Workshop

Communication and System Relevance in Liver Damage and Regeneration

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Workshop

COMMUNICATION AND SYSTEM RELEVANCE IN LIVER DAMAGE AND REGENERATION

Düsseldorf (Germany)
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Session I

System relevance in liver damage
Role of the liver for systemic immunity

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The liver has unique immune functions capable of modulating local immune responses but also influencing systemic immunity. Liver-resident antigen presenting cell populations, in particular liver sinusoidal endothelial cells, take up and (cross)present these antigens to T cells thus initiating antigen-specific immune responses. While MHC II-restricted local antigen presentation in the liver has been reported to lead to the generation of regulatory T cells, the impact of cross-presentation of soluble antigens to CD8 T cell immunity has been less clear. We have discovered that cross-priming of naive CD8 T cells by LSECs leads to their differentiation into memory CD8 T cells. Such liver-primed memory T cells relocate to lymphoid tissues and can be re-activated upon microbial infection in the context of inflammation. Then, these liver-primed T cells provide protection from these pathogens. Thus, liver-primed memory CD8 T cells generated under non-inflammatory conditions contribute to systemic immune surveillance.

Furthermore, the liver engages in the expansion of CD8 T cells outside of lymphoid tissues. Systemic distribution of TLR-ligands triggering sustained TNF receptor signaling in the liver leads to formation of so-called intrahepatic myeloid cell aggregates involved in T cell expansion (iMATE). These iMATEs represent cocoon-like structures that support massive expansion of those CD8 T cells that had recently been activated in lymphoid tissues. Such iMATE-induced expansion has proven to be important for the development of therapeutic vaccination against chronic viral infection of the liver. Taken together, these results further strengthen the unique immunological functions of the liver and will allow for novel therapeutic vaccination strategies.
Balancing viral replication in spleen and liver determines the outcome of systemic virus infection

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The innate immune system limits virus replication during systemic infection by producing type I interferons (IFN-I). Consistently, the lack of type I interferon receptor (IFNAR) promotes virus replication and results in virus persistence and the death of virus-infected animals. Macrophages are key players during IFN-I-mediated virus suppression. Macrophages capture virus particles and suppress their replication in an IFN-I-dependent manner. This mechanism suppresses the spread of virus but obviously limits the amount of antigen that is available for priming adaptive immune response.

We used the lymphocytic choriomeningitis virus (LCMV) and the vesicular stomatitis virus model system to analyze the role of different macrophage subsets in liver and spleen. Our findings demonstrated that liver resident Kupffer cells are essential for the efficient capture of infectious virus and for preventing viral replication. The latter process involved fast induction of an antiviral status in Kupffer cells by IFN-I and prevented viral spread to neighbouring hepatocytes. In the absence of Kupffer cells, hepatocytes were not able to suppress virus replication, even in the presence of IFN-I, leading to prolonged viral replication and severe T cell-dependent immune-pathology.

In contrast to liver resident macrophages, spleen and lymph node resident antigen presenting cells showed limited response to IFN-I due to expression of the endogenous interferon inhibitor *Usp18*. Therefore, virus in these spleen niches replicated despite high levels of IFN-I. This enforced viral replication led to an exorbitant propagation of viral antigens and viral RNA. Viral antigen led to massive activation of the adaptive immune system, whilst viral RNA activated innate immunity. Lack of replication of virus in the spleen abolished the innate and adaptive immune response and thereby resulted in limited control of the virus.

In conclusion, liver resident macrophages capture virus after infection and suppress its replication whilst spleen and lymph node resident antigen presenting cells promoted viral replication and thereby activated the innate and adaptive immune response.
Chemokine signaling in liver injury

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Hepatic inflammation is a key force in promoting the progression or regression of chronic liver diseases related to various types of injury, including hepatitis C or metabolic disorders. Chemokines, chemotactic cytokines, orchestrate the hepatic recruitment, migration or activation of immune cells but also other cell types in the liver (e.g., Kupffer cells, hepatic stellate cells, or endothelium). Depending on the type of injury, either activation of innate immune mechanisms or adaptive immune mechanisms might be the focus of chemokine pathway activation. In chronic viral hepatitis, chemokines such as CCL5 or CXCL10 balance cytopathic versus antiviral immune responses by T and NK cells. In non-alcoholic steatohepatitis (NASH), chemokines such as CCL2 promote infiltration of inflammatory monocytes into liver and adipose tissue. Several chemokine pathways also actively endorse hepatic fibrosis. Besides, recent data have identified anti-inflammatory and anti-fibrotic actions mediated by specific chemokines and immune cell subsets. For instance, studies are ongoing to pharmacologically target chemokines in liver fibrosis by inhibition of inflammatory monocyte influx, e.g., by blocking CCL2 or CCR2, or augmenting the differentiation of restorative macrophages. Thus, chemokines fundamentally contribute to the pathogenesis of liver diseases, making them promising candidates for novel biomarkers as well as pharmaceutical targets for liver diseases.
Hepatocellular carcinoma (HCC) is a major health problem being fifth most common malignancy worldwide and resulting in over 1.4 million fatalities per year. The quest for novel putative therapeutical strategies is of great importance. Epidemiological studies and animal models have linked dysregulation of bile acid (BA) metabolism to an increased incidence of HCC. While it has long been recognized that BAs are required to facilitate lipid digestion and cholesterol absorption in the intestine, the discovery that they serve as ligands for the nuclear farnesoid X receptor (FXR), opened a new chapter in the characterization of BAs as signaling molecules. FXR is the master regulator of BA homeostasis because it controls BA synthesis, influx, efflux, and detoxification in the gut/liver axis. Hepatocarcinogenesis has been associated with both FXR gene deletion and BA-mediated metabolic abnormalities after inactivation of FXR transcriptional activity. The landmark discovery of the role of a FXR target gene, the intestine-derived hormone fibroblast growth factor 15/19 (murine and human, respectively – FGF15/19), in the feedback regulation of hepatic bile acid synthesis shed light on the physiological relevance of the crosstalk of the gut-liver axis in the context of BA homeostasis. FGF15/19 is atypical fibroblast growth factor that functions as a hormone. Via the enterohepatic circulation it travels from the small intestine to the liver, where it acts on a cell surface receptor complex to repress BA synthesis and gluconeogenesis and activate glycogen and protein synthesis. The BA-FXR-FGF19 signaling pathway regulates several aspects of the postprandial enterohepatic response and in conditions of BA metabolic derangement it especially restore BA homeostasis through the FXR-FGF19 axis and prevent progression of liver damage to HCC even in the absence of hepatic FXR.
Pathomechanisms of hepatic encephalopathy

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Hepatic encephalopathy (HE) is a clinical manifestation of a low-grade cerebral edema, which exacerbates in response to ammonia and other precipitating factors, such as inflammatory cytokines, hyponatremia and sedatives and induces an oxidative/nitrosative stress response\textsuperscript{1,2}. There is an auto-amplificatory signaling loop between astrocyte swelling and oxidative stress. The action of different HE-precipitating conditions integrates at the level of astrocyte swelling and the production of ROS/RNS, thereby explaining why fairly heterogeneous conditions can trigger episodes of HE. The consequences of the oxidative/nitrosative stress response are manifold and include protein tyrosine nitration (PTN)\textsuperscript{3}, RNA oxidation\textsuperscript{4}, induction of astrocyte senescence\textsuperscript{5} and zinc-dependent transcription of genes\textsuperscript{6} such as the peripheral benzodiazepine receptor. Its upregulation enhances the synthesis of neurosteroids which not only positively modulate GABA\textsubscript{A} receptor activity, but also activate TGR5 in the brain\textsuperscript{7}, which exerts anti-inflammatory activity in microglia. Microglia is activated in HE \textit{in vivo} and by ammonia \textit{in vitro}, but is not reactive\textsuperscript{8,9}. Ammonia-induced astrocyte senescence is mediated by a miRNA-dependent upregulation of heme oxygenase (HO-1)\textsuperscript{10}. RNA oxidation occurs in astrocytes and neurons and oxidized RNA is also found in neuronal RNA granules, which participate in postsynaptic local protein synthesis which is involved in memory formation. RNA oxidation and PTN liver-specific knockout of glutamine synthetase is sufficient to induce hyperammonemia and to trigger PTN and RNA oxidation in the brain\textsuperscript{11}. Most importantly, markers of oxidative/nitrosative stress, PTN, RNA oxidation and senescence can also be shown in human brains from patients with liver cirrhosis and HE, but not from cirrhotic patients without HE\textsuperscript{5,8,12}. Gene expression profiling from cerebral cortex from patients without liver disease and cirrhotic patients with and without HE revealed HE-specific sets of 434 and 204 genes which are up- or downregulated in HE, respectively\textsuperscript{9}. These genes are predominantly related to oxidative stress and DNA damage defense, cell signaling, apoptosis and anti-inflammation. There is no evidence for an upregulation of inflammatory cytokines in HE. In addition, ammonia also induces via glutamine formation an increased N-acetyl-glucosaminidation of proteins\textsuperscript{13}. RNA oxidation, PTN and changes in gene transcription are thought to affect synaptic plasticity in the brain which may explain the slowing of oscillatory networks in the brain which finally accounts for motoric and cognitive symptoms in HE\textsuperscript{14}. In line with this, prevention of PTN and RNA oxidation by indomethacin in portal vein ligated rats, an animal model of HE restored locomotor activity in these animals\textsuperscript{15}.

References:


Session II

Communication and signaling in liver damage and regeneration
Molecular mechanisms of hepatic cell fate decisions in liver disease

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The normal liver regenerated through hepatocyte cell division. 2 recent populations have been described in the mouse: (1). Wnt responsive Axin2+ hepatocytes which are restricted to a single layer directly adjacent to the central vein. These cells expand radially from the central vein to replace approximately hepatocytes across the liver lobule. (2). Hybrid periportal hepatocytes (HybHP) adjacent to the portal veins and in the terminal branches of the biliary tree. These HybHPs express hepatocyte markers together with low levels of Sox9, which is also expressed by adjacent biliary ductules—over time the cells spread through the parenchyma in liver turnover and injury.

There is increasing evidence of cell plasticity in response to injury. Notch signalling can drive hepatocytes through to a ductular phenotype. Over-active Notch signalling can eventually drive biliary cell expansion and even cholangiocarcinoma formation. Inactivation of Hippo pathway signalling causes adult hepatocytes to undergo a ductular fate switch, again Notch is involved in this.

In severe injury, where hepatocyte replication is impaired then biliary ductular cells can become activated under the influence of TWEAK, Notch and other signals. In many mouse models, the ductular reactions stay as biliary ductules, but when hepatocyte replication is impaired the cells can undergo a switch towards a hepatocyte fate.
Beyond fibrosis: Stellate cells as liver stem cells

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Research on hepatic stellate cells (HSC) is mainly restricted on their contribution to fibrogenesis in chronic liver diseases, but effective therapeutic strategies for patients are still not available. Knowledge about the relevance of HSC for normal liver homeostasis may facilitate the development of suitable approaches to attenuate fibrosis and to support regeneration of injured liver. The expression of stem cell markers by HSC and their potential to undergo developmental processes indicate that functions beyond their contribution to extracellular matrix deposition and wound healing exist. Stellate cells possess an expression profile and differentiation potential related to bone marrow mesenchymal stem cells (bmMSC) and support hematopoiesis of hematopoietic stem cells. These typical features of bmMSC indicate that HSC represent liver-resident MSC. This classification is further supported by engraftment of transplanted HSC not only in the liver but also in the bone marrow of host animals. Transplantation and cell lineage-tracing studies in rodents further indicate that HSC can contribute to regeneration of injured liver by forming bile duct cells, hepatocytes and mesenchymal tissue. Collectively, the data available thus far indicate a dual role of stellate cells in the liver by supporting stem/progenitor cells such as hematopoietic stem cells and to form new cell types through differentiation. The ultimate behavior of stellate cells is presumably context-dependent and controlled by internal or external signals. Bile acids are such environmental signals, since they are not only involved in metabolism but also in cell signaling. After liver injury, elevated levels of bile acids are detectable in the blood serum and can promote liver regeneration, while exceptionally high concentrations exert adverse effects. Interestingly, when isolated HSC and bmMSC from various species are exposed to low amounts of bile acids, their differentiation into hepatocytes is initiated. Bile acid-mediated hepatic differentiation of MSC-populations involves farnesoid X receptor and transmembrane G-protein-coupled bile acid receptor 1, but requires also supportive activity of notch, hedgehog and transforming growth factor-β family signaling. In contrast to this, β-catenin-dependent canonical Wnt signaling known to counteract non-canonical Wnt cascades inhibits bile acid-initiated hepatic differentiation and represents a mechanism to avoid unnecessary cell differentiation in normal liver.

