UNIVERSITÉ DE STRASBOURG

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

INSERM U1110

THÈSE présentée par :

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soutenue le : **19 avril 2016**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline/ Spécialité : Virologie

IDENTIFICATION DES CIRCUITS BIOLOGIQUES INDUITS PAR LE VIRUS DE L'HÉPATITE C ET LEURS IMPLICATIONS DANS LE DÉVELOPPEMENT DU CARCINOME HEPATOCELLULAIRE

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ECOLE DOCTORALE 414

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19/04/2016

UNRAVELING HEPATITIS C VIRUS-INDUCED BIOLOGICAL CIRCUITS CONTRIBUTING TO THE DEVELOPMENT OF HEPATOCELLULAR CARCINOMA

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This thesis was supported by the international PhD program of the University of Strasbourg with an initiative of excellence (IdEx) scholarship, within the framework of the Investment for the future program of the French national research agency (ANR).

TABLE OF CONTENTS

ABBREVIATIONS

aa	Amino acids
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
BC	Bile canaliculus
CD81	Cluster of differentiation 81
CLDN1	Claudin-1
CypA	Cyclophilin A
DC-SIGN	Dendritic cell specific intercellular adhesion molecule 3 grabbing non-integrin
DGAT1	Diacylglycerol acyltransferase 1
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ECMV	Encephalomyocarditis virus
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
GBV-B	GB virus B
GSEA	Gene set enrichment analysis
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCVcc	Cell culture-derived HCV
HCVpp	HCV pseudoparticles
Hg	Mercury (hydrargyrum)
HIV	Human immunodeficiency virus
HCC	Hepatocellular carcinoma
HDL	High density lipoprotein
HSC	Hepatic stellate cell
Huh7	Human hepatoma 7
IFN-α	Interferon-alfa
IFN-γ	Interferon-gamma

IDL	Intermediate density lipoprotein
IRES	Internal ribosome entry site
kb	Kilobase
kDa	Kilodalton
LD	Lipid droplet
LDL	Low density lipoprotein
LDLR	Low-density lipoprotein receptor
LPL	Lipoprotein lipase
L-SIGN	Liver/lymph node specific intercellular adhesion molecule 3 grabbing non-integrin
LVP	Lipoviral particle
MAPK	Mitogen activated protein kinase
MTP	Microsomal triglyceride transfer protein
NAFLD	Non-alcoholic fatty liver disease
NANBH	Non-A non-B hepatitis
NASH	Non-alcoholic steatohepatitis
NEFA	Non-esterified fatty acid
NIH	National Institutes of Health
NPC1L1	Niemann-Pick C1 like 1
NPHV	Non-primate hepacivirus
OCLN	Occludin
OSBP	Oxysterol binding protein
PHH	Primary human hepatocyte
PI4KA	Phosphatidylinositol 4-kinase A
PTPRD	Protein tyrosine phosphatase receptor type D
Rb	Retinoblastoma protein
RTK	Receptor tyrosine kinase
SCID	Severe combined immunodeficiency syndrome
SR-BI	Scavenger receptor class B type I
STAT1	Signal transducers and activators of transcription 1
STAT3	Signal transducers and activators of transcription 3
$TCID_{50}$	50% tissue culture infectious dose
TGF-β	Transforming growth factor beta

TJ	Tight junction
uPA	Urokinase-type plasminogen activator
UTR	Untranslated region
VLDL	Very low density lipoprotein

ABSTRACT

Hepatitis C virus (HCV) is a major cause of chronic liver disease and hepatocellular carcinoma (HCC) worldwide, but how HCV causes disease remains only partly understood. One of the obstacles for HCV pathogenesis research is the lack of suitable cell culture models.

The aims of this thesis therefore consisted in (i) establishing an *in vitro* system using affinity purified contaminant-free cell culture-derived HCV (HCVcc) inoculum amenable to the study of HCV pathogenesis and (ii) uncovering virus-specific biological changes upon HCV infection *in vitro* and *in vivo* with relevance for chronic liver-disease. A particular interest was the expression of phosphatases in chronic HCV-infected patients, and its impact on cell signaling and disease development.

By combining a novel cell culture system of hepatocyte-like cells with purified virus, we effectively simulated chronic infection *in vitro*. We found that this infection model induces a transcriptomic profile of chronic HCV patients at high risk of developing HCC. Using this model, we have uncovered the functional role of EGFR as a pan-etiology driver of the HCC risk signature and revealed candidate drivers of the molecular recalibration of hepatocytes leading to liver cancer. In an approach to study liver disease *in vivo*, we opted to screen for protein phosphatase expression in liver biopsies of chronic HCV patients. We observed a downregulation of PTPRD, a well-known tumor suppressor. We demonstrated that this effect is mediated by an increase in miR-135a-5p which targets PTPRD mRNA. Moreover, *in silico* analysis shows that PTPRD expression in adjacent liver tissue of HCC patients correlates with survival and reduced tumor recurrence after surgical resection.

In conclusion, our state-of-the-art *in vitro* model offers a unique opportunity to investigate the molecular mechanisms and cell circuits that drive HCC across the distinct etiologies. Moreover, our model is capable to assess HCC chemopreventive agents allowing fast-track drug discovery. Secondly, we uncovered that chronic HCV infection induces a knockdown of PTPRD, which is a phosphatase of HCV co-factor STAT3. Our results imply that impaired PTPRD expression in infected hepatocytes is a cornerstone of prolonged infection, which may render the infected hepatocyte more susceptible to malignant transformation. Taken together, this work sheds new light on the cellular circuitry driving chronic hepatopathology.

1. INTRODUCTION

Hepatitis C is a liver disease inflicted by the hepatitis C virus (HCV), a virus that chronically infects the human liver and is mainly transmitted through direct blood-blood contact. It is a major cause of chronic liver disease and hepatocellular carcinoma (HCC) worldwide. More than 170 million people are infected, which makes it one of the most pressing health crises of our time. The discovery of HCV in 1989 kick-started the development of diagnostic and therapeutic strategies and is therefore generally considered as one of the great breakthroughs of 20th century medicine.

1.1 IDENTIFICATION OF HCV AS THE ETIOLOGICAL AGENT OF TRANSFUSION-ASSOCIATED HEPATITIS

Before the emergence of human immunodeficiency virus (HIV), viral hepatitis was the main infectious hazard of blood products for medical use. By the mid-1970s, attaining hepatitis-free blood transfusion constituted a true challenge. Fortunately, the arrival of sensitive serological tests allowed efficient screening of donor blood for hepatitis A virus (HAV) and hepatitis B virus (HBV), resulting in a drastic reduction in the incidence of transfusion-associated hepatitis. Concurrently, it became evident that apart from HAV and HBV at least one additional agent was responsible for posttransfusional hepatitis: the so called non-A non-B hepatitis (NANBH) (Alter et al., 1975; Feinstone et al., 1975). In the decade that ensued, evidence accumulated that NANBH was a significant and fatal illness. It was widely accepted that acute NANBH was generally followed by a chronic stage without apparent clinical signs, rendering infected individuals asymptomatic carriers (Dienstag, 1983). At the same time, reports showed that non-A non-B posttransfusional hepatitis caused a concatenation of liver disease progressing from chronic persistent hepatitis, over liver cirrhosis, to ultimately HCC (Kiyosawa et al., 1984).

The first real breakthrough in unraveling NANBH pathology was the establishment of the chimpanzee animal model system, which could recapitulate acute and chronic hepatitis after experimental inoculation with blood from NANBH patients (Tabor et al., 1978). However, the disease remained an etiological enigma throughout the 1980s. In other seminal studies, blood plasma from a chronic NANBH-positive chimpanzee remained infectious after filtration through a 80 nm-filter (Bradley et al., 1985), and in addition, the inoculum could be successfully inactivated with chloroform (Feinstone

et al., 1983). This all pointed in the direction of a relatively small enveloped virus as the agent responsible for NANBH.

Scores of research groups around the globe tried relentlessly to identify the elusive causative agent of NANBH, all without great success. It was only in 1989 that a team led by Michael Houghton at the American biotech company Chiron Corporation (nowadays part of Novartis) determined the exact viral etiology of NANBH. In short, they derived a cDNA library from highly infectious plasma of a NANBH-positive chimpanzee. The cDNA was subsequently cloned in a lambda gt11 expression vector, which allowed the amplification of cDNA-encoded polypeptides in *Escherichia coli*. Next, these polypeptides were screened with polyclonal serum obtained from a NANBH patient, and eventually one positive clone was singled out. Sequencing revealed it was a positive-strand RNA virus of the *Flaviviridae* family, with a genome approximating 10 kilobases (kb) in length. At last the NANBH agent had been identified: HCV (Choo et al., 1989). Since no closely related viruses were known, a new genus within the *Flaviviridae* family was especially created for this virus: the *hepacivirus* genus.

