irradiated with monochromatic UVB radiation at 308 nm (Xe-Cl-laser). Singlet oxygen is directly detected by its luminescence in the near infrared spectrum at 1270 nm time- and spectral resolved. For most of the investigated molecules, a clear luminescence signal could be obtained by time-resolved measurements. Spectral-resolved measurements in the range of 1150 to 1350nm revealed a clear signal maximum at 1270 nm, which confirmed the generation of singlet oxygen. By comparison with well-known photosensitizers like PNS, TMPyP or PN the singlet oxygen quantum yields of endogenous photosensitizers could be estimated ranging from 5 up to 40 %. By measuring the absorption spectra during UVB irradiation a change of UVB absorption could be estimated. The photosensitizers obviously changed due to peroxidation leading to a change of UVB absorption.

Discussion and Perspective

The absorption of UVB radiation in the investigated endogenous photosensitizers can lead to the generation of singlet oxygen that in turn changes the absorption of those molecules. The effect of UVB absorption and hence singlet oxygen production is either reduced or increased. The outcome of this effect needs further observation.

## 2. Group 2

TALKS

**Prediction of therapy response in short-term treated primary tumor-initiating cells in vitro**
Katharina Meyer

**The role of MIA/CD-RAP in chondrogenesis**
Rainer Schmid

**Adipocyte hypertrophy and non-alcoholic steatohepatitis in beta 2 syntrophin deficient mice on a high fat diet**
Sabrina Bauer

**KGF-induced OSM secretion by keratinocytes induces the active fibroblast phenotype in keloid and scleroderma**
Johanna Canady

POSTER

**Investigation of (new) photosensitizers for self-disinfection of surfaces**
Ariane Felgenträger

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Sylvia Möckel

**Regulation of the transcription factor c- Jun in malignant melanoma**
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**Molecular Function and Transport of MIA**
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**The functional role of AP-2ε in chondrocytes and osteoarthritis**
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**The role of sensory neuropeptides and catecholamines in callus differentiation**
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**Biomarker Detection For Non-Alcoholic Fatty Liver Disease (NAFLD)**
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**Utilizing Patient Specific Induced Pluripotent Stem Cell Derivatives to Functionally Characterize Cardiovascular Risk Alleles**
Maya Fürstenau-Sharp

**Alpha syntrophin deficienct mice are protected from high fat diet mediated obesity**
Kristina Eisinger

## 2.1 Prediction of therapy response in short-term treated primary tumor-initiating cells in vitro

**Katharina Meyer**

Glioblastoma are the most common and aggressive brain tumors in adults. They are associated with a high degree of vascularisation and angiogenesis. Patients have a short live expectancy despite aggressive therapeutic approaches. This emphasizes the need for novel therapeutic strategies. One strategy is to inhibit angiogenesis directly by inhibiting the VEGF receptor with e.g. sunitinib. The problem is that glioblastoma show very heterogeneous expression profiles, so it seems unlikely to find one treatment for all patients. Therefore it is necessary to connect treatment response with the expression profiles of a given glioma.

Within this study tumor-initiating cell lines are treated over a short period of time with different potential treatments, among others sunitinib is used. Before and after treatment functional assays and microarrays are done.

The aim is to integrate microarray data with phenotype data to compare the different treatment responses and to develop a method to predict treatment outcome. To get the overall effect of sunitinib it is necessary to transform the microarray data such that the individual patient effect does not play the main role but the overall treatment effect does. The ability to combine microarray data from different patients and to evaluate the effects of the treatment excluding the differences between the patients is very important. We used the method ComBat to adjust for patient effects, the method uses an empirical Bayes strategy assuming an additive and multiplicative "patient effect". After this transformation it was possible to find genes being differentially expressed between Sunitinib treatment and controls. The genes found were evaluated using GSEA: gene set enrichment analysis.

The data is to be augmented by the functional phenotype data and by using other treatments for comparison. Thus it is going to be necessary to compare the microarray datasets based on fold change profiles rather than on the normal gene expressions. Our Results could lead the way to gene signatures making it possible to predict the response of a glioma to different treatments.

## 2.2 The role of MIA/CD-RAP in chondrogenesis

**Rainer Schmid**

MIA/CD-RAP (melanoma inhibitory activity/cartilage-derived retinoid acid-sensitive protein) is a small soluble protein secreted from malignant melanoma cells and chondrocytes. In skin MIA promotes progression and metastasis of malignant melanoma by mediating cell detachment from extracellular matrix molecules. In chondrogenesis MIA/CD-RAP is expressed throughout cartilage development and it was revealed to be a specific marker for chondrocyte differentiation. Elevated MIA/CD-RAP serum levels can be monitored after cartilage damage as a result of MIA release from chondroid matrix. Analysis of transgenic mice harbouring mouse CD-RAP promoter linked to ß-galactosidase gene revealed expression of ß-galactosidase in all cartilage in embryos and adult animals. These data suggest that MIA may play an important role in cartilage differentiation and specific events during embryogenesis.

To study the consequences of MIA/CD-RAP deficiency in detail we used mice with a targeted gene disruption of MIA/CD-RAP and analysed cartilage organisation and differentiation. Cartilage formation and regeneration was determined in models for fracture healing and osteoarthritis *in vivo* in addition to *in vitro* studies using mesemchymal stem cells of MIA-/- mice. Interestingly, all models suggest enhanced chondrocytic regeneration in MIA-/- mice, modulated by enhanced proliferation and delayed differentiation. To further investigate modulation of differentiation in MIA-/- mice, we studied chondrogenesis *in vivo* in femoral limb cartilage by *in situ* hybridization against the chondrogenic markers Collagen type II, type X and Sox9 at different embryonic stages and revealed differing expression between MIA-/- and WT mice.

Expression analysis of cartilage tissue derived from MIA-/- mice indicated strong down regulation of $p54^{nrb}$, a recently described modulator of Sox9. Since Sox9 is an important transcription factor in cartilage development, $p54^{nrb}$ seems to be the missing link between MIA/CD-RAP and chondrogenesis. To investigate whether the induction of $p54^{nrb}$ expression by MIA was due to promoter regulation, we subcloned the promoter region of *$p54^{nrb}$* into a luciferase reporter gene plasmid. Luciferase measurement after transient transfection into murine mesenchymal stem cells showed strong activity, suggesting that induction of $p54^{nrb}$ is due to transcriptional regulation.

Taken together, our data indicate that MIA/CD-RAP is required for differentiation in cartilage by modelling signalling processes during differentiation.

## 2.3 Adipocyte hypertrophy and non-alcoholic steatohepatitis in beta 2 syntrophin deficient mice on a high fat diet

**Sabrina Bauer**

**Objective:** Beta 2 syntrophin (SNTB2) is a multidomain adaptor protein which interacts with a variety of molecules due to its modular structure. Although SNTB2 interactive proteins are expressed in adipocytes, the role of SNTB2 in these cells has not been studied so far.

**Results**: SNTB2 was found expressed in adipocytes and was strongly upregulated during differentiation. In 3T3-L1 preadipocytes SNTB2 knock down lowered cell proliferation. Differentiation of these cells to mature adipocytes led to the formation of large lipid droplets, with no effect on the expression of lipid droplet-associated proteins which are the regulators of lipid droplet size. Uptake of free fatty acids and insulin signalling were not altered whereas stimulated lipolysis was increased in SNTB2 siRNA treated cells indicating a regulatory role of SNTB2 in adipocyte lipid storage. Lipid droplets in subcutaneous but not visceral fat depots of SNTB2-/- mice kept on a high fat diet were significantly enlarged. Interestingly, this fat cell hypertrophy was not associated with increased inflammation. Inappropriate fat storage in adipose tissue causes lipid accumulation in peripheral organs like the liver. SNTB2 deficient mice kept on a high fat diet have excessive hepatic steatosis, inflammation and fibrosis. Further these animals are characterized by reduced glucose tolerance and impaired insulin sensitivity.

**Conclusion:** These experiments demonstrate that SNTB2 and the pathways influenced by this adaptor protein which still have to be identified in adipocytes alter fat cell function and thereby whole body glucose homeostasis.

## 2.4 KGF-induced OSM secretion by keratinocytes induces the active fibroblast phenotype in keloid and scleroderma

**Johanna Canady**

Cutaneous wound healing is a highly complex process, which depends on the perfectly well coordinated interplay between various cell types and cytokines. Disruption of this orchestrated process has severe pathological consequences as it is the fact in keloid and skin sclerosis. Both diseases are characterized by an overproduction of extracellular matrix components, mainly collagen, by activated fibroblasts. To date there is no effective treatment against both conditions, but a genetic origin is presumed as family clustering is observed. We aim to reveal novel important players in normal and pathological wound healing and therefore lead to new therapeutic approaches.

KGF (keratinocyte growth factor), a strong, paracrine acting mitogen for epithelial cells with an important function in wound healing, was found to be constitutively overexpressed in keloid and skin sclerosis derived fibroblasts (KHDF and SHDF) compared to normal human dermal fibroblasts (NHDF) on both mRNA and protein level. Functional assays revealed that primary keratinocytes, cultured with conditioned media from keloid and skin sclerosis fibroblasts, respectively, displayed a higher proliferation rate compared to keratinocytes maintained with conditioned media from normal fibroblasts. *In vivo*, increased KGF expression could be confirmed on keloid and skin sclerosis tissue using immunofluorescence staining. Skin sclerosis patients even show elevated blood KGF levels. The enhanced KGF expression induces increased OSM (Oncostatin M) secretion in keratinocytes. In return OSM has an activating effect on the underlying fibroblasts. When cultured with KGF-treated keratinocyte conditioned media or OSM alone, fibroblasts showed enhanced migration and collagen contraction. Furthermore, OSM treated fibroblasts are more resistant to apoptosis. These activating effects in the fibroblasts are most likely mediated by enhanced STAT3 phosphorylation.

This work shows KGF to be involved in keloid and skin sclerosis by activating fibroblasts by means of a double paracrine loop with Oncostatin M.

## 2.5 Investigation of (new) photosensitizers for self-disinfection of surfaces

**Ariane Felgenträger**

Infections with pathogens like multiresistant bacteria or fungi often lead to long and expensive therapy. Immunosuppressed patients have a high risk to infect themselves with pathogens through contact to other people or material in the hospital. The fast growing development of resistance of the pathogens against antibiotics and the slow development of new classes of antibiotics are contrarian. Countries like Holland have a quote of resistance against antibiotics of around 1% due to their quarantine procedure. The MRSA statistic in Germany - like in other European countries - gives a reason for concern because of a resistance rate of 22% (2004) and more of multiresistant *S. aurei*. As one possibility for killing pathogens the photodynamic treatment can close the therapeutic gap. Therefore a photosensitizer (PS) works as an intermediator and transfers via a short excitation with light its energy to oxygen. This excited oxygen is called singlet oxygen ($^1O_2$) and is the toxic agency. This method is used when an infection is already present.

As a new approach within this Ph. D. study a method will be developed in which a direct contact between PS and pathogen is no longer necessary but the toxic property of $^1O_2$ is still used. The aim is to obviate infections and thus to decrease the number of infected people. This will be done with embedding the PS in materials. When the PS are chemically bound on or fixated within surfaces and a diffusion of oxygen is possible then the activated $^1O_2$ can affect the adherent pathogens. The process will work as long as light is illuminating this material and thus prevents microorganisms to settle down these surfaces.

It will be clarified how $^1O_2$ can affect pathogens, specifically when there are inter-connected cells (biofilm). In reality surfaces, e.g. material of catheters, are colonized with biofilms. It has to be clarified how these react on photodynamic inactivation. For this purpose the cells are in direct contact to the PS, to activate $^1O_2$ directly at the site of the pathogens. In addition cells will be put on different surfaces to test the photodynamic activity. Different surfaces will be tested with different chemical connections to the various PS, and with different diffusion possibilities for oxygen. The photostability of these materials is investigated by absorption spectroscopy. In order to clarify if $^1O_2$ is generated, the luminescence of $^1O_2$ will be measured time resolved at 1270 nm in near-backward direction using an infrared-sensitive photomultiplier. The PS are excited with a frequency doubled Nd:YAG-laser.