In conclusion, bile acids represent important signaling molecules during liver regeneration, which can promote hepatic differentiation of adult stem cells such as MSC. Multipotent MSC occur in all organs and are represented by stellate cells in the liver. Since the therapeutic potential of MSC from various sources has become obvious in recent years, research on stellate cells as mediators of liver regeneration should be addressed by future research.
Vascular adhesion protein-1: An adhesion molecule and enzyme that promotes liver inflammation and drives hepatic fibrosis

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Vascular adhesion protein-1 (VAP-1) is an adhesion molecule and membrane-bound amine oxidase that promotes leukocyte recruitment to the liver via a novel enzyme-dependent mechanism. A soluble form (sVAP-1) accounts for most circulating monoamine oxidase activity, has insulin-like effects and can initiate oxidative stress. We have shown that both the membrane bound and soluble forms of VAP-1 are implicated in the pathogenesis of hepatic inflammation and fibrosis. They activate several pathological mechanisms including an amine-oxidase dependent change in the hepatic sinusoidal microenvironment to promote inflammation, direct effects on lymphocyte transendothelial migration and profibrogenic effects on hepatic stellate cells. Particularly high levels of sVAP-1 are detected in in non-alcoholic fatty liver disease (NAFLD) when compared to controls matched for age, sex and metabolic phenotype and levels correlated with histological inflammation, fibrosis and survival. Thus sVAP-1 may be a useful biomarkers of liver inflammation and fibrosis. Thus VAP-1 links hepatic inflammation and fibrosis via amine oxidase-dependent mechanisms suggesting that it is a good therapeutic target in inflammatory liver diseases including NAFLD.
Hippo signaling: An emerging pathway in liver growth, injury and repair

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The Hippo pathway is an evolutionarily conserved tumor suppressor pathway that regulates organ size across multiple tissues. In the liver, Hippo signaling loss triggers rapid cellular proliferation resulting in a gradually increasing mass that often results in an organ that is several times its usual size. Hippo signaling has been directly implicated in liver regeneration and tumorigenesis, particularly hepatocellular carcinoma, cholangiocarcinoma, and hepatoblastoma. YAP and TAZ, the primary target proteins of the Hippo pathway are commonly enriched in these tumors. Potentially, downregulating these molecules or restoring normal Hippo signaling to downregulate YAP/TAZ activity can be important therapeutic cancer targets. We will discuss current strategies and new potential avenues of drug discovery. A large number of signaling inputs into the Hippo pathway have been identified, but how these affect YAP/TAZ activity to regulate what can be interpreted to be disparate phenotypes remain to be clarified. Many questions remain and continue to emerge from the study of this pathway such as, “What is the importance of Hippo pathway zonation in the liver?” or “What drives YAP activity during liver regeneration?” Answers to these and other questions will facilitate a better understanding of disease development and therapy.
The nuclear receptor CAR drives hepatocyte proliferation and tumorigenesis

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The nuclear xenobiotic receptor CAR (NR1I3) is the target for the potent tumor promoting effects of phenobarbital and other non-genotoxic carcinogens in rodent models. Aberrant β-catenin activation contributes to a third or more of human hepatocellular carcinoma (HCC), but β-catenin activation alone is not sufficient to induce liver cancer in mice. Mouse hepatocytes proliferate upon acute activation of either β-catenin or CAR. These responses are strictly limited to an approximate doubling of liver size. They are also tightly linked by the previous demonstration that β-catenin is activated in nearly all of the CAR-dependent tumors generated by phenobarbital. We have found that full activation of β-catenin in the liver induces senescence and growth arrest, which is overcome by combined CAR activation. This combined activation results in uncontrolled hepatocyte proliferation, hepatomegaly and rapid lethality despite maintenance of normal liver function. Combining CAR activation with limited β-catenin activation induces tumorigenesis, and the tumors share a conserved gene expression signature with β-catenin-positive human HCC. This is not consistent with the widely held view that human CAR is not relevant for HCC, based primarily on the lack of epidemiologic evidence for increased liver cancer in phenobarbital treated patients, and on negative results of phenobarbital induction of proliferation in human hepatocytes in vitro and in transgenic mice expressing only human CAR. To directly assess the ability of human CAR to induce proliferation in vivo, we used a chimeric mouse model in which human hepatocytes replace mouse hepatocytes. We found that the highly specific human CAR agonist CITCO induces a strong proliferative response in the engrafted human hepatocytes. Overall, these results reveal an unexpected route for hepatocyte proliferation and support the relevance of the rodent models of tumor promotion to human HCC.
Session III

Contribution of different liver cell populations for liver damage and regeneration
Liver fibrosis: Role of hepatic stellate cells

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Hepatic stellate cells (HSC) are pericyte-like cells that are responsible for storage of retinoids in the healthy liver, but differentiate into myofibroblasts following liver injury. Here, we will present data on their contributions to liver fibrosis and hepatocarcinogenesis. Using LratCre transgenic mice, which label up to 99% of HSC, we demonstrate that HSC represent the main source of myofibroblasts in the injured liver. Surprisingly, HSC were not only the dominant source of myofibroblasts in toxic but also in multiple models of biliary liver injury, both at early and late time points. Using multiple models of liver injury, partial hepatectomy or hepatocarcinogenesis, we did not find evidence for HSC as a source of newly formed hepatocytes or tumor cells. As most hepatocellular carcinomas (HCC) develop in fibrotic livers, we also sought to investigate the contribution of HSC to hepatocarcinogenesis. Using a genetic model of HSC activation, we observed a significant increase in HCC formation in mice with genetically activated HSC. In summary, HSC are key contributors to fibrosis and promote hepatocarcinogenesis, thus making them attractive therapeutic targets for patients with chronic liver injury.
Molecular mechanisms underlying the contribution of liver sinusoidal endothelial cells to liver growth and regeneration

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Zaret and colleagues have demonstrated that endothelial cells are essential for embryonic liver development, and Rafii and colleagues have recently shown that angiocrine signals from the endothelial cells of the liver are required for liver regeneration. Here we investigate the molecular mechanisms by which the endothelial cells respond to changes in blood perfusion taking place during both embryonic liver growth and adult liver regeneration. We demonstrate that liver sinusoidal endothelial cells mechanically respond to an increased blood perfusion, and that this mechano-transduction enhances the expression of angiocrine signals, such as the hepatocyte growth factor and intercellular adhesion molecule-1. Therefore, we provide a new paradigm in that mechanical signals, in particular increases in hepatic blood flow, make endothelial cells produce angiocrine signals strictly needed for liver growth and survival.
Session IV

Stem cells in liver regeneration
Signaling pathways and gene regulatory networks in hepatic cell differentiation

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During embryonic development and regeneration in adult liver, precursor cells differentiate towards hepatocytes or cholangiocytes. Cell fate decisions and differentiation depend on intercellular signaling and on cell-intrinsic gene regulatory networks (GRN) controlled by hepatocyte- or cholangiocyte-specific transcription factors.

Hepatocyte differentiation is controlled by a core of six liver-enriched transcription factors (HNF1a, HNF1b, HNF4a, HNF6, LRH-1, Foxa2) which are organized as cross- and autoregulatory motifs and which regulate hepatocyte functions. During liver development the expression of most core factors progressively rises. This leads to an increased number of cross-regulations among the core factors and stabilizes the hepatocyte GRN. It also enables synergistic regulation of hepatic functions by core factors and co-activators.

We have identified microRNAs that control hepatocyte differentiation and will show how they control the dynamics of core transcription factor expression during development. Furthermore, using data from cultured hepatoblasts and mutant mice, we have generated a quantitative mathematical model of the hepatocyte GRN. This model reproduces the dynamics of core transcription factor expression during development and enabled us to propose targets for microRNA-mediated modulation of the GRN.

Our data impact on the understanding of hepatocyte differentiation and identify candidates that may regulate cell differentiation during hepatic regeneration.
Organ transplantation is the only curative method for treating end-stage organ failure. Over the past decade, there has been increased demand for organ transplantation throughout the world owing to the increased incidence of organ failure. To address such important clinical issue, a critical shortage of donor organs highlights the urgent need for generating organs from human pluripotent stem cells. Here we show the generation of functional human organ from human induced pluripotent stem cells (iPSCs) by transplantation of organ buds created in vitro. Human vasculatures in iPSC-organ bud transplants became functional by connecting to the recipient’s vessels. The formation of functional vasculatures stimulated the maturation of iPSC-organ bud into tissue resembling the adult organ. This method is the alternative way to the generation of functional human organ from pluripotent stem cells. Although efforts must ensue to translate these techniques to treatments for patients, this proof-of-concept demonstration of organ-bud transplantation provides a promising new approach to a critical shortage of donor organs for treating end-stage organ failure.

References:


The utility of human iPS cells for studying hepatogenesis and treatment of Crigler-Najjar syndrome type 1

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We have generated integration-free iPSCs from human fetal foreskin fibroblast cells (HFF1) and differentiated these into hepatocyte-like cells (HLCs) by a three-step protocol-, undifferentiated iPSC to definitive endoderm (DE), to hepatic endoderm (HE) and ultimately HLCs. The HLCs were fully characterized biochemically for glycogen storage, ICG uptake and release, urea production, and CYP3A4 activity. Ultrastructure analysis by electron microscopy revealed the presence of lipid and glycogen storage, tight junctions and bile canaliculi – all typical features of hepatocytes. Furthermore, the transcriptome of the HLCs are much closer to that of fetal liver than adult primary hepatocytes (PHH) though they express both AFP and albumin. For liver disease-related cell replacement therapies, expansion of HLCs to numbers necessary for transplantation is crucial and can be a stumbling block. To circumvent this, the generation of MSCs from induced pluripotent stem cells (iMSCs) has been reported as a possible solution. To this end we induced pluripotency in human bone marrow-derived MSCs from fetal femur (55 days post conception), these and the embryonic stem cell line-H1 were then differentiated into iMSCs and transplanted into the spleen of GUNN rats (without immunosuppression) after partial hepatectomy. After a regeneration period of 7 days to 2 months, livers were extracted, molecular and biochemical (secretion of human albumin and reduced levels of bilirubin) analyses provided evidence of trans-differentiation of the iMSCs into hepatocytes. In summary, we have shown the utility of iPS cells for studying hepatogenesis and the potential of iMSCs as an alternative cell source for liver regeneration and treatment of Crigler-Najjar syndrome type 1 which is characterized by increased levels of unconjugated bilirubin in plasma due to a mutation within the UGT1A1 gene which encodes hepatic uridine 5'-diphospho-glucuronosyltransferase for which the Gunn rat serves as an in vivo model of this disease.
Essential roles of FGF receptor and integrin signaling in liver regeneration

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The liver has a unique regenerative capability, which involves signaling via different receptors for growth factors and extracellular matrix proteins. To study the role of the latter and their interaction with growth factor signaling, we used Cre/loxP-mediated gene deletion or intravenous delivery of siRNA formulated into nanoparticles to ablate β1-integrin expression in hepatocytes of mice. While short-term loss of β1-integrin was not detrimental for the non-challenged liver, severe liver necrosis and reduced hepatocyte proliferation were observed after partial hepatectomy. This resulted from impaired ligand-dependent phosphorylation of the epidermal growth factor and hepatocyte growth factor receptors and down-stream signaling in vitro and in vivo. These results identify a crucial role and novel mechanism of action of β1-integrins in liver regeneration. We also studied the role of fibroblast growth factor receptor (FGFR) signaling in the regenerating liver using the same experimental approach. siRNA-mediated knock-down of FGFR4 severely affected liver regeneration due to impairment of hepatocyte proliferation combined with liver necrosis. The proliferation defect resulted from inhibition of an FGF15-FGFR4-STAT3 signaling pathway, which is required for injury-induced expression of the FOXM1 transcription factor and subsequent cell cycle progression, while elevated levels of intrahepatic toxic bile acids were identified as the likely cause of the necrotic damage. Most importantly, we showed that knock-down of FGFR4 in mice lacking FGFR1 and FGFR2 in hepatocytes caused liver failure after partial hepatectomy due to severe liver necrosis and a defect in regeneration. These results demonstrate that FGFR signaling in hepatocytes is essential for liver regeneration and suggest activation of FGFR signaling as a promising approach for the improvement of the liver’s regenerative capacity.
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POSTER ABSTRACTS

Poster Numbers 1 – 48

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Mitochondrial DNA damage analysis in hepatocellular carcinoma progression by ultra-deep sequencing of the entire mitochondrial genome

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Due to the lack of protective histones and limited DNA repair activity, the mitochondrial (mt) genome is very susceptible to oxidative damage, leading a high mt-DNA mutation rate. In order to identify the intratumor heterogeneity, mt-DNA alterations can therefore be used for tumor tracking and evolution analysis. Here, we designed a unique approach based on ultra-deep sequencing of mt-DNA to seek how hepatocellular carcinoma (HCC) originate, develop and progress.

In total, 48 HCC nodules representing different tumor grades, and adjacent non-cancerous tissues were macrodissected. DNA was used for automatic single PCR set-up with newly designed primers. Target enriched libraries of mtDNA were deeply sequenced and NGS data interpreted by the CLC Software.

Whole mt-genome analysis revealed a wide spectrum of mt-alterations, additionally validated by Sanger sequencing. High frequency of variants was observed in the D-Loop region and in respiratory chain complex genes.

Interestingly, a wide spectrum of mt-variants was identified in HCC nodules of non-cirrhotic origin. Furthermore, mt-variants occur not only in HCC nodules but also in peri-tumorous parenchymal foci suggesting that mitochondrial genome is susceptible at earliest stage of hepatocarcinogenesis.

In particular, most tumor nodules harbor identical mutations indicating a monoclonal origin of HCC. The increasing numbers of mt-variants as well as the increasing frequency of a particular mt-hot-spot mutation refer to the progression of the HCC dedifferentiation.

In conclusion, screening of mitochondrial genome by ultra-deep sequencing is a reliable and useful molecular tool to identify pre-tumorous nodules with high transformation potential and to illustrate tumor clonality and history.
Tauroursodeoxycholic acid activates the ubiquitin-proteasome system in hepatitis B transgenic cells

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Introduction: Accumulation of HBV envelope proteins (HBs) in the endoplasmic reticulum (ER) of hepatocytes in patients suffering from chronic HBV infection may cause a direct, intrinsic mechanism of cell damage. “Unfolded protein response”, ER-Stress, and apoptosis could be the outcome of intracellular HBs accumulation. The ubiquitin-proteasome system and molecular chaperones represent an intracellular quality control for the correct folding of proteins. The aim of the present study was to investigate the effect of the chemical chaperone Tauroursodeoxycholic acid (TUDCA) on the ubiquitin-proteasome system in HBs transgenic cells.

Methods: We overexpressed HBs in NIH 3T3 cells in order to study the effect of TUDCA. Cell culture experiments were analyzed by immunohistochemistry and Western blot.

Results: TUDCA caused an increase in ubiquitination and a decrease of intracellular HBV envelope proteins. The decreased of intracellular aggregation of HBV envelope proteins by TUDCA was skipped by inhibition of the proteasome. TUDCA reduces cell stress by the activation of the ubiquitin-proteasome system.

Discussion/Conclusion: TUDCA affects the ubiquitin-proteasome system. The identified properties of the results of our study increased the knowledge about cellular pathophysiology induced by HBV surface proteins and storage diseases (“ER storage disease”). TUDCA might be important for the development of new therapeutic options for the treatment of protein storage disease.
Role of new pathways in liver regeneration after acute and chronic liver damage

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Introduction: The liver is a vital organ that performs many biological functions (e.g. synthesis of bile and blood proteins, detoxification, glycogen storage, innate immunity). Interestingly, the liver also has a unique ability to regenerate following the loss of liver mass. Loss of at least 30% of liver mass, leads to synchronized proliferation of mature hepatocytes and rapid restoration of liver mass via compensatory hyperplasia. When a liver has recovered after chronic damage caused by e.g. viral infection the whole complex architecture of the liver must be restored. Hepatic stellate cells (HSCs) are mainly known for their contribution to fibrogenesis in chronic liver diseases, but their role and function in liver regeneration remains unclear. In summary, both processes are orchestrated by distinct signaling cascades involving components of the innate immune system, cytokines, bile acids and growth factors.