1.2 NATURAL HISTORY AND ORIGIN OF HEPATITIS C VIRUS

To this day, the origin of HCV remains a mystery. HCV exhibits a very restricted host-range with natural infections only occurring in humans, although chimpanzees can also be experimentally infected (Bradley et al., 1985; Kolykhalov et al., 1997). No proximately related viruses were known until 1995, when GB virus B (GBV-B) was isolated from a single tamarin, a small New World monkey (Muerhoff et al., 1995; Simons et al., 1995). In the early days of viral hepatitis research, scientific progress was seriously hindered by the lack of a model system. So when a surgeon with initials G.B. contracted acute hepatitis in 1967, his serum was inoculated in tamarins in an effort to recapitulate the disease. When these lab animals developed acute hepatitis as well, it was hailed as a robust *in vivo* model system (Deinhardt et al., 1967). In 1995, almost three decades later, two viruses were isolated from serum of the 11th tamarin passage. They were designated GB virus A and GB virus B, and GBV-B was subsequently identified as the agent responsible for developing acute hepatitis in tamarins. This obscure virus was never isolated again, neither in humans nor in tamarins, and its host range is restricted to New World monkeys (Bukh et al., 2001; Bukh et al., 1999). So if it is nigh impossible that the surgeon is the source of GBV-B, then where did it come from? Current understanding dictates that GBV-B is most likely to stem from the tamarin originally inoculated with

the serum of the surgeon. The designation of this virus by GBV is therefore probably a misnomer, and its nomenclature is sometimes questioned (Stapleton et al., 2011). Nevertheless, GBV-B was subsequently classified in the genus of *hepacivirus*, together with HCV. For years it was the only known virus similar to HCV.

The virtual absence of more commonly occurring HCV-related viruses was a big disappointment for the HCV community, especially since the discovery of a putative animal reservoir for HCV could explain some epidemiological unknowns: where did this virus come from? Does it have a natural reservoir, and if so, could this be a source of new infections? To make matters even more mystifying, the main mode of HCV transmission is through direct blood-blood contact such as needle-applied vaccinations, intravenous drug use and blood transfusions. Given that all of these procedures were quite uncommon before the Second World War, how could this virus circulate among humans before the dawn of the modern era?

Some revamped excitement arose in the early 2010s when novel closely related hepaciviruses were discovered in dogs (Kapoor et al., 2011) and horses (Burbelo et al., 2012), currently both grouped as non-primate hepaciviruses (NPHV). Not much later, hepaciviruses were also found to circulate in rodents, bats (Drexler et al., 2013; Kapoor et al., 2013; Quan et al., 2013), and also in the black-and-white colobus, an Old World monkey (Lauck et al., 2013). Phylogenetic analyses demonstrated that NPHV was even more closely related to HCV than the enigmatic GBV-B (Burbelo et al., 2012). This revitalized the possibility of zoonotic transfer, but no evidence has been found so far to support horse-to-man transmission (Pfaender et al., 2015b).

At the time of writing, two mutually exclusive visions on the origin of HCV are still standing. It is not improbable that more extensive screening will finally reveal a link between animals and humans for explaining HCV prevalence around the globe. On the other hand, the ability of HCV to establish a persistent infection in humans without any clinical signs in the first decades of infection argues more in favor of an extended co-evolution of both virus and host.

1.3 EPIDEMIOLOGY

1.3.1 HCV PREVALENCE AND GENETIC DIVERSITY

It is estimated that three to four million people are infected with HCV each year, and that currently around 170 million people are chronically infected (Mohd Hanafiah et al., 2013). However, there are big geographical differences, both in prevalence and in genetic diversity (Fig. 1a and 1b). HCV strains can be subdivided into genotypes 1 through 7, each boasting a variable number of subgenotypes designated with a lower-case letter. This forms the conceptual basis of classifying HCV strains into genotypes such as 1a, 1b, 2a etc. (Simmonds et al., 2005; Smith et al., 2014). Genotype 1 dominates in Europe and the Americas, and from a global perspective it is the most common genotype, representing almost half of all infections. Genotype 3 is the second most common with 20 % - 30 % of infections. Genotype 2 and 4 are less widespread with around 10 % of infections (Gower et al., 2014; Messina et al., 2015) (Fig. 1).

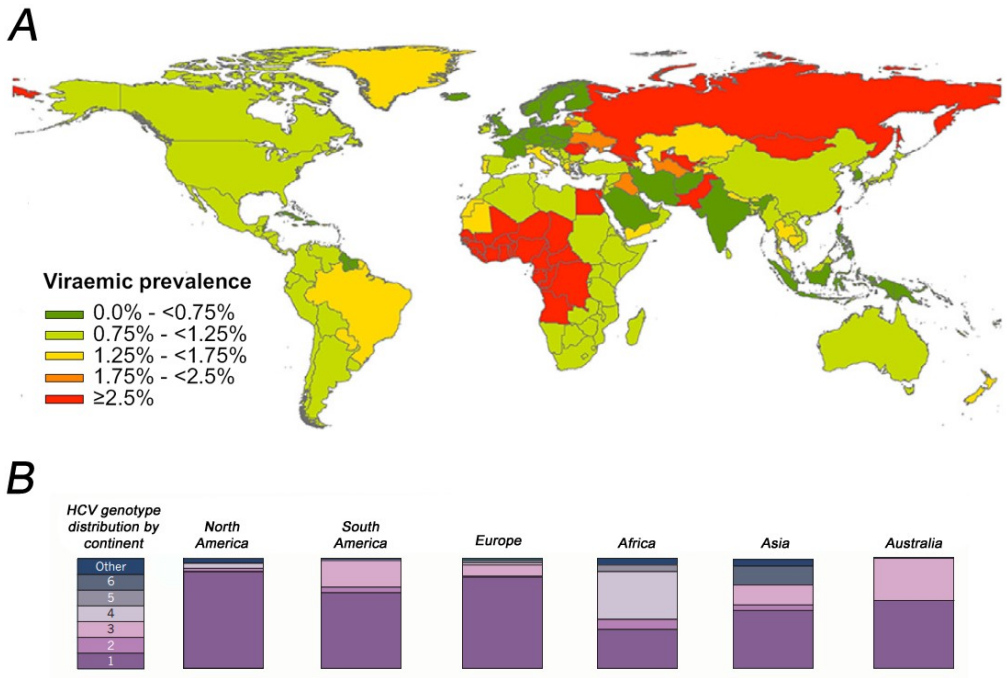

Fig. 1: (A) Estimated global HCV prevalence in 2014 (Gower et al., 2014). **(B)** HCV genotype distribution by continent. Adapted from Gravitz, 2011.

1.3.2 RECENT SPREAD OF HCV: THE SILENT EPIDEMIC

The regionally different patterns of HCV genotypes arose from both 'ancient' endemic circulation, and the relatively recent epidemic spread. The main mode of HCV transmission responsible for the current pandemic is quite straightforward: direct blood-blood contact. Historically, this happened mostly by patients receiving contaminated blood transfusions and through medical treatment with unsterilized needles. By the 1960s, HCV transmission rates accelerated even further by intravenous drug-use and needle sharing, which is nowadays the primary route of transmission after the introduction of donor blood screening (Drucker et al., 2001; Nelson et al., 2011). Egypt is the quintessential example of how HCV can spread explosively throughout the population. Historically, the main cause of liver disease in this developing nation was schistosomiasis, a parasitic disease inflicted by a flatworm. From the 1950s onwards, large-scale health campaigns were undertaken to combat *Schistosoma* infections by intravenous administration of tartar emetic, the standard of care before the arrival of the orally administered praziquantel. Although the campaigns helped to reduce schistosomiasis, the collateral damage was enormous: massive reuse of glass syringes and inadequate sterilization practices caused widespread infection with HCV (Strickland, 2006). Nowadays, a staggering 14 % of the Egyptian population is chronically infected with HCV, which is ~10 fold higher than in other countries. As a result, HCV has replaced schistosomiasis as the main cause of chronic liver disease in Egypt (Cuadros et al., 2014).

From an epidemiological perspective, transmission of HCV through sexual contact is negligible (Terrault et al., 2013). Vertical transmission however remains an important pediatric burden, especially in countries with high HCV prevalence such as Egypt. Studies demonstrated that between 4 % - 15 % of newborns are infected, but approximately half of them clear the infection spontaneously in the first two years after birth (Ceci et al., 2001; Jhaveri et al., 2015; Kanninen et al., 2015; Mast et al., 2005; Shebl et al., 2009).

Most people with chronic HCV are unaware that they are carriers of the virus since acute infection is mostly subclinical and the virus is notoriously underdiagnosed. As a result, the rampant HCV problem is sometimes referred to as *"the silent epidemic"* (Edlin, 2011).

1.3.3 ANCIENT CIRCULATION OF HCV

Given that none of the parenteral routes responsible for the current HCV epidemic were commonplace before the Second World War, the nature and transmission routes of 'ancient' endemic circulation remain a mystery. Sexual and vertical transmissions are not efficient enough to explain endemic circulation over entire generations. Possible explanations include cosmetic and religious practices such as circumcision, ritual scarification, tattooing, body piercings, and even something as ordinary as commercial barbering (Shepard et al., 2005). Another possibility might be an unrecognized arthropod vector (Pybus et al., 2007), but until now this has never been demonstrated.

1.3.4 THE ULTIMATE SOURCE OF HCV

The recently discovered HCV-like *hepacivirus* strains in rodents, bats, dogs and horses reveal a wide host range and ample genetic diversity for hepaciviruses, which increases the possibility that HCV ultimately stems from a zoonotic reservoir. However, only very few species have been tested so far for *hepacivirus* seropositivity, and it is likely that additional species will be identified as hosts for related viruses (Scheel et al., 2015). All the available phylogenetic data so far suggests that if HCV originates from an animal host, it jumped species only once, and that all subsequent genotypes arose from intra-human evolution (Pybus and Gray, 2013). Thus, the viral ancestor of all HCV strains may even predate the human race, and HCV could have simply co-evolved with its host from the time of proto-apes and beyond.