## 2.6 Prediction of therapy response in short-term treated primary tumor-initiating cells *in vitro*

**Sylvia Möckel**

The increasing knowledge of the pathogenesis of tumor formation and progression in high grade gliomas has led to the development of a novel group of therapeutic agents which includes small molecule kinase inhibitors. These novel agents often directly interfere with growth factor signaling pathways which are upregulated in brain tumors and are supposed to interfere with oncogenesis by influencing autocrine and paracrine mechanisms. However, and despite promising preclinical studies, results of pilot trials have been generally disappointing. One reason may be the enormous molecular and genetic heterogeneity between individual tumors which often contributes to the varied and often poor treatment response. Therefore, it is demanding to correlate individual response to the general susceptibility of a certain tumor entity to certain substances.

In our preclinical study we examined the effect of the small molecule receptor-tyrosin-kinase (RTK) inhibitor Sunitinib in 18 Tumor-initiating cell lines which were derived from 18 sequential patients. Cells were treated with Sunitinib or DMSO (control) alone or together with VEGF-A/PDGF-AB for 6 hours. The phosphorylation of signaling molecules downstream of these growth factors receptor tyrosine kinases (RTKs) (Erk, Akt, STAT3) was assessed by Western-Blots. The treatment response is currently analyzed by migration and proliferation assays (Spheroid-Expansion, BrdU Incorporation and XTT viability assay). The results reveal that stimulation as well as inhibition of particular RTKs has different downstream effects in distinct cell lines. We are on our way to further correlate this data to the expression profiles obtained from microarrays and the outcome of functional assays.

Our aim is to define a molecular signature which can predict treatment response *in vitro*. So far our data clearly demonstrate the heterogeneity of treatment response on a molecular level and underlines the importance to preselect patients for clinical trials. We mandate that the concept of a personalized treatment requires an *in vitro* drug testing tool that enables the prediction of therapy response within a single short-term assay.

## 2.7 Regulation of the transcription factor c- Jun in malignant melanoma

**Melanie Kappelmann**

Malignant melanoma is an aggressive tumor of melanocytes and is resistant to current therapeutic approaches. Crosstalk between melanocytes and keratinocytes is important in human epidermis. It is known that normal melanocytic phenotype and controlled proliferation of melanocytes is strictly regulated by keratinocytes via E-cadherin. Malignant transformation of melanocytes frequently coincides with loss of E-cadherin expression and the upregulation of N- Cadherin. Recent studies have shown that c- Jun (member of the AP-1 transcription factor family) plays an important role in malignant melanoma and is activated by a novel pathway which is enabled in melanoma beside the Ras/Raf/MEK/ERK (MAPK) and the PI3K/AKT (AKT) signaling pathways. A deregulation of transcriptional activity is often found during tumor development. The constitutive activity of the AP-1 transcription factor family influences the expression of a variety of regulators of cell proliferation, migration and survival, which are significantly involved in melanoma development and metastasis. Furthermore it is known that epithelial cell adhesion proteins, E- cadherins, play important roles in melanoma development, especially in metastasis.

Thus, in this project the function, stabilization and regulation of the transcription factor c- Jun should be determined.

Recently, we discovered that the loss of E-Cadherin induces c-Jun protein expression. Evidently the mRNA level c-Jun is not affected; hence c-Jun is regulated at post-transcriptional level. Here, we present data that show that the dynamic cytoskeletal network, linked to E- cadherin, is involved in the regulation of the c-Jun protein and transcriptional activity. Moreover, we demonstrate whether there is a direct interaction between tubulin or actin and c-Jun as it is necessary to reveal if c- Jun is stabilized via the cytoskeletal network. In a novel signaling cascade, the loss of E-cadherin activates the transcriptional regulator ETS-1 and consequently leads to the induction of RhoC expression that stabilizes c-Jun in melanoma. The link between RhoC and c-Jun seems to be indirect via the cytoskeleton. We conclude that the loss of E-cadherin mediated cell-adhesion induces c-Jun protein expression in a multistep process, offering several possibilities for therapeutic intervention.

## 2.8 Molecular Function and Transport of MIA

**Matthias Molnar**

MIA (melanoma inhibitory activity) is a globular, secreted protein with a molecular weight of 11 kDa and is highly expressed in melanoma cells but not in normal melanocytes. MIA is involved in the development, progression and metastasis of malignant melanoma and is already used as a prognostic marker. Previous studies showed that MIA is secreted in a calcium-dependent manner at the trailing edge of melanoma cells and thereby promotes cell migration.

The main goal of my PhD project is to clarify the mechanism by which the MIA-transport and MIA-secretion is regulated. Therefore, I investigate different melanoma (Mel Im, Mel Ju, Mel Juso) as well as non-tumoric (HEK293) cells.

In the beginning of my thesis I checked the expression and regulation of various proteins potentially involved in MIA transport. Therefore, I compared melanoma cells to normal melanocytes. Afterwards I knocked down a number of interesting proteins like Rab3A, SYT11 or FYCO1 by siRNA transfection. To determine the influence of these proteins on MIA transport/secretion I analyzed cell culture supernatants via ELISA. In another approach, I designed a couple of MIA-mutants with respect to MIA-transport. I investigate their effects by the combination of different techniques like Western Blot, ELISA and immunofluorescence.

To find out more about the mechanism of MIA transport/secretion I am also engaged in isolating MIA-containing vesicles. Therefore, I perform a variety of experiments like SEC, different centrifugation steps and affinity chromatography. Additionally, I am going to investigate the MIA-containing vesicles to define sequences/amino acids of MIA and other adapter- and transport proteins involved in the directed intracellular transport.

Taken together, I hope that my work will contribute to a deeper understanding of the role and function of MIA.

## 2.9 The functional role of AP-2ε in chondrocytes and osteoarthritis

**Stephan Niebler**

The family of AP-2 (activating enhancer-binding protein-2) transcription factors consists of five members: AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε. They have a conserved DNA binding and dimerisation domain and a less conserved transactivation domain. For the regulation of target gene expression AP-2 proteins bind to the palindromic recognition sequence 5´-$GCCN_{3/4}GGC$-3´ or variations. *In vitro* and *in vivo* analyses of AP-2 knockout mice demonstrated the importance of *AP-2* genes in numerous physiological processes during development, cell-cycle regulation and cell survival.

AP-2ε is the last identified AP-2 transcription factor. Expression of *AP-2ε* was first described in the olfactory system and skin. In previous studies we could show that AP-2ε is also expressed in chondrocytes and up-regulated in osteoarthritis (OA).

To better understand the function of AP-2ε in chondrocytes we compare the AP-2ε k.o. with wt mice concerning abnormalities in cartilage development and differentiation. To address this we use *in-situ* hybridization against prominent cartilage differentiation markers – *e.g. Collagen II* and *Collagen X* – at various embryonic stages. So far the area of *Collagen X* expression in the thigh seems to be enhanced in AP-2ε k.o. mice compared to wt mice at E15.5 and E16.5. This suggests a modulation of cartilage development due to the lack of the transcription factor. Additionally, we utilize other approaches to reveal discrepancies between k.o. and wt mice like micro-mass and hanging drop differentiation assays with embryonic limb-bud cells.

Another hint for the regulative role of AP-2ε in cartilage differentiation is derived from analysis of the *Collagen II* promoter activity. Interestingly, *Collagen II* seems to be negatively regulated by AP-2ε indicating a specific regulatory function of this transcription factor in cartilage development.

As it is known that in some cases abnormalities of k.o. specimen only become apparent under pathological conditions we established an animal model, where an OA is induced in the knee joint via destabilization of the medial meniscus. OA development was significantly enhanced in AP-2ε k.o. mice compared to wt mice after 17 days, suggesting an important function of AP-2ε in cartilage homeostasis.

In the future, we will further investigate the molecular role of AP-2ε in cartilage development and disease.

## 2.10 The role of sensory neuropeptides and catecholamines in callus differentiation

**Tanja Niedermair**

*Introduction:* During fracture healing, bone and fracture callus become innervated by sensory and sympathetic nerve fibers. The absence of sensory innervation alters callus size and bone formation and may result in non-united fractures. If sympathetic nerve fibers and therefore innervation from catecholamines as noradrenalin (NA) get lost in the tissue the proinflammatory role of the sensory neurotransmitter substance P (SP) might lead to the development of immune mediated diseases. We know that SP will promote cell proliferation *in vitro* and that chondrocytes express SP and its receptor NK1 during the time course of callus formation. NA by contrast induces apoptosis *in vitro.* Aim of this research is to analyze an impact of SP and NA on callus formation and differentiation on IL-1ß stimulated chondrocytes in monolayer cultures and in a mouse fracture explant model.

*Methods:* Costal chondrocytes, isolated from new born C57bl/6N mice, were cultured in 2D-monolayer until cells attained a confluence of 80% and were then stimulated with IL-1β (5ng/ml) , SP ($10^{-9}$, $10^{-10}$ and $10^{-11}$ M) and NA ($10^{-6}$, $10^{-7}$ and $10^{-8}$ M) for 24h. In a murine fracture model, tibial fractures were set in the left hind leg, fracture calli were isolated on day 8 and cultured as explants for 7 days under steady state conditions. Stimulation was implemented on day 6 for 24h with IL-1β (10ng/ml), SP ($10^{-9}$ - $10^{-11}$ M) and NA ($10^{-6}$ - $10^{-8}$ M). Gene expression of selected genes (*timp-1, -2, -3; mmp2, -3, -14; IL6, -8; iNos, cox2*) was determined by quantitative RT-PCR. MMP3 and Timp-3 proteins in the supernatant of the callus explants were quantified by ELISA.

*Results:* Both, SP and NA have a dose-dependent effect on the IL-1β stimulated expression of some genes (*mmp-2, -3, timp-3 and Cox2)* in monolayer cultures. Gene expression of *timp-3* was significantly reduced at a concentration of $10^{-11}$ M SP and equally after stimulation with $10^{-8}$ M NA, likewise behave *mmp-2* and *Cox2.* The expression of *mmp-3* in monolayer cultures was significantly reduced in the presence of $10^{-11}$ M SP but increased after addition of $10^{-6}$ M NA. First results of the gene expression patterns of the 3D explant cultures show similar dose-dependent effects. Timp-3 protein in the supernatants of the fracture explants was significantly reduced after treatment with $10^{-8}$ M NA and tends to be reduced after stimulation with $10^{-10}$ M SP compared to untreated controls. In the case of MMP3 no significant changes in protein level were detected so far.

*Discussion:* Stimulation of chondrocytes in monolayer cultures or 3D-explant cultures with the sensory neurotransmitter SP or the catecholamine NA has an impact on expression of genes that are involved in callus differentiation like *timp-2, -3, mmp-2 -3* ,and *-14*. SP and

NA might exert opposite effects as for *mmp-2* or *timp-2* expression, however they also act in concert.

## 2.11 Investigation of proteomic changes in liver affected by non-alcoholic steatohepatitis

**Anja Thomas**

**Introduction:**

The increasing number of obesity in western society is a major cause for higher incidence of metabolic aberrations as insulin resistance and non-alcoholic fatty liver disease (NAFLD). Non-alcoholic steatohepatitis (NASH) is the most severe form of NAFLD, characterized by liver steatosis and inflammation leading to a significant deterioration in liver function, possibly culminating in liver cirrhosis.

The exact mechanism of NASH development is still unclear. A potential cause for inflammation is a higher rate of β-oxidation in the liver mitochondria induced by elevated levels of plasma free fatty acids. Due to the harmful effect of elevated formation of reactive oxygen species, damage particularly on the mitochondrial proteome may occur resulting in significant impairment of cellular respiration.

**Methods:**

Here, an established mouse model system for NASH is used to assess the early changes in hepatic protein regulation. Wild-type mice are fed with a high-fat diet facilitating the development of NASH. Proteomic changes in different compartments of murine liver cells are investigated, thereby focusing on mitochondria as one of the key players in disease progression.

The analyses of protein-related changes are accomplished by means of two-dimensional gel electrophoresis, followed by protein identification using liquid chromatography-mass spectrometry. In a complementary approach, changes in protein abundances are quantified using stable-istotope labelling. Furthermore, results will be confirmed using functional assays.

**Results and Discussion:**

To assess changes in early NASH development, female C57/BL6 mice were fed with a high fat diet for 10 to 40 days. At each of these time points, livers were perfused for blood removal and dissected. The grade of steatosis was determined by histological examination. During the course of time, a microvesicular steatosis with mild inflammatory infiltration was confirmed.