Methods: For this reason, our studies aim to investigate the role of different signaling pathways and factors that are essential in both, acute and chronic liver damage. To analyze the dynamic processes during liver regeneration, we performed two common mouse models: PHx (acute liver damage) and BDL (chronic liver damage) in different Knockout-mice with deficiencies in innate immune responses. Using kinetics we examined the gene expression profile of liver tissue after PHx and BDL. H/E-staining of liver sections and cytokine ELISAs were performed and serum protein levels and bile acid concentrations were analyzed.

Results/Conclusion: Taken together, we have found new factors that may be essential for liver regeneration after acute and chronic liver damage, however, their precise role in liver regeneration has to be fully elucidated.
Alpha5beta1 integrins are receptors for bile acids with a (nor-) ursodeoxycholane scaffold

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Introduction: Integrins are ubiquitously expressed cell adhesion receptors and the most prevalent bidirectional signaling molecules on the cell surface. A recent study combined immunofluorescence staining (IFS) experiments and molecular dynamics (MD) simulations to identify taouroursodeoxycholic acid (TUDC) as potent agonist of alpha5beta1 integrins in hepatocytes. Activation of alpha5beta1 leads to choleresis by FAK/c-Src/MAPK dependent signaling events. TUDC-induced integrin activation and subsequent signaling is sensitive to inhibition by the trihydroxylated taurocholic acid (TC), which tightly binds to alpha5beta1 in MD simulations. However, effects of other bile acids on alpha5beta1 integrin activation have not been investigated at the molecular level.

Methods: Molecular dynamics (MD) simulations were used to predict conformational changes associated with integrin activation. Results from MD were compared to immunofluorescence stains of the active beta1 integrin subunit in rat liver slices after perfusion with nor-ursodeoxycholic acid (norUDCA), tauro-nor-ursodeoxycholic acid (TnorUDCA), glycoursoxycholic acid (GUDC) and ursodeoxycholic acid (UDCA).

Results: Our results indicate that alpha5beta1 integrins are not exclusively activated by TUDC. Nor-ursodeoxycholic acid (norUDCA), a side chain-shortened homologue of UDCA, induces conformational changes in the βA domain of alpha5beta1 similar to the ones evoked by TUDC, but overall less pronounced. Conformational changes observed with TnorUDCA and glycoursoxycholic acid (GUDC) were significantly less pronounced. Unconjugated UDCA, similar to the inhibitory TC, only showed insignificant alterations in the structure of the integrin ectodomain and was considered fully inactive. A ranking based on the extent of structural changes observed during the MD simulations correlates with results from IFS experiments on the efficacy of the bile acids.

Discussion/Conclusion: These results indicate that norUDCA activates alpha5beta1 integrins and that MD simulations are able to predict different degrees of bile-acid induced integrin activation. Minor structural changes in the bile acids strongly influence their efficacy.
Ad5-CMV-CCN gene transfer in primary liver cells induce endoplasmic reticulum stress-related cellular apoptosis

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Introduction: The endoplasmic reticulum (ER) is primarily recognized as the site of synthesis and folding of secreted, membrane-bound, and some organelle-targeted proteins. Several factors are required for optimum protein folding, including ATP, Ca2+ and an oxidizing environment to allow disulphide-bond formation. The ER is highly sensitive to stress factors that perturb cellular energy levels, the redox state or Ca2+ concentration. Such stresses reduce the protein folding capacity of the ER, which results in the accumulation and aggregation of unfolded proteins, a condition referred to as ER stress. Matricellular proteins of the CCN (CYR61, CTGF, NOV) protein family play crucial roles in extracellular matrix (ECM) signaling and turnover [1]. The six known CCN proteins exhibit a similar type of organization and share a closely related primary structure including a series of 38 conserved cysteine residues. They need proper disulphide-bond formation and protein folding before secreted to the extracellular space. We have recently shown that CCN1/CYR61 overexpression in hepatic stellate cells induces ER stress-related apoptosis [2].

Methods: We here investigated whether adenovirus-mediated gene transfer of other CCN members incur ER stress in primary HSC, portal myofibroblasts, and hepatocytes.

Results: We found Ad5-CMV-CCN2, -CCN3 and -CCN4 to induce ER stress or unfolded protein response (UPR) comparable to Ad5-CMV-CCN1. The UPR is a pro-survival response to reduce the accumulation of unfolded proteins and restore normal ER functioning. However, if protein aggregation is persistent and the stress cannot be resolved, signaling switches from pro-survival to pro-apoptotic.

Discussion/Conclusion: We conclude that the observed CCN-induced UPR is relevant in wound healing response and necessary for hepatic tissue regeneration following liver injury.

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Differential modulation of vesicular and non-vesicular associated microRNAs isolated from sera of partially hepatectomized rats

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Introduction: Cell-free circulating microRNAs are protected from degradation by their association with either component of the RNAi machinery or vesicles. Although increasing evidences shows that different types of vesicles are capable of transporting microRNAs, current research mainly focuses on the characterization of exosomal microRNAs. Reason for this is that exosomal-microRNAs are thought to directly participate in intercellular communication. However, is yet unclear whether exosomal-microRNAs are also the most reliable source for discovering disease-associated biomarker. In this study, the distribution of circulating microRNAs associated to either the vesicular or non-vesicular fractions of sera isolated from partially hepatectomized rats was measured.

Methods: The aim of this study was to evaluate to which extent the levels of vesicular and non-vesicular miRNAs are modulated in sera of animals recovering from partial hepatectomy (PHx). To circumvent obvious limitations associated to ‘standard’ vesicle isolation protocol (i.e., long preparative intervals and low throughput sample number), a combination of nanoparticle-tracking analysis (NTA) and miRNA specific PCR (miQPCR) was used to measure and characterize the size and distribution of vesicles isolated under different conditions.

Results: Here we show that independently from their origin, levels of cell-free miR-122, miR-192, miR-194 and Let-7a are upregulated two days after partial hepatectomy. The inflammation-associated miR-150 and miR-155 are upregulated in the vesicular-fraction only, while the regeneration-associated miR-21 and miR-33 are upregulated in the vesicular- and downregulated in the non-vesicular fraction.

Discussion/Conclusion: Our study shows for the first time the modulation of microRNAs contained in the non-vesicular fraction. Overall, these findings suggest that, in the search for novel disease-associated biomarkers, the investigation of either vesicular or non-vesicular microRNAs may be more informative compared to the analysis of microRNAs isolated from unfraccionated serum.
In 374 unrelated patients with suspected intrahepatic cholestasis, FIC1, BSEP, and MDR3 sequencing revealed 135 different genetic variants

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Introduction: Familial intrahepatic cholestasis 1 (FIC1; ATP8B1), the bile salt export pump (BSEP; ABCB11), and multidrug resistance protein 3 (MDR3; ABCB4) are essential for bile formation. Mutations in these transporters can lead to cholestatic liver diseases of different severity ranging from intrahepatic cholestasis of pregnancy (ICP), benign recurrent intrahepatic cholestasis (BRIC) or low phospholipid-associated cholelithiasis (LPAC) to progressive familial intrahepatic cholestasis (PFIC).

Methods: To confirm diagnosis 374 blood samples of unrelated patients with cholestasis of varying manifestation were obtained. All coding exons with adjacent intron regions of either ATP8B1, ABCB11 or ABCB4 were analyzed by sequencing.

Results: In 119 patients with assumed FIC1 deficiency, 27 variants comprising 6 new ones were detected. DNA sequencing from 187 patients with putative BSEP mutations revealed 70 different variants, 36 of them new. MDR3 analysis in 171 cases identified 38 genetic variants including 18 novel ones. In a number of patients, only one heterozygous mutation (FIC1: 4/119, BSEP: 31/187, MDR3: 27/171), common polymorphisms or synonymous variants were found, which cannot completely explain a severe cholestatic phenotype but can provide the basis for milder forms like BRIC, ICP or LPAC. In patients with no other mutation, the common FIC1 variants c.3531+8G>T or p.R952Q were detected in 39/105 specimen. Both BSEP polymorphisms p.V444A and p.A1028A were shown in 75/108 samples. All three typical synonymous MDR3 variants p.L59L, p.N168N, and p.I237I appeared in 24/116 cases.

Discussion/Conclusion: In this patient population, 135 genetic variants were detected. Numerous cases carry only one heterozygous or even no mutation demonstrating that other genes like TJP2, as described by Sambrotta and colleagues, as well as non-genomic factors contribute to the phenotype of some of these patients. Here, we focused on the common FIC1, BSEP, and MDR3 variants, which possibly have a significant effect on the manifestation of a cholestatic phenotype.
MAPKAP kinases 2 and 3 control the initiation as well as the resolution of the inflammatory response of macrophages

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Introduction: Via the blood flow the liver is frequently exposed to pathogens, thereby it plays a pivotal role in innate and adaptive immunity. A large pool of tissue macrophages reside in the liver. These macrophages are important for the elimination of opsonized immune complexes by phagocytosis and they are also sources of inflammatory cytokines, which induce the production of acute phase proteins in hepatocytes. Within macrophages the expression of inflammatory cytokines is tightly controlled by a comprehensive network of regulatory molecular pathways. On the hand the involved mechanisms should ensure the initiation of the inflammatory process, but on the other hand they should also guarantee its resolution, therefore counteracting an overwhelming inflammatory response. The MAPKAP kinases (MK)2 and 3, which are activated by the MAP kinase family member p38MAPK, are essential for the coordination of the inflammatory response of macrophages. They regulate the expression of several cytokines upon contact with viral as well as bacterial components like the TLR4-activating molecule lipopolysaccharide (LPS). Moreover, for the control of transcriptional or post-transcriptional regulation as well as for counterregulating mechanisms in terms of a negative feedback, many mediators are essential, among others signal transducer and activator of transcription (STAT)3, suppressor of cytokine signaling (SOCS)3, dual-specificity phosphatase (DUSP)1, Inhibitor of NFκB (IκB)β or tristetraprolin (TTP). Conditional or complete deletion of these molecules leads to a high susceptibility towards septic shock or liver damage. The impact of the interplay of MK2 and MK3 on a variety of negative feedback mediators is largely unknown.

Methods: For the experiments bone marrow derived macrophages were used and stimulated with LPS. RNA and protein was isolated and subjected to rtPCR or immunoblot analysis respectively.

Results: We observed, that in the context of the inflammatory response towards LPS the interplay of MK2 and MK3 controls the protein stability of IκBβ, which regulates NFκB-mediated expression of IFN-β. Sustained IL-10 expression is dependent on IFN-β synthesis and both activate STAT3. Furthermore, we suggest that MK2 utilizes TTP to regulate mRNA-stability of IL-10, which therefor leads to STAT3-mediated expression of SOCS3 and DUSP1. In summary, MK2 and MK3 exert not only direct, but also indirect effects on the negative feedback regulation mainly mediated via IL-10.
**Discussion/Conclusion:** Understanding the molecular mechanisms of LPS-induced regulation of the macrophage’s response is important as they suggest new potential targets for therapeutic interventions in the context of inflammatory diseases. Our observations reveal that the interplay of MK2 and MK3 not only controls the function of molecules that maintain the expression of inflammatory cytokines, it further controls the function of negative feedback mediators, which are important for the resolution of the inflammatory response.
Analysis of different histone deacetylase isoforms in hepatocellular carcinoma and hepatic stellate cells

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Introduction: Histone deacetylase (HDAC) inhibition is a promising option for treatment of various metabolic or neurodegenerative diseases. More recently, HDAC inhibition appeared as therapeutic strategy in hepatic fibrosis and the development and progression of hepatocellular cancer (HCC). Still, mostly unspecific HDAC inhibition has been applied and the knowledge about the expression and role of individual HDACs during the progression of chronic liver disease is very limited. The aim of this study was to systematically analyze the expression of HDAC families class I (HDAC1/2/3/8), IIa (HDAC4/5/7/9), IIb (HDAC6/10) and IV (HDAC11) in liver steatosis, fibrosis and hepatocellular carcinoma.

Methods: HDAC expression levels were analyzed in primary hepatocytes, 4 different human HCC cell lines (Hep3B, HepG2, PLC, HuH7) by qPCR. Furthermore, primary hepatic stellate cells (HSC) and hepatic tissues from different murine fibrosis models were analyzed in comparison to control liver tissue.

Results: Expression levels of all 4 HDAC classes were significantly increased in HCC cells compared to primary hepatocytes. In primary HSC we observed an upregulation of all HDAC classes during the course of in vitro activation. HDACs class IIa showed the most prominent increase. Similarly, HDAC levels were increased in fibrotic liver tissue. Here, class I and IIa showed the highest increase. To imitating anaerobic tumor environment in vitro, Hep3B and HepG2 cells were cultivated for 24 h under hypoxia (< 0.1% oxygen) resulting in a strong upregulation of all HDAC expression levels. Especially, class IIa HDACs 5 and 7 and Class IV HDACs revealed a significant increase.

Discussion/Conclusion: During liver disease HDAC expression levels appear to generally increase. There are differences with regards to cell type, activation level and different HDAC classes. The reported changes in HDAC concentrations in HCC cells and HSCs can contribute to the understanding of the role of different HDACs in the pathology of hepatic fibrosis and the development and progression of hepatocellular carcinoma.
Determining the molecular consequences of clinically relevant glutamine synthetase mutations

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Introduction: Glutamine synthetase (GS) catalyzes the conversion of ammonia and glutamate to glutamine and, thus, is essential for nitrogen metabolism. Loss of hepatic GS activity has been linked to serious clinical conditions. In particular, two mutations of human GS (R324C and R341C) were connected to congenital glutamine deficiency resulting in neonatal death. Another GS mutation (R324S) was identified in a neurologically compromised patient. However, the underlying molecular mechanisms of GS deactivation by these mutations remain unclear.

Methods: First, molecular dynamics simulations, free energy calculations, and rigidity analyses we used to investigate the molecular effects of the GS mutants. Subsequently, after GS overexpression in HEK293 cells, dot-blot analyses were used to further corroborate our findings.

Results: We found that all mutations influence ATP binding, the first step of GS glutamine formation. In the case of the R324S/C mutants, we found a loss of direct interactions that hampers ATP binding. Remarkably, in the case of the R324S mutant, we observed water-mediated interactions with ATP that reduce this effect and may explain the suggested higher GS activity. The R341C mutation, first, destabilizes residue R340 that is important for ATP binding and, second, was predicted to result in a significant destabilization of helix H8, which should hamper glutamate binding. We introduced an additional GS variant through alanine mutagenesis of amino acids interacting with R341, mimicking the loss of interactions in the R341C mutant. Dot-blot analyses revealed that the structural stability of H8 was impaired in the case of the newly introduced GS mutant. This results in a loss of masking of the epitope in the glutamate binding pocket for a monoclonal anti-GS antibody by L-methionine-S-sulfoximine.