1.4 BASIC HCV VIROLOGY

1.4.1 GENOMIC ORGANIZATION OF HCV RNA

HCV is a single-stranded positive-sense RNA virus within the *Flaviviridae* family. Its genome comprises approximately 9.6 kb encoding a single polyprotein of over 3000 amino acids (aa) (Fig. 2). The 5' untranslated region (UTR) of the genome contains an internal ribosome entry site (IRES) allowing cap-independent translation of the polyprotein (Tsukiyama-Kohara et al., 1992; Wang et al., 1993), while the 3'-UTR promotes translation efficiency (Bradrick et al., 2006; Song et al., 2006). Of note, the 5'-UTR also contains two binding sites for miR-122, a crucial liver-specific host factor which

enhances viral RNA replication (Jopling et al., 2005), translation (Henke et al., 2008) and stability (Sedano and Sarnow, 2014; Shimakami et al., 2012), and is believed to be partly responsible for HCV's strict hepatotropism.

Upon synthesis, the polyprotein undergoes co- and post-translational processing by cellular and viral proteases into ten viral proteins. They comprise three structural proteins that build up the virion: Core, E1 and E2, and also seven nonstructural (NS) proteins implicated in replication and assembly: p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Fig. 2).

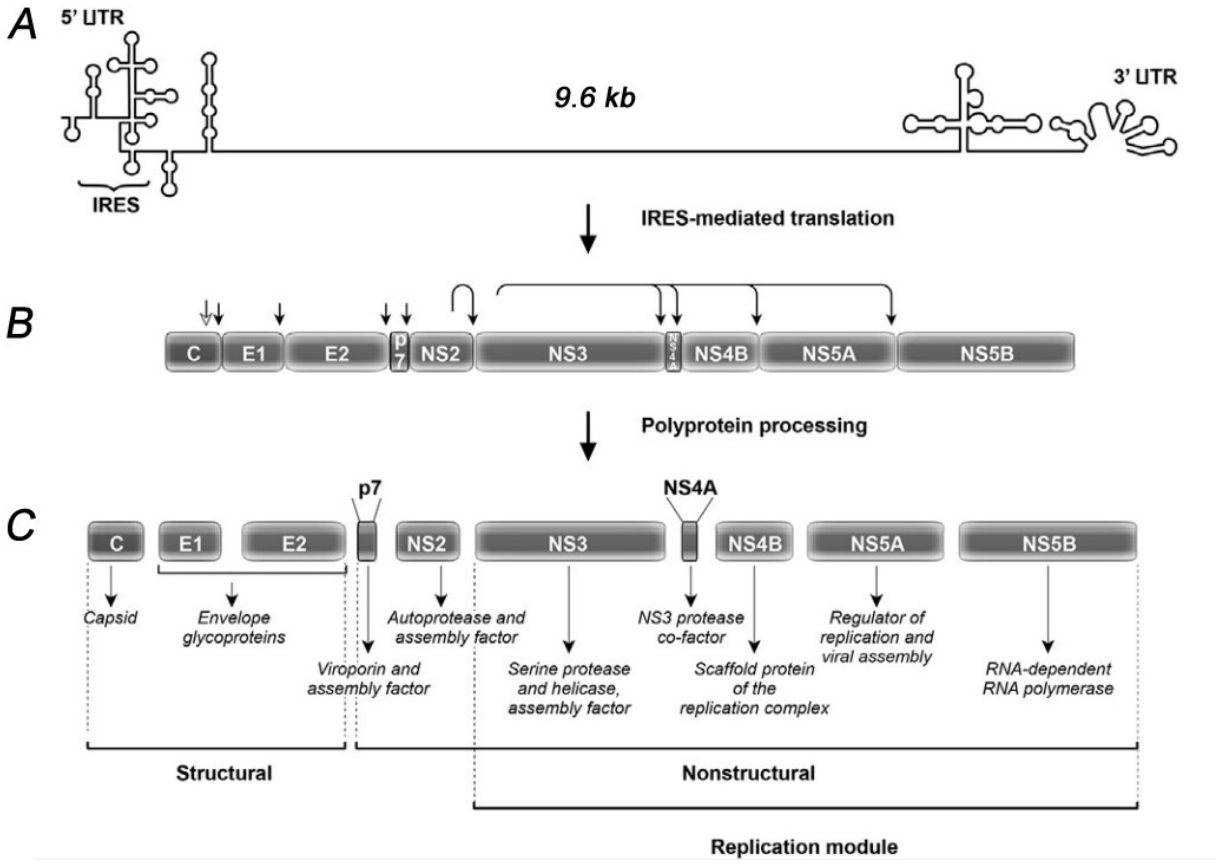

Fig. 2: Schematic overview of **(A)** the HCV genome encoding **(B)** the polyprotein, proteolytically processed into **(C)** individual viral proteins. UTR: untranslated region; IRES: internal ribosome entry site; C: Core; NS: nonstructural. Adapted from Dubuisson and Cosset, 2014.

1.4.2 HCV VIRION STRUCTURE: THE LIPOVIRAL PARTICLE

HCV particles are enveloped viruses containing core proteins forming a capsid around the viral genome. The envelope is derived from the host cell's endoplasmic reticulum (ER) upon biogenesis, and incorporates two separate viral glycoproteins, E1 and E2, which mediate binding and entry into the host cell. The most outstanding characteristic of HCV particles however, is that they are inextricably bound to serum lipoproteins as lipoviral particles (LVPs) (Andre et al., 2002). Lipoproteins are biological cargo ships filled with lipids that circulate in the bloodstream. They are manufactured by hepatocytes and secreted in the blood for redistributing fatty acids and cholesterol to peripheral tissues. Lipids are hydrophobic and therefore normally insoluble in an aqueous environment. Lipoproteins emulsify the hydrophobic content and thus enable fat to be transported through the blood. The lipoprotein particles are decorated with different apolipoproteins, proteins that allow interaction with cellular surfaces. HCV is unique in that it associates with lipid-rich lipoproteins as LVPs in order to escape neutralization by the humoral immune responses. As a consequence, HCV particles display an unusually low and heterogeneous buoyant density compared to other enveloped RNA viruses (Table 1).

Notwithstanding intense research, the exact nature of LVPs is still unclear. HCV may either be transiently attached to lipoproteins, or it may be embedded inside a lipoprotein while sharing part of

Virus or lipoprotein	Buoyant density range (g per ml)	
Enveloped RNA viruses		
HCV	1.03–1.10	
Pestiviruses	1.12–1.15	
Flaviviruses	1.20–1.23	
Alphaviruses	1.19–1.22	
Rubiviruses	1.18–1.19	
Arteriviruses	1.13–1.17	
Retroviruses	1.16–1.18	
Bornaviruses	1.18–1.22	
Rhabdoviruses	1.18–1.20	
Bunyaviruses	1.16–1.18	
Serum lipoproteins		
VLDL	0.950–1.006	
LDL	1.019–1.063	
HDL	1.061–1.210	

Table 1: Buoyant density of different enveloped viruses. The lipoprotein-mediated enrichment in lipids gives HCV an unusually low density compared to other viruses. VLDL: Very low density lipoprotein; LDL: High density lipoprotein; HDL: High density lipoprotein. Taken from Lindenbach and Rice, 2013.

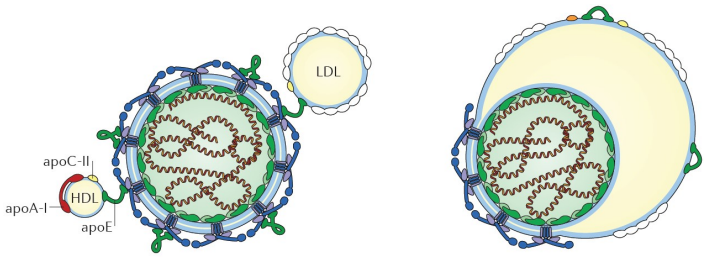

Fig. 3: Two mutually exclusive visions for LVPs. The two-particle model where HCV binds to serum lipoproteins (left), and the single particle model where HCV is firmly lodged inside a lipoprotein (right). LDL: Low density lipoprotein. HDL: High density lipoprotein. Apo: Apolipoprotein. Taken from Lindenbach and Rice, 2013.

the envelope (Fig. 3). HCV particles are thus asymmetrical and vary in shape and size, ranging from 40-80 nm in diameter. Unlike other enveloped viruses, they lack spike-like features protruding from the viral envelope (Catanese et al., 2013; Merz et al., 2011).

1.4.3 HCV PROTEINS

The HCV genome encodes ten viral proteins. They can be divided into three functional cassettes: (i) structural proteins Core, E1 and E2 build up the virus particle, (ii) viroporin p7 and nonstructural protein NS2 are involved in HCV virion assembly, while (iii) the remaining nonstructural proteins NS3, NS4A, NS4B, NS5A and NS5B assemble into the HCV replicase necessary for genomic replication (Fig. 4).

