Abstracts of Invited Lectures
Poster Abstracts

Falk Research Workshop

MORPHOGENESIS AND CANCEROGENESIS OF THE LIVER

Göttingen (Germany)
January 25–26, 2007

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G. Ramadori, Göttingen (Germany)
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Session I

Morphogenesis of the liver
HNF4α orchestrates expression of cell adhesion proteins to control epithelial formation within the fetal liver

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We have previously shown that fetal livers lacking the transcription factor HNF4α have severe developmental abnormalities including an inability to generate an epithelial parenchyma. In our original studies we found that expression of E-cadherin, a cell adhesion molecule with an integral role in forming adhesion junctions, was severely depressed. We, therefore, tested whether loss of E-cadherin during liver development was sufficient to disrupt junction formation in fetal hepatocytes. Surprisingly, mice lacking E-cadherin in their livers were normal. Based on this result, we postulated that HNF4α might regulate expression of multiple proteins involved junction formation and that loss of multiple adhesion or junction proteins was necessary to block formation of the hepatic epithelium during embryogenesis. We tested this proposal by examining global changes in gene expression associated with loss of HNF4α in fetal livers. Oligonucleotide array analyses revealed that HNF4α was required for expression of 27 genes with defined or predicted roles in cell adhesion or junction formation. Moreover, we demonstrated that several of these genes had HNF4α-binding sites that were occupied by HNF4α in fetal livers.

Since HNF4α has been shown to regulate expression of other transcription factors, such as HNF1α, we attempted to define the extent to which HNF4α was necessary for expression of the network of transcription factors that are required for normal hepatic function. Using a combination of bioinformatics and oligonucleotide array studies we found that 773 transcription factors could be detected in E18.5 fetal livers. Analyses of these factors in HNF4α null livers revealed that expression of 26 were reduced and that 18 had binding sites within their promoters that were occupied by HNF4α in fetal livers.

In summary, we conclude that HNF4α regulates cell junction protein gene expression to coordinate epithelial formation during hepatogenesis and that it utilizes both direct and indirect mechanisms to control hepatocyte differentiation.
**Endothelial cells in liver and pancreas development**

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The liver and pancreas arise from adjacent domains of the endodermal epithelium in the vertebrate embryo. We previously found that interactions between newly specified hepatic endoderm cells and nascent endothelial cells in embryos are crucial for the endoderm's subsequent growth and morphogenesis into a liver bud. Reconstitution of endothelial cell stimulation of hepatic cell growth with embryonic tissue explants demonstrated that endothelial signaling occurs independent of the blood supply. The development of the pancreas also requires endothelial-derived inductive signals. We found that interactions between the midgut endoderm and aortic endothelial cells induce endodermal expression of *Ptf1a*, a crucial pancreatic determinant. Endothelial cells also have a later effect on pancreas development, by promoting the survival of the dorsal mesenchyme, which in turn produces factors supporting pancreatic endoderm. Both of these pancreatic inductive events by endothelial cells have been reconstituted in vitro, demonstrating that they result from direct signaling by endothelial cells. A major goal of our laboratory is to determine the endothelial-derived signaling molecules and transcriptional regulatory responses involved in these inductive events in the liver and pancreas.

Our data show that cultured endothelial cells can promote the growth of liver bud explants and can induce *Ptf1a* in dorsal endoderm explants lacking an endogenous vasculature. We are purifying the endothelial cell line product(s) responsible for these effects. We are also identifying endothelial-responsive regulatory elements in *Ptf1a* and other genes by genetic mapping and chromatin-based assays. These latter approaches will allow us to track endothelial-responsive signal pathways starting from DNA targets within progenitor cells. The diversity of organogenic steps dependent upon endothelial cell signaling suggests that the cross-regulation of tissue development with its vasculature is a general phenomenon.
Plasticity of hepatic cell differentiation: bipotential adult mouse liver clonal cell lines competent to differentiate in vitro and in vivo

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In fetal liver, bipotential hepatoblasts differentiate into hepatocytes and bile duct cells (cholangiocytes). The persistence of such progenitor cells in adult mouse liver is still debated. In damaged liver of adult animals, when hepatocyte proliferation is compromised, bipotential oval cells emerge, probably from the bile ducts, proliferate and differentiate to regenerate the liver. However, treatment to elicit oval cell proliferation is not necessary to obtain normal bipotential stem cells from adult liver. Here, we have isolated bipotential clonal cell lines from healthy liver of 8–10 week old C57 BL/6 mice. Primary cultures established from hepatocyte enriched suspensions were characterized by time lapse image acquisition, immunocytology and RNA transcript analysis. While hepatocytes dedifferentiated with loss of apical polarity and of other hepatocyte markers, they rapidly activated expression of bile duct/oval cell markers. Reversibility of these processes was achieved in part by culture in dilute Matrigel or by aging of confluent cultures. Cell lines were obtained at high frequency, from mass cultures, from isolated colonies or by primary cloning of the hepatocyte enriched suspensions. Cells of the clonal cell lines do not grow in soft agar, are non-tumorigenic, and they express CK19, A6 antigen and α6 integrin as well as a large panel of hepatocyte functions. Furthermore they can participate in liver regeneration in Alb-uPA/SCID mice, where they differentiate in clusters of hepatocytes and occasionally bile ducts. These results demonstrate the existence in normal young adult mouse liver of a significant pool of clonogenic cells that are (or can become) bipotential.
Growth control of intrahepatic biliary epithelium

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The intrahepatic biliary tree is a complex three-dimensional network of interconnected ducts. The finest proximal branch of the intrahepatic biliary tree is the canal of Hering, which represents the link between the bile canaliculus and the biliary tree. The canal of Hering is lined by hepatocytes and cholangiocytes, contains the bipotential hepatic progenitor cells (HPC) and serves as the site for influx and localization of intra- and extra-hepatic adult stem cells (1, 2). The canal of Hering continues into bile ductules, entirely lined by cholangiocytes, which converge into interlobular bile ducts located in the portal space, and then continue into ducts of major size (1, 2). It is now quite accepted that the intrahepatic bile duct system originates from the endodermal cells already within the liver (1–4). The key event in formation of the intrahepatic biliary tree occurs at the interface between the developing hepatic parenchyma and the mesenchyme of the portal tracts (1–4). Beginning around the 6th week of gestation, the hepatoblasts immediately adjacent to the portal tract mesenchyme flatten slightly and become a continuous layer of biliary-type cuboidal cells, the ductal plate. The hepatoblast differentiation into cells destined for the biliary tree (cholangiocytes) or into cells destined for the hepatic parenchyma (hepatocytes), appears to occur at the time of ductal plate formation and depends from a number of different, and not yet completely defined, agents and mechanisms (1–5). Certainly, different matrix components specifically enriched in the connettive tissue near portal vein play a major role. Recently, the Notch signaling has been shown to play an important role in the differentiation of biliary epithelial cells and in tubular formation (6). Also in the differentiation of hepatoblast toward cholangiocytes the Notch signalling plays a major role by controlling a network of transcription factors (1–6). Suppression of “hepatocyte specific” genes (C/EBPa, HNF6→HNF1beta cascade) and activation of “cholangiocyte specific” genes (CK19) promote the transitions of hepatoblasts into cholangiocytes. Finally, different hormones, growth factors and cytokines (parathyroid hormone related peptide, glucocorticoids, TGFα, HGF/scatter factor) are also involved in the remodeling of ductal plate and in bile duct developments (1–6).

In the adult liver, cholangiocytes lining the intrahepatic biliary tree are mitotically dormant supported on a conventional basement membrane and surrounded by the connective tissue matrix and the peribiliary plexus (1, 2). The constitutive expression of the cyclin dependent kinase inhibitors p27, bcl2, Bcl-xL and Mcl-1 are some of the factors responsible for the maintenance of this resting state. Cholangiocytes display primary cilia in their apical pole, which could be of relevance for maintaining cholangiocytes in a quiescent state since cilia bending maintains intracellular Ca^{2+} at a threshold where cAMP, an intracellular modulator of proliferation, is inhibited (1). Many different insults determining cell damage, disruption of the normal cell/matrix interaction or release of cytokines or soluble mediators trigger cholangiocyte proliferation. In the last 15 years, the intrahepatic biliary tree has become the object of extensive studies, which highlighted the extraordinary biological properties of cholangiocytes involved in bile formation, injury repair, fibrosis, angiogenesis and regulation of blood flow (1, 2, 7, 8). Proliferation is a “typical” property of
cholangiocytes and is key as a mechanism of repair responsible for maintaining the integrity of the biliary tree (1, 2, 7). Cholangiocyte proliferation occurs virtually in all pathological conditions of liver injury where it is associated with inflammation, regeneration and repair, thus conditioning the evolution of liver damage. In most of these conditions, cholangiocyte proliferation is a part of the so called “ductular reaction”, a term coined by Popper to identify the expanded population of epithelial cells at the interface of the biliary tree and the hepatocytes, and which refers to proliferation of pre-existing ductules, progenitor cell activation and appearance of intermediate hepatocytes (1, 2, 7). Recently, the term “ductular proliferation” has been suggested (8) instead of “ductular reaction”, however, this term is not completely satisfactory since the proliferating reactive ductules may not simply arise from proliferation of pre-existing bile ductular cells, as they may also originate from activated and differentiated progenitor cells, from cells which entered from the circulation and differentiate towards liver cells or, more rarely, from biliary metaplasia of hepatocytes (1, 2, 7).

Interestingly, proliferating cholangiocytes acquire the phenotype of neuroendocrine cells, and secrete different cytokines, growth factors, neuropeptides and hormones, which represent potential mechanisms for crosstalk with other liver cells (1, 2, 7). Many studies suggest the generation of a neuroendocrine compartment in the injured liver, mostly constituted by cells with cholangiocyte features, which functionally conditions the progression of liver disease. It is now clearly evident that cholangiocyte proliferation is modulated by a huge number of agents including hormones (estrogens, prolactin, gastrin, somatostatin...), neuropeptides (acetylcholine, catecholamines, dopamine, serotonin...), growth factors (IGF1, NGF, VEGF, HGF), cytokines and different bile salts (1, 2, 7). Most of these agents act on cholangiocyte proliferation by modulating cAMP-related pathways, Ca$$^{2+}$$-related signalling and/or the phosphatidylinositol 3-kinase (PI3K) pathway.

In conclusion, recent insights on cholangiocyte pathophysiology and the emerging role of proliferating cholangiocytes as neuroendocrine compartment (8) in the diseased liver could provide new potential strategies for the management of chronic liver diseases.

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Session II

Damage and repair
Modulation of hepatic apoptosis by inhibitors of histone deacetylases

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Our previous studies were focused in vivo on cytokine-mediated hepatic apoptosis of the TNF and CD95 system, the role of caspases, as well as the role of the energy (J Exp Med 191, 1975–1985, 2000) and the glutathione redox status (J Biol Chem 277, 5588–5595, 2002) for the activation of caspases and the execution of the apoptotic program. Recently we addressed the mechanism of action of Melphalan and found that this anti-neoplastic drug mediates its hepatotoxicity via both TNF receptors by expressing membrane-bound TNF on Kupffer cells (J Immunology 175, 4076–4083, 2005).

While in healthy cells, the balance between apoptosis and proliferation provides the intactness of tissue homoeostasis, this process is disturbed in tumor tissue. A novel approach in cancer therapy comes from the use of epigenetic mechanisms in order to target tumor tissue via the apoptotic pathway, in particular in the pharmacological manipulation of gene expression by inhibiting histone deacetylases. Histone deacetylase inhibitors (HDIs) are a chemically heterogeneous group of compounds which lead globally to hyperacetylation of histones with the consequence of a general promotion of gene expression. HDI-mediated modulation of an increased expression level of pro-apoptotic together with a decreased expression of anti-apoptotic proteins represents therefore a potentially very efficient tool to amplify the cell death process.

In the in vitro study presented here, we examined whether and how malignantly transformed liver cells, primary mouse hepatocytes, whole mouse liver, and human primary liver cell cultures are sensitized by different HDIs to death receptor agonists, i.e. TNF, TRAIL and CD95L, respectively. In the human liver cancer cell line HepG2 and in primary mouse liver cell cultures, treatment with Apicidin or M344 or CBHA Valproic acid (VPA) selectively caused a sensitization towards CD95L- and TRAIL-triggered apoptosis, but not towards the one initiated by TNFα. The relative rank order of the sensitizing potency of the various HDIs examined was similar in all cell types: Apicidin > M344 ≥ CBHA >> VPA. VPA was examined in primary human hepatocytes. In fact, a similar sensitization towards CD95 was found as in HepG2 cells.