Discussion/Conclusion: Our analyses show complex molecular effects underlying GS deactivation in clinically relevant mutants. Furthermore, our findings could stimulate the development of ATP binding-enhancing molecules by which the R324S mutant can be “repaired”.
Mutational mapping of the transmembrane binding site of the G-protein coupled receptor TGR5 and binding mode prediction of TGR5 agonists

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Introduction: TGR5 is the first bile acid sensing G-protein coupled bile acid receptor (GPCR) GPCR and directly interacts with several G-protein subtypes. High expression levels of TGR5 are found in the brain, the liver, and the gastrointestinal tract. TGR5 is an emerging target for the treatment of metabolic diseases. Therefore, developing selective and potent agonists of TGR5 is of high importance. However, without an x-ray crystal structure or an experimentally determined binding mode, the rational design of compounds is difficult.

Methods: Our strategy includes multi-template homology modeling, molecular docking, and structure-based 3D-QSAR with subsequent mutational analysis and molecular dynamics simulations.

Results: Here, we present an experimentally validated binding mode of 68 natural and synthetic bile acids and neurosteroids with agonistic activity towards TGR5. After application of this strategy, our binding mode model of the 68 TGR5 agonists results in a good 3D-QSAR model ($q^2 = 0.50$), thus indicating that differences in the agonist structures correlate with differences in experimentally determined pEC$_{50}$ values. Subsequently, nine mutants of binding site residues were suggested for experimental validation of the binding mode. Activity analysis with functional read-out and FACS analysis for membrane localization confirmed these predictions in all cases.

Discussion/Conclusion: Hydrogen bonding between Y240 and TGR5 agonists play an important role, which could be shown by a severe impairment of receptor activity upon Y240F mutation. Additionally, we identified the epimer selectivity determining residue Y89 for hydroxyl-groups in position seven on the cholane scaffold. This provides strong support to the validity of the binding mode. Through this, our binding mode could ease the structure-based design of new TGR5 agonists.
Evidence for mesenchymal stem cells in liver regeneration

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Introduction: The liver is known for its high regenerative potential, which is normally achieved by induction of hepatocyte proliferation and leads to an efficient recovery of acute liver injury. When the hepatocyte proliferation is impaired the liver can regenerate by an alternative stem-cell based mechanism, which employs the proliferation and differentiation of liver progenitor cells, so-called oval cells, to hepatocytes. But recent lineage-tracing experiments in mouse challenged this view and showed only a minor contribution of stem cells to liver repair. To address this controversy, we searched for hints of stem cell activity during rat liver regeneration.

Methods: Hepatocyte-based liver regeneration was induced by 70% partial hepatectomy (PHx) and stem-cell based liver regeneration by 2-acetaminofluorene (2AAF) treatment prior to PHx. Stem/progenitor cell-associated factors were analysed by quantitative real-time PCR and immunofluorescence staining during the first two weeks of liver regeneration.

Results: Quantitative real-time PCR analysis revealed not only an up-regulation of oval cell marker (CK19, Epcam) but also an increase of diverse mesenchymal stem cell (MSC) factors like CD90, CD140B, CD146 and CD248 during stem-cell based liver regeneration (2AAF/PHx), indicating an enhanced MSC activity during this process. Especially factors of the PDGF signaling like the ligands PDGFb and PDGFd as well as their receptor PDGFRb/CD140B showed strong differences in the two models and were associated with the ductular reaction during stem-cell based liver regeneration. In contrast, hepatocyte-based liver regeneration only showed minor changes in the expression of MSC associated-factors during liver regeneration.

Discussion/Conclusion: Comparative analysis of different liver regeneration models in rats revealed an increased expression of MSC marker in the stem-cell based liver regeneration and led to the identification of the PDGFRb pathway, which might be important for this process. Further analysis are required to gain more insight into the function of MSC during liver regeneration.
Modelling of human non-alcoholic fatty liver disease with hepatocyte-like cells derived from pluripotent stem cells

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Introduction: Non-alcoholic fatty liver disease (NAFLD) is an increasingly common diagnosis in the Western Hemisphere. It is defined by an accumulation of lipid droplets in more than 5% of hepatocytes. In the beginning the disease is rather benign, but later on patients develop steatohepatitis, cirrhosis and up to 27% of these patients end up with hepatocellular carcinoma. The molecular reasons of this disease are still questioned, but it is well-recognized that NAFLD is strongly associated with obesity and insulin resistance. In this metabolism-based field of research, results obtained from rodent model systems cannot be easily extended to humans as both organisms differ in their metabolisms. Unfortunately, liver cells from steatosis patients are very rarely available and not suitable for longer experiments as hepatocytes rapidly dedifferentiate in culture.

Methods: We have established a human model system for NAFLD based on hepatocyte-like cells (HLCs) generated from pluripotent stem cells. We are able to induce the accumulation of lipid droplets (LDs) in these cells by adding oleic acid (OA) into the medium. LD formation has been documented by staining with Oil Red O or BODIPY.

Results: After fat induction with OA the expression of PLIN2, a protein covering LDs, was consistently up-regulated. As PLIN2 knockout mice are protected against the development of steatosis, we selected PLIN2 expression as a molecular marker for the successful induction of steatosis. We thoroughly investigated the consequences of LD accumulation on the level of gene expression. We found that many GO categories related to lipid, glucose and sterol metabolism were up-regulated in HLCs after OA induction. Interestingly, many members of the Peroxisome proliferator-activated receptor (PPAR) pathway, which is important for the regulation of lipid metabolism, were up-regulated after fat induction. Modelling PPARα in HLCs with small molecules resulted in profound gene expression changes. Inhibition of PPARα with GW6471 resulted in down-regulation of genes involved in lipid catabolism, while activation via fenofibrate reduced expression of AGPAT2 and HMGCR, which are involved in biosynthesis of phospholipids and cholesterol, respectively. Also insulin signaling was affected by PPARα modulation.

Discussion/Conclusion: Although obesity and NAFLD are increasing health problems worldwide, there is no specific treatment for NAFLD at the moment. Our HLC-based NAFLD model can be used in the future to screen for drugs that might interfere with LD accumulation.
Ammonia inhibits LPS-induced inflammation in cocultured astrocytes and microglia

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Introduction: Hepatic encephalopathy (HE) in liver cirrhosis is associated with microglia activation but not with neuroinflammation in brain. In the present study we analyzed effects of ammonia, a main toxin in HE, on lipopolysaccharide (LPS)-induced microglia activation and cytokine production in mono- and cocultured microglia and astrocytes.

Methods: Gene expression levels were quantified by real-time PCR and cytokine concentrations were measured by Luminex® Multiplex cytokine assays.

Results: LPS (100 ng/ml, 18 h) strongly enhanced the transcription of the microglia activation marker CD14 and the cytokines interleukin (IL)1α/β, IL6 and TNFα in cultured microglia. Cotreatment with NH₄Cl (5 mmol/l) had no effect on LPS-induced upregulation of CD14, IL1α/β and IL6 mRNA but enhanced LPS-induced transcription of TNFα. In cocultured microglia and astrocytes LPS-induced upregulation of CD14, IL1α/β IL6 but not of IL-10 was inhibited by cotreatment with NH₄Cl. MCP1, TNFα, IL1α/β, IL6 and IL10 protein level quantified in the supernatant differed between LPS-treated mono- and cocultured astrocytes and microglia. LPS-treatment strongly enhanced MCP1 and IL6 protein levels in the supernatant of astrocytes and of IL1α/β, IL6 MCP1 and TNFα in the supernatant of cultured microglia. Cotreatment of microglia with NH₄Cl inhibited LPS-induced upregulation of MCP1, IL6 and IL10 but had no effect on MCP1, TNFα, IL1α/β IL6 and IL10 level in the supernatant of microglia cocultured with astrocytes.

Discussion/Conclusion: Ammonia inhibits LPS-induced microglia activation and transcription of pro-inflammatory cytokines in cocultured astrocytes and microglia. This anti-inflammatory effect of ammonia might relate to recent findings indicating enhanced microglia activation but unchanged brain cytokine production in cirrhotic patients with HE.
Therapeutic effects from modulation of the cannabinoid receptor 1 signaling

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Introduction: The endocannabinoid system is involved in the development and control of chronic inflammatory liver disease. Previous studies, e.g. in mouse models of toxic liver injury, could demonstrate an antifibrotic effect of cannabinoid receptor 1 (CB1) antagonism. Our studies aimed to show the potential profibrotic effect of CB1 agonist ACEA and the potential antifibrotic effect of CB1 antagonist rimonabant in the Abcb4−/− mouse model.

Methods: After weaning, male Abcb4−/− mice where treated with ACEA and rimonabant until the age of 16 and 52 weeks. Liver, serum and abdominal fat were isolated and analyzed by serum analysis, microarray, RT-PCR, Western blot and immunohistochemistry. As a control we used Abcb4−/− and Balb/C wild type mice.

Results: Microarray analysis revealed that approximately 60% of the genes which were regulated due to the Abcb4-knockout, showed a wild type-like level in the liver after treatment. ACEA treatment as well as rimonabant ameliorated liver integrity (GPT) and fibrosis (hydroxyproline). Focusing on the metabolic level, similar effects of agonism and antagonism were observed (PPARα, PPARγ, PEPCK, FASN, triglyceride measurements in serum und liver).

Discussion/Conclusion: Interestingly, both stimulation and antagonism of the CB1 receptor have protective effects on the fibrotic liver in Abcb4−/− mice. These results will be subject for future studies in order to understand the underlying mechanisms.
Interplay of insulin, cytokines and prostaglandin E$_2$ in the development of hepatic insulin resistance and NAFLD

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Introduction: Hepatic insulin resistance and NAFLD are hepatic hallmarks of the metabolic syndrome. Insulin resistance and steatosis are interlinked and are most likely caused by a complex interplay between metabolic, hormonal and inflammatory signals. Thus, affluent fatty acids together with a persistent hyperinsulinemia may favor lipid accumulation in hepatocytes and a low grade inflammation may expose the hepatocyte to elevated levels of cytokines and small lipid mediators, amongst others prostaglandin E$_2$ (PGE$_2$). It was the goal of this study to unravel the role of PGE$_2$ in the development of hepatic insulin resistance and steatosis.

Methods: Gene expression in liver samples of genetic and diet-induced obesity mouse models and human biopsies as well as cell culture samples were analyzed by RT-qPCR. Cytokine levels as well as insulin and cytokine receptor signal chain activities were monitored by western blot with phospho-specific antibodies. Metabolite levels and flux rates were determined biochemically or with radioactive tracers.

Results: In a large number of genetic and diet-induced mouse obesity models as well as in humans with NASH key inducible enzymes of PGE$_2$ synthesis, namely cyclooxygenase 2 and microsomal PGE synthase 1, were induced, implying that PGE$_2$ levels might be elevated in NAFLD. PGE$_2$ was shown to directly impair insulin signaling in rat and human hepatocytes binding to EP3 and EP1/EP4 receptors, respectively via an ERK1/2-dependent inhibitory serine phosphorylation of the insulin-receptor substrate. Thereby, PGE$_2$ impaired the insulin-dependent incorporation of glucose into glycogen. At the same time, PGE$_2$ inhibited β-oxidation and VLDL-secretion and thereby enhanced insulin-dependent lipid accumulation in hepatocytes. In addition, PGE$_2$ enhanced the release of cytokines from macrophages and Kupffer cells. These cytokines in turn impaired insulin signaling in hepatocytes. Cytokines induced PGE$_2$-synthesising enzymes in Kupffer cells and macrophages and the cytokine-mediated impairment of insulin signaling was further aggravated by the simultaneous presence of PGE$_2$. Cytokines, in particular IL-1β, were released from macrophages also by prolonged exposure to high concentrations insulin independently of other stimuli, indicating the hyperinsulinemia per se might contribute to the low grade inflammation found in the liver of insulin-resistant patients.

Discussion/Conclusion: The results indicate that cytokines and PGE$_2$ might cooperate to drive hepatic insulin resistance and NAFLD by mutual induction of their formation and synergistic impairment of insulin signaling in hepatocytes. Hyperinsulinemia, which results from the body's attempt to compensate peripheral insulin resistance, might trigger cytokine production independently of other signals, in particular in the portal circulation where much higher insulin concentrations prevail than in the systemic circulation.
Development of a new modified western diet to induce NASH with obesity and insulin resistance in mice

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Introduction: The alarming increase of obesity becomes a major global health issue. Obesity is often associated with insulin resistance, type II diabetes and non-alcoholic fatty liver disease (NAFLD) and may result in the metabolic syndrome. The diet is one of the most relevant contributing factors to obesity, but in rodent feeding studies the applied high-fat-diets often did not induce the same phenotype like in human metabolic syndrome. Here we designed a new modified western diet that caused obesity, insulin resistance and NASH in mice.

Methods: Male C57BL/6 mice were fed chow, high-fat-diet (HFD) (25 g/100 g lard) or modified western-diet (mWD) containing high fat (25 g/100 g soybean-oil) and cholesterol for 20 weeks.

Results: Mice fed a mWD significantly gained weight and increased their body fat mass 2.5-fold compared to chow fed mice. In an oral glucose intolerance test mice fed mWD or HFD were glucose intolerant with slightly increased insulin levels. In comparison to chow fed animals, serum parameters for liver inflammation like ASAT, ALAT and cholinesterase were elevated after feeding a mWD but not after feeding a HFD. Histological scoring of the liver revealed steatohepatitis (NASH) in mWD-fed mice and only steatosis without inflammation in HFD-fed mice. Gene expression analysis detected an up-regulation of chemokines (CCL2), pro-inflammatory cytokines (IL-1\textbeta, TNF\textalpha) and immune cell infiltration (CD68) in livers of mWD-fed, but not HFD-fed mice. Still, both mWD and HFD feeding was accompanied by enhanced expression of markers of hepatic insulin resistance (PTP1B, FGF21).