1.4.3.1 STRUCTURAL PROTEINS

1.4.3.1.1 CORE

Core is the first protein encoded by the polyprotein, and it forms the capsid protecting the viral genome upon packaging. After synthesis and processing on the ER-membrane, Core proteins are transported away from the ER and sequestered on the outer leaflet of lipid droplets (LDs) by diacylglycerol acyltransferase 1 (DGAT1) (Herker et al., 2010). LDs are cytoplasmic organelles found in most eukaryotic cells and they function as dynamic transport vehicles for neutral lipids such as triglycerols and cholesterolesters. Their hydrophobic core is surrounded by a phospholipid

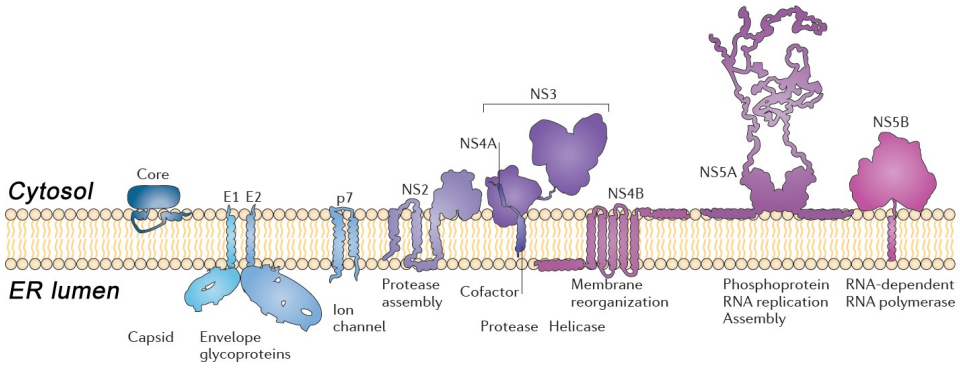

Fig. 4: Overview of HCV-encoded proteins and their orientation in the endoplasmic reticulum membrane. NS: Nonstructural protein. Adapted from Bartenschlager et al., 2013.

monolayer derived from the outer leaflet of the ER bilayer membrane (Farese and Walther, 2009). LDs are generally tethered to the ER (Ozeki et al., 2005) where they provide the lipids that are packaged into lipoproteins for redistribution to peripheral tissue. HCV uses LDs as a platform to concentrate Core subunits awaiting assembly (Fig. 6) (McLauchlan et al., 2002; Okamoto et al., 2008).

1.4.3.1.2 GLYCOPROTEINS E1 AND E2

Glycoproteins E1 (~30 kilodalton (kDa)) and E2 (~70 kDa) are embedded in the viral envelope as trimeric E1-E2 heterodimers (Falson et al., 2015). They are responsible for receptor binding on the hepatocyte, and after endocytosis of the viral particle they mediate membrane fusion for endosomal escape. When freshly formed particles are trafficked through the Golgi system, the glycoproteins obtain a complex glycan shield (Goffard et al., 2005) and become covalently cross-linked through disulfide bridges (Vieyres et al., 2010). Even though part of the E2 glycoprotein structure was recently characterized by crystallography (Kong et al., 2013), the exact mechanism of how E1 an E2 mediate fusion is still unknown.

1.4.3.2 NON-STRUCTURAL PROTEINS

1.4.3.2.1 P7

p7 is a small 63 aa integral membrane polypeptide, which assembles into hexa- or heptameric ion channels (Clarke et al., 2006; Luik et al., 2009). Its presence is essential for viral assembly and

infectivity, both *in vitro* (Steinmann et al., 2007) and *in vivo* (Sakai et al., 2003). p7 acts in close concert with NS2 to recruit Core proteins back from the lipid droplets towards the ER where assembly takes place, but this process happens independently of its ion channel activity (Boson et al., 2011) which can counteract the generation of pH gradients in intracellular vesicles. It is hypothesized that such hindering of acidification can prevent premature conformational changes of nascent virions upon assembly (Wozniak et al., 2010). The classical view on the HCV virion dictates that p7 is not incorporated in the virion itself. However, recent experiments demonstrate that p7 ion channel blockers can effectively impair HCV entry, and preliminary data support a presence of p7 in the HCV particle (Shaw et al., 2015).

1.4.3.2.2 NS2

NS2 is a cysteine protease of ~23 kDa firmly anchored in the ER by three transmembrane domains. It uses its autocatalytic activity to cleave the NS2-NS3 junction, and release itself from the polyprotein (Fig. 2B) (Grakoui et al., 1993). In association with p7, NS2 recruits glycoproteins and other viral proteins of the replication complex to the assembly site on the ER which is situated in close proximity to LDs (Boson et al., 2011; Jirasko et al., 2010; Popescu et al., 2011).

1.4.3.2.3 NS3-4A COMPLEX

NS3 is a bifunctional protein of ~70 kDa. First and foremost, it is a serine-dependent protease responsible for proteolytic separation of the polyprotein at four different sites: NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B (Fig. 2B) (Tomei et al., 1993). After release from the polyprotein, the ~8 kDa NS4A serves as a cofactor for NS3, enhancing its proteolytic activity (Failla et al., 1994; Lin et al., 1995). Secondly, NS3 boasts a superfamily 2 helicase domain with ATPase activity, which has the capacity to unwind the viral dsRNA intermediate with a ratchet-like mechanism in a 3'-5' direction (Gu and Rice, 2010).

NS3-4A is also an important viral tool for evading innate immunity. It cleaves critical adaptor proteins in viral sensing (Li et al., 2005a; Li et al., 2005b; Meylan et al., 2005). Since blocking protease activity hampers both polyprotein processing and viral immunoevasion, the NS3-4A protease was identified as a target for therapeutic intervention (see 1.9: Hepatitis C treatment).

1.4.3.2.4 NS4B

NS4B is a highly hydrophobic membrane protein of ~27 kDa and it functions as a critical organizer of the HCV replication complex. Together with other nonstructural proteins it promotes formation of the membranous web: juxtanuclear double-membrane vesicular rearrangements of the ER where viral replication takes place (Fig. 5) (Gouttenoire et al., 2010; Romero-Brey et al., 2012).

1.4.3.2.5 NS5A

NS5A is a RNA-binding phosphoprotein of ~447 aa serving a dual role in both replication and assembly. After translation, NS5A proteins are anchored to the ER membrane with their N-termini. They are responsible for starting the vesicular rearrangements of the ER by recruiting phosphatidylinositol 4-kinase A (PI4KA) (Reiss et al., 2011). PI4KA in turn activates its downstream effector oxysterol binding protein (OSBP) (Wang et al., 2014) which promotes cholesterol enrichment of the ER. The fluidity and integrity of the ER membranes change and the membranes transform into the vesicle-like structures of the membranous web (Fig. 5A). Inside the membranous web reside all the viral proteins of the replication complex replicating viral RNA (Fig. 5B).

Some NS5A molecules however are transported away from the ER and loaded on LDs by DGAT1, where they guide newly synthesized viral RNA to the assembly site on the interface between the ER and LDs (Fig. 6) (Camus et al., 2013; Miyanari et al., 2007).

NS5A boasts three domains of which domain I assembles into dimeric complexes (Tellinghuisen et al., 2005). Domain II increases its RNA binding capacity (Foster et al., 2011) and interacts with cyclophilin A (CypA), a crucial host factor necessary for instigating the vesicular rearrangements of the membranous web (Kaul et al., 2009; Liu et al., 2009; Madan et al., 2014). Domain III is involved in particle assembly, and is a prerequisite for close interaction with Core proteins on the LD surface (Appel et al., 2008; Masaki et al., 2008). Given its extensive role for HCV, NS5A constitutes an efficient target for antiviral therapy (see 1.9: Hepatitis C treatment).

1.4.3.2.6 NS5B

NS5B is an RNA-dependent RNA polymerase of 591 aa responsible for synthesizing a negative-strand template of the viral genome, and subsequently copying the viral genome based on the negative-strand template (Behrens et al., 1996). Since its indispensable role as a polymerase, it emerged as a major target for antiviral intervention (see 1.9: Hepatitis C treatment).

1.4.3.2.7 F-PROTEIN

The HCV genome boasts an alternative reading frame overlapping with the Core region encoding the F-protein. It lacks a start codon, and is therefore translated through a +1 ribosomal frame-shift. As a result, the first 10 aa of the F-protein are similar to Core (Xu et al., 2001). The F-protein is not essential for HCV infection and replication, and its function remains poorly understood (McMullan et al., 2007).

1.4.4 VIRAL LIFECYCLE

The complete life cycle of HCV occurs mainly in hepatocytes. Extrahepatic replication in astrocytes, microglial cells and endothelial cells of the central nervous system has been reported but remains controversial (Fletcher et al., 2012; Wilkinson et al., 2009). In general HCV is believed to be strictly hepatotropic because (i) its receptors are mainly expressed in hepatocytes, (ii) its dependency on liver-specific miR-122 and (iii) the close association of HCV particles with lipoproteins.

1.4.4.1 VIRION ATTACHMENT AND ENTRY

In general, interplay between enveloped viruses and the host cell surface during attachment and entry are largely determined by the viral proteins inserted in the envelope. For HCV, these interactions are unique since the virus circulates covertly in the bloodstream as a LVPs (Andre et al., 2002). In other words, HCV hides in lipoproteins, which have their own set of cellular receptors. As a consequence, there is no arbitrary line between a truly 'viral' receptor and a 'lipoprotein' receptor: HCV makes use of both. HCV attachment and entry is therefore a puzzlingly complex process of which the exact sequence of events and details are still not entirely clear.