Using a transfection approach it was shown that HDIs enhance caspase-8 mediated caspase-3/-7 activation. Our data showed that in this system overexpression of caspase-8 in HepG2 cells is sufficient to trigger the apoptotic cascade. We also examined caspase-3/-7 activity in a cell-free systems using dATP/cytochrome c in cytosolic extracts of HepG2 cells and found no influence of the HDIs tested on this part of the intrinsic pathway of apoptosis. In a death receptor-independent but mitochondria-dependent model of apoptosis i.e. UV light irradiation of HepG2 cells, the HDIs tested had essentially no influence on cell death. These results indicate that the HDIs used here preferentially modulate the extrinsic pathway of apoptosis.
After exposure of HepG2 cells to the various HDIs, a strong downregulation of the distal receptor protein cFLIP, which inhibits the assembly of the death-inducing signalling complex (DISC) was found. The expression of the anti-apoptotic protein XIAP was decreased, while pro-apoptotic adapter protein FADD was increased. It is concluded (i) that HDIs preferentially modulate the extrinsic death receptor signalling pathway in HepG2 cells and (ii) that the major site of action of HDIs is located at the activation from caspase-8 to caspase-3/-7. Experiments using the isolated, recirculating mouse liver perfusion as an in situ model confirmed the sensitization by the HDI apicidin towards CD95-dependent apoptosis.

Our results extend the biochemical and pharmacological basis for HDIs and provide new evidence for potential limitations in clinical use in patients with a liver disease history where the CD95 or TRAIL system might be pre-activated.
Antiapoptotic effect of ursodeoxycholic acid in hepatocytes

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Ursodeoxycholic acid (UDCA), an endogenous bile acid, is widely used in the treatment of liver disorders. UDCA acts as a potent inhibitor of the classical mitochondrial pathway of apoptosis, in part, by directly stabilizing membranes. Furthermore, as a cholesterol derived molecule, UDCA interacts with nuclear steroid receptors (NSR), such as the glucocorticoid receptor (GR), suggesting that it may also modulate gene expression. In these studies, we investigated whether regulation of upstream mitochondrial events by UDCA prevents hepatocyte apoptosis. In addition, we explored the role of NSR in the antiapoptotic function of UDCA. Our results showed that UDCA inhibited TGF-β1-induced apoptosis of primary rat hepatocytes, by modulating the expression of E2F-1, Mdm-2, p53 and Bcl-2 family members, in a caspase-independent manner. Moreover, UDCA specifically prevented induction of p53 and Bax by overexpression of E2F-1 and p53, respectively. We also demonstrated that UDCA modulated the E2F-1/Mdm-2/p53 apoptotic pathway in hepatocytes through a NSR-dependent mechanism. Indeed, pretreatment with UDCA upregulated NSR expression. UDCA was further shown to promote GR/hsp90 dissociation, thus inducing subsequent NSR translocation. However, when the C-terminal region of GR was deleted, UDCA no longer induced GR/hsp90 dissociation and GR nuclear translocation nor protected against apoptosis. Surprisingly, the bile acid does not require NSR transactivation for preventing apoptosis in hepatocytes. Finally, UDCA appeared diffuse in the cytosol but aggregated in the nucleus of hepatocytes; nuclear trafficking of UDCA occurred through a NSR-dependent mechanism.

In conclusion, this work revealed additional pathways for the antiapoptotic function of UDCA, demonstrating that the bile acid regulates the expression of specific targets upstream of mitochondrial commitment. Further, our results suggest that UDCA interacts with a specific region of NSR to translocate to the nucleus and inhibit apoptosis-related genes in hepatocytes.

(Supported by FCT, Lisbon, Portugal)
Mechanisms of liver damage in Con A-induced hepatitis

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Concanavalin A (Con A) induces acute immune-mediated liver injury upon a single intravenous injection to mice, which depends on CD4\(^+\) T cell activation and which is associated with a typical Th1 cytokine response. The mechanisms by which T cells contribute to hepatic inflammation and liver damage in Con A hepatitis have been intensively studied. They interact directly with hepatic sinusoidal endothelial cells and activate Kupffer cells to produce TNF\(\alpha\), which might result in endothelial cell damage thereby giving access of FasL-expressing lymphocytes (probably NKT cells) to hepatocytes. Alternatively, TNF\(\alpha\) might also contribute directly to hepatocyte apoptosis. Both bioactive forms of TNF\(\alpha\), i.e. its membrane-bound precursor as well as in its soluble form have been shown to mediate Con A-induced liver injury via cooperative signaling of both TNF receptors, i.e. TNFR1 and TNFR2. Adoptive transfer studies with TNFR2\(^{-/-}\) mice suggested that TNFR2 is critical for down-modulation of the NF-\(\kappa\)B-dependent anti-apoptotic pathway in the liver thereby facilitating TNFR1-induced activation of apoptosis.

In addition to CD4\(^+\) conventional T cells, glycolipid activated, CD1d-dependent NKT cells are also involved in the pathophysiology of Con A-hepatitis. These cells produce large amounts of IFN\(\gamma\) and IL-4 upon activation in vivo. Indeed, IFN\(\gamma\) has been shown to mediate Con A hepatitis. Studies using knockout mice for several components of the IFN\(\gamma\) signaling pathway provided evidence for a critical role of IFN\(\gamma\) for induction of the cytokine response, rather than for induction of apoptosis. IL-4 seems to contribute to Con A-induced liver injury by activation of eosinophils.

We have observed recently, that C57Bl/6 mice developed resistance towards a second Con A injection within 8 days after the first application. This state of tolerance was characterized by significantly reduced serum transaminase activities and improvement of liver morphology as well as by an anti-inflammatory cytokine milieu, i.e. down-modulation of IFN\(\gamma\), TNF\(\alpha\), IL-6 and IL-2 production and a concomitant increase of IL-10 release. Experiments using IL-10\(^{-/-}\) mice argued for a critical role of IL-10 for induction of tolerance. Moreover, the tolerogenic effect was reduced upon in vivo depletion of CD25\(^+\)CD4\(^+\) regulatory T cells (Tregs) prior to re-stimulation, and Tregs from Con A tolerant mice displayed a higher immune-suppressive potential in vitro and in vivo compared to those from non-pretreated animals.

Finally, Con A-induced hepatitis has often referred to represent a model of autoimmune hepatitis. Although Con A activates a wide variety of T cells irrespective of their antigen specificity, thus not representing a model for autoimmune liver disease in the strict sense, several features of autoimmune hepatitis are observed in this model. These include the Th1-response, strain differences with respect to susceptibility, the recently observed immunosuppression in state of remission and a good responsiveness to immunosuppressive drugs. Hence Con A-induced hepatitis in mice serves as a model to study basic mechanisms of T cell-mediated destruction of hepatocytes in vivo, immune-regulator functions of the liver, as well as to develop new strategies for immunosuppressive treatment.
Inflammation, damage repair, and liver fibrosis – Role of cytokines and different cell types

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Liver fibrosis is defined as an excessive deposition of extracellular matrix. It is the main complication of chronic liver damage and its endpoint, the liver cirrhosis is responsible for impressive morbidity and mortality. To the current knowledge liver inflammation in many animal models (e.g. after CCl₄ administration or bile duct ligation and in ischemia-reperfusion models) but also in humans may not be initiated by death (apoptosis or necrosis) of liver parenchymal cells but by liver resident and by recruited inflammatory cells. The hepatocellular stress, induced by hepatotoxins or maybe also by viruses, may lead to activation of liver resident macrophages on one side and to release of chemokines on the other side. Proinflammatory cytokines like the “immune” interferon, namely IFN-γ, whose tissue concentration increases early after toxin administration, followed by TNF-α (later), are released by NK-cells as well as by the Kupffer cells. They induce an increased expression of cell adhesion molecules like intracellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on sinusoidal endothelial cells and a downregulation of platelet endothelial cell adhesion molecule-1 (PECAM-1) allowing the recruitment and sinusoidal transmigration of inflammatory cells. The increased expression of lymphocyte function-associated antigen-1 (LFA-1) on mononuclear phagocytes may enable them to invade into the space of Disse, which precedes the death of hepatocytes. However, IFN-γ may also have a protective function e.g. against liver injury during extrahepatic cholestasis. Inflammation perpetuates as long as the damaging noxae remains present or is repeatedly administered. Leucocytes can enter the liver tissue by different routes (portal tracts, sinusoids, and the hepatic vein) which may determine distribution of the hepatic infiltration. The inflammatory infiltrate may include T-lymphocytes (more peripheral), B-lymphocytes (mainly central), plasma cells, histiocytes (granuloma), eosinophils, neutrophils, macrophages, NK-cells and mast cells. Resident and recruited inflammatory macrophages can stimulate matrix synthesis by activated (myo)fibroblastic cells and its deposition by the action of cytokines, especially TNF-α, TGF-β and reactive oxygen intermediates/ lipid peroxides. The accumulation of extracellular matrix proteins in liver fibrosis and cirrhosis may be due to different cell types which acquire a myofibroblastic phenotype – (i) the hepatic stellate cells, located in the space of Disse and widely thought to be the major producers of extracellular matrix in the liver, (ii) the portal fibroblasts as well as (iii) myofibroblasts of the portal tracta and the pericentral areas. Further studies also suggest an impressive role of (iii) bone marrow derived myofibroblasts. Differences have been reported between the different cell populations with respect to myofibroblastic differentiation, activation and “deactivation”, proliferation and apoptosis. However further studies are required to biologically and biochemically characterize these cells, to determine their interactions with inflammatory cells and to reveal the composition of cytokine environment necessary for their activation or cell death. These data are essential to understand the mechanisms underlying the progressive development of excessive scarring in the liver as well as the ability of the liver for tissue repair and regeneration.
The stages of liver repair as well as that of liver fibrogenesis resemble that of a wound healing process - inflammation, formation of a provisional clot influencing the invasion and proliferation of inflammatory and matrix producing cells, finally a complete restauuration or scar formation (septa). In fact, when the injury is recurrent (or “chronic”), matrix deposition occurs in excess of resorption as a result of an imbalance between fibrogenesis and fibrolysis leading to scar formation. As scarring progresses from bridging fibrosis to the formation of complete nodules it results in an architectural distortion and ultimately in liver cirrhosis.

From the toxic animal models of liver fibrosis we learned that fibrogenesis may result form recurrent small liver injuries, which per se would result in complete tissue repair, suggesting that activation of the fibrosis process with matrix deposition may not be a primarily cellular problem but of recurrence of the damaging noxae within a certain time window. Furthermore, the progression of fibrosis and probably also the susceptibility might be influenced by genetic polymorphisms. Different types of disease, however, lead to different patterns of fibrosis as the disease progresses. These can be divided into portal and central based fibrosis. The major portal based diseases that lead to cirrhosis include chronic viral hepatitis, chronic cholestatic diseases and hemochromatosis. Central based diseases include steatohepatitis (alcoholic or non alcoholic) and chronic venous outflow obstruction. The septa can be divided into porto-portal (e. g. following cholestatic liver injuries) or porto-central (e. g. in case of viral hepatitis) or centro-portal (e. g. alcoholic liver disease). Data available now propose that in the different patterns of fibrosis also different types of myofibroblastic cells seem to be the predominant producers of extracellular matrix, suggesting that there is a need to reconsider the role of hepatic stellate cells in development of liver fibrosis.
Biliary cells in liver disease

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In addition to classical biliary disorders, “bile duct proliferation” can be observed in a wide variety of liver diseases. This histological reaction used to be one of the most neglected fields of liver pathology. The exact origin, function and importance of these structures are still largely unknown. They have been proposed to participate in liver regeneration, re-absorption of biliary constituents or to provide route for the escape of bile. However, biliary proliferation has been mentioned in connection with adverse reactions as well e.g. liver fibrosis and carcinogenesis.