Conclusion: In contrast to mice, which receive a HFD based on saturated fatty acids, mice fed a mWD with high amounts of unsaturated fatty acids and cholesterol developed obesity, insulin resistance and hepatic inflammation. Mice fed a mWD therefore are a potential better model for human metabolic syndrome and NASH than mice fed a HFD.
Ammonia induces oxidative stress through NADPH oxidase 4 in cultured rat astrocytes

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Introduction: Hepatic encephalopathy (HE) is a neuropsychiatric syndrome frequently accompanying acute or chronic liver dysfunction. HE in liver cirrhosis is seen as a consequence of a low grade cerebral edema (Häussinger et al. Gastroenterology. 1994;107:1475–80) which is associated with cerebral oxidative/nitrosative stress (Görg et al. Hepatology. 2010;52:256–65) and senescence (Görg et al. Glia. 2015;63: 37–50). The current study investigated effects of ammonia on NADPH-oxidase (NOX) 4 expression and analyzed the role of NOX4 for ammonia-induced oxidative stress and senescence in cultured rat astrocytes.

Methods: NOX4 and Growth Arrest and DNA Damage (GADD)45α mRNA level were quantified by real-time PCR. NOX4 protein expression and the oxidative stress marker oxidized DNA/RNA (8-oxo-(deoxy)-guanosine/8OH(d)G) were detected by immune-fluorescence analysis. Astrocyte proliferation was quantified by fluorimetric detection of Hoechst34580 fluorescence.

Results: NH4Cl (5 mmol/l) strongly increased nuclear anti-NOX4 immunoreactivity and concurrently downregulated NOX4 mRNA level after 24, 48 and 72 h in cultured rat astrocytes. As shown by immunofluorescence analysis, anti-8OH(d)G immunoreactivity strongly increased 24, 48 and 72 h after treating cultured astrocytes with NH4Cl (5 mmol/l). Both, NH4Cl-induced upregulation of NOX4 as well oxidation of RNA was sensitive against glutamine synthetase inhibition by methionine-sulfoximine. siRNA-mediated downregulation of NOX4 prevented NH4Cl-induced upregulation of anti-8OH(d)G immunoreactivity as well as enhanced transcription of the senescence biomarker GADD45α in cultured rat astrocytes.

Discussion/Conclusion: The data suggest that NOX4 is involved in NH4Cl-induced oxidative stress and senescence in cultured rat astrocytes. Further studies are required to analyze the role of NOX4 for the pathogenesis of hepatic encephalopathy.
Effects of lactic acid on primary hepatocytes *in vitro*

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**Introduction**: Tumor cells utilize glycolysis instead of oxidative phosphorylation for energy production, even under oxygen-rich conditions, the so-called “Warburg effect”. High glycolytic activity and consecutive secretion of lactic acid are known to promote the progression of a wide range of tumors, including hepatocellular carcinoma (HCC). It has already been shown that lactic acid affects tumor-associated immune cells thus providing a “tumor-friendly” environment. Further mechanisms by which lactic acid promotes tumor growth are yet to be discovered.

The aim of this study was to analyze whether HCC secreted lactic acid affects surrounding liver parenchyma in a way to promote tumor growth.

**Methods**: Primary murine and human hepatocytes were isolated by two-step collagenase perfusion procedure and incubated with different concentrations of L-lactic acid. After 24 h, cell culture supernatants were collected and mRNA expression was analyzed by qRT-PCR.

**Results**: Microscopical analysis of transaminases in the cell culture supernatants revealed that lactic acid did not affect viability of hepatocytes up to a concentration of 100 mM. Under these conditions, lactic acid did not affect mRNA expression levels of glucose transporters (GLUTs) or monocarboxylate transporters (MCTs), which are responsible for transmembraneous transport of glucose and lactate in hepatocytes. However, incubation with lactic acid increased the mRNA expression of chemokine (C-X-C-motif) ligand 2 (CXCL2) in murine hepatocytes as well as its human analogue interleukin-8 (IL-8) in human cells.

**Discussion/Conclusion**: Lactic acid causes a significant increase of CXCL2/IL-8 expression in hepatocytes. These cytokines are potent promotors of angiogenesis and expression levels correlate with a poor prognosis in HCC patients. Thus, our findings indicate a potential mechanism by which tumor-derived lactic acid promotes HCC progression.
Bile acids for the generation of artificial liver tissue

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Introduction: Due to the shortage of donor organs, artificial liver tissue is urgently needed for patients to bypass loss of liver function in acute or chronic liver diseases. First attempts were made with bone marrow mesenchymal stem cells (MSC) placed into decellularized rodent liver scaffolds and treated with growth factors to initiate their hepatic differentiation and to generate new liver tissue. Since bile acids can also induce this differentiation process, their use for the production of liver tissue was tested with stellate cells as liver-resident MSC.

Methods: Rat livers were decellularized by perfusion with Triton X-100 and sodium dodecyl sulfate. Stellate cells isolated from rat liver were added to liver scaffolds and treated up to 4 weeks with 2 µM tauroursodeoxycholic acid (TUDCA) by constant perfusion via the portal vein. Albumin and bile acid production as well as vesicle release was measured in the perfusion medium. The expression profile of the artificial liver was analyzed by Affimetrix GeneChip Array.

Results: Engraftment of isolated stellate cells in liver scaffolds and their differentiation into hepatocyte-like cells in response to TUDCA treatment was observed as indicated by the induction of albumin and bile acid production. The cells further released vesicles and initiated the expression of hepatocyte-specific genes such as sodium-taurocholate-cotransporting polypeptide, organic anion-transporting polypeptide 4 and multidrug resistance protein 2. Although markers of activated stellate cells were still elevated, GeneChip analysis revealed high relation of artificial liver tissue to normal rat liver. The expression profile of stellate cell-derived artificial liver clearly differed from freshly isolated stellate cells, indicating cell differentiation processes.

Discussion/Conclusion: Bile acids such as TUDCA can replace growth factors for establishing artificial liver from MSC populations such as hepatic stellate cells and could provide an economic tool to generate liver tissue from patient-derived MSC for therapeutic strategies.
Common NPC1L1 variants in gallstone diseases: Combined analysis of genetic risk and cholesterol homeostasis

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Introduction: Individuals with gallstone disease (GSD) secrete increased amounts of cholesterol into bile¹. Since NPC1L1 mediates sterol uptake in hepatocytes and enterocytes², we now investigate common NPC1L1 polymorphisms as known determinants of serum cholesterol³ for their association with GSD.

Methods: Serum surrogate markers of cholesterol synthesis (lathosterol and desmosterol) and transport (sitosterol and campesterol) were measured by GC-MS in a German cohort of 99 patients with GSD (42 males, age 39–84 years) and 127 controls (56 males, age 31–89 years). Three NPC1L1 polymorphisms (rs17655652, rs2072183, rs41279633)² were genotyped in Germans as well as in a Romanian cohort of 235 sibs with GSD (30 males, age 24–80 years) and 260 controls (19 males, age 21–78 years).

Results: We detected a potential association between GSD and the NPC1L1 variant rs41279633. German individuals with genotype [GG] were at increased GSD risk as compared to [TT] carriers (OR = 3.7, 95% CI: 1.0–14.1, p = 0.042). Moreover, we identified a departure from Hardy-Weinberg equilibrium in German cases (p < 0.01) and a trend for an association in the Romanian cohort (common OR = 1.4, p = 0.06). However, serum markers of cholesterol homeostasis were not affected by NPC1L1 genotypes, and the non-parametric linkage analysis sib-pairs did not provide evidence for linkage between NPC1L1 and GSD.

Discussion/Conclusion: Our results point to a potential association between the NPC1L1 variant and GSD. Apparently, the increased gallstone risk is not reflected by distorted systemic cholesterol homeostasis. Further studies are warranted to investigate the intestinal and/or hepatobiliary effects of additional rare NPC1L1 variants on GSD.

References:
Anti-fibrogenic effects of silymarin vary significantly in human hepatic stellate cells from different donors depending on the expression of OATP transporters

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Introduction: Silymarin derived from the milk thistle plant Silybum marianum is composed of six major flavonolignans and is widely used for self-treatment of liver diseases. However, scant information is available on the direct effect of silymarin on activated hepatic stellate cells (HSC), the key players of hepatic fibrosis. The aim of this study was to assess the effect of a defined silymarin extract (SE) on human HSCs in vitro from different donors.

Methods: Human HSCs were isolated from liver specimens of 5 different human donors and were activated by culture on plastic. Subsequently, cells were incubated with serial concentrations of SE for 24 h.

Results: SE has exhibited no cytotoxic effects up to 200 microM as assessed by microscopic documentation and assessment of LDH in cultured media. Moreover, SE inhibited dose-dependently (10–100 microM) proliferation, as well as pro-inflammatory (MCP-1) and pro-fibrogenic (collagen I, TGF-beta) gene-expression in HSCs as assessed by qPCR. Notably, anti-fibrogenic effects of SE varied significantly between HSCs from 5 different human donors. In search for the underlying mechanism of this variation we analyzed the expression of different organic anion-transporting polypeptides (OATPs) transporters in the 5 different HSCs. Interestingly, we observed high variation of OATPs expression in different HSCs, and SE effect were more pronounced in HSCs with high OAT2B1 expression.

Discussion/Conclusion: Our study provides novel insights into the potential anti-fibrogenic action of silymarin in chronic liver disease. Furthermore, our study indicates OATP expression as potential mechanism explaining silymarin's varying therapeutic efficacy, which may also be relevant for other anti-fibrogenic drugs and which may lead to individualized, targeted therapeutic strategies in chronic liver disease.
Comparative induction of pluripotency in human umbilical vein endothelial cells and dermal fibroblasts and further differentiation into hepatocytes

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Introduction: Generating induced pluripotent stem cells (iPSCs) is a standardized technique. To date iPS cells have been generated from HUVECs using viral-based approaches.

Methods: We employ an episomal plasmid-based approach to generate integration-free iPSCs (E-iPSCs) from human somatic cells. In this study, we compared the generation of E-iPSC lines from human umbilical vein endothelial cells (HUVEC) and fetal foreskin derived fibroblast (HFF1).

Results: The efficiency of inducing pluripotency in HFF1 was 0.03% compared to 2.5% in HUVEC. This implies that the efficiency of reprogramming HUVECs is 83-fold higher than in HFF1 cells. Additionally, the kinetics of reprogramming was much faster using HUVECs, i.e. three weeks for the stabilization of E-iPSC colonies compared to HFF1 cells which needed four months. The E-iPSCs from both somatic cell types were fully characterized and are comparable to human embryonic stem cells (hESCs). Both E-iPSC lines express pluripotency associated transcription factors as OCT4, NANOG, SOX2 and also the surface markers SSEA-4, TRA-1-60, TRA-1-81 and TRA-2-49 but not SSEA-1. Additionally, they have the ability to differentiate to all cell types representative of the three germ layers endoderm, ectoderm and mesoderm in vitro (by formation of embryoid bodies) and in vivo (teratoma formation in immunodeficient mice).

Discussion/Conclusion: We have already shown that E-iPSCs generated from HFF1 cells could be used to study hepatogenesis and represent a tool for studying human gastrulation at the molecular and cellular levels in vitro. We now demonstrate that HUVEC-derived E-iPSCs can be used to study hepatogenesis. A careful comparative analysis to determine which cell type would be better for generating hepatocyte-like cells with a more mature phenotype is in progress.
A disintegrin and metalloprotease 10 (ADAM10) is a central regulator of murine liver tissue homeostasis

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Introduction: A Disintegrin and Metalloprotease (ADAM) 10 exerts essential roles during organ development and tissue integrity in many organs. However, only little is known about its implication in liver tissue physiology.

Methods: We generated mice deficient for Adam10 in hepatocytes, cholangiocytes and liver progenitor cells, which are referred to as ADAM10Δhep/Δch. Functional studies were performed in the murine liver progenitor cell line BMOL.

Results: In contrast to its role in other tissues, ADAM10 is dispensable for the Notch2-dependent biliary tree formation in ADAM10Δhep/Δch animals. However, we observed areas with hepatocyte necrosis. This correlated with an impaired expression of bile acid transporters. Decreased numbers of necrotic areas in 15-week old ADAM10Δhep/Δch mice were clear evidence for on-going regenerative processes in ADAM10-deficient livers. Interestingly, we observed a strongly augmented ductular reaction that was accompanied by activation of hepatic stellate cells in 15-week old ADAM10Δhep/Δch mice, resulting in fulminant liver fibrosis. We show in cell-based assays that lack of ADAM10 in liver progenitor cells leads to enhanced c-Met signaling.

Discussion/Conclusion: Our data demonstrate that ADAM10 is a central regulator of murine liver tissue homeostasis by regulating bile acid transporter expression and liver progenitor cell activation.
Ammonia-induced miRNA expression changes in cultured rat astrocytes

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Introduction: Hepatic encephalopathy (HE) is a severe complication of acute or chronic liver failure. HE is a clinical manifestation of a low-grade cerebral edema associated with oxidative stress and senescence. In the present study we investigated effects of ammonia on miRNA expression level in cultured rat astrocytes.

Methods: miRNA or gene expression levels were analyzed by Agilent Rat miRNA Microarray or Affymetrix Rat Genome Microarray and validated by quantitative real-time PCR. Potential miRNA target genes were predicted by bioinformatic analysis (miRWalk) and validated by transfection with inhibitors inactivating the respective miRNA species. Astrocyte proliferation was measured by fluorimetric quantification of Hoechst34580 fluorescence. Heme oxygenase (HO)-1 protein and RNA expression was analyzed by Western-blot and real-time PCR.

Results: Using microarray analysis we identified 43 miRNAs downregulated and 142 genes upregulated by NH₄Cl (5 mmol/l, 48 h) in cultured astrocytes, respectively. NH₄Cl-induced miRNA and gene expression changes were validated by real-time PCR. Among 43 predicted target genes of miRNAs downregulated by NH₄Cl HO-1 was selected for further analysis. Upregulation of HO-1 mRNA in NH₄Cl-treated astrocytes was accompanied by downregulation of 6 individual miRNA species predicted to target HO-1 mRNA. Transfecting astrocytes with inhibitors of HO-1-targeting miRNA species which were downregulated by NH₄Cl increased HO-1 mRNA and protein levels and impaired astrocyte proliferation in a HO-1-dependent manner. HO-1 inhibition by Sn-protoporphyrine IX as well as blocking HO-1 upregulation by taurine (5 mmol/l) prevented inhibition of astrocyte proliferation by NH₄Cl.

Discussion/Conclusion: The data suggest that ammonia induces senescence through downregulation of miRNA species targeting HO-1 in cultured rat astrocytes.
The liver-specific microRNA-122 modulates hepatic response to infection and inflammation by antagonizing YY1, FoxP3, Nfr1 and E2F4 molecular networks

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Introduction: The liver-specific microRNA miR-122 is essential for the maintenance of liver functionality, e.g. by regulating iron homeostasis and cholesterol biosynthesis. miR-122 deficient mice develop a pro-inflammatory hepatic phenotype with increased tendency in developing HCC. miR-122 expression is dysregulated in a number of human diseases including HCC, viral hepatitis, and NASH. Although a great number of miR-122 targets has been validated, the global set of genes regulated by miR-122 and its actual function remain unknown.