The first contact between HCV and the basolateral membrane of hepatocytes occurs when LVPs sieve through the fenestrated endothelium of the hepatic microcirculation into the space of Disse (see Fig. 9). Here, initial attachment depends on heparan sulfate-bearing proteoglycans syndecan-1 or syndecan-4 (Lefevre et al., 2014; Shi et al., 2013). However, attachment can also occur through lipoprotein receptors binding the lipoproteins associated with HCV. They include low-density lipoprotein receptor (LDLR) (Agnello et al., 1999) and scavenger receptor BI (SR-BI) (Dao Thi et al., 2012; Scarselli et al., 2002).

Interestingly, SR-BI can also bind directly to E2 glycoproteins (Scarselli et al., 2002), but this interaction takes place in a critical post-binding step which primes the viral particle for interaction with other host cell receptors (Dao Thi et al., 2012; Zeisel et al., 2007). Apart from SR-BI also CD81 can interact with E2 (Pileri et al., 1998). Next, tight junction protein claudin-1 (CLDN1) was discovered as an important post-binding entry factor (Evans et al., 2007). Current understanding holds that CD81 binding triggers epidermal growth factor receptor (EGFR) autophosphorylation (Diao et al., 2012; Lupberger et al., 2011), which results in basolateral diffusion of CD81 so it can associate with CLDN1 to form the CD81-CLDN1 co-receptor complex (Harris et al., 2010; Harris et al., 2008). The absence of EWI-2wint in hepatocytes in this process is considered crucial, as EWI2-wint would interact with CD81 and restrict HCV entry (Rocha-Perugini et al., 2008).

Intriguingly, EGFR is not an integral part of the co-receptor complex, nor does E2 or any other part of the LVP appear to bind to EGFR. It is the intracellular HRas downstream the EGFR signaling pathway which is responsible for communication between the EGFR axis and CD81-CLDN1 (Zona et al., 2013). In addition, other crucial host factors have been identified for HCV entry: occludin (OCLN) (Ploss et al., 2009), Niemann-Pick C1-like 1 (Sainz et al., 2012) and transferrin-1 (Martin and Uprichard, 2013). However their precise roles remain to be elucidated. In addition, the HCV E2 glycoprotein is also known to bind to dendritic cell-specific intercellular adhesion molecule 3 grabbing non-integrin (DC-SIGN) (Pohlmann et al., 2003) and liver/lymph node specific intercellular adhesion molecule 3 grabbing non-integrin (L-SIGN) (Gardner et al., 2003). However, these interaction partners are not present on hepatocytes but rather on dendritic cells and endothelial cells respectively, which questions their involvement in HCV entry in hepatocytes. Recently, also CD63 was found to facilitate HCV entry through a direct interaction with E2 (Park et al., 2013). CD63 is a tetraspanin expressed on the cell surface and in endosomes. CD63 is therefore thought to participate in HCV endocytosis, but its precise role in this process remains unclear.

After co-receptor complex formation on the basolateral membrane of the hepatocyte, CD81-CLDN1 bound HCV particles are endocytosed in a clathrin- and dynamin-dependent manner (Blanchard et al., 2006; Farquhar et al., 2012). When the resulting HCV-bearing endosome gets acidified, the viral glycoproteins mediate membrane fusion (Douam et al., 2014; Lavillette et al., 2006; Sharma et al., 2011). This paves the way for uncoating and cytoplasmic release of the genome, processes that are still not very well characterized.

1.4.4.2 GENOME TRANSLATION AND COTRANSLATIONAL PROCESSING

Once the viral genome is released into the cytoplasm, it serves as a messenger RNA instructing viral polyprotein synthesis. Its 5'-UTR constitutes a functional IRES which allows it to dock on the ribosome and instigate cap-independent translation (Lukavsky, 2009). Shortly after translation initiation, a signal sequence between Core and E1 halts protein synthesis and targets the ribosome to the translocon complex of the ER. Once relocated, translation proceeds and the signal peptidase of the ER cleaves the nascent polypeptide, releasing a 191 aa Core precursor on the cytosolic side of the ER (Santolini et al., 1994). The Core precursor is further processed into the mature 21 kDa Core protein of ~177 aa by signal peptide peptidase, an ER-residing cellular protease (McLauchlan et al., 2002).

After release of the Core segment, polyprotein synthesis continues with the N-terminus of E1 which is now pumped inside the internal cistern of the ER. The C-terminus of both E1 and E2 display cleavage sequences embedded inside transmembrane domains. The moment these sites are processed, the C-termini of E1 and E2 are reoriented towards the cytosolic side and become firmly anchored in the ER membrane (Cocquerel et al., 2002). As a result, both E1 and E2 protrude into the ER lumen with their ectodomains (Fig. 4).

The remaining nonstructural protein NS2, NS3, NS4A, NS4B, NS5A and NS5B are also attached to the ER with transmembrane domains, but unlike E1 and E2, they are oriented towards the cytosolic side. NS2 uses its autocatalytic activity to release itself from the polyprotein (Grakoui et al., 1993), while the remaining viral proteins are cleaved by the NS3-4A protease (Tomei et al., 1993).

1.4.4.3 GENOME REPLICATION

After the polyprotein has been processed, NS5A recruits PI4KA, whose activity leads to membrane invaginations of the ER, eventually forming double membrane vesicles hosting the replication complex (Ferraris et al., 2013; Paul et al., 2013; Reiss et al., 2011). This morphological feature of HCV-infected hepatocytes known as the membranous web is visible through electron microscopy imaging (Fig. 5A). The vesicles reside in close proximity of the ER and can even surround entire LDs.

The active replication complex comprises viral proteins NS3-4A, NS4B, NS5A and NS5B and host factor CypA. The initial step of RNA synthesis creates a negative strand of the viral genome which serves as a template for progeny genomes. Newly generated positive-strand RNA copies are either

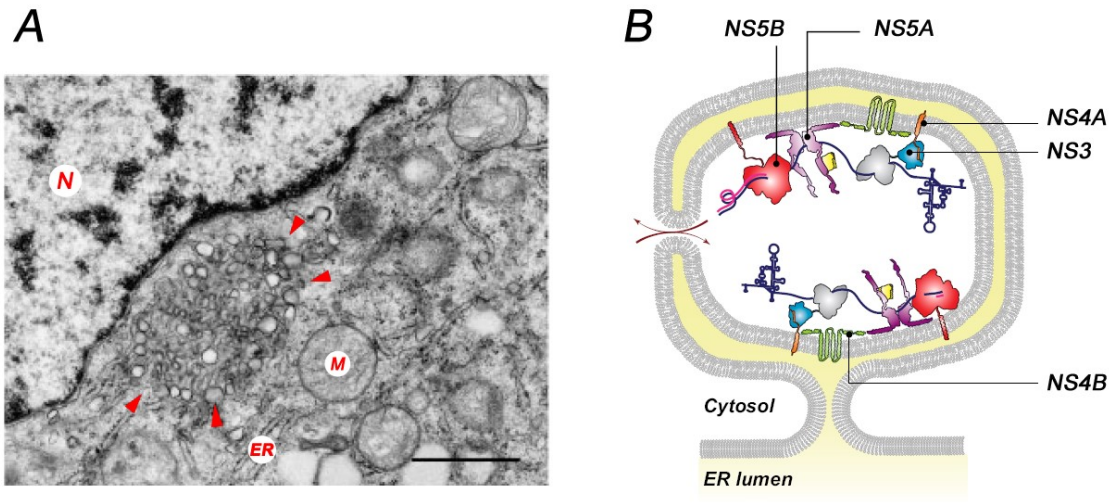

Fig. 5: (A) Electron microscope imaging of the membranous web: HCV-induced vesicular rearrangements in close proximity to the endoplasmic reticulum (ER). Arrowheads: membranous web. N: nucleus; M: mitochondrion; Bar: 1 µm (Gouttenoire et al., 2010). **(B)** Cartoon showing a cross section of a double-membrane vesicle of the membranous web where active replication takes place. The vesicles may display pores for the viral genome to exit towards the cytoplasm for subsequent translation or packaging. Adapted from Paul et al., 2014.

used for secondary rounds of replication, or exported from the membrane vesicle for translation or packaging into new virions (Fig. 5B), probably through pores in the vesicles (Romero-Brey et al., 2012). Recent data suggest that such pores may actually be nuclear pore-like complexes, reminiscent of nuclear pore complexes that serve as gatekeepers to the nucleus (Levin et al., 2014; Neufeldt et al., 2013). By cloaking dsRNA intermediates inside membrane vesicles of the membranous web, viral replication goes unnoticed by innate pattern recognition receptors, and hence serves as a mechanism of immunoevasion.

1.4.4.4 VIRAL ASSEMBLY

Detailed information on the different steps of HCV assembly is currently lacking, but it is assumed that viral RNA export from the replication complex towards the site of assembly is mediated by NS5A. Concomitantly, Core is recruited back to the ER with the aid of p7 and NS2 (Boson et al., 2011). The HCV particle then buds inside the ER, incorporating the E1-E2 glycoproteins in the process.

HCV morphogenesis shares another specific feature with lipoprotein synthesis, which is lipid droplet-derived lipidation. LDs are generally tethered to the ER (Ozeki et al., 2005) where they provide the lipids that will be incorporated into nascent apoliporotein B (ApoB) bearing very low density lipoproteins (VLDL) by microsomal triglyceride transfer protein (MTP). For HCV it is not ApoB however, but apolipoprotein E (ApoE) which is essential for virion formation (Benga et al., 2010; Da Costa et al., 2012; Jiang and Luo, 2009). In addition, the implication of MTP for HCV lipidation is controversial (Paul et al., 2014).