Similarities between the oval cell proliferation of rodent liver and ductular reactions suggest the stem cell origin for (at least some of) the ductules in the human liver. The recently increased attention for the field resulted in confusion in the terminology of these lesions. This anarchy led a panel of experts to publish a consensus paper (Hepatology 39, 1739, 2004) proposing to use the unified term “ductular reaction” and discouraging further classifications e.g. typical and atypical ductular proliferation. In one respect, the application of simplified terminology is certainly useful. On the other hand, the enforcement of all histological reactions with the appearance of ductules in one category may be oversimplification which does not help to understand the biological and clinical complexity of this tissue response.

In order to establish a rational classification of “ductular reactions” the morphology and immunophenotype of biliary ductules were analysed in various liver diseases: PBC, PSC, secondary biliary cirrhosis, viral hepatitis and cirrhosis, hepatic necrosis, peritumoral reaction etc. The ductules in these diseases can be divided into three categories based on the expression of epithelial membrane antigen (EMA), NCAM/CD56, CD 10 and Claudin 2. The distribution of these antigens on the different ductules showed similarity with the expression pattern of these genes on particular segments on the biliary tree in the normal liver. This observation suggests that different forms of ductular reactions may derive from defined portions of the biliary system.

The rational classification of ductular/biliary reactions may assist in development of a differential diagnosis and can contribute to better understanding the role that cholangiocytes play in biliary and non-biliary liver diseases.
Session III

Cancer development
Genomic decoding of human liver cancer

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Much is known about both the sequential cellular changes that precede the formation of hepatocellular carcinoma (HCC) and the etiological agents (i.e. HBV, HCV infection, and alcohol) responsible for the majority of HCC. Nevertheless, the molecular pathogenesis of HCC is not well understood. Also, a staging system that reliably separates patients with early HCC as well as intermediate to advanced HCC into homogeneous groups with respect to prognosis does not exist. This is important because the natural course of early HCC is unknown and the progression of intermediate and advanced HCC are quite heterogeneous. Thus, improving the classification of HCC patients would at minimum improve the application of currently available treatment modalities and at best provide new treatment strategies. We have investigated the possibility that variations in gene expression of HCC at diagnosis would permit the identification of distinct subclasses of HCC patients with different prognoses.

We applied three independent but complementary approaches for data analysis to uncover subclasses of HCC and the underlying biological differences between the subclasses. Unsupervised classification methods based solely on gene expression patterns revealed two subclasses of HCC strongly associated with the length of patients’ survival. Also, when the classifiers used in a training set to optimized classification of the tumors were applied to the validation set, all the classifiers successfully separated poorer survival patients (cluster A) from longer survival patients (cluster B). Furthermore, application of a univariate Cox regression model was used to identify individual genes whose expression is highly correlated with the length of survival. Application of survival associated genes for subclass prediction was highly accurate as illustrated by the fact that averaged gene expression indices from the selected 406 “survival genes” were sufficient to segregate the two subclasses even without the use of sophisticated prediction models.

The variability in the prognosis of individuals with HCC may result from activation of different oncogenic pathways during tumorigenesis and/or from a different cell of origin. We have addressed whether the transcriptional characteristics of HCC can provide insight into the cellular origin of the tumor. We integrated gene expression data from rat fetal hepatoblasts and adult hepatocytes with HCC from human and mouse models. Individuals with HCC who shared a gene expression pattern with fetal hepatoblasts had a poor prognosis. The gene expression program that distinguished this subtype from other types of HCC included markers of hepatic oval cells, suggesting that HCC of this subtype may arise from hepatic progenitor cells. Analyses of gene networks showed that activation of AP-1 transcription factors in this newly identified HCC subtype might have key roles in tumor development.
These findings support the notion that multiple molecular pathways dictate the development and different clinical outcomes of HCC. These findings also indicate that the molecular features of HCC such as prognostic gene expression signatures are present at the time of diagnosis. Therefore, the use of gene expression profiling promises to improve molecular classification and prediction of outcomes in HCC. Furthermore, molecular stratification of individuals with HCC into homogeneous subgroups may provide opportunities for the development of new treatment modalities.
Antitumoral effect of thyroid hormone on rat liver carcinogenesis

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Several evidences indicate that cell proliferation is associated with increased HCC development. Indeed, agents that do not induce liver cancer when given to adult rats, are carcinogenic when given to newborn rats, whose liver is actively dividing, or when given shortly before or after surgical heptectomy of 2/3 of the liver (PH); moreover, in humans, as well as in experimental animals, conditions associated to liver damage and regeneration (HBV/HCV infection, alcohol, hemochromatosis, copper-accumulation, a choline-methionine deficient diet) have been shown to be associated with increased incidence of HCC. All these evidences have led to the hypothesis that cell proliferation per se may be carcinogenic and carcinogens that increase cell proliferation may be operating exclusively by this mechanism.

The thyroid hormones influence a variety of physiological processes, including cell growth and metabolism in mammals, metamorphosis in amphibia, and development of the vertebrate nervous system. As far as the liver is concerned, T3 has been shown to be a powerful inducer of hepatocyte proliferation in rats, and its mitogenic capacity has been utilized for experiments in gene therapy and repopulation of hepatocytes.

Our recent work has shown that treatment with thyroid hormone (T3), in spite of its mitogenic activity, exerts an antitumoral effect. Indeed, short treatment with T3 accelerates the regression of nodules and adenomas induced by genotoxic carcinogens, and inhibits the incidence of hepatocellular carcinoma and lung metastases; the anticarcinogenic effect of T3 is maintained also when treatment begins late in the process, and, its antitumoral property appears to be specific and not shared by other liver mitogens, such as members of the class of peroxisome proliferators. Interestingly, disappearance of liver preneoplastic lesions occurs in the absence of apoptosis, suggesting that the induction of differentiation of preneoplastic hepatocytes towards a normal, fully differentiated phenotype is the main mechanism involved in this process. As most, if not all, of the effects induced by T3 are mediated by nuclear receptors (TRs) encoded by two genes (α and β) and expressed as several isoforms, additional experiments were performed aimed at determining the TR isoform responsible for the antitumoral effect of T3. Preliminary results indicate that GC-1, a selective agonist of the TR-beta isoform, accelerates the disappearance of hepatic preneoplastic lesions, suggesting an important role of TR-beta isoform in this process. Identification by microarray analysis of the critical target genes involved in the remodelling of preneoplastic hepatic nodules is currently under study.

(Supported by funds from Associazione Italiana Ricerca sul Cancro, and Ministero dell’Università e della Ricerca Scientifica, Italy)
Alpha-fetoprotein in liver regeneration and cancer

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Alpha-fetoprotein (AFP) was first detected by electrophoresis of human fetal serum in the first (a1) position next to serum albumin while in normal adult serum this protein was not detected. AFP is a polypeptide of about 600 amino acids and consisting of 4% carbohydrate residues. It is a secretory protein with structure and physicochemical properties similar to serum albumin.

AFP functions and its role in development and carcinogenesis are still far from being understood. The main properties of AFP are a high affinity for polyunsaturated fatty acids (105 times higher than albumin) and the ability to bind estrogens. Some data indicate that AFP participates in immune response regulation.

AFP is the main embryonic plasma protein synthesized by the fetal liver and yolk sac. Its serum concentration falls rapidly after birth to reach low, barely detectable levels in the adult. In contrast, albumin is the dominant plasma protein synthesized by the adult liver and its serum concentration increases from low levels early in liver development to high levels after birth and through adult life. Hence, the synthesis of albumin and AFP undergoes sequential changes during embryonic and postnatal development.

Elmaouhoub et al. could show that at the time of initiation of hepatic specification (in rats’ embryonic day 10), the complete program controlling regulation of mRNA expression as well as synthesis and secretion of albumin and AFP are already established.

Several molecular analyses have clearly demonstrated that the differential expression of the AFP and albumin gene during liver development is mainly regulated at the transcriptional level. Down-regulation of AFP expression seems to be mediated by an interaction of all-trans-retinoic acid (RA), hepatocyte nuclear factor (HNF) 1 and HNF 4 signals.

In adults, AFP expression can resume in the liver under several pathophysiological conditions, mainly (I) proliferation of adult hepatic progenitor cells termed “oval cells”, or (II) development of hepatocellular carcinoma (HCC).

(I) Oval cells may emerge and proliferate in the adult liver when the proliferative capacity of hepatocytes is severely impaired. This scenario can experimentally be mimicked in rats by exposure of the animal to a carcinogen (2-acetylaminofluorene, AAF), and subsequently applying a strong proliferative stimulus, e. g. performing a partial hepatectomy (PH). Since the proliferative capacity of hepatocytes is severely impaired by AAF, oval cells start to proliferate, and eventually may differentiate to hepatocytes or cholangiocytes. AFP mRNA and protein are strongly induced in this rat model of liver regeneration, and AFP is the most specific marker of oval cells, so far. Notably, AFP seems not to be induced in liver regeneration without involvement of oval cells.
(II) HCC is a common cancer in patients with chronic liver disease. In these patients, serum AFP is the most widely used oncomarker for suspecting HCC. However, modest elevations of AFP levels (between 10 and 500 ng/ml) may also occur in benign liver diseases (hepatitis of any cause or liver cirrhosis), thus presenting a major clinical problem. Chronic liver disease finally leads to severe impairment of the functional and proliferative capacity of hepatocytes. Presence of oval cells has been demonstrated in patients with chronic liver disease, and there is now increasing evidence that activation of oval cells may also give rise to HCC.
Session IV

Cancer prevention and therapy
Epidemiology and prevention strategies of hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is a disease linked to environmental and life style factors. Hepatitis B and C account for most cases of the tumor, worldwide, the former virus being the main risk factor in mainland China and Africa while the latter virus together with alcohol is the prevalent cause of HCC in Europe, USA and Japan. In many developed countries, time trends of HCC incidence are on the rise as a consequence of the accumulation of patients with chronic liver disease of either viral or alcoholic origin being at increased risk of developing HCC. Prevention is the only realistic approach for reducing mortality rates associated with this tumor. The substantial reduction in hepatitis B and C transmission achieved among the general population through screening of blood donations and anti-hepatitis B vaccination programs, is expected to significantly reduce the HCC burden in the near future. Preliminary data in Taiwan indicates, in fact, that HBV vaccination of all newborns has been effective in preventing perinatal transmission of HBV together with the onset of infantile HCC. Primary prevention includes also approaches which alter susceptibility to HCC, as well as treatments slowing progression to cirrhosis (chemoprevention). The only evidence that chemoprevention may reduce HCC risk is a multicenter randomized controlled study in Asian patients with advanced hepatitis B treated with the oral nucleoside analogue lamivudine. Results of this trial, however, have been criticized by many and were not reproduced in the West. It is even more controversial whether interferon reduces the risk of HCC in patients with hepatitis B both during the early HBeAg positive and the late anti-HBe positive phase of hepatitis B. The reanalysis of a large database in Japan and a randomized controlled study suggested that a reduction of HCC in interferon treated patients with chronic hepatitis C may occur independently from a virological response. More recently, two meta-analysis revealed a modest (~10%) reduction of HCC risk occurring only in patients with chronic hepatitis C who achieved a sustained virological response, with doubtful results in cirrhotic patients. In both HBV and HCV setting evidence for secondary prevention of HCC is still inconclusive due to poor methodologies and scientific background of the studies.

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Medical treatment of liver cancer

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Medical treatment of liver cancer in terms of local ablative measures, systemic cytotoxic chemotherapy, biological treatment or new targeted therapy approaches, as well as combinations thereof may be considered for patients who do not qualify for surgical, i.e. curative, therapies and will be discussed here both for hepatocellular carcinomas (HCC) and cholangiocarcinomas of the liver (CC). Since cirrhosis has to be viewed as precancerous condition of the liver it seems natural that most HCCs are diagnosed in cirrhotic livers, however, mostly in advanced stages. Impaired liver function due to cirrhosis not only limits surgery in these patients, but also pharmacotherapy, in particular of cytotoxic agents that may further impair liver function and obtain increased toxicity due to reduced hepatic detoxification. Even those agents passing the hurdle of a randomized clinical trial like doxorubicin failed to achieve objective response rates high enough to feed the hope for a survival benefit. Increase of response rates by combining other cytotoxic agents only was obtained at the expense of an (unacceptable) increase of adverse events. Alternative treatment approaches employing biological agents like anti-estrogens or octreotid, all encouraged by the presence of respective receptors in HCC initially produced encouraging results not confirmed later. Recent progress in treatment of solid tumors by small molecules or by antibodies targeting cell signalling molecules involved in tumorigenesis or tumor cell growth induced the hope for new treatment options in HCC. In fact, targeting of the receptor tyrosine kinases kit, PDGF-R or EGF-R by respective molecules like imatinib, erlotinib or even by sorafenib, a multikinase inhibitor, suggested efficacy in HCC in recent studies, but further investigations have to find the right place of these drugs for the right patient in the right combination.