Methods: Here we employed an approach to isolate and fractionize polyribosomes on sucrose gradient to identify novel miR-122 targets. Microarray analysis of polysomes isolated from cells treated with miR-122 mimic or inhibitor enable target analysis on genome-wide level. Moreover, we used luciferase reporter assay to characterize the human MIR122 promoter.

Results: Our microarray data identify a large number of putative miR-122 target genes and we show that many of these genes are downstream to YY1, FoxP3, E2F4 and Nfr1 transcription factors, which in turn are associated to liver malignancies. We further demonstrate a significant downregulation of 12 putative target genes in response to elevation of miR-122 levels. Remarkably, we also show that miR-122 transcription is regulated by the activity of the immunoresponsive cytokines TNFα, IL10, BMP6 and TGFβ.

Discussion/Conclusion: Our data from microarrays on polysomes and promoter analyses indicate that miR-122 suppression of its targets is crucial in modulating the response of liver cells to infection and inflammation. Nevertheless, how miR-122 modulates liver functionality in response to hepatic injury or during liver regeneration remains elusive.
Role of tumor necrosis factor in the pathogenesis of hepatic encephalopathy

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Introduction: Hepatic encephalopathy is a frequent neurological complication of acute or chronic liver failure. Ammonia is considered as the main precipitating factor for hepatic encephalopathy. During liver failure hepatocytes fail to metabolize ammonia from the blood stream. This allows high concentrations of ammonia to affect the brain which induces astrocyte swelling. Swelling of astrocytes results in increased formation of reactive nitrogen and oxygen species. Consequently, this leads to protein tyrosine nitration, RNA oxidation and alteration in gene expression. TNFα can increase ammonia induced RNA oxidation in vital brain slices. This directly indicates the involvement of TNFα in the pathogenesis of hepatic encephalopathy, however, detailed mechanism of action of TNFα remains unknown.

Methods: To further investigate the role of TNFα in hepatic encephalopathy, we used TNFα knockout animals. We analyzed levels of ammonia in vivo and performed behavioral studies on this model. We studied the effects of acute ammonia intoxication on Tnf⁻/⁻, Tnfr1⁻/⁻ and Tnfr2⁻/⁻ animals. In order to study the permeability of blood brain barrier, we injected TNFα, followed by injection of fluorescein sodium salt and analyzed fluorescence intensity in different areas of the brain.

Results: We found that elevated levels of TNFα in vivo lead to increased permeability of blood brain barrier in TNFR1 dependent manner. We showed reduced sensitivity of Tnf⁻/⁻ animals to ammonia intoxication. We observed behavior abnormalities in naïve conditions of TNFα knockout animals together with elevated ammonia levels and reduced protein tyrosine nitration.

Discussion/Conclusion: Taken together, we found that Tnf⁻/⁻ animals are protected from ammonia intoxication, and TNFα is involved in pathogenesis of hepatic encephalopathy by regulating blood brain barrier permeability through TNFR1.
Loss of the extracellular matrix molecules tenasin C or periostin results in impaired regeneration after cerulein-induced pancreatitis

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Introduction: Extracellular matrix molecules, such as tenasin C and periostin are secreted by activated pancreatic stellate cells, which are thought to be mainly responsible for the fibrotic response during pancreatic injury and following regeneration processes. Both molecules are also highly expressed in liver fibrosis contributing to the severity of the fibrotic response. However, the functional role of various secreted extracellular matrix molecules on regenerative pancreatic or liver epithelial cells has not been addressed so far. Here, we analyzed the function of tenasin C and periostin in pancreatic exocrine regeneration after the induction of a severe acute pancreatitis.

Methods: Tenascin C and periostin-deficient mice and wildtype control animals received repetitive cerulein injections, and a detailed histological analysis of pancreatic tissues was performed.

Results: Although there was no difference in pancreatitis severity in the acute inflammatory phase, the recovery of the exocrine pancreas was massively impaired in tenascin C and periostin deficient mice. Depletion of tenasin C results in prolonged acinar-to-ductal metaplasia and loss of periostin expression was accompanied by strong pancreatic atrophy and acinar-to-adipocyte differentiation, which was also reflected in gene expression patterns.

Discussion/Conclusion: Our data suggest that tenasin C and periostin are crucial factors for proper exocrine lineage-specific regeneration after severe acute pancreatitis. The functional relevance of both extracellular matrix molecules in liver regeneration will be addressed in future studies.
TGR5 deficiency renders mice more susceptible to cholestatic liver injury

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Introduction: TGR5 (Gpbar-1) is a G-protein coupled bile acid (BA) receptor expressed in various non-parenchymal cells of human and rodent liver. Here, the receptor has anti-inflammatory, antiapoptotic, proliferative and choleretic effects. Thus, activation of TGR5 may reduce liver injury induced by infection, inflammation and cholestasis. To address this hypothesis, cholestasis was induced in TGR5 knockout and wildtype mice by common bile duct ligation (CBDL, 1–7 days), cholic acid (CA, 0.5% for 7 days) and lithocholic acid (LCA, 1% for 4 days) feeding.

Methods: Liver injury was investigated by serum biochemistry and liver histology. Hepatocyte and cholangiocyte proliferative response was monitored by PCNA and Ki67 staining, cyclin D1 and cytokeratin-19 expression levels. Changes in gene expression were analyzed using an affymetrix mouse whole genome array as well as real-time PCR. Changes in protein levels were determined by western blotting and ELISA.

Results: Liver injury following BA feeding and CBDL was more severe in TGR5 knockout mice, which also showed a significantly reduced hepatocyte and cholangiocyte proliferation. While serum bile acid levels and composition of the serum BA pool were similar between genotypes after CBDL, LCA-feeding significantly raised serum BA concentrations and CA-feeding led to a more hydrophobic composition of the serum BA pool. However, cholestasis-induced suppression of BA synthesis genes was comparable in livers from both genotypes. Using mouse whole genome expression arrays differentially regulated genes were identified between genotypes and comprised genes involved in cell proliferation, detoxification and inflammation. Cytokine and chemokine expression was elevated in livers from TGR5 knockout mice as compared to their wildtype littermates after 3 days of CBDL and LCA-feeding, however, serum cytokine levels showed not genotype-specific differences at the time points studied.

Discussion/Conclusion: TGR5 deficiency renders mice more susceptibile to cholestasis induced liver damage and suppresses the regenerative, proliferative response of both hepatocytes and cholangiocytes. While higher BA concentrations and a more hydrophobic BA pool composition in TGR5 knockout mice could be responsible for the more severe liver injury following LCA- and CA-feeding, the significant elevation in liver cytokine mRNA expression observed in TGR5 knockout mice may contribute to liver damage following CBDL.
Role of the signaling protein reelin in chronic liver disease and regeneration

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Introduction: The secreted neuronal protein reelin, which regulates neuronal positioning in the developing nervous system and modulates synaptic plasticity in the adult brain, is also expressed in non-neuronal tissues, including the liver. However, the functions of hepatic reelin are unknown. Here, we analyze the spatial and temporal expression of reelin in isolated liver cell populations and models of liver damage and regeneration.

Methods: Hepatic stellate cells and additional liver cell populations were prepared from livers of Wistar rats by enzymatic digestion followed by density gradient centrifugation. Expression of reelin was detected by qPCR, Western blotting and immunocytochemistry. Stem cell-based liver regeneration was induced by pretreatment of rats with 2-AAF 1 week before partial hepatectomy to inhibit hepatocyte proliferation. Reelin expression in liver tissue was assessed by qPCR and immunohistochemistry using the monoclonal reelin G10 antibody.

Results: In tissue sections and isolated cell cultures, reelin expression was almost exclusively found in hepatic stellate cells, which have a profound impact on liver development and regeneration. Moreover, we found that reelin expression in the liver is reciprocally regulated in samples of CCl4-induced liver fibrosis and stem cell-based liver regeneration, suggesting a functional role in different physiological and pathophysiological conditions. As essential mediators of lipoprotein receptor-mediated reelin signaling are not present in the liver to a significant extent, non-canonical signaling pathways must be involved. Studies in polarized hepatocyte cell lines suggest that integrins might be involved in mediating hepatic functions of reelin.

Discussion/Conclusion: Our data demonstrate that reelin is expressed at high levels by hepatic stellate cells. Its reciprocal expression in conditions of liver fibrosis and regeneration suggest a functional significance of hepatic reelin expression during liver repair.
Development and optimization of 3D liver organoids

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Introduction: We previously published a new 3-dimensional tissue culture model that allows the in vitro generation and culture of human liver organoids (LO). Based on these data we have now further optimized and developed this model.

Methods: Our "LO-prototype" (Ramachandran et al., PlosOne 2015) was composed of hepatocytes, LSECs and MSC (mesenchymal stem cells). These LOs spontaneously formed and remained vital and functional (e.g. in terms of Cyp450 activities) for 10 days in the dynamic system. While the hepatocytes nicely formed parenchymal areas making such LOs already a promising approach for toxicity studies, the LSECs seemed to clump together in central areas of the LOs without lining the "pores". Obviously the conditions were not yet optimal for the formation of real sinusoids. We therefore further optimized the initial conditions of LO-formation.

Results: 1) The previously used matrigel was replaced by Agarose and defined, FCS-free medium is used. 2) We optimized the ratio of cell types to a more physiological one of 70% hepatocytes, 25% LSECs and 5% MSC. 3) We replaced the MSC with upcyte® hepatic stellate cells (HSC), which are the "real" pericytes of human liver. LOs with HSC instead of MSC also formed spontaneously and now after 72 h the LSECs are nicely distributed throughout the whole LO and do not any more form unphysiologic clumps in the center (as they mainly did in the "prototype").

Discussion/Conclusion: Our “2nd generation” LOs, composed of differentiated, human liver cells are supposed to serve as valuable new 3D system for numerous applications like toxicity testing, basic science approaches (including replacement of animal models) and finally generation of ex-vivo human "livers".
The synthetic chaperon 4-PBA induces an acute phase response in a mouse model of protein storage diseases

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Introduction: HBs transgenic mice aggregate HBV surface proteins (HBs) within the endoplasmic reticulum of hepatocytes. This causes an accumulation of misfolded proteins leading to ER stress whereby the unfolded protein response is activated. Since ER stress and the storage of misfolded proteins are responsible for a part of the liver pathology of chronic hepatitis B the aim of the project was to attenuate the ER stress within the hepatocytes by treatment with the synthetic chaperon 4-PBA (4-phenylbutyrate).

Methods: A HBs transgenic mouse on BALB/c genetic background and the HBs expressing murine hepatocyte cell line AML 12 were treated with 4-PBA and were investigated by microarray, quantitative real-time PCR (qPCR) and Western blot analysis.

Results: The hypothesis supposing that 4-PBA reduces ER stress in HBs transgenic mice was not confirmed. However, microarray analysis showed an increase of acute phase protein gene expression such as lipocalin 2 and serum amyloid A (SAA). The results were proofed by qPCR of hepatocyte cell line lysates and liver tissue of transgenic mice. Western blot analysis of liver total protein lysates demonstrated a strong expression of lipocalin 2 protein. Moreover, the gene expression of certain cytokines (CCL-3, CCl-4, MCP-1, CXCL-1) was increased in the liver of HBs transgenic mice.

Discussion/Conclusion: The synthetic chaperon 4-PBA induced an acute phase response in mouse liver and in HBs expressing hepatocyte cell line. Lipocalin 2 may play an important role in this response.
DNA methylation changes during hepatic stellate cell activation

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Introduction: Beside their well established function in liver fibrosis, hepatic stellate cells (HSC) were identified as mesenchymal stem cells with remarkable differentiation potential. Cell fate decisions are associated with dynamic epigenetic modifications affecting gene expression. Therefore, the aim of this study is to identify DNA methylation changes and their correlation to gene expression during HSC activation.

Methods: Quiescent and culture activated primary HSC from rats were examined for genome-wide gene expression with Affymetrix GeneChip Rat Gene 2.0. The results were compared with genome-wide data for DNA methylation obtained by Methyl-Mini Seq (Zymo Research).

Results: Microarray analysis identified almost 1300 differentially expressed genes during early HSC activation, the majority of them displaying a down-regulation of gene expression. Likewise more than 5000 differentially methylated regions were detected in intragenic and promoter regions. Hypermethylation was detected much-over hypomethylation. Out of these, approximately 1000 genes show a change in gene expression. Furthermore, gene ontology (GO) term analysis revealed that genes with DNA methylation changes and those with differential expression can be allocated to similar GO terms. The enriched GO terms include cell stress, differentiation, migration and secretion. In contrast to genome-wide investigations, global DNA methylation decreases by 60% during HSC activation, which is mediated by an active DNA demethylation process.

Discussion/Conclusion: In summary we identified vast changes in the methylome during HSC activation that coincide with alterations in gene expression, indicating a functional connection. The affected genes are, amongst other processes, involved in cell differentiation. These properties make HSC a promising model for the investigation of epigenetic regulation of adult stem cells.
Identification of atypical cadherin FAT1 as protein interaction partner of MIA2 in hepatocellular carcinoma

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Introduction: Melanoma Inhibitory Activity 2 (MIA2) is a 451 amino acid protein exclusively expressed by hepatocytes in the liver. In hepatocellular carcinoma (HCC), however, MIA2 expression is strongly downregulated and acts as a tumor suppressor. The molecular mechanisms underlying MIA2's tumor suppressive effect are not known until today. The aim of this study was to identify protein interaction partners of MIA2 which may impact the function of MIA2 in HCC cells.

Methods: To identify potential interaction partners of MIA2, a yeast-two-hybrid assay was performed, using MIA2 as "bait" and a liver protein library as "prey". In this screening, FAT1, an atypical member of the cadherin family, was identified as possible interacting partner of MIA2. To verify the MIA2-FAT1 interaction, co-immunoprecipitation analysis have been performed using protein lysates of HepG2 hepatoma cells, which had been stably transfected with a MIA2 expression plasmid (HepG2_MIA2). Protein lysates were incubated with an anti-MIA2-antibody and antibody-protein complexes had been pulled down using sepharose G-coupled beads. Subsequent western blot analysis using an anti-FAT1 antibody revealed a clear band, indicative for specific MIA2-FAT1 interaction.