Two other important questions regarding HCV assembly into LVPs are still unresolved: (i) what is the molecular basis of ApoE association with LVPs? And (ii) are LVPs hybrid particles sharing the same membrane, or rather a transient association where lipoproteins only stick to the virion for a given period of time? (Fig. 6).

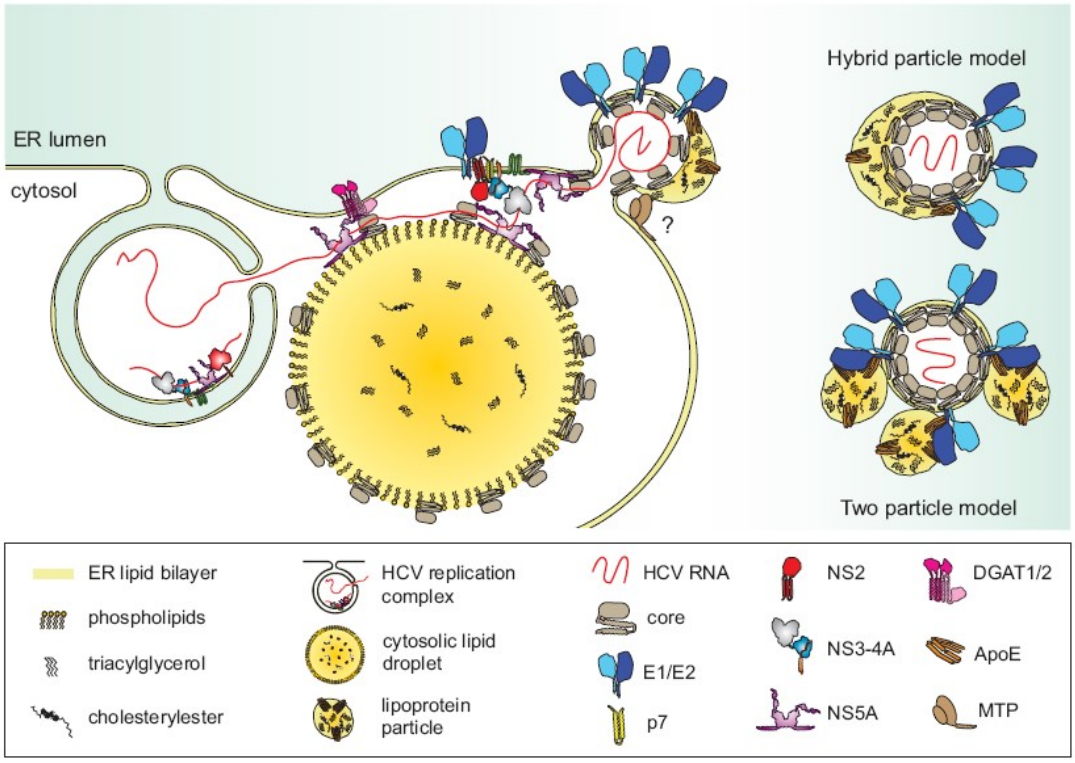

Fig. 6: Model of HCV packaging according to Paul et al., 2014. Viral RNA is shunted from the replication complex to the site of assembly on LDs in close proximity to the ER. It is thought that when Core is recruited back to the ER membrane, it triggers budding of the nascent virion inside the ER lumen.

1.4.4.4 VIRUS EGRESS

The events following HCV assembly are not very well understood. Viral particles are assumed to exploit the conventional secretory system for trafficking from the ER lumen to the Golgi system. In these compartments E1 and E2 are covalently crosslinked and further glycosylated into mature viral glycoproteins (Vieyres et al., 2010). Eventually, HCV is released at the plasma membrane after transportation in VAMP1 associated secretory vesicles (Coller et al., 2012).

Apart from cell-free transmission stemming from secretion of VLPs in the bloodstream, HCV also spreads to neighboring cells through cell-cell transmission (Timpe et al., 2008; Witteveldt et al., 2009). This mode of infection implicates all main entry factors and is actually much more efficient than cell-free transmission (Xiao et al., 2014). As a result, infected hepatocytes are clustered in discrete pockets inside the liver (Wieland et al., 2014).

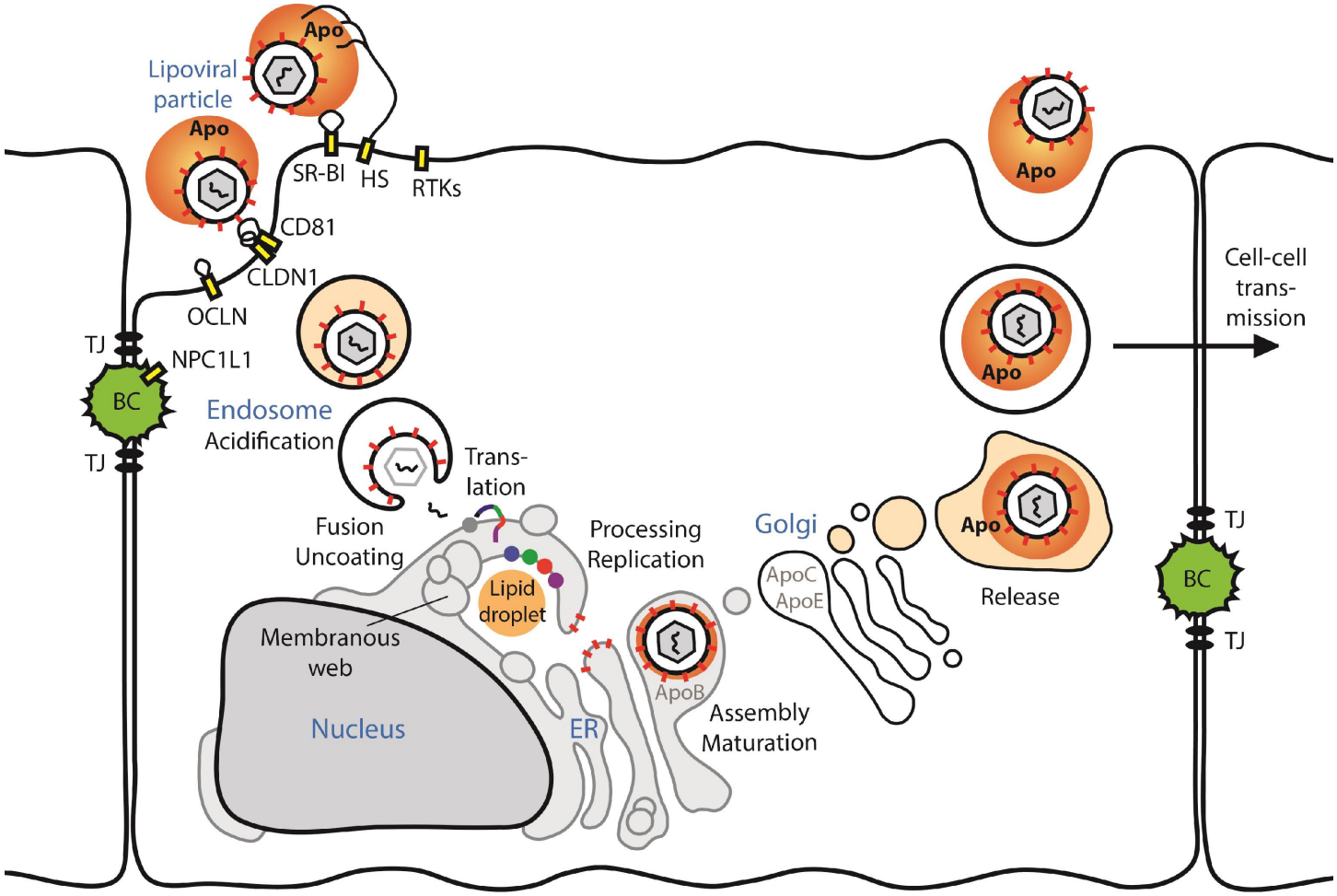

Fig. 7: Round-up of the complete lifecycle of HCV in polarized hepatocytes showing entry, endosomal escape, membranous-web associated HCV replication, assembly at the LD-ER interface and viral egress through the Golgi system. HS: heparan sulfate proteoglycans; RTKs: receptor tyrosine kinases; SR-BI: scavenger receptor BI; CD81: cluster of differentiation 81; CLDN1: claudin-1; OCLN: occludin; NPC1L1: Niemann-Pick C1-like 1; Apo: apolipoprotein; BC: bile canaliculus; TJ: tight junction. Adapted from Zeisel et al., 2013.

1.5 *IN VITRO* MODEL SYSTEMS OF HCV

When HCV was discovered in 1989 (Choo et al., 1989), initial attempts to grow the virus *in vitro* ended in failure, and they would continue to do so over the ensuing years. The development of a cell-culture permissive HCV system occurred step by step, but three key achievements constitute the bedrock on which most of current understanding of HCV biology is built: the replicon, retroviral pseudoparticles, and finally in 2005 the cell culture system recapitulating the complete HCV life cycle (Fig. 8).