The degree of inflammation and fibrosis was assessed by quantitative real-time-PCR of relevant marker genes. The expression analysis showed an up-regulation of the pro-inflammatory cytokine TNFα as well as the fibrosis marker Collagen-1, which indicates the onset of inflammation and fibrosis in hepatic tissue.

A slight increase in total cholesterol content was observed during the time course. In contrast, the triglyceride content of liver tissue increased significantly with a peak at 20 d, with a slight decrease afterwards.

Purification techniques for different compartments of murine liver cells were established. Mitochondria were isolated from whole tissue by differential centrifugation and ultracentrifugation using a sucrose density gradient. For purity control of mitochondria, immunoblotting against endoplasmatic reticulum and cytoplasmatic proteins was performed. Furthermore, liver cells were partitioned in a soluble and a membrane fraction. In a first differential analysis, the soluble fraction was examined for differentially regulated proteins by DIGE. After 10 days, an upregulation of various proteins involved in lipid metabolism could be shown. Also, some proteins participating in primary energy metabolism were shown to be regulated in early disease progression. For several proteins, a graduated regulation over the experimental course was observed.

## 2.12 Biomarker Detection For Non-Alcoholic Fatty Liver Disease (NAFLD) using In Silico Modeling

**Torsten Schön**

Detection and evaluation of biomarker candidates requires knowledge extraction from newly generated or existing data. One of the major challenges in this area is the robust extraction of knowledge in addition to making reliable predictions in a field where a high degree of freedom is accompanied by a relatively small set of data. Different machine learning techniques have been used to extract knowledge from measured data [1]. Analysis of data often leads to a set of key features that can differ between groups contained in the dataset. For biomarker detection for non-alcoholic fatty liver disease, features are searched that differentiate between three major groups, patients having a non-alcoholic fatty liver disease, patient that suffer from alcoholic fatty liver disease and a control group having no know liver disease. Common used feature selection techniques are compared to a group of newly developed feature selection methods based on the analysis of graphical networks. A graphical network is represented by a set of nodes indicating variables and a set of edges between those node representing connections and interaction between the nodes. Learning the structure of a graphical network that represents the underlying dataset structure is a challenging task itself. Therefore, preliminary to the analysis of the structure, different structure learning algorithms are compared. Two established problems with a known network structure, the alarm network [2] and the hailfinder network [3] are used to generate random datasets. A set of structure learning algorithms is applied to the datasets and the learned structure is compared to the original structure. The method leading the best results is used to learn the structure of a biomarker dataset determined at the Medical University of Graz. A node representing the state of NAFLD is marked as target node and the network topology is analyzed with respect to ascertain the nodes that have a high impact on the target node and that have the potential to build reliable biomarkers for NAFLD.

## 2.13 Utilizing Patient Specific Induced Pluripotent Stem Cell Derivatives to Functionally Characterize Cardiovascular Risk Alleles

**Maya Fürstenau-Sharp**

Through genome wide association studies (GWAS) and exome sequencing several cardiovascular risk alleles have been identified. However, to date the function of most of these risk genes is unclear. To functionally characterize two promising candidate genes, patient specific Induced Pluripotent Stem (iPS) cell derivatives will be used as an *in vitro* system to model cardiovascular disease. Instead of utilizing dermal fibroblasts, which have routinely been used for somatic cell reprogramming, we chose human peripheral blood T cells as our starting material. These cells can easily be isolated from a few millilitres of patient blood eliminating the need for invasive skin biopsies. A few recent publications have already demonstrated the successful *in vitro* reprogramming of T cells to an undifferentiated pluripotent state.

We first isolated peripheral blood monocyte cells (PBMC) from whole human blood and cultured them in a lymphocyte medium containing Il-2 and CD3 antibody. This method resulted in the preferential expansion of mature CD3 + T cells as confirmed by flow cytometry analysis. Upon their activation, the T cells were subjected to retroviral transduction with four separate vectors, each encoding one of the Yamanaka reprogramming factors (OCT4, KLF4, SOX2 or c-Myc). Using this method, we have thus far generated 4 iPS cell lines from 2 different individuals. In addition, we have isolated several iPS colonies from a third individual that are currently under expansion. The next step will be to further characterize these iPS cell lines to verify the expression of human embryonic stem cell (hESC) pluripotency and genetic markers as well as the silencing of the viral reprogramming genes. Subsequently, the differentiation potential of the iPS cell lines will be evaluated by subjecting them to cardiac and endothelial differentiation protocols. Once these target cells have been generated in sufficient quantities, the identified cardiovascular risk alleles will be functionally characterized.

## 2.14 Alpha syntrophin deficienct mice are protected from high fat diet mediated obesity

**Kristina Eisinger**

Objective: Alpha syntrophin (SNTA) is a multidomain adaptor protein which interacts with other molecules due to its modular structure. SNTA interactive proteins are expressed in adipocytes but the role of SNTA in these cells has not been studied so far.

Results: SNTA was found expressed in adipocytes and was modestly upregulated during differentiation. SNTA knock-down in 3T3-L1 preadipocytes increased cell proliferation. Differentiation of these cells to adipocytes led to the formation of small lipid droplets and reduced cellular cholesterol and triglyceride concentrations. Insulin mediated phosphorylation of AKT was not impaired excluding reduced insulin activity as explanation for lower fat storage. SNTA deficient mice kept on a high fat diet for 24 weeks were protected from obesity. Adipocyte diameter was significantly reduced in subcutaneous but not in visceral fat depots, and triglyceride and cholesterol concentrations tended to be lower. The mRNA and protein levels of fatty acid synthase were increased and adipophilin was reduced. Expression of antiinflammatory CD163 was lower whereas mRNA levels of inflammatory proteins were elevated. Higher concentrations of free fatty acids in fasting serum indicate increased lipolysis. Hepatic and systemic triglycerides were higher but apolipoprotein B100 in serum was similar in wild type and SNTA deficient mice indicating formation of triglyceride enriched lipoprotein particles. Glucose tolerance was only modestly impaired.

**Conclusion**: These experiments demonstrate that SNTA and the so far unknown pathways influenced by this adaptor protein in adipocytes have a function in fat cell lipid storage and adipose tissue inflammation.

# 3. Group 3

TALKS

**Strept. agal. infection in humanized mice**
Wolfgang Ernst

**Mouse ficolin B is produced by immature myeloid cells**
Katja Hunold

**9p21 CAD risk haplotype shows altered up-regulation of IL12B and IL1B in macrophages after inflammatory stimuli**
Christa Zollbrecht

**Functional characterization of mouse beta-defensin 14 (mBD14) in inflammation**
Nicola Barabas

POSTER

**Defining Immunological Parameters to be used as End- Point Measures in Clinical Transplant Studies**
Anja Kammler

**Cyclophilin A dependent HIV-1 replication of Cyclosporine A dependent viral variants can be interchangeably restored by structurally distinct in vivo and in vitro mutations**
Benedikt Grandel

**Basophils as regulators of CD4 T cell homeostasis**
Fabian Herman

**The homing of myeloid-derived suppressor cells and interaction with leucocytes differs between C57BL/6 and BALB/c after infection with *L. major***
Maximilian Schmid

**Characterization of the anti-inflammatory impact of molecular fragments derived from the adipokine CTRP-3 in adipocytes and monocytes**
Andreas Schmid

## 3.1 *Strept. agal*. infection in humanized mice - a novel model to improve neonatal sepsis treatment in clinic?

**Wolfgang Ernst**

In 2002 the triple deficient NOD/*scid*-IL2$\gamma^{-/-}$ (NSG) mice were generated. Upon transplantation of human $CD34^+$ hematopoietic stem cells NSG mice develop all human subsets of the immune system like T-, B-, and NK-cells as well as myeloid cells and are able to mount a humoral and cellular immune response to antigens. After engraftment with human $CD34^+$ cells they are called humanized mice. These mice were infected with *Streptococcus agalactiae* (group B streptococcus; GBS) which can lead to sepsis and bacteraemia in human neonates causing a mortality rate of up to 10%. GBS is also frequently responsible for meningitis in newborn infants. Despite prophylactic treatment of colonized pregnant women with antibiotics, this pathogen remains a serious threat to neonates and newborn infants especially in the western world. Therefore there is a need of an appropriate animal model that mimics the naivety and reduced capabilities of the neonatal immune system, in order to optimize and test treatment strategies for GBS infected neonates.

Humanized mice were infected with different doses of *GBS* and various parameters, like survival, change in weight, T cell memory response, numbers of human immune cells (T cells, B cells, NK cells, granulocytes, myeloid cells) in different organs (peripheral blood, spleen, bone marrow, liver, lung, peritoneum) were analyzed. In addition we determined the colony forming units in various organs, cytokine response in peripheral blood, histological changes and organ damage. Next we analyzed the influence of commonly used treatments in the clinic like Betamethason and Indometacin on the parameters mentioned above.

Overall, this novel model of neonatal sepsis in humanized mice offers the possibility to optimize “old” or introduce new therapy strategies in clinic in the context of an early human immune system.

### 3.2 Mouse ficolin B is produced by immature myeloid cells

**Katja Hunold**

Ficolins activate the complement system via the lectin pathway upon binding to carbohydrate pattern on pathogens. Like its human orthologue M-ficolin, the mouse lectin ficolin B (FcnB) is cell associated and has been found to co-localize in peritoneal macrophages with Lamp1 to lysosomes and late endosomes. We studied in detail the expression of FcnB in macrophages and dendritic cells during differentiation from bone marrow cells, and during differentiation of granulocytes derived from ER-Hoxb8 precursor cells. FcnB expression in macrophages, dendritic cells, and granulocytes declined during terminal differentiation in culture and was low when cells were fully differentiated. Also, primary $CD11b^+$ splenocytes showed a loss of FcnB expression during maturation into macrophages. Maximal expression of FcnB was observed in granulocytes with surface markers $CD11b^+CD11c^-Ly6C^{int}Ly6G^+$ isolated from the bone marrow. FcnB expression was observed exclusively in the immature granulocytic fraction when primary granulocytes from bone marrow and spleen were separated according to their Ly6C and Ly6G expression. While FcnB expression was further reduced upon stimulation of macrophages or dendritic cells with LPS, FcnB expression was enhanced in ER-Hoxb8-derived granulocytes after stimulation. Thus, these results reveal immature myeloid cells as producers for the mouse lectin FcnB. The recombinant FcnB was found to bind to well known ficolin ligands such as acetylated BSA, N-acetylglucosamin, and fetuin. In addition, besides binding to a wide range of different bacteria, FcnB also binds to necrotic and late apoptotic cells and to apoptotic blebs indicating recognition and binding of FcnB to DNA. The biological relevance of this FcnB production by immature granulocytes is under current investigation.

## 3.3 9p21 CAD risk haplotype shows altered up-regulation of IL12B and IL1B in macrophages after inflammatory stimuli

**Christa Zollbrecht**

**Background**

Genome-wide association studies identified a risk haplotype on chromosome 9p21 to be associated with coronary artery disease (CAD) and myocardial infarction (MI). Since this region does not contain a clear candidate gene with known pathophysiology, we performed an allele-specific expression study in human macrophages during proinflammatory stimulation to investigate the function of this risk locus in the development of atherosclerosis and MI.

**Methods**

Blood samples were taken from 40 stable MI patients either homozygous for risk (n = 20) or protective haplotype (n = 20) as well as from 28 healthy male individuals (n = 14 for each haplotype). Monocytes were isolated and differentiated into macrophages via M-CSF. Besides control macrophages, cells were incubated with a proinflammatory IFNγ/LPS cocktail. After 24 hours, RNA was isolated and applied to Affymetrix Human Exon 1.0 ST Arrays. Resulting expression data of MI patients and healthy individuals were analyzed separately as well as in a combined dataset using Partek Genomics Suite. Robust multi-array averaging (RMA) was used for summarization of fluorescence signals and quantile normalization was included. To identify differentially expressed genes, single exons were summarized to transcript cluster values and a 3-way analysis of variance (ANOVA) was performed for *group, treatment* and *subject* (individuals) as factors.