Results: Analysis of the MIA2 structure revealed that it harbors an SH3 domain, which is known to mediate protein-protein interactions. This study identified FAT1 protein as a new interaction partner of the MIA2 protein, which acts as a tumor suppressor in HCC.

Discussion/Conclusion: Conversely, FAT1 expression is upregulated in HCC and promotes proliferation and migration of HCC cells. Further studies need to investigate the functional effect of MIA2-FAT1 interaction on the tumor promoting effect of FAT1. However, it can be hypothesized that an inhibitory effect of MIA2 on FAT1 may explain its tumor suppressing function in HCC. Furthermore, the MIA2-FAT1 interaction site may serve as a target for the development of novel therapeutics for this highly aggressive tumor.
**In vivo reprogramming of myofibroblasts into induced hepatocytes during liver fibrosis**

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**Introduction:** Myofibroblasts are the main driver of liver fibrosis, and hence, targeting of myofibroblasts is considered as one of the plausible approaches for the treatment of liver fibrosis. Here, we aim to convert myofibroblasts into induced hepatocytes (iHep) by \textit{in vivo} reprogramming, in mouse models of liver fibrosis.

**Methods:** We generated \textit{in vivo} iHep by forced expression of four transcription factors in myofibroblasts using an adenoviral vector. We isolated \textit{in vivo} generated iHep from mice and compared their functions with endogenous hepatocytes. In addition, we examined whether \textit{in vivo} expression of four transcription factors ameliorates liver fibrosis.

**Results:** We found that overexpression of four transcription factors convert myofibroblasts into iHep via \textit{in vivo} reprogramming. The isolated iHep exhibited functional characteristics similar to endogenous hepatocytes, such as albumin
secretion, urea synthesis, cytochrome activity and drug responsiveness. Furthermore, the targeted expression of our reprogramming factors in myofibroblasts reduced liver fibrosis.

**Discussion/Conclusion:** We demonstrate conversion of pro-fibrogenic myofibroblasts into hepatocyte-like cells by combination of four transcription factors *in vivo*. Our study suggests that *in vivo* reprogramming may open new perspectives for the treatment of diseases such as liver fibrosis.
Regenerative potential of human pluripotent stem cell-derived MSCs in a Gunn rat liver injury model

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Introduction: The Crigler-Najjar syndrome type 1 is characterized by massively increased levels of unconjugated bilirubin in the plasma because of the lacking of hepatic uridine 5’-diphospho-glucuronosyltransferase activity (due to a mutated UGT1A1 gene) which is crucial for bilirubin conjugation and excretion. The Gunn rat is an in vivo model for this disease. Current treatments have several limitations therefore alternative therapy strategies like (stem) cell transplantations are needed. Transplantation of primary hepatocytes is limited due to the dependence on donated organs. An alternative approach would be the increment of functional hepatocytes by cell transplantation. Studies have shown that co-transplanting hepatocytes with bone marrow-derived MSCs improves homing-in, engraftment and survival of the donor cells and that MSCs can transdifferentiate into hepatocytes. These properties make MSCs attractive tools in the treatment of acute liver injuries. However, the downside of procuring MSCs is that the number of cells that can be generated from a single human donor is limited due to their restricted long-term proliferation.

Methods: To circumvent these drawbacks iMSCs (induced MSCs) were generated from (i) iPSCs derived from human fetal bone marrow MSCs and (ii) human embryonic stem cells- line H1. These were compared to human fetal bone marrow-derived MSCs. The iMSCs and the parental MSCs were transplanted into the spleen of partially hepatectomized Gunn rats (without immunosuppression). After a regeneration time of 1 week to 2 months the organs and sera were examined.

Results: Molecular, immunohistochemical and immunofluorescent-based analysis of rat liver tissue showed integration of human cells. Expression of human HNF4α as well as measureable human albumin levels in the sera provides evidences of trans-differentiation into hepatocytes. Further analysis revealed reduced levels of bilirubin.

Discussion/Conclusion: To summarize, human iMSCs are an alternative source for treating inherited liver diseases such as Crigler-Najjar syndrome type 1.
TGR5 protein levels are reduced in cholangiocytes of livers from patients with primary sclerosing cholangitis or from mice deficient for Abcb4

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Introduction: The membrane-bound, G-protein coupled bile acid receptor TGR5 (Gpbar-1) is highly expressed in cholangiocytes from of human and rodent liver. Stimulation of TGR5 in cholangiocytes promotes fluid secretion, induces cell proliferation and protects these cells from bile acid toxicity and cell death. Thus, changes in TGR5 expression, localization or function may contribute to biliary diseases, such as primary sclerosing cholangitis (PSC) or primary biliary cholangitis (PBC).

Methods: TGR5 mRNA expression was studied in human liver samples from PSC patients by real-time PCR and compared to control samples. TGR5 protein localization and amount were studied by immunofluorescence staining and quantification of the fluorescence intensity of TGR5 in relation to the intensity of the cholangiocyte marker protein cytokeratin-7 (CK-7). Abcb4 knockout mice were analysed as an animal model for PSC. Again, mRNA expression was studied by real-time PCR and TGR5 protein localization and levels were determined by immunofluorescence staining in comparison to CK-19. To study potential mechanisms of TGR5 regulation, macrophages were derived from blood mononuclear cells and incubated with bile acids and cytokines. Furthermore, cell lines derived from human cholangiocytes/ cholangiocarcinomas were used.

Results: TGR5 fluorescence intensity in bile ducts from PSC liver biopsies (n = 15) was significantly reduced as compared to control livers (n = 24), while CK-7 fluorescence intensity was unchanged. In livers from Abcb4 knockout mice (n = 6) TGR5 fluorescence intensity was also significantly lower as compared to wildtype controls (n = 6). Again the staining for the cholangiocyte marker protein CK-19 was unaffected. In chronic inflammatory biliary tract diseases bile salt and cytokine levels are increased. Analysis of whole liver mRNA did not reveal a significant reduction in TGR5 mRNA in livers from PSC or Abcb4 knockout mice as compared to the respective control samples, however, mRNA levels for CD14 as a marker of inflammatory cells as well as for CK-19 were upregulated in PSC and Abcb4 knockout livers. Since both bile acids and inflammatory cytokines are elevated in PSC, we tested whether these agents could explain the downregulation of TGR5 in the diseased livers. Interestingly, incubation of human macrophages with the inflammatory cytokines TNFα and IL1β significantly reduced TGR5 mRNA expression in these cells. In contrast, no such effect was observed in the cholangiocyte-derived cell lines.
Discussion/Conclusion: Analysis of TGR5 protein levels by immunofluorescence staining revealed a significant reduction in TGR5 staining in bile ducts of PSC and Abcb4 knockout livers. In contrast the fluorescence intensity for the cholangiocyte marker proteins CK-7 and CK-19 was unchanged. Whether the reduction in TGR5 protein amounts results from a decreased mRNA expression of the receptor in diseased cholangiocytes is elusive. While TNFα and IL1β significantly suppressed TGR5 mRNA levels in human macrophages, this effect was not observed in cholangiocyte-derived cell lines. Thus, further studies are needed to identify the mechanism of TGR5 downregulation in cholangiocytes of PSC livers. However, the finding that TGR5 protein levels are reduced in cholangiocytes of PSC and Abcb4 knockout livers, may explain why liver damage in Abcb4 knockout mice could not be alleviated by stimulation of the TGR5 receptor using specific agonists (INT-777, INT-767; Baghdasaryan et al., 2011).
Characterization of monoclonal BSEP-reactive antibodies associated with antibody-induced BSEP disease (AIBD)

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Introduction: Antibody-induced Bile Salt Export Pump (BSEP) Disease (AIBD) is caused by inhibitory IgG-class antibodies directed against BSEP in some patients following liver transplantation for Progressive Familial Intrahepatic Cholestasis Type 2 (PFIC-2). Clinically, the AIBD phenotype mimicks the pre-transplant BSEP deficiency (PFIC-2). While BSEP-reactive antibodies are the underlying cause of this rare disease, the pathogenesis of AIBD as well as the molecular mode of action of the BSEP-reactive antibodies remains uncharacterized.

Methods: Using fluorescence-activated single cell sorting, BSEP-reactive B cells were isolated from peripheral blood of an AIBD patient. By single cell RT-PCR, corresponding full-length immunoglobulin heavy and light chain variable region gene transcripts were amplified and cloned into eukaryotic expression vectors for in vitro production of the encoded monoclonal antibodies (mAbs) and their corresponding monovalent Fab fragments (mFabs). BSEP-reactive mAbs and mFabs were characterized by immunofluorescence (IF) staining of human and rat liver sections and BSEP-expressing cell lines, Western blot, FACS analysis and in vitro BSEP transport assays.

Results: BSEP-reactive mAbs and mFabs recognized transiently expressed human BSEP on the surface of human embryonal kidney (HEK293) cells while yielding clear canalicular staining patterns on human and rat liver slices. When used in Western blot experiments, the mAbs specifically detected human BSEP. FACS analysis of unpermeabilized cells transiently expressing BSEP showed clear extracellular BSEP recognition by all mAbs and mFabs. BSEP-reactive mAbs and mFabs reached the canalicular space of in situ perfused rat livers within two hours. In vesicular transport studies, a clone-dependent influence on transport activity was detected, and selected mAbs and Fabs were tested for any differences in transport inhibition.

Discussion/Conclusion: Our data demonstrate that the serum of an AIBD patients contains different BSEP-reactive antibodies, which bind to an extracellular epitope of human BSEP and cause AIBD through direct inhibition of BSEP transport activity. Interestingly, we also identified one BSEP-stimulatory mAb/mFab antibody. Comparison of BSEP-inhibition by each mAb and its derived mFab suggests that next to direct BSEP inhibition, the crosslinking of BSEP molecules by the bivalent mAbs could also contribute to impaired transport activity of BSEP.
Cytokines and microbial endotoxin in experimental model of liver injury

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Introduction: Liver injury causes extensive immunological pro-inflammatory response characterized by significant growth of IL-1β, TNFα, IL-6 and other pro-inflammatory mediators. Following idea that microbial endotoxins cause similar immune reactions we hypothesize that this effects may combine and/or even multiply. The aim of the study is to determine the dominating etiology factor for immunologic changes following liver injury.

Methods: Liver injury was modelled intraoperatively in 87 Wistar rats under general anesthesia; sutures were applied immediately. 45 rats (51.72%) additionally received S. typhimurium endotoxin intraperitoneally. Liquid chromatography and ELISA were used for determination of cytokines levels in liver tissue homogenates taken 24 hours after injury.

Results: Aseptic liver injury alone cause minor changes in cytokines levels in liver homogenates compared to control: IL-1β grew insignificantly (55.72 ± 8.30 pg/g of homogenated hepatic tissue under liver injury compared to 45.37 ± 5.82 pg/g in healthy control rats, p > 0.05); TNFα – 39.26 ± 4.89 pg/g and 47.63 ± 6.47 pg/g, p > 0.05, respectively; gamma-interferon (γ-INF) – 112.9 ± 7.62 pg/g and 123.9 ± 10.75 pg/g, p > 0.05; TGF-β1 – 204.5 ± 12.17 pg/g and 196.2 ± 6.42 pg/g, p > 0.06. Endotoxin added intraperitoneally caused dramatic growth of cytokines in liver tissue: IL-1β increased over 80% to 74.27 ± 8.09 pg/g, p < 0.01; TNF-α grew 45% to 56.91 ± 6.53 pg/g, p < 0.05; γ-INF level (419.16 ± 30.68 pg/g) raised 3.7 times compared to liver injury without endotoxin, p < 0.001. In contrast, TGF-β1 remained almost unchanged – 236.16 ± 25.68 pg/g, p > 0.05.

Discussion/Conclusion: Although liver injury is generally accompanied by proinflammatory tendencies, they are generally insignificant in case of isolated aseptic and immediately cured trauma. However, this presents ideal, theoretic condition; raised endotoxin level is inevitable in case of real clinical situation in relation to trauma. Obtained data shows that better control of microbial lipopolysaccharide-endotoxin influence must be ensured in order to achieve sufficient results and prevent further liver inflammation. This may be valid not only for trauma itself but any liver injury including surgery and possibly autoimmune disease.
The gut-liver axis as systemic expression of relationship between gut microbiota, immunity and vascular dysfunction

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Introduction: Multiple organ deficiency/dysfunction syndrome (MODS) is the leading course of hospital associated mortality in Europe. Hepatic failure involvement into this malicious statistics is unquestionable. We hypothesized that acute enteral dysfunction syndrome (EDS) may contribute into liver dysfunction by means of a vicious circle, which includes disorders of gut microbiocenosis with associated immunity (EndoCAb), and NO (endothelium) levels changes. The aim of the study was to find whether changes of gut microflora, EndoCAb and nitric oxide levels are somehow related in pathogenesis of EDS and MODS.

Methods: Study included 87 patients with clinically proven EDS and liver dysfunction, mean age – 49.06 ± 8.34. EndoCAb assessed by ELISA, NO (nitrite/nitrate) by IEA.

Results: Colonic flora changes dramatically under EDS: significant decrease (p < 0.05) or elimination of autochthonic anaerobic microorganisms and hyperproliferation of conditionally pathogenic Enterobacteriacea: E. coli, including Hly+ – 9.31 ± 0.62 lg CFU/g against 7.39 ± 0.56 lg CFU/g in control; Klebsiellae – 5.17 ± 0.40 lg CFU/g against 3.48 ± 0.49 lg CFU/g in control, Proteus – 6.23 ± 0.35 lg CFU/g, and Serratia – 5.49 ± 0.74 lg CFU/g (not found in control). EndoCAb changes were not uniform. In patients with negative (complicated or lethal) clinical course of disease EndoCAb IgM (1.05 ± 0.02 MMU/ml) and IgG (2.51 ± 0.11 GMU/ml) were significantly lower than in control (p < 0.01). However, positive course of the syndrome, even accompanied by MODS, gives higher figures of IgM (2.98 ± 0.23 MMU/ml) and IgG (9.57 ± 0.84 GMU/ml). In most cases (83–95.4%) significant (p < 0.05) EndoCAb growth was observed only after 5th day of disease. In 4 (4.6%) cases only IgM increased, while IgG level remained low. NO levels rose reliably (p < 0.05) in all observed patients with EDS (42.96 ± 2.75 mmol/l vs 34.61 ± 3.07 mmol/l in healthy subjects). Strong negative correlation (r = -0.79, p < 0.05) between EndoCAb and NO levels was found only in negative course cases.