The circumscribed nodular growth of HCC favours local ablative therapies like radiofrequency ablation (RFA), laser-induced thermotherapy (LITT) or percutaneous injection of toxic compounds like ethanol (PEI) that were shown to induce a survival benefit for patients with small tumors or for single larger tumors. However, radiofrequency may be granted a minor superiority in respect to response rates and survival benefit, but not to cost effectiveness. Large tumors, in particular, that are in risk for incomplete ablation by RFA, LITT or PEI may be treated by transarterial chemoembolisation (TACE) that was shown to improve survival in these patients. It may also be effective in patients on the transplantation waiting list reducing losses from the list and tumor recurrence after transplantation.

In contrast to HCC cholangiocarcinomas mainly erupt from non-cirrhotic livers. Liver function generally is close to normal with the exception of bile duct obstructions that may be managed by interventional therapies like internal or external stent drainage. Although pharmacotherapy of CC therefore is less troublesome, effectiveness remains poor except for gemcitabine that may be effective as concluded from the limited data available. However, local ablative therapies are not effective due to diffuse growth of CC.
In summary, medical treatment both of HCC and CC remains individual to date. In HCC local therapies like RFA or PEI may be preferred for small, TACE in combination with RFA or PEI may be preferred in larger tumors for patients who do not qualify for curative surgery. In the near future present therapies may be expanded by targeted therapy molecules.
Surgical therapy of liver cancer: resection or transplantation?

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Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and is estimated to cause approximately half a million deaths per year. Most tumors (80%) develop in cirrhotic livers caused by viral hepatitis C or B and alcoholic liver disease. In the Western World only a minority of patients is amenable to surgery.

In our experience comprising more than 650 patients, less than 20% were suitable for liver resection. Liver resection was performed in 114 patients. In 67 of 114 patients HCC occurred in a noncirrhotic liver. 5-year survival in cirrhotic patients was 20% compared to 40% in noncirrhotic patients. In 120 patients liver transplantation (LT) was performed. In the majority of these patients (n = 87) the HCC was identified during the preoperative workup. The rate of incidentalomas was 22%. The latter figure hints to an important problem: diagnostic workup is highly unreliable. Number and even size of lesions cannot be predicted due to the underlying cirrhosis. Lesions of less than 1 cm in diameter are usually missed.

Liver surgery is hampered by the functional impairment of the liver. In addition to that, up to 100% of the patients experience recurrent disease within 5 years after hepatic resection. Long-term survival is influenced by cirrhosis: More than 30% of the patients die from cirrhosis and not from recurrence. Five-year survival following liver resection ranges from 10% to 50% depending on the underlying disease and on the respective tumor features.

LT was assumed to be superior to conventional surgery. Early results including patients with advanced disease did not exceed a five-year survival of 20% to 25%. Presently, Milan criteria defining the indication of LT in HCC (≤ 3 nodules, ≤ 3cm) are generally accepted. Based on these criteria the 5-year survival is 60% to 70%, whereas patients with more than one small nodule are supposed to be treated best by LT. The crucial question remains the indication for resection versus LT in patients with small and singular nodules. If the HCC may be cured by hepatic resection, sparse liver grafts might be saved and LT may be an option for those patients who experience tumor recurrence. Unfortunately, LT as a rescue approach was possible only in a few resected patients according to several authors. Nevertheless, liver resection is performed in many centers if feasible. We prefer to perform LT as the first treatment option even in singular tumors.

Due to the lack of donor organs, patients with HCC who are candidates for LT, have to wait until an organ is available. During waiting time the tumor may exceed the listing criteria and the patients have to be delisted. There are several approaches to bridge this period: radiofrequency ablation, ethanol injection, chemoembolization or even liver resection.

The crucial issue remains to select those patients whose tumor is suitable for LT. Even if tumors with vascular invasion and poorly differentiated tumors are usually excluded, reliable biological markers capable of selecting appropriate tumors are urgently needed.
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POSTER ABSTRACTS

Poster Numbers 1 - 33
Diseases of pancreatobiliary system at chronic virus hepatitis B and C – Syntropy or extrahepatic manifestations?

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Introduction: One of actual problems of clinical medicine is studying co-morbidities. The problem of combination cholelithiasis with chronic viral hepatitis is discussed.

Methods: 485 cases of viral hepatitis B and C are analysed, including clinical, morphological data.

Results: 485 patients with chronic viral hepatitis and cirrhoses of a liver B and C were observed (without dependence from etiology). In this group is more often, than in other population of region had a chronic cholecystitis and cholelithiasis ($\chi^2 = 137.47$, p < 0.0001), chronic pancreatitis ($\chi^2 = 104.42$, p < 0.0001), diabetes ($\chi^2 = 199.1$, p < 0.0001). Pathology of biliary system was revealed in 30.5% of cases, chronic pancreatitis at 12.4%. Diseases correlated with markers HBV- and HCV-infections (HBsAg, HBcorAb IgG, HBeAg, HCV Ab IgM, HCV Abcore, NS3, NS4, NS5, DNA HBV, RNA HCV), specific (AT to islet of pancreas, insulin, GAT) and nonspecific autoimmune parameters (C-reactive protein, ESR, RF, antibody to cardiolipin, to DNA, ANA, LE cells). Cholelithiasis more often accompanied HCV (14%, p < 0.001) and HCV-cirrhosis (37%, p < 0.001).

Discussion/Conclusion: Chronic cholecystitis and cholelithiasis at viral hepatitis at all stages of infectious process we consider as syntropy, and in this case they are integrated by a morphological basis. The chronic pancreatitis and diabetes at chronic viral hepatitis B and C at all stages of infectious process can be related either to syntropy or to autoimmune damages of pancreas.
Clinical features and outcomes of hepatocellular carcinoma

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Introduction: To estimate clinical manifestations and outcomes of HCC at patients in Ulyanovsk region for the period 1994–2003 years.

Methods: 6478 patients were hospitalized with pathology of liver in gastroenterology department of regional clinical hospital. Primary (HCC, cholangiocarcinoma) and metastatic tumours of a liver were diagnosed in 1.56% of all cases. 1127 patients with primary liver cancer were included in the cancer register, 516 from which died during 3.75 ± 0.35 months. Standard criteria of diagnostics consisted of sonographic, laboratory (AFP), histologic parameters, nodal and diffuse growth pattern; tubular, trabecular, acinar histologic forms. For the 10-years period yearly mortality from HCC had increased in 5.6 times.

Results: HCC prevailed among primary liver cancer. Metastatic lesions of a liver were revealed 11 times more often, than primary. The age of patients with HCC varied from 55 till 73 years, median age – 65.7 ± 0.49 years (95% CI: 58.7–69.4 years). Sex ratio of men and women was 61.2 to 38.8%. HCC at 60% of patients debuted within 1.5–2 months. Clinical presentation of HCC was characterized by symptoms of intoxication, malaise, progressive weight loss, haemorrhagic manifestations, fever, progressing portal hypertension, jaundice, cholestasis, elevated erythrocyte sedimentation, anaemia in 43–60% of cases. HCC developed in right lobe of liver, in VI–VII segments 36–50, 67–60, 110–80 mm and more in diameter. It metastasized in a liver gate, and in 19% of cases had plural metastases in internal organs. HCC was diagnosed at HBV-associated cirrhosis of a liver in 69%. Cholangiocarcinoma had manifestations of progressing hepatic failure, portal hypertension. In one case cholangiocarcinoma was associated with chronic HCV-infection and had clinic of paraneoplastic dermatomyositis/polymyositis. The cause of death at cholangiocarcinoma was hepatic failure. HCC was complicated by intoxication, cachexia, acute hepatic failure, hepatorenal insufficiency, obstructive jaundice, paraneoplastic pulmonary embolism, cardiopulmonary decompensation.

Discussion/Conclusion: Most patients with viral hepatitis and cirrhosis have clinically unknown general paramalignant symptoms. It is necessary to have in view primary liver cancer since HCC has an extremely poor prognosis.
Prognostic factors at hepatocellular carcinoma

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Introduction: Prevalence of HBV-HCV-infection, progressing morbidity rate of chronic viral hepatitis and hepatocellular carcinoma (HCC) are registered in Ulyanovsk region. The risk of HCC neoformation repeatedly increases, especially at liver cirrhosis stage.

The aim: To define prognostic factors of clinical course and outcomes of HBV-associated HCC.

Methods: 11 patients with HCC were examined, in 9 cases it developed at patients with HBV-associated cirrhosis, in 2 – at mixed HBV + HCV-infection. HCC was diagnosed according to CLIP scale, including standard sonographic, laboratory (AFP), histologic parameters. B and C stages of HCC were registered mainly. HCC was revealed at Child-Pugh grade B with portal hypertension III, and in 70% cases combined with massive ascites. Significance of prognostic criteria was estimated by correlation, cluster and logistical regression types of statistical analysis.

Results: The age of patients with HCC varied from 50 till 73 years, median age was 60.9 ± 2.43 years (95% CI: 55.4–66.4 years). One of 11 patients HCC had acute hepatitis B 34 years ago, 2 were observed with CHB over a long period (9 and 11 years). The level of alpha-fetoprotein (AFP) exceeded norm 2.85 times more. HCC had extremely unfavorable prognosis at long-term infection, active cirrhosis, high degree portal hypertension, mixed infection, abusing alcohol. Prognostic value of a fever, cancer intoxication, progressive weight loss, trophic disorders, haemorrhagic manifestations, decreasing of serum iron, lymphopenia, HBsAg, accompanying somatic pathology (diabetes), extrahepatic manifestations (vasculitis, glomerulonephritis) were evaluated in clinical outcome.

Discussion/Conclusion: The estimation of prognostic value of factors is possible only at realization of complex clinical research. Prognostic importance of mixed infection B + C, alcohol, age of patients and activity of cirrhosis is confirmed.
Reactivation of embryonic signal transduction pathways (Hex, HNF3α, and HNF3β) during liver regeneration via adult hepatic progenitor cells (oval cells)

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Introduction: The homeobox transcription factor Hex and transcription factors HNF-3α and HNF-3β are required during early hepatogenesis. We postulated that the signal transduction pathways necessary for liver development may be reactivated during adult liver regeneration.

Methods: Liver regeneration was studied in adult rats after acute toxic liver injury by CCl₄, and after 2/3rd partial hepatectomy (PH). Corn oil-treated or sham-operated animals were used as controls. Liver regeneration via hepatic progenitor cells (oval cells) was activated by PH and simultaneous administration of 2-acetylaminofluorene (2-AAF; modified Solt-Farber protocol). RNA was extracted from regenerating livers at different time points by Guanidin/CsCl₂ gradient ultracentrifugation. cDNA was generated and amplified by Real-time PCR with SYBR-Green as detecting reagent (ABI Prism 7000). β-Actin and ubiquitin c were used as internal controls and the relative expression level was analyzed using normal liver as a calibrator.

Results: Hex, HNF-3α and HNF-3β were transiently down-regulated at 12 hours after acute liver injury by CCl₄ (Hex -24.1 ± 4-fold; HNF3α -11.2 ± 2.5-fold; HNF3β -7.9 ± 3-fold), and reached control levels again at 48 hours. Similarly, Hex, HNF-3α and HNF-3β gene expression were down-regulated after standard PH (Hex -12.7 ± 3-fold at 4 hours; HNF-3α -18.4 ± 4-fold at 16 hours; HNF-3β -20 ± 2.8-fold at 4 hours), and remained below control level until 72 hours after PH. All three factors were early up-regulated in sham-operated animals (Hex 2.8-fold, HNF-3α 6-fold, HNF-3β 3.5-fold), and fell to control levels at 24 hours (Hex) or 48 hours (HNF-3α, HNF-3β), respectively. In AAF-treated rats, Hex, HNF-3α and HNF-3β were significantly up-regulated after PH (Hex +5 ± 2-fold at day 7; HNF3α +1.6 ± 0.6-fold at day 3; HNF-3β +4 ± 1-fold at day 7). In AAF/sham-operated rats, Hex, HNF-3α and HNF-3β gene expression were significantly below PH-operated rats (Hex +2.4 ± 1-fold at day 7; HNF-3α +0.5 ± 0.8-fold at day 3; HNF-3β +0.2 ± 1-fold at day 7).