**Results**

In macrophages without stimulation only marginal expression differences could be detected between MI patients with protective or risk haplotype. No significant changes were observed in healthy individuals and the combined data analysis. Addition of IFNγ/LPS up-regulated several interleukins, chemokine ligands and other known inflammatory markers. Since no significant differences between the haplotype groups could be detected in the stimulated state, we focused on differences in gene regulation due to the stimulus. As one of the most prominent genes IL12B showed higher up-regulation on basis of the 9p21 risk haplotype compared to the protective haplotype in all 3 datasets (MI 33 %, healthy 37 %, combined 37 %). IL1B was less up-regulated in the risk group in all datasets (29 %, 13 % and 23 %, respectively). For candidates with inconsistent expression regulation among MI patients and healthy individuals it remains to be proven if this was a disease specific effect.

**Conclusion**

We found different up-regulation of two interleukins in macrophages based on the different 9p21 haplotypes. We suppose that inducing inflammation in these cells is a good model for the *in vivo* situation and may help to reveal the impact of the 9p21 locus on development of atherosclerosis. These findings support the concept that IFNγ influences gene expression depending on 9p21 haplotype.

## 3.4 Functional characterization of mouse beta-defensin 14 (mBD14) in inflammation

**Nicola Barabas**

Mammals and other higher organisms generate antimicrobial peptides in response to pathogenic microorganisms during the early phase of an immune response. Mouse β-defensin 14 (mBD14) is a small cationic antimicrobial peptide, which belongs to the family of β-defensins. In addition to its direct antimicrobial effects mBD14 induces chemotactic activity of dendritic cells and monocytes/macrophages via CCR6 and CCR2. Thus, β-defensins form a link between the innate and the adaptive immune response. mBD14 is the mouse orthologue of human β-defensin 3 showing 68% homology on protein level and is expressed in a variety of tissues including liver, spleen, lung and colon. We analyzed the expression of mBD14 *in vitro* in macrophage and epithelial cell lines after stimulation with TLR ligands e.g. LPS, CpG and poly(I:C). While in epithelial cell lines no induction of mBD14 expression was detected, expression of mBD14 was significantly reduced after stimulation with TLR ligands in the macrophage cell line J774 as well as in bone marrow derived macrophages (BMDM).

In order to characterize mBD14 *in vivo* an mBD14:Ig transgenic mouse line was generated expressing the mBD14 transgene under an ubiquitinous promoter. Analysis of mBD14 mRNA expression in the transgene revealed strong expression of mBD14 mRNA in all tissues as well as in cells of hematopoietic origin. Interestingly, stimulation of BMDM derived from mBD14 transgenic mice with TLR ligands resulted in a reduced expression of pro-inflammatory cytokines, e.g. TNF and IL-6 when compared to non-transgenic littermates. These results were further verified by stimulation of J774 cells with recombinant mBD14 protein prior to the stimulation with TLR ligands. These results provide first evidence of an immuno-modulatory function of mBD14 *in vitro*. In order to verify these observations on a molecular level, analysis of mBD14 binding partners, signaling pathway activation in target cells and transcription factors regulating mBD14 expression will be further characterized.

## 3.5 Defining Immunological Parameters to be used as End- Point Measures in Clinical Transplant Studies

**Anja Kammler**

Development of donor antigen-specific regulatory T cell responses, particularly those operating through the indirect pathway, are believed to be essential for immunological acceptance of a transplanted organ between HLA-disparate, immunologically competent individuals [1]. No clinically applicable assay of immunological regulation of the indirect pathway currently exists, primarily because existing assays are too complicated and technically demanding to be standardised [2]. One part of my project is concerned with the further development of an assay of indirect immunological regulation in both human and mice which, being based on the *bystander suppression* principle, is simplified by referencing suppressive activity against the *internal standard* of a strong, memory effector response. To test for donor-specific regulation, recipient blood cells are pulsed with antigens such as purified protein derivative (PPD) from BCG against which the recipient has specific T cell immunity, and acellular antigen from the donor. Recipient T cell reactions against donor antigen are gauged by measuring cytokine and chemokine release by enzyme-linked immunosorbent assays (ELISA). The assay development will be done in both human and mouse models. It is planned to demonstrate bystander suppression of memory T cell responses in normal, healthy humans using sibling pairs who regulate against non-inherited maternal antigens to provide a clinically applicable assay to be used e.g. in *The ONE Study*, we expect to correlate the degree of donor-specific regulation observed in an *in-vitro* assay with the minimal amount of immunosuppressive therapy. The use of a mouse model provides a clean and specifically induced system in which to define the immunological mechanisms of the bystander suppression and the cells that are involved. It is planned to do a phenotyping of the cells responsible for *bystander suppression* via flow cytometry and gene expression analysis. This method will also be useful to investigate the activation and specificity of the cells, where ELISPOT-assays will be done as well. Once there is evidence for certain cell-types to have a major role in the bystander suppression assay, functional testing will be performed by depleting of the cells of interest using AutoMACS and FACS-Sort or with blocking Antibodies and siRNA or the enrichment and activation of the cells of interest via transfection or stimulation with cytokines.

## 3.6 Cyclophilin A dependent HIV-1 replication of Cyclosporine A dependent viral variants can be interchangeably restored by structurally distinct in vivo and in vitro mutations.

**Benedikt Grandel**

**Introduction:** Due to its association with delayed disease progression, the human leukocyte antigen (HLA)-B27 is considered to be a protective allele during HIV infection. The reason for this phenomenon lays in the limitation of HIV to overcome the cytotoxic T cell (CTL) response, which is immunodominantly directed against the HLA-B27 restricted KK10 epitope. KK10 is a ten amino acid long peptide (K263-K272) within the N-terminus of HIV p24 of the Gag polyprotein.

The major escape mutation R264K alters the epitope in a way that it is no longer presented by HLA-B27, causing a substantial reduction in viral replicative capacity at the same time. This fitness defect is due to an altered interaction between HIV p24 and the cellular protein Cyclophilin A (CypA). By introducing R264K, the viral phenotype changes from CypA dependency towards a viral variant whose replication is blocked by CypA. This “toxic” effect of CypA was demonstrated by treating host cells with Cyclosporine A (CsA), a competitive inhibitor of CypA. The *in vivo* compensatory mutation S173A, strongly associated with R264K, restores the CypA dependent phenotype and replication capacity of the R264K variant. Here, we analysed the compensatory function of S173A in the context of three additional *in vitro* mutations in p24 (A224E, G226D, and T186A), which have been shown to display the same CsA-dependent phenotype as the R264K variant.

**Material and methods:** Viral variants were constructed by site directed mutagenesis of the HIV-1 lab strain pNL4-3. Infectivity of viral variants was analysed by flowcytometry, using a Tat-inducible GFP-reporter cell line.

**Results:** We demonstrate that S173A is capable of compensating at least two of the three variants A224E, G226D, and T186A. Furthermore, we show that the replication capacity of the R264K variant can be enhanced through the alternative *in vitro* compensatory mutations A237T and V218A.

**Conclusion:** This interchangeability leads to the conclusion that a similar mechanism at structural distinct sites of the p24 molecule acts in conveying CypA- and CsA-dependency. Further studies will have to analyse the mechanism of the p24-CypA interaction, contributing to a better understanding of the strong *in vivo* selection for the S173A-R264K combination.

### 3.7 Basophils as regulators of CD4 T cell homeostasis

**Fabian Hermann**

Until recently basophils were considered mainly as effector cells of an innate immune response linked to allergy and parasite infection. Only in the past few years they were recognized as important regulators of adaptive immunity.

Homeostasis of T cells is an important part of the immune system it is important to understand the mechanisms of T cell homeostatis especially in the context of repopulating a T cell depleted host after radiation therapy as part of hematopoietic stem cell transplantation and leukaemia treatment.

It is already known that basophils are important inducers of a Th2 response secreting large amounts of the Th2 cytokine IL-4. The data presented here demonstrate the impact of basophils on the homeostasis of $CD4^+$ T cells.

Basophils appear to be negative regulators of homeostatic proliferation as well as in T cell homeostasis *in vivo*. We were able to show *in vitro* that this regulation is independent of Fas and is transferrable with the supernatant of cell cultures.

## *3.8* The homing of myeloid-derived suppressor cells and interaction with leucocytes differs between C57BL/6 and BALB/c after infection with *L. major*

**Maximilian Schmid**

Myeloid-derived suppressor cells (MDSC) represent a heterogenous cell population characterized by an immature differentiation state and immunosuppressive potential. According to the surface molecules Ly6G and Ly6C, MDSC can be further dissected in monocytic (MO-MDSC, $CD11b^{+}/Ly6G^{-}/ Ly6C^{high}$) and polymorphonucleated suppressor cells (PMN-MDSC, $CD11b^{+}/Ly6G^{+}/Ly6C^{int}$). Myeloid cells with suppressive capacities were described for the first time in patients suffering from cancer or autoimmune diseases. However little is known about the impact of MDSCs in innate and adaptive immunity against parasitic infections.

Thus, we used the experimental model of leishmaniasis to assess the potential role of MDSCs in modulating the immune response against Leishmania major. Two different mouse strains were included in the study: i) C57BL/6, which are known to cure the parasite infection with a cellular $T_H1$-type immune response ii) BALB/c mice, which develop a $T_H2$-driven humoral immune response incapable of the elimination of the parasite. The site of infection and the secondary lymphoid organs were analyzed regarding inflammation and micro-compartmentation of MDSC subtypes.

Our data revealed an unexpected localization of PMN-MDSC in the skin-draining lymph nodes of infected mice. These cells co-localize with $CD4^{+}$ T cells in the germinal center (GC), indicating a potential suppressive function of these cells on GC formation. We showed that homing of PMN-MDSC`s differs in C57BL/6 and BALB/c mice. In BALB/c mice the ratio of $Ly6G^{+}$ cells localizing with T cells in the germinal center was lower compared to infected C57BL/6 mice. Furthermore we demonstrated that the total number of infiltrating MO-MDSC in the skin draining lymph nodes is increased in C57BL/6 compared to BALB/c mice. In contrast, the number of PMN-MDSC was significantly lower in BALB/c mice. Similar tendencies were observed in the spleens of infected mice.

In conclusion we showed that the localization and infiltration of MDSC`s differs between resistant and susceptible mouse strains after infection with *L. major* parasites. Further studies will be necessary to characterize the MDSC-leukocyte interaction in detail and a possible impact on the course of leishmaniasis.

## 3.9 Characterization of the anti-inflammatory impact of molecular fragments derived from the adipokine CTRP-3 in adipocytes and monocytes

**Andreas Schmid**

**Introduction**

C1q/TNF-related protein-3 (CTRP-3) is a newly discovered adipokine which has been shown to have anti-inflammatory impact on monocytes by decreasing the secretion of pro-inflammatory cytokines, e.g. interleukine 6 (IL-6) and tumor necrosis factor alpha (TNF-alpha), in both adipocytes and monocytes. So far it has remained unclear which kind of interactions and molecular mechanisms are underlying the observed effects. It is our aim to characterize the differential effects of the globular and the collagen domain of CTRP-3 and to compare these with the effects exerted by the full length protein.

**Methods**

Recombinant DNA of full-length CTRP-3, its globular domain, and its collagen domain were transfected into and expressed in H5 insect cells via the baculovirus expression system. The recombinant proteins were extracted from the cell culture supernatant and purified by affinity chromatography.

For stimulation experiments, differentiated 3T3-L1 and monocyte-like THP-1 cells were used. Release of pro-inflammatory cytokines IL-6, monocyte chemotactic protein 1 (MCP-1), resistin and TNF-alpha was induced by addition of lipopolysaccharide or free fatty acids to the medium. Costimulation experiments were performed with these stimuli plus CTRP-3 or its globular domain, respectively, to analyse and to compare their impact on the secretion of pro-inflammatory cytokines into the medium, being measured by ELISA techniques.

The protein levels of intracellular components of pro-inflammatory signal transduction pathways were analysed by Western blot.

**Results**

Both full-length CTRP-3 and its globular domain alone were shown to significantly decrease the amount of secreted IL-6, MCP-1 and resistin after costimulation with LPS in differentiated 3T3-L1 cells. In THP-1 cells, these effects have not been verified so far.

**Conclusion and outlook**

The present results suggest that the observed anti-inflammatory impact of CTRP-3 is mediated by its globular domain, whereas the collagen like domain is still to be tested on these effects. As a next step we plan to investigate the anti-inflammatory effects of CTRP-3 in an animal model of LPS-induced SIRS.