1.5.1 HCV REPLICON SYSTEM (1999)

Following molecular cloning of HCV in the early 1990s, several *in vitro* HCV systems were reported. Unfortunately, they lacked robustness and were most of times fraught with abysmal replication efficiency (Bartenschlager and Lohmann, 2000) which rendered detailed molecular studies of the HCV replication cycle as good as impossible. The establishment of subgenomical replicons of other *Flaviviridae* viruses such as classical swine fever virus (Mittelholzer et al., 1997) and West-Nile virus (Khromykh and Westaway, 1997) inspired the creation of a similar replicon for HCV. A genotype 1b strain was derived from a chronic HCV patient and cloned into a plasmid as an artificial genomic construct, devoid of viral proteins core, E1, E2, p7 and NS2. The resulting replicon encoded the 5'-UTR of HCV, the first 12 codons of the core protein fused with a G418 selection cassette, the IRES from encephalomyocarditis virus (EMCV), the HCV non-structural proteins NS3, NS4A, NS4B, NS5A, NS5B and finally the HCV 3'-UTR (Fig. 8). After *in vitro* transcription, this construct was transfected into Human hepatoma 7 (Huh7) cells (see 1.5.4: HCV-permissive cells). Finally, colonies displaying high level HCV RNA replication could be isolated by simple G418 selection (Lohmann et al., 1999). This model enabled the very first *in vitro* studies of HCV genome replication and was further extended over the years to different HCV genotypes and full-length reporter replicons. It was key to the development of direct-acting antivirals (see 1.9: Hepatitis C treatment).

1.5.2 HCV PSEUDOPARTICLES (HCVPP) (2003)

The approach to establish surrogate models to study only part of the viral life cycle continued with the development of pseudotyped retroviral particles (or pseudoparticles). Pseudoparticles are basically enveloped retroviral capsids with viral glycoproteins of choice embedded in their envelopes. As a consequence, they behave as the virus whose glycoproteins they incorporate, at least for what

attachment and entry is concerned. The addition of a reporter gene inside the pseudoparticle allows an easy quantification of viral entry. In 2003, the HCV glycoproteins E1 and E2 were successfully integrated in retroviral particles: the HCV pseudoparticles (HCVpp) (Bartosch et al., 2003; Hsu et al., 2003). HCVpp serve as a proxy for host cell entry, elegantly independent of HCV replication. There are some limitations however. Since HCVpp do not associate with lipoproteins, they do not completely resemble *bona fide* HCV particles. Moreover, HCVpp derive their envelope from the cell membrane, while the true HCV envelope is derived from the ER. HCVpp therefore constitute an approximation of HCV entry that is somewhat different of genuine HCV VLP entry.

1.5.3 CELL CULTURE DERIVED HCV (HCVcc) (2005)

Notwithstanding the progress made with the replicon and the pseudoparticle system, the challenge for establishing a HCV permissive cell culture system remained. While patient-derived consensus genomes led to productive infection after intrahepatic inoculation in chimpanzees (Kolykhalov et al., 1997; Yanagi et al., 1997), transfection of the same genomes in cell lines were unsuccessful. The big breakthrough stemmed from research on the HCV replicon system. *In vitro* selection for replicon-bearing cells produced a handful of replication-enhancing mutations in the replicon genome. Such mutations were enriched in the replicon gene pool when kept under selection pressure, but when full-length viral RNA of these mutated genomes was used to inoculate chimpanzees, *in vivo* virion production was impaired (Bukh et al., 2002; Pietschmann et al., 2009). This demonstrated that these mutations were only helpful in the replicon setting.

When a certain genotype 2a strain called JFH1 -shorthand for Japanese Fulminant Hepatitis 1, a strain isolated from a Japanese patient with acute viral hepatitis (Kato et al., 2001)- was found to replicate without adaptive mutations (Kato et al., 2003), it was clear this was a particular strain with massive replicative potential. In 2005, three groups published a robust *in vitro* system recapitulating the complete life cycle of the JFH1 strain (or derivatives) in Huh7 cells (or derivatives) (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). This genotype 2a-based HCV cell culture system (HCVcc) became the laboratory standard for *in vitro* HCV studies ever since. A related HCV strain commonly used in research is Jc1. This strain is a chimeric construct based on JFH1 but with Core-E1-E2-p7 and partially NS2 derived from J6, another genotype 2a virus. Jc1 thus contains the replication complex of JFH1 and the glycoproteins and viroporin of the J6 strain. The hybrid Jc1 strain yields infectious titers 100 to 1000-fold higher than the original strains (Pietschmann et al., 2006), and is

easier to use in biomedical research than the classical JFH1 strain. In a later stage, several other JFH1-based chimeras have been developed to study the effect of different genotypes on entry, replication, neutralization, assembly and activity of antiviral compounds (Gottwein et al., 2007; Gottwein et al., 2009; Scheel et al., 2011), but none of them compare to JFH1 when it comes to infectivity.

It took another decade before it was elucidated why JFH1 displayed such an exceptional replicative power. In short, HCV replicases are very sensitive to lipid peroxidation, a process stimulated by the infection itself (Huang et al., 2007). It is believed that HCV applies this mechanism to downtone its own replication to promote persistence. Lipid peroxidation sensitivity is completely absent in the JFH1 strain, and as a consequence, infection-induced peroxidation does not hinder its fierce replication (Yamane et al., 2014). Since this mechanism prohibits the study of non-JFH1 strains *in vitro*, the bulk of knowledge derived from *in vitro* experiments is thus based on JFH1. It was only recently appreciated that introducing SEC14L2 in the Huh7 cell line confers permissivity for all HCV strains by promoting vitamin E mediated inhibition of lipid peroxidation (Saeed et al., 2015).

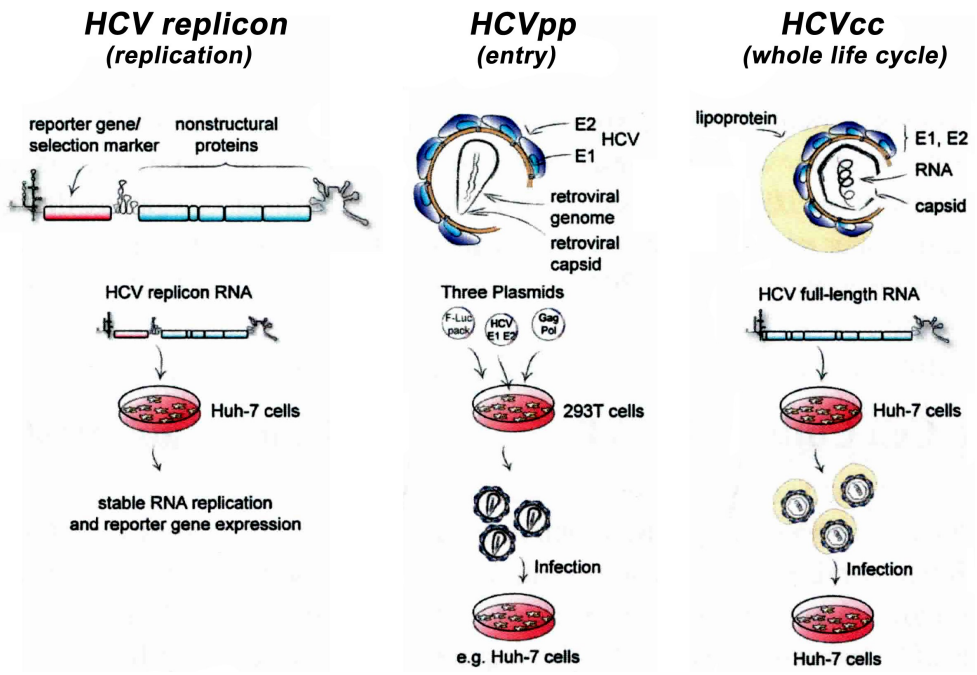

Fig. 8: Outline of the HCV replicon (left), HCV pseudoparticle (middle) and full-length recombinant cell culture-derived HCV (right) system. These different systems are suitable to study viral replication (replicon), entry (pseudoparticle) or the complete lifecycle (cell-culture derived HCV). Adapted from Steinmann and Pietschmann, 2013.

1.5.4 HCV-PERMISSIVE HOST CELLS

HCV replicates exclusively in human hepatocytes, and hence cultured primary human hepatocytes (PHH) are the *in vitro* model closest to the natural host. They are quiescent and do not proliferate. However, PHH are difficult to obtain and display a high donor-dependent variability. In addition, once seeded on collagen-coated plates they start dedifferentiating and lose their cuboid morphology (Shulman and Nahmias, 2013). Moreover, they can only be cultured for approximately two weeks before cell death occurs, which makes them unsuitable for long-term experiments. Another disadvantage is that primary cells are notoriously difficult for standard biomedical research procedures such as transfection, transduction and infection. Indeed, when infected with HCV PHH generally yield irreproducible low-level replication (Fournier et al., 1998; Molina et al., 2008; Rumin et al., 1999). Improved replication could be obtained by using JFH1-derived HCVcc particles (Podevin et al., 2010), but practicality precludes wide usage of this system. Instead, almost every HCV laboratory uses Huh7-derived cells for *in vitro* experiments and PHH are punctually used to confirm findings generated from cell lines.