Discussion: Hex, HNF-3α and HNF-3β gene expression are down-regulated during liver regeneration via adult hepatocytes, but are up-regulated during liver regeneration via adult progenitor cells (oval cells). We suppose that embryonic signalling pathways involved in hepatogenesis are reactivated during liver regeneration via oval cells.
Nuclear accumulation of the E3-ubiquitin ligase SIAH-1 promotes tumor cell growth

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SIAH-1 (Drosophila *seven in absentia homologue*-1) is an E3-ubiquitin ligase that facilitates the degradation of different proteins like transcription factors (e. g. c-myb), coactivators (e. g. β-catenin) and membrane proteins (e. g. synaptophysin). However, only little is known concerning the function of this protein in cancer development and tumor cell growth.

Transcript levels of SIAH-1 were found to be reduced (> 2-fold) in 70% of all analyzed human hepatocellular carcinomas (HCC; n = 71). Using HCC tissue microarrays (25 normal livers, 35 pre-malignant lesions, 155 HCCs) we detected SIAH-1 in the cytoplasm of normal hepatocytes; however, a nuclear accumulation was observed in tumor cells (Spearman correlation: r = 0.288; p < 0.001). In HCC tumor cell lines Western-blot-analyses of subcellular protein fractions and immunofluorescence-analyses revealed a nuclear and cytoplasmic expression of SIAH-1. Inhibition of SIAH-1 expression using gene-specific siRNAs was associated with a significant reduction of tumor cell viability as compared to cells transfected with nonsense-siRNA. SIAH-1 inhibition also resulted in decreased expression of the transcription factor *fuse-binding protein* (FBP), which may in part mediate oncogenic function of nuclear SIAH-1. In contrast to other *in vitro* model systems SIAH-1 was not regulated by p53<sup>wt</sup> in HCC cells.

Together these data demonstrate that down-regulation, and nuclear translocation of SIAH-1 is associated with HCC tumor cell functionality. However, the function of SIAH-1 in HCC cells presumably differs from previously described model systems, since this ligase is not modulated by p53<sup>wt</sup> and its expression positively correlate with the accumulation of other oncogenic factors.
Thy-1 is the first cell surface marker to identify myofibroblasts in the liver

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Identification of the different mesenchymal cells within the liver lobule, especially, under pathological conditions is still difficult, as reliable cell surface markers are not available. Thy-1 (CD 90) a glycoposphatidylinositol-linked outer membrane leaflet glycoprotein is already described in human myometrial, orbital and lung fibroblasts. In this work Thy-1 was used to distinguish among liver mesenchymal cell populations. Thy-1\(^+\) cells were detected in the periportal area of rat and human liver specimens in normal, in injured and in regenerative conditions. In regenerating rat liver Thy-1 remained periportal, while smooth-muscle-alpha-actin showed periportal, and also perisinusoidal reactions. In the terminal stage of human liver cirrhosis and in chronic CCl4-induced liver cirrhosis of the rat: Some Thy1-and SMA-positive cells were observed also in the sinusoids of the cirrhotic nodules. Thy1-and SMA-positive cells were also observed in the regenerating liver of 2-acetylaminoferuene-treated rats after partial hepatectomy. However, no co-localisation with the oval cell markers (Cytokeratin 19 and alpha-fetoprotein) was detectable. Cultured and passaged rat liver myofibroblasts (rMF) showed a constant Thy-1-gene-expression, while Thy-1-specific transcripts were neither detected in freshly isolated nor in activated hepatic stellate cells (HSC). These results provide strong evidence that Thy-1 is the first cell surface marker to distinguish between rMF and HSC in the liver tissue, and provides a tool for flow cytometric sorting of the different liver mesenchymal cells.
Comparison of cytochrome P450 activities of blood monocyte derived Neo-Hepatocytes and primary hepatocytes

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Altering the molecular structure of chemicals to increase polarity is essential for their elimination from the human body. Most bio-activation reactions are catalyzed by a group of hepatic microsomal monooxygenases, which reaction specificity is defined by cytochrome P450 (CYP). Thus, CYPs are essential for the determination of pharmacologic and toxic effects, which are conventionally measured in primary human hepatocytes. However, their availability is limited by to donor organ scarcity. Thus, hepatocyte-like (NeoHep) cells generated by trans-differentiation of monocytes might be used as an alternative.

NeoHep cells were generated from monocytes of peripheral blood and compared to primary hepatocytes in terms of their morphology and metabolic behavior. CYP (1A2, 2A6, 2B6, 2C9, 2E1, and 3A4) expression and activity was investigated by RT-PCR, Western blot, and fluorescence-based activity assays.

After 13–15 days of differentiation NeoHep cells form a confluent layer with cell-cell contact displaying the typical hexagonal shape of primary hepatocytes, expressing hepatocyte marker genes like transferrin and connexin 32. The basal CYP activity is increasing throughout differentiation, reaching a stable level after approximately 15 days. Over a culture period of 5 days primary mouse hepatocytes undergo typical morphologic changes in collagen monolayer culture and basal activity of all CYP isoforms decreases. Responsiveness to inducers (e.g. Rifampicin) and inhibitors (e.g. Verapamil) of CYP activity is comparable in both cell types.

Our data show phenotypic and metabolic similarities of NeoHep cells and primary hepatocytes. Thus, NeoHep cells might be used as an alternative for primary hepatocytes in measuring bioactivation of substances.
Kinetics for biosynthesis and secretion of albumin and alpha-fetoprotein during rat liver development

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Albumin and alpha-fetoprotein (AFP) are the main hepatic markers and are the earliest synthetic products of hepatoblasts during liver development. In this study, we established a reliable method using a sensitive radioactive biosynthetic labelling, to analyse the albumin and AFP synthesis and secretion capacity of endoderm cells derived from ventral foregut region (E10). It seems that the regulation of gene expression, synthesis and secretion of albumin and AFP already acts at the earliest developmental stage, when specification of hepatic endoderm appears. We were interested in the development of the liver after it was clearly identifiable as a separate organ (from E12 to adulthood). Morphometric analysis showed the number of albumin and alpha-fetoprotein producing cells develop in a similar way up to E18. During this time there is an increase of the ratio of albumin and alpha-fetoprotein producing cells to proliferating cells as the liver develops and increases in size. Despite this, we found the ratio of albumin and alpha-fetoprotein to the total number of liver cells remained at 50% throughout liver development. After 18 days of gestation the ratio of albumin producing cells to proliferating cells continues to increase until adulthood. In contrast, the ratio of alpha-fetoprotein producing cells to proliferating cells reaches a maximum and thereafter decreases. Quantitative analysis revealed that at 18 days of gestation, albumin and alpha-fetoprotein mRNA production reaches a maximum and a high level of synthesis and secretion of albumin and alpha-fetoprotein was observed. Additionally, it was found that at the embryonic stage (from E12 up to E16) alpha-fetoprotein was synthesized and secreted at a higher rate than albumin even though the number of albumin and alpha-fetoprotein producing cells is similar. After 18 days of gestation to birth the kinetics for synthesis and secretion of albumin is similar to that in mature hepatocytes.
Detection of a cholangiocyte cell population in the early rat liver development

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Besides albumin and α-fetoprotein (AFP), Prox1 is an early marker of hepatoblasts. In humans, mice and rats Prox1 expression in hepatocytes persists into adulthood. Prox1 is not expressed in bile duct epithelial cells, which are positive for cytokeratin (CK)-19 and 7. The early hepatoblasts are supposed to be bipotent, giving rise to hepatocytes and intrahepatic cholangiocytes. Intrahepatic bile ducts are supposed to start to differentiate from perportal hepatoblasts, which express AFP and albumin at embryonic day (ED) 15.5 in the rat. In this study the question was addressed, whether Prox1 is expressed in cells that start to differentiate to the biliary cells.

Embryonic liver was isolated at embryonic days (ED) 12–22, used for in vitro culture of adherent and non-adherent cells as well as for direct studies on the whole tissue. Embryonic liver tissues and cultured embryonic liver cells were analysed by real-time RT-PCR, by immunocyto- and histochemistry, by flow cytometry and cell sorting.

At ED14–16 the majority of Prox1⁺ cells in the developing rat liver showed cytokeratin 19 in their cytoplasm, and they were AFP⁺ as well. At this stage Prox1⁺/CK19⁺/AFP⁻ small cells, and Prox1⁺/AFP⁺/CK19⁻ cells were identified as well. Culture and flow cytometric sorting of adherent liver cells from this stage of embryonic development proved the existence of separated Prox1⁺/CK19⁻ and Prox1⁺/CK19⁺ cell populations. In fetal liver (ED 18–22) hepatoblasts were Prox1⁺/CK19⁻/AFP⁺. CK7⁺ cholangiocytes were detected only at this stage, and they were Prox1⁻/AFP⁺. In the adult liver hepatocytes were Prox1⁺/CK19⁺/CK7⁻/AFP⁻, cholangiocytes were CK19⁺ and/or CK7⁺ and AFP⁻/Prox1⁻.

This study defines a cell population showing markers of the biliary lineage and the absence of hepatocyte markers at an early stage of rat liver development. The existence of such a cell population challenges the view that all bile duct cells derive from the bipotent hepatoblasts.
Extracellular matrix configuration modulates TGF-β effects in primary hepatocytes

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From all in vitro models to study TGF-β effects on the liver, primary hepatocytes resemble closer most of the features of an intact liver. We compared the behaviour of primary hepatocytes on collagen type I as a dry monolayer or as gel sandwich and their response to TGF-β. Hepatocytes cultured on dry matrix displayed a typical cuboidal shape for the initial 24 h, but started to spread and dedifferentiate constantly thereafter. Bile canaliculi was absent in this culture condition. Cells cultured in collagen sandwich form bile canaliculi from day 1 persisting after 5 days. Bile canaliculi function was evaluated by CMFDA secretion. Cell morphology was better preserved as compared to dry matrix, as judged by nuclear/cytoplasmic ratio, cytoplasm granulosity and cell refringence. Upon TGF-β stimulation, cells in dry matrix displayed slight enhanced dedifferentiation. Weak apoptosis induction was observed. In collagen sandwich system, TGF-β treated cells showed strong morphological changes in time, progressing through a clear dedifferentiation resembling EMT. These changes were accompanied with expression of profibrogenic markers like CTGF. After 72 h, strong apoptosis was observed. This was confirmed by PARP degradation and Caspase-3 activation. Antiapoptotic proteins Bcl-2 and Bcl-xL were downregulated by TGF-β treatment. The p38 inhibitor SB203580 strongly attenuated apoptosis induction, but had no effect on dedifferentiation and CTGF expression, while ALK5 inhibitor SB431542 inhibited every feature induced by TGF-β. Our findings indicate that hepatocytes cultured on gel matrix preserve their features better and for a longer time than on dry matrix, which allows a more sensitive response to TGF-β.
Oxidative stress tolerance induced by selenium deficiency in hepatocellular carcinoma cell lines

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Selenium is a trace element that is involved in regulation of oxidative stress in mammalian cells. Its deficiency is associated with different diseases including liver cancer (1). Selenium-deficiency in a subset HCC-derived 'hepatocyte-like' cell lines causes oxidative stress and apoptosis in vitro (2). These HCC cells, particularly HBV-related ones, tolerate selenium deficiency and escape its deadly consequences. Such malignant cells have acquired a selective survival advantage, which is prominent under selenium-deficient and oxidative-stress conditions. In addition selenium deficiency sensitive cells become tolerant to oxidative-stress by Vitamin E supplementation. Because both selenium depletion and the excess of ROS are common in chronic liver diseases serving as a reservoir for hepatic malignancies we assume that the acquired tolerance of HCC cells to selenium deficiency and ROS may occur in vivo. Therefore, we further investigated the underlying mechanism of oxidative stress tolerance by comparative large-scale transcriptome analysis. In this respect, we selected two hepatoma cell lines, which give opposite responses to selenium deficiency for microarray expression analysis. We compared the expression profiles of these cells under selenium deficiency. Statistically significant gene list were analyzed by using bioinformatics tools such as Webgestalt, R-bioconductor. Highly significant candidate genes, which were overexpressed in selenium deficiency tolerant cell lines, were verified by RT-PCR.