# 4. Group 4

TALKS GROUP 4

**Functional role of natriuretic peptides in compensatory renal hypertrophy**
Andrea Schreiber

**Cellular Mechanisms of Renin secretion**
Dominik Steppan

**Localization and Regulation of the Angiotensin II Type 1 Receptor-associated Protein Arap1 in the Kidney**
Elisabeth Doblinger

**Fibrocyte development: role of monocytes recruited via the chemokine receptor CCR2**
Barbara Reich

POSTER

**MicroRNAs in the kidney and their relevance in renal diseases**
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**The impact of the basolateral potassium channel KCNJ10 for salt resorption in the distal tubule of the kidney**
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**The role of TASK3 $K^+$ channels for the regulation of aldosterone secretion in neonatal mice**
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**The physiological and pathophysiological relevance of the inwardly rectifying potassium channel Kir5.1 (KCNJ16) in the kidney**
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**The role of Ecto-5'–nucleotidase (CD73) –mediated extracellular adenosine generation in renal fibrosis**
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**The role of WNK3-kinase in activation of NKCC2 in vivo**
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**The impact of mutated Fanconi-associated protein on the mitochondrial proteome**
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**Chemokines and Their Receptors as Potential Regulators of Renal Development and Function**
Simone Wurm

## 4.1 Functional role of natriuretic peptides in compensatory renal hypertrophy

**Andrea Schreiber**

Loss of renal tissue results in an increase in function and mass of the remaining intact nephrons. The initial signals that induce this compensatory renal hypertrophy are largely unknown. Since plasma concentration of atrial natriuretic peptide ANP increases after uninephrectomy (UNx), we investigated kidney growth and function in response to UNx in mice lacking the ANP receptor guanylyl cyclase-A (GC-A).

Except for heart weight, which was higher in GC-A-/- compared to wildtypes, no differences in organ weights were detected (kidneys, spleen, liver, brain) between the genotypes under control conditions. Moreover, glomerular filtration rate, urine volume and sodium excretion were not different between the genotypes. UNx of the left kidney for 4 days resulted in a compensatory growth of the remaining right kidney in GC-A+/+ (right/left ratio of kidney weight: 1.20$\pm$0.01, $p<0.001$, n=12) but not in

GC-A-/- (ratio: 1.04$\pm$0.02, n.s., n=12). GFR of wildtypes 4 days post UNx was 70 $\pm$ 2 % of baseline values before uninephrectomy indicating functional adaptation of kidney function, while in GC-A-/- GFR was 56 $\pm$ 3 % of baseline values. 24h urine volume and sodium excretion did not differ between genotypes. 18 days and 6 weeks after UNx no significant differences of kidney weight, urine and sodium excretion were detected between GC-A-/- and GC-A+/+.

In conclusion, natriuretic peptides that signal via GC-A are involved in the functional adaptation of GFR and renal mass of the remaining kidney 4 days after UNx. Additional mechanisms contribute to the long term adaptation to renal tissue loss as well as in the regulation of urine and salt excretion.

## 4.2 Cellular Mechanisms of Renin secretion

**Dominik Steppan**

Active renin is exclusively secreted into the circulatory system by the renal juxtaglomerular epitheloid cells (JG-cells). The release process of renin vesicles is assumed to be similar to other endocrine cells, although morphological evidence for this is rather limited. It is well established that an increase of cAMP in JG-cells stimulates renin release and that increased $Ca^{2+}$-concentrations are paralleled by inhibition of release what stands in contrast to classical secretion processes, in which exocytosis is stimulated by $Ca^{2+}$. This unusual behaviour of renin cells is known as "calcium paradoxon of renin release." The mechanisms by which cAMP stimulates and $Ca^{2+}$ inhibits secretion in renin producing cells remain largely obscure. The aim of our work is to obtain more direct information about the cellular events triggering the release of renin. For this purpose we have analyzed the structure of renin storage vesicles.

We analyzed renin cells of unstimulated kidneys of 129SVJ mice. In addition, we isolated and perfused kidneys of these mice (IPMK-method) for 3, 10 and 15 minutes with a solution containing isoproterenol and EDTA (creating high cAMP and low $Ca^{2+}$ conditions). The structure of renin storage vesicles was analyzed by 3-dimensional electron-microscopical reconstruction of single JG-cells with corresponding secretory vesicles. Reconstructions were made with 70nm thick serial slices of kidney tissue. Slices were photographed and reconstructed with the AMIRA program. In unstimulated renin cells and in renin cells stimulated for 3 minutes, we so far found no exocytotic events. Some, but not all of the renin containing vesicels appeared to be interconnected to mesh-like structures. In JG-cells stimulated for 10 minutes, we found multiple exocytotic events. In addition to normal exocytotic vesicles, some vesicles appeared to be less electron-dense. Moreover, the percentage of interconnected vesicles appeared to be larger. In JG-cells stimulated for 15 minutes, we again found no exocytotic events, however, nearly all of the secretory vesicles featured a significantly low electron densitiy. All vesicles with low electron density appeared to be connected, forming a single mesh-like structure.

Our results support the theory of high cAMP and low $Ca^{2+}$ conditions stimulating renin exocytosis, because we noticed multiple exocytotic events in renin cells stimulated for 10 minutes. In JG-cells stimulated for 15 minutes nearly all vesicles showed a low electron densitiy, which almost certainly means that they are empty. The fact that these vesicles form a single mesh-like structure could point to the so-called “compound-exocytosis” mechanism. According to this theory, vesicles first fuse homotypically with each other forming a mesh-like compound, which then fuses with the cell membrane.

Now, the next step would be to separately stimulate isolated kidneys with solutions containing only isoproterenol on the one hand and EDTA without isoproterenol on the other hand, identifying whether observed effects are caused by a high cAMP concentration or a low concentration of $Ca^{2+}$, or a combination of these factors.

## 4.3 Localization and Regulation of the Angiotensin II Type 1 Receptor-associated Protein Arap1 in the Kidney

**Elisabeth Doblinger**

Arap1 is an interacting protein of angiotensin II type 1 (AT1) receptors and it facilitates increased AT1 receptor surface expression in vitro. In the present study we assessed the tissue localization and regulation of Arap1 in vivo. Arap1 was found in various organs of the mouse with an order of expression of heart>kidney>aorta>adrenal gland≈liver≈testis≈spleen>brain, as determined by RT-PCR. In the kidney, Arap1 was found along a cortical-medullary gradient, with cortical expression levels exceeding those of the inner medulla by 130%. Similar like in mice, Arap1 mRNA was detected in all kidney zones of the human kidney. Arap1 expression was regulated by various stimuli. Thus, a high salt diet (4% NaCl [w/w], 7d, n=5) upregulated renal Arap1 expression in mice by 47% compared to controls (.6% NaCl [w/w], n=5, p=.01). Similarly, AT1 antagonism (losartan, 30 mg/kg/d, 7d), enhanced Arap1 mRNA expression by 52% (n=5 each, p<.01), whereas Ang II infusion (osmotic mini pumps, 7d, 2µg/kg/min Ang II) reduced Arap1 mRNA levels compared to vehicle by 34% (p<.01, n=7 each). Conditions of high Ang II levels, like unilateral kidney artery stenosis (48 hrs, n=5) or water restriction (48 hrs, n=6), all suppressed Arap1 levels compared to controls (-64% and -62% in the clipped and contralateral kidney, respectively, and – 28% after water restriction; p<.01 vs. control each). Changes in Arap1 mRNA expression were paralleled by changes in Arap1 protein levels. Similar like in vivo, Arap1 mRNA and protein were suppressed by Ang II in a time- and dose-dependent manner in cultured mesangial cells (down to 32% of control), and this could be blocked by losartan. In summary, Arap1 expression appears to be suppressed by AT1 receptor activation. Thus, Arap1 may serve as a local modulator of vascular AT1 receptor function in vivo.

## 4.4 Fibrocyte development: role of monocytes recruited via the chemokine receptor CCR2

**Barbara Reich**

**Purpose**

Fibrocytes are bone marrow-derived and type I collagen producing spindle-shaped cells which express markers of hematopoietic and stromal cells. Fibrocytes are associated with fibrosis (e.g. pulmonary and renal fibrosis) and inflammation. Using a depleting monoclonal antibody against CCR2 (MC-21) and CCR2-/- mice we investigated whether CCR2 dependent recruitment of monocytes is correlated with fibrocyte development in the kidney and renal fibrosis.

**Methods**

Unilateral ureteral obstruction (UUO) is a well-described model of renal fibrosis in mice. After 1 week of UUO blood, spleen and kidneys were harvested and tissues were analyzed for expression of CD45, CD11b and collagen type I by flow cytometry. Collagen type I was also quantified by RT-PCR and immunostaining.

**Results**

Daily application of anti-CCR2 mAb (MC-21) resulted in depletion of $CCR2^+$ monocytes and significantly reduced the number of these monocytes in the peripheral blood on day 0 and 3 compared to controls. However, on day 7 the monocyte frequency in blood, spleen or kidneys was no longer reduced in MC-21 treated mice. The development of fibrocytes measured on day 7 in the obstructed kidney was not affected by MC-21 mediated short term depletion of $CCR2^+$ monocytes.

In contrast, CCR2-/- mice showed markedly reduced numbers of fibrocytes and expression of collagen I mRNA in the obstructed kidneys. In the contralateral kidneys the number of fibrocytes was low and unchanged. The number of infiltrating monocytes was significantly decreased in CCR2-/- mice by 81 % in the contralateral and 67 % in the UUO-kidney. CCR2-/- mice also displayed reduced numbers of Gr1+ monocytes in the peripheral blood (10 % of controls) and the spleen (60% of controls) throughout the course of UUO.

**Conclusion**

Recruitment of monocytes via CCR2 plays a critical role for development of fibrocytes and renal fibrosis. Lower numbers of renal monocytes in CCR2-/- mice and reduced migration of monocytes into the kidneys results in reduced numbers of fibrocytes and diminished fibrosis.

### 4.5 MicroRNAs in the kidney and their relevance in renal diseases

**Susanne Baumgarten**

**Introduction.** MicroRNAs (miRNAs) are short non-coding RNA molecules (19-25 nucleotides) which are found in all eucaryotic cells. They are involved in regulation of gene expression by either inhibiting translation or triggering mRNA degradation, thus regulating several biological processes. In contrast to the growing number of identified miRNAs, the knowledge about their respective target mRNAs and thus their functional role is rather scarce. Some miRNAs are ubiquitously expressed, whereas others are highly enriched in certain cell types or developmental stages.

**Methods and results.** Aim of the project is to examine the effect of varying miRNA levels, either by knock-down or overexpression of miRNAs in the kidney, in order to investigate the effects on the molecular level and to finally identify miRNA-mRNA interaction pairs. Deep sequencing profiles identified the present miRNAs in mouse glomeruli and tubuli, and these data have been validated by Northern blot analysis.

To get an idea about the physiological miRNA functions, a knockdown of specific miRNAs, which are enriched in glomeruli, was performed by injection of antisense oligonucleotides in mice. As a second approach, miRNA-“sponge” constructs have been designed to specifically bind their target miRNAs in a competitive manner. First results from luciferase assays show that miRNA activity in HeLa cells can be lowered successfully by these “sponges” and the principle of this technique will be presented. Additionally, 2D gelelectrophoresis has been established to examine changes in the protein profile of treated cells and animals. As a starting point, a first protein gel of wildtype C57BL/6 mouse glomeruli will be presented.

**Discussion.** We have established two techniques which allow the knockdown of specific miRNAs. While the intravascular injection of antisense oligonucleotides in animals is only suitable for short-term treatments, a stable decrease of miRNA levels can be reached by sponge expression. For in vivo studies, cell type specific promoters will be used for sponge expression in order to prevent systemic effects and ensure a high level of “sponge”-mRNA at the desired site-of-action.

**Perspective.** We have shown that miRNAs can be knocked down in cell culture expressing sponge mRNAs. The next steps will be to construct sponges for several different miRNAs and examine their effect on the protein profile of the cells. The promising candidates can then be transferred to animal models (kidney disease models) using lentiviral vectors to investigate *in vivo* effects.