Discussion/Conclusion: Systemic interference of liver and other organs is often under evaluated. There is no doubt concerning existance tight ties between hepatic dysfunction and EDS. Excessive growth of conditionally pathogenic Enterobacteriacea and endotoxin release is associated with insufficient production of antinuclear anti-endotoxin antibodies (EndoCAb). NO aggravate EDS gravity by means of decreased motility and increased gut' permeability. This "intestinal leakage" plays role as pathophysiologic vicious circle including endotoxin and inflammatory changes liver.
Hepatocyte transfection in small pigs after weaning by hydrodynamic intraportal injection of naked DNA/minicircle vectors

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Introduction: Non-viral and non-plasmid-based vectors that express a therapeutic protein from a non-integrating (episomal) element have the potential of robust and sustained transgene expression while bearing a lower risk of insertional mutagenesis. While hydrodynamic injection into the tail vein of mice to target the liver is an efficient but experimental approach, delivery of naked DNA/minicircle vectors into larger animals or humans remains a challenge. Here we show successful hepatocyte transfection in domestic small pigs treated after weaning with portal vein catheterization and hydrodynamic injection of naked DNA/minicircle vectors expressing the luciferase reporter gene from a liver-specific promoter.

Methods: A surgical method was established allowing hydrodynamic portal vein pressurization up to 200 mmHg and infusion of naked DNA/minicircle vectors with long-term transgene expression potential.

Results: No acute adverse effects or signs of liver cell damage were observed in pigs. Stable hepatocyte transfections at 10 and 28 days in single experiments were found in up to 98% of samples (74/75 PCR-positive for minicircle-DNA), and 13% of the positive specimens (6/45) showed low but stable luciferase expression with up to two vector copies per diploid hepatocyte genome.

Discussion/Conclusion: While further optimization to enhance transfection efficiency and transgene expression is required, we conclude that hydrodynamic gene delivery using naked DNA/minicircle vectors in small pigs is a safe procedure for stable gene transfection. Moreover, the established surgical method for hepatocytes transfection in small pigs is not only a prerequisite to potentially treat infants with genetic liver diseases but also to test novel therapeutic approaches in our transgenic pigs.
Optimizing safety and long-term efficacy of liver gene therapy with non-integrating naked-DNA minicircle vectors expressing their transgene from a natural promoter

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Introduction: We have reported successful therapy of phenylketonuria (PKU) in the PKU mouse model (C57Bl/6-Pahu2) by using non-viral naked DNA vectors, so-called minicircles (MCs) which are devoid of any viral or bacterial sequences, upon liver-directed phenylalanine hydroxylase (Pah) gene transfer via hydrodynamic vein injection (Viecelli et al. Hepatology. 2014;60:1035–43).

Methods: To further address the biosafety of these vectors for gene therapy, we performed partial hepatectomy to investigate the non-integrating (episomal) state of MCs. MC-corrected PKU mice were challenged by resecting up to 70% of the liver, and blood L-Phe levels were followed upon regeneration of the remaining liver lobes.

Results: We found a re-rise of blood L-Phe values back to pre-treatment levels as well as the loss of Pah-transgene expression, indicating that treated PKU mice had lost therapeutic MC vectors during liver regeneration. These results corroborate previous observations that MC-DNA do not integrate – or only at a very low frequency that cannot be detected by our assays – and thus express their transgene predominantly as episomal vectors. We then further optimized the expression cassette to improve transgene expression which may allow treatment at lower vector doses. We found that the therapeutic doses of MCs could be significantly lowered by using a codon-optimized murine Pah cDNA in combination with a truncated 5’-intron. Moreover, when using the natural or endogenous 3.6 kb murine Pah-promoter to drive the Pah transgene, vector doses could again be lowered compared to two other “minimal” liver-specific promoters, a synthetic hybrid enhancer/promoter (P3) or the classical CBA (modified cytomegalovirus enhancer/chicken β-actin) promoter.

Discussion/Conclusion: In conclusion, MC-vectors which do not have a defined size-limitation, offer a favorable safety profile due to their non-integrating behavior, and at the same time have the potential for long-term gene-therapy of liver defects.
Characterization of liver damage and regeneration in C5 receptor-deficient mice

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Introduction: Complement factor C5 contributes to hepatic fibrogenesis as C5-deficient mice display less fibrosis after challenge with CCl₄ (Hillebrandt et al. 2005). During inflammation C5 is cleaved and the small chemoattractive peptide C5a binds to the receptors C5aR and C5L2. Our aim was to assess the specific roles of the C5 receptors during CCl₄-induced liver damage and regeneration.

Methods: C5aR- and C5L2-deficient mice and wild-type (WT) controls were treated with CCl₄ for 6 weeks. In addition, mice were challenged with CCl₄ for 6 weeks and left untreated for another 6 weeks (regression model). Expression of Th1 and Th2 cytokines was determined by qRT-PCR, and hepatic collagen contents were measured via hydroxyproline. Mice were also objected to bile duct ligation (BDL) and unilateral ureteral obstruction (UUO) to compare fibrosis progression in liver and kidney.

Results: Chronic fibrosis in liver and kidney was least pronounced in C5aR-deficient mice in comparison to the other lines. Of note, 6 weeks after the last injection C5aR-deficient mice developed highest hepatic collagen levels, indicating response and ongoing damage after cessation of fibrotic stimuli. This is resembled by cytokine profiles, with IL6, IL10, IL12, IL23 and IL27 being reduced in mice deficient for C5 receptors in the chronic model but elevated in C5aR⁻⁻ mice during fibrosis regression in the liver.

Discussion/Conclusion: C5aR is critical during chronic fibrogenesis. The novel observation of fibrosis progression in C5aR-deficient mice the after removal of the fibrotic stimulus points to its critical role during wound healing and fibrosis regression.
A human hepatic in vitro co-culture system for the analysis of DILI-related signaling

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Introduction: The interaction between immune cells and hepatocytes during a sterile inflammation has come into focus in Drug-Induced Liver Injury (DILI) research during recent years. Therefore, this crosstalk was analyzed in a novel indirect co-culture system with HepG2 and differentiated THP-1 cells using the antifungal drug ketoconazole as a known hepatotoxic model compound.

Methods: The metabolism of ketoconazole, changes in the proteome, cytokine expression and secretion upon treatment in single- and co-culture were analyzed.

Results: HepG2 metabolized ketoconazole to several metabolites which differed from those found in THP-1 cells both in single and co-culture. In the supernatant, the ketoconazole concentration decreased time-dependently while the concentration of several metabolites increased. The global proteomics analysis of HepG2 cells in co-culture identified the activation of the “Nrf2 mediated stress response” and the “Integrin linked kinase signaling” pathways after treatment with ketoconazole. Further upregulated proteins belonged to the NFκB, CXCL8 and sterol pathway. ELISA and qPCR assays revealed the upregulation of several pro-inflammatory cytokines including CXCL8, TNF-α and CCL3 in treated, co-cultured but not single-cultured HepG2 cells.

Discussion/Conclusion: The advantage of this indirect system with inserts is that both cell lines can be separated and analyzed individually after co-culture and the results can be compared to single-cultured cells. The activation of signalling pathways related to the hepatotoxicity of ketoconazole occurred in the co-culture at lower concentrations, compared to the single-culture. In conclusion, the novel indirect co-culture system represents a promising new tool to study hepatotoxic drug effects in vitro.
MiR-198 regulation in response to oncogenic stress in hepatocellular carcinoma

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Background and aim: In response to oncogenic stress, miR-198 is significantly downregulated in hepatocellular carcinoma (HCC). Therefore, we focused on molecular mechanisms of miR-198’s regulation in hepatoma cells.

Methods: For conditional, doxycycline-induced miR-198 expression, the miR198 encoding sequence has been inserted in a Tet-on vector system and stable, transgenic huh7 hepatoma cell line was generated. Cell growth and invasion was determined by MTT and scratch assays, respectively. RNA from cells, supernatants, and from exosomal fractions and used for transcriptional quantification by qPCR. Impact of miR-198 on protein expression was studied by immunoblotting.

Results: Hepatoma cells growth and migration were inhibited after mimic miR198 transfection. MiR-198 overexpression induces upregulation of cell adhesion proteins such as E-cadherin and Claudin-1. Interestingly, upregulation of adhesion molecules was associated by a pronounced inhibition of transcription factors Zeb1 and Zeb2, known to regulate epithelial-mesenchymal transition (EMT). After establishment of hepatoma cell lines harboring an inducible miR198 inducible expression system, miR-198 expression pattern was studied. After doxycycline treatment, miR198 was massively expressed in the first 12 hours, but followed by a marked decrease in the next 36 hours, involved with lysosome associated degradation and prominent exosomal release. qPCR and immunoblotting results showed that both mRNA and protein level of the HnRNPA2B1, highly promoting miR-198 secretion, but also involved in EMT, were strongly downregulated in response to miR198 induction.

Conclusion: In hepatoma cells, intracellular levels of tumor suppressor miR198 are tightly controlled meanwhile its overexpression increases cell-to-cell adhesion and conjunction, which contribute to epithelial mesenchymal transition inhibition.
Improved transdifferentiation of adipocyte-derived mesenchymal stem cells into hepatocyte-like cells

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Background: The low availability of human liver tissue causes a limited supply of much needed primary human hepatocytes (pHH) for cell transplantation or in-vitro liver research. Recently many groups have targeted hepatocyte-like cell differentiation using various stem cell types. However, many protocols failed showing a sustained hepatocyte phenotype. Therefore, intention of the present work was to optimize the hepatic differentiation from human adipocyte mesenchymal stem cells (AD-MSC) comparing different established protocols in gene expression and metabolic characteristics compared to primary human hepatocytes.

Methods: Ad-MSCs were isolated from fresh fat tissue according to the ethical guidelines of the Eberhard-Karls-University Tübingen. After three or four passages, the cells were incubated with 5-azacytidine in absence or presence of vitamin C or trichostatin A for epigenetic changes. The following 18 days, the hepatic differentiation was carried out using miscellaneous protocols applying four-step combined culture steps with different supplements to improve hepatic metabolic and enzymatic activities. After day 1, 10 and 18, expression levels of different genes responsible for urea-, protein- and phase-I-enzyme-synthesis were measured by RT-PCR. Ammonium chloride detoxification and urea synthesis were measured by photometry and the secretion of albumin in supernatant by ELISA. Primary human hepatocytes served on day 2 as positive controls.

Results: Ad-MSCs survived in a static culture during the whole differentiation time. HLCs expressed enzymes necessary for urea- and protein-synthesis as well for phase-I/II-enzyme genesis. The rate of urea production and albumin secretion was higher after 18 days of differentiation in combination with 5-azacytidine or Trichostatin A than in non-treated or undifferentiated cells. The phase-I/II-enzyme activities were higher after treatment and hepatic differentiation than untreated cells. The correlative difference between pHHs and HLCs is lower compared to the difference between pHHs and undifferentiated cells.

Conclusion: Our data clearly show that epigenetic changes of Ad-MSCs significantly improve the hepatic-like cell differentiation and may serve as a possible alternative for cell-based therapy or for in-vitro liver research to the scarcely available human hepatocytes. The low health detriments of these cells represent an important benefit compared to other artificial alternatives.
Dysregulation of Bmi1 promotes malignant transformation of hepatic progenitor cells

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Introduction: Adult hepatic progenitor cells (HPCs) participate in a broad range of human liver diseases, including hepatocellular carcinoma (HCC). The polycomb group gene B cell-specific Moloney murine leukemia virus integration site 1 (Bmi1) has been reported to play a vital role in the self-renewal and malignant transformation of stem cells. This study was designed to investigate the effects of Bmi1 on the biological properties of rat HPCs.

Methods: Bmi1 was silenced or enhanced in two HPCs cell lines (WB-F344 and OC3) using a small interfering RNA targeting Bmi1 or a Bmi1 forced expression retroviral vector, respectively. The biological functions of Bmi1 in HPCs were investigated through cell proliferation assays, colony formation assays, cell cycle analysis, invasion assays and xenograft formation assays.

Results: In this study, genetic depletion of Bmi1 in HPCs repressed the proliferation, colony formation and invasion of both HPCs in vitro compared with normal controls. Conversely, the forced expression of Bmi1 in two HPCs cell lines promoted cell proliferation, colony formation and invasion in vitro. An aldehyde dehydrogenase (ALDH) assay revealed a significant increase in the number of ALDH positive cells after the forced expression of Bmi1 in HPCs. Most importantly, transplantation of Bmi1 forced expression HPCs into nude mice resulted in the formation of tumors with histological features of poorly differentiated HCC.

Conclusions: Taken together, our findings indicated that dysregulation of Bmi1 promoted malignant transformation of HPCs, supporting the hypothesis that HCC could derive from hepatic progenitor cells.
Hepatic expression of oncogenes Bmi1 and DKK1 is upregulated in HBs antigen-transgenic mice in vivo and can be induced by LPS treatment in hepatoma cell line Hepa1–6 in vitro

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Background: Chronic hepatitis B virus (HBV) infection is a major risk factor for the development of hepatocellular carcinoma (HCC). The oncogenic role of HBV might involve a combination of direct and indirect effects. Recent studies have shown that Toll-like receptor 4 (TLR4) signaling links innate immunity and HCC progression. Here, we investigated, the oncogenic role of HBV using an HBV transgenic mouse model that shows liver inflammation and tumor progression.

Methods: The HCC markers AFP, hepatic stem cell markers EpCAM, HCC oncogene Bmi1, DKK1 and β-catenin were analyzed by immunohistochemistry in 16 cases of HBs antigen-transgenic mice and wild type littermates mice liver specimens. Mice hepatoma cell line Hepa1–6 was used for in vitro studies. Lipopolysaccharide (LPS), a specific ligand of TLR4, was used to activate TLR4 signaling. The effects of LPS-TLR4 signaling on cell proliferation and cell colony formation were examined by cell counting kit 8 (CCK-8) assays and cell colony formation assay.

Results: Immunohistochemistry revealed that the oncogenes Bmi1 and DKK1 were highly upregulated in 100% (16/16) of liver specimens from 12-month-old HBs-transgenic mice compare to wild type littermates, whereas β-catenin appeared in 18.75% (3/16) and AFP appeared in 43.7% (7/16) of specimens obtained from HBs-transgenic mice. In vitro LPS treatment of Hepa1–6 cells led to increased expression of oncogenes Bmi1 and DKK1 as well as enhanced cell proliferation and colony formation in vitro.

Conclusion: Taken together, these findings suggest that Bmi1 and DKK1 might link inflammation and tumor progression during chronic HBV infection.
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