References:

Inhibition of protein kinase C by the NTPase/helicase domain of hepatitis C virus nonstructural protein 3 (NS3)

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A broad range of biological and biochemical studies document that disturbing protein kinase C (PKC) function induces tumor promotion and may promote carcinogenesis in vivo. In our foregoing works we have demonstrated that fragments of the NTPase/helicase domain of the nonstructural protein 3 (NS3) of hepatitis C virus (HCV) reduce the catalytic activity of the PKC and block its shuttling between cell compartments. Thus, NS3-mediated PKC-inhibition may be involved in the carcinogenesis leading to HCV-induced hepatocellular carcinoma. The inhibition is mainly mediated by a short arginine rich amino acid stretch of the protein (so called motif VI) localized on the surface of Domain 2 of the NS3-NTPase/helicase. The HCV amino acid sequence of this motif strongly resembles the pseudosubstrate sequence within the autoregulatory domain of PKC. In the presented studies we confirm that catalytically active NS3-NTPase/helicase as well as an NTPase-inactive mutant act as potent PKC-inhibitors in vitro with IC$_{50}$-values in the submicromolar range for most PKC isoforms. To measure the PKC-activity in intact cells, we determined the phosphorylation of the PKC-substrate p80/MARCKS in NIH-3T3 cells with stable expression of the NS3-NTPase/helicase after stimulation with the PKC-activator TPA (12-O-Tetradecanoyl-phorbol 13-acetate). The NS3-expression had no significant effect in this assay, however the stably transfected cells were morphologically changed and showed a decreased doubling time. Proliferative and morphological changes are often observed in the course of neoplastic transformation. To our knowledge, this is the first report that attributes the induction of these phenotypic changes to the NTPase/helicase portion of HCV-NS3.
Continuous cell injury promotes hepatic tumorigenesis in Cdc42-deficient mouse liver

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The Rho small GTPase Cdc42 is critical for diverse cellular functions, including regulation of actin organization, cell polarity, intracellular membrane trafficking, transcription, cell cycle progression, and cell transformation. To address the role of Cdc42 in liver in vivo, we generated mice lacking Cdc42 expression in hepatocytes and bile duct cells (Cdc42HeKO). These mice were born at Mendelian ratios. They did not exhibit increased mortality but they showed chronic jaundice. They developed hepatomegaly soon after birth, and signs of liver transformation, such as nodule and tumor formation, became macroscopically visible at age six months. Hepatocellular carcinoma was observed eight months after birth. Immunofluorescent examination and electron microscopy revealed severe defects. At the age of two months, the canaliculi between hepatocytes were greatly enlarged, although the tight junctions flanking the canaliculi appeared normal. Regular liver plates were absent. The E-cadherin expression pattern and gap junction localization were distorted. Analysis of serum samples indicated a cholestatic phenotype. This new mouse model, in which a chronically diseased liver leads to development and progression of hepatocellular carcinoma (hepatocarcinogenesis), may contribute to the understanding and treatment of human liver diseases.
Oxygen free radical scavengers reduce superoxide production
but not morphological changes induced by hepatic
hypoxia/reperfusion injury

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Reperfusion of a previously ischemic/hypoxic tissue is known to be associated with
additional injury leading to anatomical and physiological alterations in many organs
including the liver. During the past two decades, much attention has been paid to the
potential role of reactive oxygen species (ROS) on hepatic parenchymal and
non-parenchymal cell injury. The present study was designed to examine the effects
of ROS scavengers on superoxide (O$_2^-$) production, liver function, and morphological
changes induced by hypoxia/reperfusion. Both anterograde and retrograde
reperfusion were used in the isolated slow-flow, reflow rat perfused liver model to
induce zonal hepatic damage and compare the feature of reperfusion injury in
different perfusion directions. Hypoxia/reperfusion of livers did not affect oxygen
consumption but caused significant increase in enzyme release and a decrease in
bile flow in both anterograde and retrograde perfusions. O$_2^-$ production quantitated
using the cytochrome C reduction method, increased markedly upon reperfusion. The
production of O$_2^-$ was significantly decreased in livers pretreated with superoxide
dismutase (SOD), gentisic acid, N-acetyl cysteine and trolox C ($p < 0.05$). These
compounds significantly reduced enzyme release (LDH, AST) and improved bile flow
in the liver exposed to hypoxia/reperfusion in both anterograde and retrograde
perfusions. However, they failed to protect the liver against the structural alterations
induced by reperfusion injury. Thus, ROS may not be the sole causative mechanism
of hepatic injury induced by hypoxia/reperfusion suggesting possible involvement of
other events and mediators in this injury.
Temporal and spatial expression of IGF-I and IGFBP-1 during acute-phase response induced by localized inflammation in rats

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Objective: The IGF-system (IGF-1 and IGFBPs) is involved in the metabolic processes taking place during infections causing an acute-phase-response. The liver is a central organ of both the IGF system and the APR because it provides most of IGF-I and IGFBP-1 in the blood and is the main target organ for the most important acute-phase-cytokines like IL-6.

Methods: In the current work the expression of IGF-I and IGFBP-1 was studied in the liver and extrahepatic tissues in a rat model of localized inflammation induced by intramuscular injection of turpentine oil (TO). The mRNA expression of IGF-I and IGFBP-1 was determined by Northern blot analysis. Circulating levels of IGF-I and IGFBP-1 were evaluated by radioimmunoassay and [125I]-IGF-I ligand blotting, respectively.

Results: Administration of TO to the rats led to the significant reduction of IGF-I gene expression in the liver and spleen. These changes were accompanied by almost twofold reduction of serum IGF-I concentrations. In contrast to IGF-I, IGFBP-1 mRNA expression was rapidly elevated in the livers of TO-treated rats. IGFBP-1 transcripts were already detectable at 30 minutes after TO injection and reached their maximal levels by 6 hours. IGFBP-1 gene expression was also increased in the kidneys. This elevation, however, was delayed and less prominent than in the liver.

Conclusions: Our data demonstrate that localized inflammation induced by intramuscular TO-injection is accompanied not only by decreased IGF-1 but also by increased IGFBP-1 gene expression explaining at least in part the catabolic consequences of the acute-phase-response.
ATP-competitive inhibition of Wnt-signalling pathway-associated protein kinases with a potential influence on hepatocellular carcinoma

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**Background:** Several protein kinases of the Wnt-signal transduction pathway play critical roles in the regulation of proliferation and apoptosis of liver cells. Accordingly, the prominent protein kinase CK2 (casein kinase 2) is quantitatively elevated in the most of proliferating tissues, particularly in tumor cells. Selective inhibition of kinase CK2 rather than of the kinases CK1 and GSK3 which are predominantly involved in metabolic regulation may represent an important advance in the field of growth control and anti-tumour therapy.

**Methods:** In silico screening of a polyphenolic compound library (flavonoids, anthranoids and their derivatives) through molecular modelling and Docking studies features a selective inhibition of protein kinase CK2 in the range of 1E-06/1E-07. The inhibitory potency of auspiciously docking structures was further measured by a radio-labelled phosphorylation assay. In addition, some of the inhibitors were tested by a cytotoxic activity assay on primary hepatocytes and the HepG2 tumor cellline.

**Results:** The IC₅₀-values for kinase CK2 ranged between 300 nM and 20 μM for more than 30 of the tested inhibitors. Some of these compounds are quite specific when compared with inhibition of kinases CK1 and GSK3 despite the high sequence similarity of the ATP-pocket. In this work, we would like to display relevant interactions between the ligands and specific residues or backbone atoms of the target enzyme, in order to reveal possible contributions to the different inhibitory efficacy and selectivity. Furthermore, we probe acetylated variants that exhibit prodrug activity through esterase cleavage, and ethylester variants which offer a higher cytotoxic selectivity against HepG2-cells compared with primary hepatocytes.

**Conclusion:** The elucidation of molecular details of specific protein-inhibitor interactions discloses further possibilities for compound modification and for target-orientated signalling pathway interference.
Identification of the transcription factors FBP-1 and FBP-3 as pro-tumorigenic factors in (hepato)-carcinogenesis

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Recognizing torsional dynamics in DNA-topology, the DNA binding transcription factors FBP (far upstream element [EUSE]-binding protein)-1 and FBP-3 modulate expression of genes such as c-MYC in some in vitro model systems suggesting pro-tumorigenic function of these factors. However, nothing is known concerning the functional relevance of FBP-1 and FBP-3 in cancer development and progression. We show that FBP-1/-3 are expressed in different human hepatocellular cancer (HCC) cell lines. Inhibition of FBP-1/-3 by siRNA significantly reduced tumor cell viability in these cell lines. However, Western-blot analyses revealed that no changes of c-MYC expression were detected after siRNA-mediated inhibition of both transcription factors. Moreover, semiquantitative PCR-analyses showed that the overexpression of FBP-1/-3 in human HCCs (70%, 19/27) did not correlate with elevated c-MYC levels. However, the expression profiles of FBP-1 and FBP-3 significantly correlate among each other. Using tissue-micro arrays (25 livers, 35 pre-malignant lesions, 155 HCCs) a highly significant correlation between the nuclear expression of FBP-1/-3 with tumor progression was observed (Spearman correlation: r = 0.355 [FBP-1] and r = 0.454 [FBP-3]; p < 10^{-6}). As described on transcript level, the nuclear accumulation of FBP-1 and FBP-3 correlated with each other (r = 0.535; p = 0.004), and the increased expression of FBP-1/-3 did not correlate with the nuclear expression of c-myc in HCCs.

In conclusion, we identified the transcription factors FBP-1 and FBP-3 as over-expressed and co-regulated in human (hepato)-carcinogenesis. Although they do not modulate the expression of previously described target structures (e. g. c-MYC), they probably exert their oncogenic properties through the activation of so far unknown pro-tumorigenic target genes.
Effectiveness of a comprehensive roentgen examination in screening for hepatocellular carcinoma

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In most cases hepatocellular carcinoma is detected in its later stages, when resection is impossible due to the tumor size, its invasion into blood vessels, portal vein thrombosis, metastases, patient’s grave condition. Resection is performed on as few as 3–30% of patients. Recurrence of tumors after resection is characteristic of 57% of patients.

Objective: Determination of the effectiveness of a comprehensive roentgen examination in screening for hepatocellular carcinoma.

Over the five-year period from 2000 through 2005 the cancer care center of Ulyanovsk region attended to 78 patients afflicted with hepatocellular carcinoma. Techniques used for early detection of this tumor type have a different success rate. For instance, the sensitivity of ultrasound tests, which are often conducted in early stages of examination, proved to be 78.2%. However, hepatocellular carcinomas whose size did not exceed 2 centimeters went undetected in 30.8% of the cases. All the patients were administered computerized tomography to identify the exact tumor location and size, sometimes along with contrast angiography. This technique proved to have high sensitivity (94–100%) in detection of multifocal and tiny hepatocellular carcinomas. To exclude remote metastases, patients were given a chest X-ray and a bone structure scan tests.

Consequently, a comprehensive examination using all current radiodiagnostic techniques ensures early detection of hepatocellular carcinoma, which is crucial to the effectiveness of further treatment.
Different strategies for efficient inhibition of IGF-signalling in hepatocellular carcinoma cells

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cDNA-microarray analyses of human hepatocellular carcinomas (HCCs; n = 43) revealed that overexpression of insulin-like growth factor (IGF)-II is associated with tumour cell proliferation, anti-apoptosis, and lower numbers of tumour-infiltrating lymphocytes in a subgroup of tumours (Breuhahn et al., Cancer Res. 2004). Since all analysed HCC cell lines (HuH-7, Hep3B, HepG2) also exhibited elevated IGF-II levels, we reduced IGF-signalling using IGF-I and IGF-II receptor (IGF1R)-selective tyrosine-kinase (RTK)-inhibitors and gene-specific siRNAs for IGF-II and its receptors.