## 4.6 The impact of the basolateral potassium channel KCNJ10 for salt resorption in the distal tubule of the kidney

**Maria Ripper**

Introduction

Mutations in the $K_+$-channel KCNJ10 (Kir4.1) are causative for the autosomal recessive EAST syndrome (also referred to as SeSAME syndrome) which is characterized by **e**pilepsy, **a**taxia, **s**ensorineural deafness and a salt-wasting **t**ubulopathy. The renal salt-wasting pathology is caused by transport defects in the distal convoluted tubule and the connecting tubules where KCNJ10 plays a pivotal role as a basolateral $K_+$-channel. While in the early parts of the nephron the vast majority of the filtrate is reabsorbed, in post-macula densa segments fine tuning of the urinary composition takes place. All mutations in KCNJ10 which have been found in EAST patients so far lead to a partial or even a total loss of channel function. As a consequence, transport processes in the distal convoluted tubules and connecting tubules are impaired.

Methods/Results

Total KCNJ10 knockout mice show failure to thrive and die within the first week after birth. To investigate the renal phenotype of adult KCNJ10$_{-/-}$ animals we are currently generating two different mouse lines with either kidney-specific or inducible kidney-specific inactivation of KCNJ10. The kidney-specific animal model is generated by crossing a Kir4.1-floxed mouse line with a ksp-cre transgenic line in which cre recombinase is specifically expressed in the renal tubular epithelial cells under control of the ksp-cadherin gene promoter (kidney-specific cadherin 16). The second, inducible kidney-specific mice are generated by crossing the Kir4.1-floxed line with the Pax8 mouse strain. In both animal models the target gene Kir4.1 is deleted at the loxP sites that flank the gene of interest (Kir4.1). Additionally, for the second model, tetracycline will be added to the drinking water which triggers the expression of cre recombinase with subsequent ablation of Kir4.1.

Discussion/Perspective

When conditional knockout animals are available we first have to verify the absence of functional KCNJ10 protein by Western-Blot, realtime PCR and immunofluorescence techniques. In further experiments we want to characterize the two mouse lines by establishing growth curves, litter statistics, mortality and fertility, telemetric blood pressure measurements, and weight of the kidneys. Moreover, functional measurements of the kidney will be done. Therefore urine composition will be analyzed by measurement of electrolytes ($Na_+$, $K_+$, $Cl_-$, phosphate, $Mg_{2+}$, $Ca_{2+}$) and kreatinin. Also plasma-aldosterone will be determined and compared to the data of the EAST patients.

Electron microscopic investigations of the distal tubule will be performed as EAST patients exhibit morphological changes in this tubular segment. After treatment of mice with thiazid and furosemid (diuretics), further histological studies might show if there are morphological changes of distal tubular cells by adaption of transport ability and efficiency to certain diuretics.

Finally an electrophysiological characterization of isolated distal tubules will give more insight about the function of Kir4.1 channels in native tissue.

## 4.7 The role of TASK3 $K^+$ channels for the regulation of aldosterone secretion in neonatal mice

**Philipp Tauber**

**Background:** Depolarisation of adrenal zona glomerulosa cells is a pivotal event for the secretion of aldosterone, which is activated by Angiotensin II and high plasma $K^+$ levels. Potassium channels like TASK1 and TASK3 are highly expressed in the adrenal cortex and determine the membrane voltage of these aldosterone-producing cells. The deletion of TASK1 or both TASK1 and TASK3 in mice results in an increase of plasma aldosterone level. Surprisingly, in TASK3 knockout mice, hyperaldosteronism could be observed only in newborn mice. Therefore, the aim of this study is to investigate the adrenal phenotype of newborn TASK3 knockout mice in detail and to find compensating age-dependent differences in the regulation of aldosterone secretion.

**Methods and Results:** Plasma concentrations of different steroids were measured in 1 and 12 day old TASK3 $^{-/-}$ (ko) and wildtype (wt) mice by using specific enzyme immunoassays. Neonatal TASK3 $^{-/-}$ mice show higher plasma aldosterone levels compared to wildtype animals, which correlates with an increased mRNA expression level of the aldosterone-synthase (Cyp11b2) in the adrenal gland (realtime PCR). These high aldosterone levels normalize within the first two weeks. Plasma concentrations of progesterone and corticosterone, also synthesized in the adrenal gland, show the same age-dependent increase. This points to a more general adrenal phenotype in newborn ko mice. Therefore, we performed a gene chip analysis (Affymetrix Microarray) to measure the differential gene expression pattern in the adrenal glands of 1 and 12 day old ko and wt mice. Among other candidates, we observed a strong upregulation of renin mRNA in newborn ko mice that normalized to the wt expression level in 12 day old mice. The higher renin expression was verified by realtime PCR. Renin-specific immunofluorescence was only detected in the adrenal cortex of ko mice and was restricted to the corticosterone-producing zona fasciculata.

**Discussion and Perspective:** These data suggest, that a transient activation of the local adrenal renin-angiotensin-system could be causative for the age-dependent hyperaldosteronism in TASK3 $^{-/-}$ mice. The next approach is to investigate the reason for an increased renin production in these animals, to assess how the deletion of TASK3 is involved in this mechanism and to explain the compensation in adult mice.

## 4.8 The physiological and pathophysiological relevance of the inwardly rectifying potassium channel Kir5.1 (KCNJ16) in the kidney

**Evelyn Humberg**

### Introduction

Mutations of the inwardly rectifying $K^+$ channel subunit Kir4.1 (KCNJ10) are causative for a novel autosomal recessive disease, which is characterized by **e**pilepsy, **a**taxia, **s**ensorineural deafness, and a **s**alt-wasting renal **t**ubulopathy (**EAST** or SeSAME syndrome). The Kir4.1 subunit is believed to form heteromeric $K^+$ channels with Kir5.1 in the kidney. Therefore we want to investigate the physiological and pathophysiological relevance of the Kir5.1 subunit and the Kir4.1/Kir5.1 heteromeric channel for the kidney function.

### Results and Methods

To study the role of the Kir5.1 subunit we are using conventional Kir5.1 knockout (*Kir5.1*$^{-/-}$) mice in a SV129 background. Under normal diet *Kir5.1*$^{-/-}$ mice appeared normal with no growth retardation and no apparent morphological abnormalities. Immunofluorescence stainings and real-time PCR experiments showed that Kir5.1 is expressed in kidney, stomach and thyroid in *Kir5.1*$^{+/+}$ mice. The localization of Kir5.1 in renal tubules seemed to be restricted to the basolateral membrane of the distal convoluted tubule (DCT), the connecting tubule and the cortical collecting duct. As expected, *Kir5.1*$^{-/-}$ mice did not exhibit any Kir5.1 expression.

### Discussion and Perspective

The next step is to characterize the phenotype of *Kir5.1*$^{-/-}$ mice in detail via measurements of electrolytes in blood and urine, blood pressure, kidney weight and kidney histology. The absence of the Kir4.1 subunit in *Kir4.1*$^{-/-}$ mice leads to an altered morphology of DCT cells: the number of infoldings of the basolateral membrane as well as the number of mitochondria is strongly reduced. Therefore we also want to investigate the cell morphology of *Kir5.1*$^{-/-}$ mice via electron microscopy. Furthermore we want to measure the glomerular filtration rate of *Kir5.1*$^{-/-}$ mice under control conditions and after treatment with diuretics to examine the relevance of Kir5.1 for the electrolyte reabsorption in the kidney. We also planned to investigate the effect of the missing Kir5.1 subunit on membrane voltage, potassium current and pH dependency of DCT cells via electrophysiological techniques.

## 4.9 Localization of Renin-producing cells in Cx40-deficient mice

**Christian Karger**

The renin–angiotensin-aldosterone system (RAAS) is a major regulatory system controlling extracellular fluid volume and blood pressure. The rate-limiting factor in this hormonal cascade is renin, an enzyme which is secreted into the circulation by the kidney from specialized renin-producing cells. These are called juxtaglomerular cells and are typically located in the walls of the afferent arteriole at the transition into the glomerular capillary network. Here they largely replace the typical vascular smooth muscle cells and form the media layer of the terminal part of the afferent arteriole. It has long been known that the different cell types of the juxtaglomerular apparatus are abundantly coupled via gap junctions. Juxtaglomerular cells form gap junctions among themselves as well as with their neighboring extraglomerular and endothelial cells (Taugner *et al*., 1978; Taugner *et al*., 1983). There are several types of connexin proteins which form the gap junctions in this region but it was shown that juxtaglomerular cells predominantly express connexin 40 (Cx40) (Arensbak *et al*., 2001; Hwan and Beyer, 2000). The role of this protein is not entirely clear but experiments revealed that mice lacking Cx40 have severe defects in the feedback control of renin secretion, rendering them massively hyperreninemic and hypertensive (Krattinger *et al*., 2007; Wagner *et al*., 2007). Furthermore, deletion of Cx40 leads to abnormalities in the location of renin-producing cells in the adult kidney. Here you can observe a disappearance of these cells from the media of the afferent arteriole. Instead you can find them located outside the vessel wall in the periarteriolar and periglomerular interstitium (Kurtz *et al*., 2007). As the reasons for this particular distribution pattern are unknown this study aims to analyze the expression of renin in Cx40-deficient mice in more detail. First experiments, using laser scanning microscopy, which helps us to obtain high resolution images of the interesting cell types, have revealed age-dependent differences in the renin localization of Cx40 knockout mice. It seems that only animals of an older age show the typical abnormal distribution pattern of the renin-producing cells whereas in younger mice the fully developed Cx40 knockout phenotype cannot be observed. In older animals you can also find indications of an advanced sclerosis of the glomeruli, a fact which might lead to an age-dependent proteinuria. To shed light on the consequences of those substantial structural changes we will use metabolic cages which allow us to observe mice under standardized conditions. Urine-analysis of wild type and Cx40-deficient mice of different ages might help us to find symptoms of an evolving glomerular disease.

## 4.10 Creation and Characterization of *Pkd2* Knock-in Mice

**Denise Schmied**

**Overview**: Autosomal-dominant polycystic kidney disease (ADPKD) is a monogenetic disorder with a prevalence of 1:1000. ADPKD is responsible for about 10% of all cases of end-stage renal disease. Approximately 50% of the patients develop chronic kidney failure at the age of 60. ADPKD is caused by mutations in *PKD1* and *PKD2*, 85% of patients carry mutations in the *PKD1* gene. Mutations in *PKD2* lead to a less progressive disease than mutations in *PKD1*. *PKD1* and *PKD2* encode the proteins polycystin-1 and polycystin-2. To date the function of polycystin-1 is not well understood. Polycystin-2 is an integral membrane protein of 968 amino acids in human, it functions as a non-selective cation channel.

**Aim**: The aim of my work is to examine the development of ADPKD in two knock-in mouse models which were generated previously. In the first mouse model, *Pkd2* (PoreL1), the pore-forming segment of polycystin-2 was replaced by that of polycystin-2L1. In the second model, *Pkd2* (L701), polycystin-2 lacks its C-terminus and the protein has just 701 instead of 966 amino acids. So, the new approach of this work is to exchange specific gene sequences instead of creating a null mutant. Thus, the function of polycystin-2 can be investigated in more detail.

**Methods**: To investigate the influence of the genetic background caused by modifier genes, the two mouse models were generated with two different strains, C57BL/6 and 129Sv. To verify that the modified mRNA and the protein will be produced in these mouse models polycystin-2 mRNA and protein will be isolated out of the kidney. Additionally, after PFA fixation of heterozygous *Pkd2* (L701) mice the kidney, liver, lung, heart, eye, aorta and pancreas were isolated and analyzed for tissue alterations. To investigate whether these mice develop renal failure, we measured the protein concentration in urine.

Results: Preliminary results show that the modified mRNA in *Pkd2* (PoreL1) mice are produced. No obvious phenotype was found in heterozygous *Pkd2* (L701) mice in lung, heart, eye and aorta but were seen in the kidney. To date, an increase of the protein concentration in urine of both models was not seen.

**Perspectives**: Now it is important to show that the modified proteins are produced in these mice. In future analysis it is planned to characterize the pore region. To determine which amino acids of the pore region are embedded into the lipid bilayer cysteine-scanning mutagenesis will be done. With a patch-clamp method the conductivity and the opening state will be analyzed. Likewise, it is known that the calcium flow is changed in modified polycystin-2 proteins. So, with the help of calcium-sensitive fluorescence-labeled fusion constructs the calcium flow of the pore region will be analyzed.