Treatment of all HCC cell lines with different RTK-inhibitors (picropodophyllin [PPP] and tyrphostins AG1024/AG538) diminished phosphorylation of pivotal IGF-downstream effectors (e.g. AKT/PKB) and tumour cell viability in a concentration-dependent manner. Furthermore, PPP but not the tyrphostins lead to a significant accumulation in the G2/M-cell cycle phase and the induction of apoptosis in HepG2 cells. siRNA mediated inhibition of the insulin receptor (IR) and IGF1R revealed that IGF-II signalling is predominantly mediated by the IGF1R. Reduced IGF-II and IGF-1R expression is associated with increased apoptosis, reduced tumour cell viability and proliferation as compared to nonsense transfected controls.

We conclude that IGF-signalling can be specifically reduced by RTK-inhibitors and RNAinterference in HCC cells. Both techniques significantly impair tumour cell functionality; however, to a different extent. Therefore the modulation of the IGF-pathway by both techniques may offer eminent prospects for therapeutic intervention for the treatment of tumour types with elevated IGF-signalling. However, while selective RTK-inhibitors to a certain degree also affect other signalling pathways (e.g. insulin/IR with potential diabetogenic effects), highly specific siRNA-mediated approaches did not enter clinical applicability, yet.
Impact of hepatitis B virus on E-cadherin/beta-catenin expression and cell distribution and its role in HBV-mediated liver carcinogenesis

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Hepatitis B virus (HBV) infection is an important risk factor for hepatocellular carcinoma (HCC). E-cadherin, a key component of adherence junctions, is reported to be a tumor suppressor. Its intracellular binding partner, beta-catenin, plays a pivotal role in the Wnt signalling-pathway and carcinogenesis. However, the contribution of HBV to liver carcinogenesis is still not completely understood.

We investigated the expression of E-cadherin and beta-catenin \textit{in vitro} in hepatoma HepG2 and corresponding HBV producing HepG2-H1.3 cells. To evaluate the \textit{in vivo} situation, we analyzed liver tissues from HBV-transgenic mice and littermates as well as tumor-peritumor tissues from HCC patients with chronic HBV infection using real-time LightCycler RT-PCR and Western blot analysis.

In HepG2-H1.3 cells, the expression levels of E-cadherin mRNA negatively correlated with the amount of HBV pregenomic RNA. In comparison to HepG2 cells, HBV replication in HepG2-H1.3 cells did not alter the total amount of E-cadherin and beta-catenin. Luciferase reporter constructs were used to analyze the impact of HBV on beta-catenin signalling. Interestingly, HBV replication did activate the beta-catenin signalling-pathway indicating a redistribution of beta-catenin towards the nucleus.

Neither in normal liver nor in HCC tumor-peritumor tissues, E-cadherin or beta-catenin correlated with HBV replication. Expression levels of E-cadherin and beta-catenin showed a high variation and seemed individual-dependent.

We conclude that HBV has a potent effect on beta-catenin-signalling-pathway probably via redistribution of beta-catenin to the nucleus. E-cadherin is not necessarily downregulated and seems to play an ambiguous role in HBV-mediated carcinogenesis.
Hepatocellular carcinoma: distribution, clinical picture and therapy

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Over the five-year period from 2000 through 2005 the cancer care center of Ulyanovsk region provided treatment to 78 patients (49 female and 29 male) diagnosed with hepatocellular carcinoma. In 11 of the patients (14.1%) the disease affected bile ducts, 30 patients (38.5%) had tumors located in the gallbladder, 37 (47.7%) in the liver. The distribution of the disease among age groups was as follows: over 60 years old – 67 patients (89.5%), 50–60 years old – 7 patients (9%), 45–50 years old – 3 (3.8%) and under 40 – 1 person (1.3%).

The clinical picture varied from absence of obvious symptoms to full-scale hepatic insufficiency. The most common symptoms were the so called “secondary” signs: general weakness, weight loss, epigastric distress, in some cases fever, pains, jaundice, ascites.

Surgical removal of the tumor was performed on 11 of the patients (14.1%), 32 (41%) were administered conservative treatment. The rest of the patients received palliative care in view of the tumor size, its invasion into blood vessels, the patient’s age and accompanying diseases.
Cancer development of the liver in geriatric patients

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**Introduction:** It is becoming increasingly clear that primary hepatocellular carcinoma (HCC) is largely a disease of ageing. In elderly people it is almost always associated with cirrhosis, regardless of the underlying cause. Hepatic metastases from distant primary sources are often in the gut.

**Material and methods:** During the last 2 years we examined 1990 patients by ultrasound. There were 60% females and 40% males: 65–90 years old. There were 5% patients with primary cancer of the liver, 43% had metastases. In our group 45% patients had alcoholic liver disease, 20% had hepatitis B or C. Main symptoms were abdominal discomfort, weight loss, vomiting, or pain. Some of them had occasionally high fever. Clinical findings were: 30% of patients had enlarged liver, 10% had ascites, 30% pain with palpation, and 20% jaundice. In 1% of patients murmurs could be found above liver.

**Results:** Laboratory findings were: high azothemia, hepatic enzymes were very high, anaemia microcytic was frequent, alpha-fetoprotein was high, also carcinoembryonic antigen (CEA). Ultrasound findings in HCC were: solid hypoechoogenic mass, diameter 1–10cm, and hepatomegaly.

**Discussion:** Findings showed that the consumption of alcohol, particularly with HBV or HCV infection, was associated with the development of HCC. Among men, the risk of development of HCC in cirrhosis ranges from 1–5% per year. Among women, development is less common.
High expression levels of HNF4α and HNF1α account for a
dependence of hepatitis B virus replication on the differentia-
tion state of hepatocytes

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The efficiency of Hepatitis B Virus (HBV) replication is discussed to depend on the
hepatocyte differentiation state. We followed the hypothesis that expression levels of
hepatocyte specific transcription factors determine hepatocyte differentiation as well
as efficiency of HBV replication. In freshly isolated primary human hepatocytes
(PHH), HepG2 and HuH7 (hepatoma) and pop10 (hepatocyte) cell lines, expression
levels of liver specific differentiation markers, transcription factors and efficiency of
HBV replication following adenovirus-mediated transfer of HBV 1.3 genomes at equal
levels were studied. PHH replicated HBV most efficiently and expressed highest
levels of HNF1α, HNF4α and HNF3γ, whereas expression of HNF1β, HNF3α,β,
C/EBPα,β and LRH-1 did not correlate. The expression of differentiation markers
OATP-C (organic anion transporting polypeptide) and LSA (liver specific antigen),
regulated by HNF4α and HNF1α, was restricted to PHH.
Next, HBV replication dynamics were studied in HBV producing cell lines HepG2.2.15
and HepG2-H1.3 under differentiating conditions. In both cell lines, the production of
HBV pgRNA, proteins and progeny HBV as well as expression levels of HNF4α,
HNF1α and HNF3γ markedly increased over 20 days at confluency.
Knock-down of HNF4α or HNF1α using specific siRNA dramatically decreased HBV
transcription, synthesis of HBV proteins and progeny HBV release.
In vitro data were confirmed by a significant correlation between the amounts of
HNF4α and core-protein in tumor-peritumor tissue of HCC patients chronically
infected with HBV.
We conclude that high expression levels of HNF4α and HNF1α, maintaining high
degree of hepatocyte differentiation, are necessary for efficient HBV replication.
The role of selected risk factors for hepatitis C virus infection in children from South Poland

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The purpose of this study was to analyze most frequent risk factors for HCV infection in children connected with hospitalization in South Poland.

The study included 86 children (3–18 years old) with chronic hepatitis C virus infection and was conducted from 1999 to 2004 at the Hepatology Center of the John Paul II Hospital in Krakow. A group of 30 children with hyperbilirubinemia, matched for age and gender in whom HCV infection was excluded, served as the controls.

Physical examination and laboratory tests were performed in all children. A complete medical history based on direct questions was obtained. To identify the source of infection questions were asked about hospital admissions, surgical operations, dental procedures, blood and blood product transfusions, previous small surgical incisions, cosmetic procedures and tattooing.

Analysis of the data revealed that in children with chronic HCV infection blood and blood product transfusions were significantly more frequent risk factors than in the controls (p < 0.05). Another risk factor was admission to hematological and oncological wards (p < 0.05). There was no relationship between HCV infection and the frequency of dental procedures, or stays at sanatoriums and non-surgical wards.

Conclusions

1. Transfusions of blood and blood products and admission to hematological and oncological wards, followed by surgical procedures are the major risk factors for hepatitis C virus infection in children aged 3–18 years.

2. HCV infection in older children is independent of risk factors present in their parents, indicating that HCV infection is acquired and associated with lifestyle, living conditions and health status.
Vascularised liver test system: A possible model for the screening of potential cytostatic drugs in tumour therapy

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Background: Liver cancer comprises primary liver cancer as well as secondary tumours caused by metastases. The choice of the right therapy for these different tumours is important. We have developed a vascularised liver test system which could be used as a matrix for the culture of hepatocytes isolated from individual cancer biopsies. After cell expansion potential cytostatic drugs could be injected over the artery into the system to screen their effects on the tumour cells as well as their metabolites.

Methods: A porcine jejunal segment with obtained vascular system is chemically acellularised. Hepatocytes and endothelial cells (EC) as well as endothelial progenitors are isolated from porcine biopsies. Firstly the vascular system of the matrix is reseeded with endothelial cells respectively progenitor cells. In the second step hepatocytes in collagen gel suspension are seeded on the matrix lumen. During the cultivation period the matrix is perfused with medium over the artery in a bioreactor system.

Results: The culture of hepatocytes on the matrix shows good results for cell growth and conservation of liver specific functions (Phase 1 and 2 metabolism, albumin and urea synthesis). EC and precursor cells were seeded successfully onto the vascular bed of the matrix sustaining their vitality and differentiation potential. Our aim is to transfer the results from porcine to human model and to integrate tumour cells into the system to screen cytostatic drugs. This test system should enable the arterial application of substances and the identification of metabolites in a venous system.
Metabolic profiling in cholangiocarcinoma: Diagnostic potential of magnetic resonance spectroscopy

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Background: The incidence of intrahepatic cholangiocarcinoma (ICC) has increased dramatically in the UK. Bile acids enhance cholangiocyte proliferation and alterations in their biliary content may serve as disease biomarkers, when screening bile fluid. Magnetic resonance spectroscopy (MRS) of bile may provide insights into cholangiocarcinogenesis.

Objective: To identify novel diagnostic and prognostic biomarkers of disease.

Methods: Four contrast free cholangiocarcinoma bile samples at physiological pH were collected. In vitro proton (1H) and phosphorus-31 (31P) MR spectra were acquired using an 11.7 Tesla spectroscopy system. Data were compared to control bile from other hepatopancreaticobiliary pathologies (seven gallstones, four sphincter of Oddi dysfunction, three pancreatic cancer and one primary sclerosing cholangitis).

Results: Taurine-conjugated bile acids were elevated in CCA bile in patients when compared to bile from pancreatic carcinoma (p = 0.034) and non-malignant disease (p = 0.004). There was no significant difference in biliary phosphatidylcholine levels.

Conclusion: Taurine-conjugated bile acids are potential biomarkers in CCA and need further evaluation. Further work aims to quantify bile acids in bile.
Quantitative proteomic analysis of intrahepatic cholangiocarcinoma

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Background: The incidence of intrahepatic cholangiocarcinoma (ICC) has increased dramatically in the UK. Cholangiocyte exposure to environmental toxins, oxysterols and bile acids has been implicated. Mass spectrometry-based quantitative proteomics may provide insights into pathogenesis and cellular signalling pathways in ICC. It may also identify novel diagnostic and prognostic biomarkers of disease.

Aim: To use stable isotope labeling tags and tandem mass spectrometry to determine proteome alterations in ICC.