## 4.11 Renal Function of cGKII

**Andrea Schramm**

Although the role of the second messenger cGMP and its effectors, cGMPdependent kinases (cGKs), is quite well known in other organs, only a few hints exist regarding the function of these signalling molecules in the kidney. There are 3 different isoforms of cGKs, namely cGKIα and cGKIβ (which are derived from the same gene and altered by alternative splicing), and cGKII, which is derived from a different gene. First results show that cGKII is associated with regulation of rennin secretion in juxtaglomerular cells, however, recent data is missing due to the fact that there exists no specific antibody. This would be an extremely useful tool to identify the localization of cGKII in the different nephron segments and further evaluate possible additional functions. Thereby, one aim of our work is to develop a specific antibody by expressing and purifying the N-terminal region of cGKII for subsequent injection in rabbits and production of antibodies against it.

It is known that natriuretic peptides, primarly the atrial natriuretic peptide (ANP), cause natriuresis and diuresis. As many functions of cGKII are ANP-induced, we want to investigate, if these natriuretic and diuretic effects of ANP are mediated through cGKII. Therefore, we performed metabolic cage experiments with WT- and cGKII-KO-mice after implantation of ANP-loaded mini-osmotic pumps. The ownstream-effectors leading to natriuresis and diuresis are diverse; however, we want to elucidate possible cGKII-regulated effectors. cGKII regulates chloridechannels in the intestinum, so it seems to be likely, that this enzyme also can regulate ion-channels in the kidney. Apparently, the anchoring protein NHERF2 binds cGKII, so that the kinase is located close to NHE3, an important channel for sodium resorption in the proximal tubular system, and thus could act on this exchanger. This could be a first hint regarding functions beyond renin-regulation and shall be examined by co-immunprecipitation-experiments of NHE3/NHERF2/cGKII.

Another possible role of cGKII in the kidney is the regulation of Aquaporin2 (AQP2)-trafficking in the collecting duct. This field is discussed controversially, while some investigators propagate a cGK-mediated inhibition of membrane assembly of AQP2, others show an activation of this process. By isolating collecting ducts of WT and cGKII-KO-mice for use in cell culture, we want to establish a cell-based model for stimulation experiments, so that the cGKII-mediated AQP2-trafficking can be analyzed in detail.

## 4.12 The role of Ecto-5'–nucleotidase (CD73) –mediated extracellular adenosine generation in renal fibrosis

**Isabel Carota**

Several factors mediate the development of renal fibrosis upon renal injury. Adenosine is known to play a key role in the regulation of tissue protection and repair and is released by injured cells. Adenosine is generated either intracellularly and subsequently transported to the extracellular space or it is formed extracellularly from AMP by the activity of dephophorylating enzymes like ecto-5'-nucleotides/CD73. One location of CD73 within the kidney are interstitial fibroblasts, which were related to the initiation of renal fibrosis. Furthermore, there is convincing evidence that adenosine modulates the formation of fibrosis in other organs, like liver and skin. Therefore, the aim of this study was to assess the relevance of CD73 for the development of renal fibrosis.

For this purpose, renal injury leading to fibrosis was induced in $CD73^{+/+}$ and $CD73^{-/-}$mice by unilateral ureter obstruction (UUO). UUO-kidneys and the contralateral unaffected kidneys were harvested after 7d. Urine trapped in the obstructed ureter was collected for determination of adenosine concentrations. Collagen content was quantified by Sirius Red/Fast Green staining of cryopreservated kidney sections. mRNA expression levels of several fibrosis mediators as well as Extra Cellular Matrix (ECM) components were quantified by real-time polymerase chain reaction (RT-PCR). Protein expression levels of specific fibrosis markers were determined by Western Blot Analysis. Immunohistochemical staining was performed on paraffin embedded sections.

As compared to fibrotic kidneys of wild type mice, kidneys from $CD73^{-/-}$ mice showed significantly lower mRNA levels of alpha-SMA, fibronectin, TGF-beta, Ki67, MMP-2, MMP-13, MMP-14, adenosine receptor A1, and of the collagens Col 1a1, Col 1a2, Col 3a1 and Col 4a3. Immunostaining of alpha-SMA, fibronectin and collagens revealed that mRNA levels of fibrotic markers were paralleled by respective protein levels. Furthermore, immuno-staining of fibrinogen that acts as a mitogen for tubulointerstitial fibroblasts and staining of macrophages, supported the assumption that renal fibrosis was less severe in $CD73^{-/-}$ compared to WT mice.

These results suggest that loss of CD73 activity has an alleviating effect on the progression of renal fibrosis. Consequently, extracellular adenosine appears to promote the development of renal fibrosis. CD73 may constitute a target for the management of renal fibrosis in humans.

### 4.13 The role of WNK3-kinase in activation of NKCC2 in vivo

**Katharina Mederle**

**Introduction**: WNK´s (=with no K [lysine]) are serine/threonine kinases with the characteristic amino acid substitution of cysteine for lysine within subdomain II of the catalytic core. Four mammalian WNK-kinases do exist (WNK1-4). In the kidney WNK3 is expressed throughout the nephron with the highest expression in the proximal tubule and the TAL. In in-vitro experiments kinase-active WNK3 was demonstrated to be a potent activator of NKCC2 activity, while kinase-dead WNK3 inhibits NKCC2 activity. The effects of WNK3 on NKCC2 are regulated by phosphorylation of N-terminal threonines. Furthermore, a chloride-sensing mechanism involving WNK3 and SPAK results in activation of NKCC2. The aim of this study is to investigate the role of WNK3-kinase in activation of NKCC2 in vivo.

**Results and Methods**: Experiments were performed in generally WNK3-deficient mice with 129/SvEv-C57BL/6 background; wild-type littermates served as controls.

Urine osmolarity and urine volume: 24-h urine was collected in metabolic cages for three days and 24-h urine volume and 24-h drinking volume were measured. Urine osmolarity was determined in spontaneous urine (male and female mice) and in urine samples obtained by transurethral catheterization of female mice following 48-h water restriction.

GFR: The glomerular filtration rate of conscious mice was measured by FITC-Sinistrin clearance after a single retro-orbital injection and consecutive blood sampling from the tail vein. Plasma renin concentration: Blood was collected puncturing the submandibular vessels. Plasma renin concentration was measured with a radioimmunoassay kit.

RBF: In anesthetized male mice an ultrasonic flow probe was placed at the renal artery to measure renal blood flow. No significant differences between knock-out and wild-type mice were observed in the performed experiments.

**Discussion and Perspectives**: So far, no relevant renal phenotype was detected in WNK3-knock-out mice. The performed experiments will be repeated after feeding low salt diet for 7 days to enhance the estimated effect of a compromised concentrating ability. Next, urea nitrogen, creatinine and electrolytes in urine and serum will be determined to identify differences in tubular reabsorption. In-vitro data indicates that WNK3 lies downstream of vasopressin signaling for activation of NKCC2. Therefore, urine osmolarity will be measured following vasopressin treatment. Micropuncture experiments will be performed to determine salt reabsorption in the TAL, especially chloride reabsorption. Immunhistochemistry will be performed to verify the lack of function of WNK3-kinase and to detect differences in the colocalization of WNK3 and NKCC2 in knock-out and wild-type mice. The expression of NKCC2-isoforms in the kidney areas of WNK3-knock-out mice will be characterized using RT-PCR.

## 4.14 The impact of mutated Fanconi-associated protein on the mitochondrial proteome

**Nadine Aßmann**

The renal Fanconi syndrome is characterized by the failure of the proximal tubules in the kidney to reabsorb small molecules causing urinary loss of amino acids, glucose, electrolytes, phosphate, and low-molecular-weight proteins.

Recently, Kleta and coworkers at the University College London identified a novel form of autosomal dominant renal Fanconi syndrome (LOD-Score > 3) in an extended family by classical linkage analysis. The gene encodes for a peroxisomal protein (Fanconi-associated protein, FAP) and upon mutation at the N-terminal end a negatively charged amino acid is replaced with a positively charged amino acid, which generates a mitochondrial targeting sequence leading to the erroneous transport of FAP into mitochondria as confirmed by immunohistochemistry.

The aim of the study is the elucidation of the molecular effects of the FAPmut-mislocalization to the mitochondria. The impact of the mislocated FAPmut is analysed using LLC-PK1 cells, which have been derived from the proximal tubulus. For this purpose, the cells were stably transfected with either FAPwt or FAPmut cDNA using the inducible Tet-On gene expression system. Cells start to express FAP within 24 hours after the addition of tetracycline and are grown for 7 days on glucose-free medium.

By means of differential proteome analysis of purified mitochondria and whole cell lysate the effect of the mutated FAP on the LLC-PK1 proteome is analysed employing two-dimensional gel electrophoresis and multi-dimensional liquid chromatography tandem mass spectrometry. Whole cell lysate was analysed by means of 2D-DIGE. First and second dimension protein separation were performed on an immobilized pH gradient (pH 3-7; pH 7-11) and a precast 24x20 cm 12.5% SDS-PAGE gel, respectively. Gels were analysed using the Progenesis SameSpots software. Preliminalry results of the comparison between the whole cell lysates of the FAPmut and FAPwt transfected cell lines show 31 regulated spots with $p \leq 0.05$ and a statistical power $P \geq 0.8$.

Ongoing characterization of the differentially regulated spots by nano-HPLC/QTOF-MS has led so far to the identification of proteins involved in remodelling of the cytoskeleton, e.g. actin, tubulin, and plastin, as well as proteins that are involved in energy metabolism, such as alpha-enolase and an electron-transfer-flavoprotein. For mass spectrometry based quantitative proteomics stable isotope labeling with amino acids in cell culture (SILAC) will be performed.

## 4.15 Chemokines and Their Receptors as Potential Regulators of Renal Development and Function

**Simone Wurm**

Chemokines represent a large group of chemotactic cytokines that are classified in four families (CL, CCL, CXCL and CX3CL). These molecules and their receptors do not only effect the progress of inflammatory responses such as leukocytes recruitment, but play also a role in various physiological processes like angiogenesis and haematopoiesis. Another example displays the relevance of chemokines during the nephrogenesis: deletion of the ligand-receptor pair CXCL12/CXCR4 leads to malformation in the developing glomerular vascular system. Previous studies in our group revealed that mesangial cells constitutively express the receptor CCR7, while podocytes express the CCR7 ligand CCL21.

To elucidate the role of non-inflammatory chemokines within the kidney, in a first step physiological expression patterns of both chemokines and chemokine receptors were comprehensively analysed within developing murine kidneys. We found (amoung others) CCL21 and its receptor CCR7 with a significant and highly regulated expression during embryogenesis and the early postpartal period.

To study the relevance of CCR7 and CCL21, we investigated the renal phenotype of CCR7 knockout mice. CCR7 $^{-/-}$ mice showed a significantly elevated proteinuria compared to wild type mice. Histological studies revealed structural changes in the murine glomeruli, while additional electron-microscopical examinations display amorphous, perimesangial deposits. Furthermore, mRNA expression levels of other chemokines and chemokine receptors were analysed aiming to identify compensatory effects. While CCL21 had a slightly lower mRNA expression level in CCR7$^{-/-}$ renal tissue, the mRNA expression of the second CCR7 ligand, CCL19, remained unchanged. In contrast to these findings several chemokine receptors and their ligands show a significant up-regulation. Several chemokine receptors (e.g. CXCR7 and CXCR2) and chemokines (e.g. CCL2, CCL7, CCL8) showed a significant up-regulation, while the mRNA expression level of CXCL12 was significantly reduced.

Based on these findings we suggest that CCL21 and CCR7 may be important for renal development and function. Further experiments will include a local overexpression of CCL21 within the glomerulus.

## 5. Freshmen

**Ultrastructural and cell-biological studies of the primary cilium**
Benjamin Salecker

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## 5.1 Ultrastructural and cell-biological studies of the primary cilium

**Benjamin Salecker**

The non-motile primary cilium [5] represents one kind of cilia on eucaryotic cells. It can be found on most mammalian cells. It is thought to act as a sensory organelle [4]. In the kidney, the primary cilium is assumed to act as a sensor for measuring flow in renal tubules. The mechanical stimulus of its bending leads to the activation of a $Ca^{2+}$-dependent messenger system. Furthermore, the cilium plays a role as a signaling center during development [1]. It is composed of a basal body located near the cell surface and the ciliary shaft that extends into the extracellular space. The area between the basal body and the shaft is called the transition zone and is believed to form a barrier between the ciliary membrane and the cytoplasmic membrane. The basal body consists of 9 triplets of microtubules and distinct appendages around them, the basal feet, with the basal cap at their. The axoneme of the ciliary shaft is made up of 9 pairs of microtubules. In contrast to motile cilia, the primary cilium does not contain an additional central pair of microtubules. Mutations in genes encoding ciliary proteins can cause a variety of diseases, which are for this reason called "ciliopathies". These are, amongst others, the Bardet-Biedl syndrome (BBS) and autosomal dominant polycystic kidney disease (ADPKD) [8]. ADPKD is caused by mutations in the *PKD1* or the *PKD2* genes. Polycystin-2 [2,3] forms a cation channel and acts as a signal transduction molecule. Polycystin-1 is mutated in about 85% of the patients. Its function is still unclear, but it is known to interact with polycystin-2. BBS is caused by mutations in genes that encode the so called BBS proteins. They are located in the basal body and the ciliary shaft and may play a role in vesicular transport.

We will further investigate the ultrastructure of the primary cilium especially by means of electron tomography. The use of high-pressure freezing [7] and freeze substitution during sample preparation results in a better structural preservation with less artifacts. 3D-models of tomograms will be generated with AMIRA®. Of special interest are the findings of Sonja Gürster who showed that vesicles can be found inside the basal body. One aim is to detect vesicles the ciliary shaft as well. To decide whether these vesicles represent anterograde or retrograde transport, different endocytosis experiments with electron microscopy as well as with fluorescence microscopy will be done.

By now, the subcellular distribution of certain proteins is still uncertain and it is unclear to which structures they contribute. To answer these questions, a new genetically encoded tag for correlative light and electron microscopy, the so-called miniSOG [6] protein, will be used for protein localization of polycystin-2 and BBS proteins. Moreover the primary cilium of inducible knock-down mutants of ciliary and BBS proteins will be examined by electron tomography and the results will be compared to those of wild type cells to find out which

structural abnormalities are caused by the knock-down of the specific protein. These mutants are about to be generated by Karin Babinger and Larissa Osten  by lentivirus-mediated RNA interference.

## 5.2 Autosomal dominant renal Fanconi syndrome caused by mitochondrial mistargeting

**Carsten Broeker**

**Introduction**

Renal Fanconi syndrome is characterized by reduced transport capacity of the proximal tubule and is accompanied by a renal loss of glucose, amino acids, bicarbonate and phosphate among other metabolites. Professor R. Kleta (University College, London) has identified a mutation in the sequence of enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase (EHHADH), an enzyme involved in peroxisomal β-oxidation, that leads to a mistargeting of the protein to the mitochondria and thereby causes an autosomal dominant form of Fanconi syndrome in affected patients. According to our hypothesis this mistargeting leads to an impaired mitochondrial function causing the observed renal phenotype.

**Methods and results**

Immunohistochemical analysis was conducted on a stably transfected inducible cell line (LLC-PK1) to determine cellular localization of mutated EHHADH. Hereby we could confirm correct localization of wildtype EHHADH in the peroxisomes and mistargeting of mutated EHHADH to the mitochondria. Metabolites were measured in the medium of induced LLC-PK1 cells to assess potential differences in energy metabolism. These measurements showed a faster decrease in glucose concentration in the medium of cells with mutated EHHADH compared to cells overexpressing wildtype EHHADH which indicated an increased glycolytic activity in these cells. Furthermore, lactate/pyruvate ratio was elevated in the medium of cells overexpressing mutated EHHADH which is highly indicative of a mitochondrial dysfunction. Additionally, activity of the respiratory chain was evaluated using high-resolution respirometry. These measurements showed significantly reduced oxidative phosphorylation capacity upon maximum stimulation of complex I+II of the respiratory chain.

**Discussion and perspective**

According to our hypothesis, mistargeting of mutated EHHADH to the mitochondria leads to reduced ATP generation caused by impaired oxidative phosphorylation in affected cells. As a consequence, ATP-dependent reabsorption of metabolites in renal proximal tubule cells is reduced. To further elucidate the mechanism leading to renal Fanconi syndrome it is important to discover the exact subcellular localization of mutated EHHADH within the mitochondria. In addition, the effect of the mutation on cellular ADP/ATP ratio is of interest. To confirm the *in vitro* data a transgenic mouse carrying the mutation is currently being generated. Future experiments include respirometric measurements on freshly prepared tissue, immunohistochemical analysis of kidney tissue, and measurement of urine metabolites among others.

## 5.3 Function of cGKI

**Franziska Limmer**

We are trying to find out whether the cGMP-dependent protein kinase I plays a role in the mechanism of the rebound-effect of furosemide.

After the administration of furosemide we can see an increased excretion of sodium and water. 6 hours later there is an amplified retention of sodium what is called the rebound effect. The result of this effect is that the excretion of sodium and water lies under the check value and that in the summary during 24 h hours after the furosemide administration more sodium is reserved then it is excreted.

The fact that the macula densa controls the GFR by measuring the concentration of the sodium ions in the distal tubule and that cGKIα is expressed in the JGA leads us to the supposition that the cGKIα takes part in the signalling of the regulation of the natriuresis.

Therefore we first ascertained basic securities about the urine excretion of wildtype mice and cGKIα-rescue mice. The mice had first been confined in metabolic cages for five days during they had been fed with enough water and food in order to adapt to the cage.

In all experiments we measured the volume and the concentration of sodium and potassium ions of the collected urine.

In order to evaluate the role of cGKI in the rebound-effect of furosemide we injected furosemide 200mg/kg KG i. p. During the following hours the mice did not receive water and food. We measured the volume of the urine and perfused the kidneys from a part of the mice with NaCl rinsing solution and removed them at different times after the injection. One kidney per mouse had been bedded in paraffin in order to carry out the immunhistochemical staining. By dyeing cGKIα, cGKIβ, NKCC, actin und renin we are looking forward to show a higher activity of cGKIα and more production of renin in mice, that had been treated with furosemide.

The other kidney of each mouse had been perfused with NaCl rinsing solution too. After that we isolated the RNA, generated the cDNA with reverse transcription and

used it for light cycler in order to find out whether we can see a higher expression of cGKIα and renin after the injection of furosemide.

We are planning to do all this experiments with cGKIα-rescue mice and cGKIα-KO-mice too.

## 5.4 Investigations on the pathophysiology of the hepatorenal syndrome using multiphoton technology

**Ina Schießl**

**Introduction:** The hepatorenal syndrome (HRS) is a severe complication of end stage liver disease, which occurs most often in cirrhotic patients. As the pathophysiology of HRS is still poorly understood, the renal function shall be investigated in an animal model by the usage of multiphoton microscopy. This technology provides the unique opportunity to judge basic renal function like capillary flow, single nephron glomerular filtration rate (snGFR), glomerular sieving coefficient (GSC) or renin activity in almost real time.

**Results and methods:** The following parameters are measured using multiphoton microscopy.

Capillary flow: A fluorescent dye, which is conjugated 70kD dextran and therefore is excluded from filtration process, is injected i.v. to stain the plasma. The red blood cells (RBCs) are not labeled. A line-scan is performed and repeated every millisecond over a certain time. In these images the RBCs are visible as dark bands showing a slope inversely proportional to their velocity.

snGFR: A superficial glomerulus showing an, at least 100µm long, initial segment of the proximal tubule(PT) is perfused with a fluorescent dye of small molecular weight. The volume of the PT can be calculated through length and diameter. The time differences between the fluorescent maximas is then used to calculate the snGFR [ml/min]

GSC: A 70Kdextran conjugated fluorescent dye is injected i.v. and a superficial glom is chosen. The fluorescent intensities in the intraglomerular capillaries and the bowman space (PUS) are measured. The GSC can then be calculated by using the following formula:

GSC = (intensity PUS – intensity background) / (intensity capillaries – intensity background)

Renin activity can be observed by administration of FRET renin substrate, which only turns fluorescent in the presence of renin activity.

At the moment I am establishing all the methods for future experiments.

**Discussion&perspectives:** HRS shall be induced by a common bile duct ligation in rats or mice. The stage of disease will be diagnosed according to the criteria of International Ascites Club comparing serum creatinine, GFR, urine and serum sodium concentrations between BDL and sham-operated animals.

## 5.5 ANO1 involvement in carcinogenesis

**Luisa Wolf**

### Introduction

Ion channels regulate a huge variety of cellular mechanisms. The transport of ions and subtracts are fundament for cell proliferation, cell migration, pH regulation, apoptosis, synaptic excitation and epithelial transport.
Carcinoma cells show an excessive cell proliferation which means a particular increase of ion channel expression. Those carcinoma ion channels are located in the cell plasma membrane. This localisation puts the ion channels in the point of view. Membrane localized ion channels could be potential pharmacological targets because of the huge amount of inhibiting / activating chemical compounds.
In previous studies the calcium activated chloride channel was identified as Anoctamin 1. Anoctamin 1 is in demand to correlate with cell proliferation, apoptosis and carcinogenesis as there is a high expression of this channel in various carcinoma cell types and cancer tissues. Anoctamin 1 is identical to DOG-1, a well known tumour marker.

### Objectives

In regard to the expression of Anoctamin 1 (ANO1) in certain carcinoma cells, the first step will be to indentify the role of ANO1 for cell proliferation and apoptosis in carcinoma cell lines. Additionally the cellular signalling pathway which regulates ANO1 expression during cell proliferation will be identified.
The second step is to investigate the impact of ANO1 for tumour development in an animal model. $APC^{min/+}$ mice carrying a mutation in the APC gene which is responsible for autosomal dominant inherited familiar adenomatous polyposis and colorectal carcinoma. These mice develop a huge amount of intestine polyps. Rapamycin treatment of these mice prevents polyp formation.
In the third step, in collaborations, the expression of ANO1 in human health and cancer tissues will be analysed correlating to survival data and in the fourth step potential associated proteins will be identified.

Finally new information about Anoctamin involvement in carciongenesis or apoptosis could be a starting point of new anti cancer therapy.

## 5.6 A Molecular and Cellular Dissection of Tumor Initiating Cells in High-Grade Gliomas

**Verena Gawrisch**

**Research Goal:**

Glioblastomas (GBM) represent one of the most devastating human tumor forms. Through the identification of molecules and pathways controlling the generation of **B**rain **T**umor **I**nitiating **C**ells (BTIC), their pathogenesis shall be investigated in this project. Main focus is the description of [1] functional differences between **N**eural **S**tem **C**ells (NSC), BTIC and **T**umor **C**ells (TC) in respect of migration in several *in vitro*, *in situ* and *in vivo* assays, [2] to search for new markers correlating to stemness in BTICs and NSCs by investigating migrated cells by microarray analysis of microdissected cells, and [3] to test the hypothesis that BTICs and TCs are able to re-differentiate into NSCs after si/shRNA transfection with STAT3 and C/EBPß, recently described as pivotal transdifferentiation transcription factors in GBM.

**Working programme:**

In order to characterize the putative transcriptional differences between NSCs and incompletely differentiated BTICs *in vivo* as well as *in vitro*, three different aspects will be further investigated:

- Primarily, the functional and molecular differences between NSC, BTIC and TC in migration using first of all live cell imaging for wound healing migration assays as well as spheroid and Boyden Chamber assays
- Moreover organotypic brain slice cultures as an *in situ* tool to monitor the migration of living cells will be used as a tool for the investigation of invasion
- a search for new markers correlating to stemness in BTICs and NSCs will be performed by investigating migrated cells through microarray analysis of microdissected cells (after predefined timepoints, non-migrating, migrating and differentiated BTIC and NSC will be harvested by laser capture microscopy (LCM))
- the hypothesis if BTICs and TCs are able to re-differentiate into NSCs after si/shRNA transfection with STAT3 and C/EBPß

**Results and perspectives:**

Currently the organotypic brain slice culture invasion model and the set up of live cell migration (wound healing) assays with BTICs transfected with siRNA are established.

Later on, newly identified targets will be either cloned, over-expressed or silenced using si/shRNA or vector based strategies in order to manipulate the tumorigenicity of NSC and BTIC. All relevant genes will be knocked down, and engineered cells will be inoculated in a nude mouse model to investigate their invasion potential in comparison to control cells.

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