Methods: Liquid nitrogen snap-frozen periductal, infiltrating ICC and normal adjacent liver tissue were homogenised and subjected to differential centrifugation to obtain microsomal fractions. 80 µg of normal and tumour microsomal protein was denatured, reduced and trypsin digested and labelled with the 114 and 117 iTRAQ reagents respectively. The combined peptide mixture was fractionated using on-line two dimensional liquid chromatography coupled with electrospray tandem mass spectrometry (QTOF Global, Micromass, UK). Database searching was performed using Proteinlynx Global Server (PLGS) 2.2.5 software against International Protein Index database.

Results: A total of 583 proteins were identified. 26 proteins which included Annexin A5, Gag protein were significantly upregulated in ICC tissue with 117:114_log(e) ratio of greater than 1.11. 23 proteins were down-regulated which included cytochrome P450 2A13, importin 9 and Glutathione-S-transferases. Annexin A5 is tumour promoting and a substrate for EGFR activated tyrosine kinases. Oxysterol binding proteins 9 and 10 were found to be in lower abundance in ICC tissue when compared to normal adjacent liver.

Conclusion: Annexins may be useful biomarkers in ICC. We aim to fully quantify further paired samples to establish a signature ICC proteome.
Protumorigenic overexpression of stathmin/Op18 by gain of function mutation in p53 in human hepatocarcinogenesis

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The microtubule-destabilizing protein stathmin/Op18 has previously been described to be negatively regulated by p53 and to be highly expressed in several tumor entities. However, little is known about its expression profile, functional or therapeutic relevance, and regulation in human hepatocarcinogenesis. Here we demonstrate cytoplasmic overexpression of stathmin in premalignant lesions (Dysplastic Nodules; DN) and hepatocellular carcinomas (HCCs), which significantly correlated with tumor progression, proliferation, and activation of other protumorigenic factors (e.g. nuclear p53). Inhibition of stathmin expression by siRNA was associated with significantly reduced tumor cell viability, proliferation, migration, increased apoptosis as well as aberrant cell cycle regulation in HCC cells. Loss of stathmin expression increased responsiveness of tumor cells to the treatment with cytostatic drugs targeting microtubule-stability (paclitaxel, vinblastine) and for DNA-cross-linking agents (cisplatin). Surprisingly, inducible expression of p53\textsuperscript{wt} in p53-negative HCC cells as well as a reduction of p53\textsuperscript{wt} by siRNA in p53\textsuperscript{wt}-positive HCC cells did not alter stathmin expression. However, stathmin expression was down-regulated after siRNA-based reduction of p53\textsuperscript{mut/Y220C} expression in a respective liver tumor cell line. In conclusion, our results demonstrate that overexpression of stathmin is an early protumorigenic event in human hepatocarcinogenesis and its up-regulation can be mediated by \textit{gain of function} mutation in p53. Thus, in HCC cells stathmin represents a potential therapeutic target e.g. by increasing responsiveness of tumor cells to treatment with chemotherapeutic agents.
Characteristics of clinical course of cholelithiasis at HBV/HCV-infection

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Introduction: The development of cholelithiasis is connected to character of feed, but the problems of etiopathogenesis are still opened. The question of an infection influence, including viruses, their role and place is discussed at the given

Methods: Clinical, morphological features of gallbladder and liver at 50 patients with surgical stage of cholelithiasis. Biopsy of a liver estimated according to Knodell’s index and the METAVIR system.

Results: Among patients with cholelithiasis were 46 women, 4 men in the middle age 57.0 ± 2.16 years with duration of disease till 22 years. The infection is verified at the first time at 22 (44%), from them HBV at 12 (54.5%), HCV at 6 (27.3%), HBV + HCV at 4 (18.2%). At 48 patients with cholelithiasis (96%) histologically diagnosed chronic hepatitis, from them HBV at 24%, HCV at 12% and HBV + HCV at 8%, and at 2 (4%) – HCV-cirrhosis, and HBV + HCV-cirrhosis (FIV). Fibrosis classified as FII (58%), FIII (20%), FI (18%), in the last case markers of a HBV/HCV-infection were absent. At 7 of 28 patients (25%) without markers HBV/HCV the histologic findings were similar to a liver at HCV.

Discussion: Chronic viral hepatitis B and C were diagnosed at 29 patients with cholelithiasis (58%), in 44% of cases confirmed with the presence of markers HBV/HCV-infection.
Ultrastructure of biliary cells of portal ducts in the child with clinically suspected AMA-negative/ANA positive primary biliary cirrhosis. The third pediatric case of the disease

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Background: Primary biliary cirrhosis (PBC) – a chronic autoimmune cholestatic liver disease of an unknown etiology is very rare in children. Up to now only two cases of PBC in children have been described in the world literature (1). PBC is characterized by a T-cell-mediated destruction of bile duct epithelial cells that line the small intrahepatic bile ducts. Although it is assumed that portal bile ducts are the primary sites of injury in this disease, morphogenesis of these disturbances, particularly in AMA-negative PBC, is not well known. Recently the presence of antimitochondrial antibodies (AMA) has been considered to be one of the major diagnostic criteria in PBC. However, in the last decade four serological groups have been distinguished among patients with histological diagnosis of PBC, based on their antimitochondrial antibody (AMA) and antinuclear antibody (ANA) status [AMA-positive/ANA-negative (typical PBC), AMA-positive/ANA-positive (PBC/AIH overlap), AMA-negative/ANA-positive (autoimmune cholangitis), AMA-negative and ANA-negative (2)].

The main aim of the present report was the ultrastructural evaluation of biliary epithelial cells of portal ducts found in percutaneous needle-biopsy samples of hepatic tissue in a 14 year old AMA negative/ANA positive girl with clinically suspected PBC. The child had a positive family history of primary biliary cirrhosis (father and father’s mother). The immunological and serological disturbances observed in the blood serum manifested themselves in: high concentration of total protein, high concentration of IgM, high titre of ANAs. However, no AMAs were found. Differential diagnostics excluded, among others, infection with HBV, HCV, Wilson’s disease, alpha-1-antitrypsin deficiency, cytomegaly.

Methodology: Fresh small tissue blocks from liver biopsy were fixed in formalin glutaraldehyde, post fixed in OsO4, stained with uranyl citrate and routinely processed for ultrastructural analysis and examined by Opton 900 PC electron microscope.

Results: The main histologic findings were bile ductular proliferation, portal and periportal fibrosis and inflammation which corresponded with stage 2 of PBC (2). The changes were sometimes accompanied by features of slight or moderate fatty changes in the liver. The ultrastructural examinations of portal bile ducts revealed damage to biliary epithelial cells – light as well as dark cells which were irregular in shape. The widened intercellular spaces and small lumina of ducts were observed. Frequently biliary epithelial cells contained cytoplasmic vacuoles, especially in the upper part of the dark cells. Sometimes epithelial cells showed increased lysosomal activity. Dark biliary cells seemed to be shrunken. In some pictures, close contacts between biliary epithelial cells and lymphocytes were noted. The basement membrane surrounding the portal perimeter of the portal bile ducts was often duplicated. The abnormalities were accompanied by hepatocellular changes,
including mitochondrial and cytophagosomal changes and focal cytoplasmic degeneration. Frequently, collagen bundles with transitional forms of stellated cells were found within the spaces of Disse, in the intercellular spaces and in dilated portal tracts.

**Conclusion:** Ultrastructural changes observed in the epithelial cells of bile ducts coexisting with histological findings, biochemical and immunological disturbances as well as with family history may be very useful for the final diagnosis of AMA-negative PBC.

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PDGFRα: A novel therapeutic target in human hepatocellular cancer

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Hepatocellular cancer (HCC) is a disease of poor prognosis due to poorly understood molecular mechanisms. Owing to the similarities between the processes of development and carcinogenesis, we utilized early developing livers to recognize differentially expressed genes and analyzed their expression in HCC. Livers from various mouse embryos (E11–E18) were utilized for gene and protein analysis. Patient HCC tissues (n = 32), normal adjacent prneoplastic tissue and normal livers were examined for PDGFRα and PDGFRβ by Western and Real-Time PCR. The upstream effectors such as PDGF-A and PDGF-C were also examined. Finally, therapeutic effect of anti-PDGFRα antibody (MAb3G3; ImClone) was investigated on human hepatoma cells.

A significantly higher expression of PDGFRα is observed during early liver development (p < 0.001). 26/32 (> 80%) of HCC samples showed a 1.5–82.6-fold increase in total PDGFRα levels as compared to controls (p < 0.01). 17/21 additional samples revealed cytoplasmic PDGFRα and activated-PDGFRα (Tyr754) staining in tumors only. Real-time PCR showed an increase in PDGFRα expression in around 30% of tumors. For PDGFRβ protein expression levels, no statistical significant difference were found in HCC samples versus normal liver tissue. PDGF-AA/CC were elevated in only a small subset. Hep3B cells, which showed elevated and activated PDGFRα, when treated with MAb3G3, showed significant decrease in cell proliferation and survival (p < 0.01).

Elevated PDGFRα levels due to multiple reasons were identified in more than 80% of patient samples (n = 53) independent of fibrosis incriminating its role in progressed HCC. Our findings suggest that HCC patients might benefit from therapeutic inhibition of PDGFRα.
Hepatocyte specific conditional knock-out – A new tool to investigate the role of E-cadherin in liver morphogenesis and carcinogenesis

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E-cadherin is involved in calcium dependent cell-cell adhesion by homophilic interactions in epithelial tissues and has a dual function. First the E-cadherin-catenin complex is the key component of adherens junctions in epithelial cells and second it sequesters cytoplasmic pools of beta-catenin, which prevents beta-catenin of entering the nucleus and starting a transcription programm.

In liver three epithelial cell types, hepatocytes, cholangiocytes and progenitor cells, exist and the spatial expression of E-cadherin in liver was firstly, but superficially described by Butz and Larue. On histochemical level a gradient expression from periportal to pericentral area is observed. In hepatocytes located immediately around central veins no E-cadherin is detectable immunohistochemically. This zonal expression of E-cadherin is already observable in neonatal mice and seems to play a pivotal role in morphogenesis of liver. In addition E-cadherin silencing and/or loss of function is a frequent incident in hepatocacinogenesis.

Because of its outstanding role in early development a constitutive E-cadherin knock-out (KO) is lethal and this prevented the investigation of its role in liver morphogenesis. Here, we present a conditional liver specific KO of E-cadherin based on the tet-system. By using the CEBP/beta(LAP) promotore the knock-out is restricted to hepatocytes and is controllable at least 10 days after insemination. In experiments with adult mice the succesfully knocking out was demonstrated on protein level by western blotting and immunohistochencistry. E-cadherin KO mice show distinct histological changes in liver like ductular reactions. Consequences of KO of E-cadherin during early postnatal period are under investigation.
Homeobox transcription factor Prox1 is a key regulator of liver morphogenesis and hepatocyte commitment

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The homeobox transcription factor Prox1 is expressed in early embryonic hepatoblasts and remains stably expressed in adult hepatocytes. Prox1-null mice show severe deficiencies of liver development, but the underlying mechanisms are unknown. Although some of the Prox1-regulated genes have been studied, a global analysis of its functions in hepatoblasts has not been performed. We investigated embryonic day-14 (ED14) Met Murine Hepatocytes (ED14-MMH). These cells express numerous hepatoblast markers, except for Prox1. We performed stable transfection of the cells with Prox1 cDNA, analyzed the transcriptome with Agilent 44k mouse whole genome microarrays and validated genes by qRT-PCR. We observed more than 12-fold up-regulation of 22 genes and down-regulation of 232 genes. Numerous of these genes are involved in metabolic hepatocyte functions and may be regulated by Prox1 either directly or indirectly, e. g. by down-regulation of HNF4α. Prox1 induced down-regulation of transcription factors, which are highly expressed in neighbouring endodermal organs, suggesting a function during hepatoblast commitment. Prox1 did not influence proliferative activity of MMH. Other Prox1-regulated genes are involved in liver morphogenesis. Specifically, we observed up-regulation of a specific type-IV collagen chain (α3) and functionally active Matrix Metalloproteinase-2 (MMP-2), which digests type-IV collagen of basal laminae. This places Prox1 in the centre of liver matrix turnover and is consistent with the observation of MMP-2 expression in hepatoblasts during liver development, and the persistence of a basal lamina around the liver bud in Prox1-deficient mice. Our studies show that Prox1 is a multifunctional regulator of liver morphogenesis, hepatocyte function and commitment.
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