The Huh7 cell line was originally isolated from a HCC of a Japanese patient in 1982 (Nakabayashi et al., 1982). By continued selection of replicon-containing Huh7 cells, certain Huh7 subclones with increased HCV permissivity were obtained. These subclones were subsequently cured from the replicon by treatment with interferon-alfa (IFN-α), interferon-gamma (IFN-γ) or selective inhibitors, and designated Huh7.5 (Blight et al., 2002), Huh7-Lunet (Friebe et al., 2005) and Huh7.5.1 (Zhong et al., 2005). It is not always known why these cell lines yield higher level of HCV replication. In case of Huh7.5 however, a mutation in the antiviral sensing protein RIG-I was found to be the cause of increased viral replication (Sumpter et al., 2005). Because of their ease of use, high permissivity and reproducibility, these cell lines have been the main workhorse for HCV *in vitro* research.

Notwithstanding their excellent track record as a model system, Huh7-derived cells do have their disadvantages. Since they constitute dedifferentiated asynchronously dividing cancer cells, they do not faithfully represent natural hepatocytes, which are differentiated and quiescent (Michalopoulos and DeFrances, 1997). It was found that adding 1 % of dimethyl sulfoxide (DMSO) to cell culture medium could induce cell-growth arrest and hepatocyte-specific gene expression in Huh7-derived cells, while maintaining HCV permissiveness (Sainz and Chisari, 2006). These hepatocyte-like cells are believed to be closer to a true hepatic physiology than continually dividing cancer cells, and because

of growth arrest, it has the advantage of allowing the study of HCV persistence *in vitro* (Bauhofer et al., 2012).

1.6 ANIMAL MODEL SYSTEMS

The contributions of *in vitro* culture systems for understanding HCV biology have been of paramount importance. However, fundamental concepts such as integrated immunological host responses or liver disease development cannot be investigated *in vitro*. Furthermore, clinical research in patients is by definition limited because of the lack of control over experimental parameters such as time and dose of infection. An ideal animal model would therefore consist of a cheap and easy to breed immunocompetent mammal. Furthermore, HCV infection needs to develop into chronicity, recapitulating liver disease progression towards cirrhosis and HCC. Lastly, a wide panel of reagents allowing high-throughput biomedical research should be available for the species in question. Unfortunately, this ideal HCV animal model does not exist. What follows is an overview of the main different animal models developed so far, with their specific advantages and drawbacks.

1.6.1 CHIMPANZEE

Natural infections occur only in humans, while the chimpanzee (*Pan troglodytes*) is the only primate susceptible to experimental HCV infection (Abe et al., 1993). Historically speaking, the chimpanzee has been the most relevant animal model, as it was crucial for the identification of HCV as the etiological agent behind NANBH (Houghton, 2009). Unlike humans however, chimpanzees only develop a persistent infection in around 40 % to 60 % of cases (Bassett et al., 1998; Major et al., 2004) and do not develop progressive hepatic fibrosis (Walker, 1997), which is testimony of a much milder hepatitis than in their human counterparts. Moreover, research on chimpanzees is only available in the US but its use is becoming more and more restricted because of ethical concerns and the cost of life-long housing ranging from 40 to 60 years in captivity (Altevogt et al., 2011). Recently the US National Institutes of Health (NIH) even announced to completely phase out chimpanzee research (Kaiser, 2015). On the other hand, the big advantage of chimpanzees is that they mount a fully competent immune response to HCV infection (Cooper et al., 1999). This makes them the only well-studied animal model suitable to study protective immunity and vaccine development.

1.6.2 TREE SHREW

Except for primates, only one species is permissive for chronic HCV: the tree shrew (*Tupaia belangeri*) (Xie et al., 1998; Xu et al., 2007). Tree shrews are small non-rodent mammals with squirrel-like appearance native to South-East Asia. They are relatively easy to house and reproduce, but their most attractive feature as an animal model is that they recapitulate HCV-associated liver disease progression when chronically infected (Amako et al., 2010). Unfortunately, infection rates and HCV titers display a lot of variation, which hampers reproducible results. Another big disadvantage is that *Tupaia*-specific reagents for biomedical research are scarce.

1.6.3 RODENT MODELS

1.6.3.1 CHIMERIC MOUSE MODELS

In the chimeric mouse models, mouse livers are xenografted with human hepatocytes which can subsequently be infected by HCV or other hepatotropic pathogens. For mouse livers to be efficiently repopulated by human hepatocytes, specific genetic defects are required. These defects have to result in (i) an accelerated hepatocyte death rate of the original mouse hepatocytes and (ii) severe deficiencies of the mouse immune system. An increased hepatocyte turnover can be induced by using mice that have been genetically modified with multiple urokinase-type plasminogen activator (uPA) genes under the control of albumin promotors. This model was established in the 1990s to study bleeding disorders since overexpression of uPA in the liver causes hypofibrinogenemia and hence blood clotting impairment (Sandgren et al., 1991). As a side effect, an increase in hepatocyte cell death is observed. When these mice are intrasplenically injected with normal mouse hepatocytes the donor hepatocytes colonize the liver and rescue the recipient mouse from liver failure (Rhim et al., 1994). Next, uPA mice are back-crossed with mice with severe combined immunodeficiency syndrome (SCID) to enable xenogeneic engraftment, resulting in uPA-SCID mice. When these mice are injected with human hepatocytes, they display significantly enhanced hepatocyte engraftment, and they can then be infected with HCV leading to chronic infection (Mercer et al., 2001). This achievement enabled important preclinical efficacy and safety trials of new antiviral compounds *in vivo* (Mailly et al., 2015; Vanwolleghem et al., 2007). Today, the uPA-SCID mice are still the state-of-the-art infection model in the preclinical setting, but they have their limitations. First of all, uPA-SCID mice are generally in feeble health given their multiple genetic defects. Moreover, they need to be

engrafted within the first weeks after birth to repopulate their livers with human xenograft hepatocytes. What is more, the uPA transgene is sometimes deleted leading to the regeneration of the original murine liver mass and concomitant rejection of the human graft. The most important drawback however is that these mice are not immunocompetent, and cannot be used for studying the adaptive immune response.

The $Fah^{-/-}$ $Rag2^{-/-}$ γ-$c^{-/-}$ (FRG) mouse is another human-liver xenograft mouse model that was established to counter the limitations of the uPA-SCID mice. These mice lack the Rag2 gene -involved in VDJ recombination, a pivotal process in generating the virtually endless repertoire of B cell receptor (antibodies) and T cell receptors- and the gene for the common γ-chain of the interleukin receptors. These genetic defects lead to severe immune system malfunctions. In addition, they lack fumarylacetoacetate hydrolase (Fah) which causes liver degeneration. This degeneration can be prevented by administration of the drug 2-(2-nitro-4-trifluoromethylbenzoyl)1,3-cyclohexanedione (NTBC) and therefore, timing of human hepatocyte injection can be controlled by simply taking away the drug. Another advantage is that the hepatocyte lethal phenotype always persists as the Fah gene is irreversibly deleted. This model is easier to handle, but because of their immune defects, the mice are no better than uPA-SCID mice to study the immune response on HCV infection.

1.6.3.2 GENETICALLY HUMANIZED MOUSE MODEL

Another approach to enable HCV infection in mice was to complement the mouse genome with all human factors essential for the HCV lifecycle. Transgenic mice expressing four human entry factors under an albumin promoter, namely SR-BI, OCLN, CLDN1 and CD81, and having knockouts of four genes involved in innate immunity (STAT1, IRF1, IRF7 and IFNAR1) allowed chronic low-level HCVcc replication yielding infectious virus (Dorner et al., 2013). In a similar approach, transgenic mice with an intact immune system expressing only the two human entry factors (CD81 and OCLN) responsible for the HCV species-specificity were found to replicate HCV derived from patient serum for over 12 months, albeit at very low levels. Interestingly these mice did show mild fibrosis levels (Chen et al., 2014). In theory, these models are suitable to study adaptive immune responses, immunoevasion mechanisms and vaccination efficacy, but further study still has to demonstrate to what extent it can be put into practice for clinical trials. It is also remains to be seen if these models can lead to progressive liver disease culminating in HCC.

1.6.4 HCV HOMOLOGS

Other potential fully immunocompetent model systems for studying HCV biology include HCV-related viruses which infect mammals other than humans such as GBV-B or NPHV. Unfortunately, GBV-B inoculation seldom progresses to persistent infection, rendering GBV-B useless as a surrogate for chronic HCV (Takikawa et al., 2010). NPHV on the other hand does cause persistent infection, with weakly elevated liver enzymes testimony to mild hepatic inflammation, and a robust cellular and humoral immune response (Pfaender et al., 2015a; Scheel et al., 2015). Practical reasons including the animal size and housing costs preclude an easy use of NPHV as an animal model. Rodent hepaciviruses would be a welcome alternative, and their evaluation as an animal model system is currently ongoing.

1.7 LIVER ANATOMY AND PHYSIOLOGY

HCV primarily replicates in hepatocytes, and therefore the principal HCV-associated diseases manifest themselves in the liver. A brief overview of normal liver physiology will provide better insight into HCV-induced pathology.

1.7.1 LIVER FUNCTION

The liver is the largest organ in the body, weighing around 2 % to 3 % of body weight. It fulfills an impressive range of functions, which can be broadly categorized as metabolic, endocrine/anabolic, exocrine, catabolic, storage capacity and hematopoiesis (Rubin and Reisner, 2009).

- Metabolic function: The hepatic parenchyma is a central organ in energy maintenance. It takes part in glucose homeostasis by performing glycogenolysis and gluconeogenesis. It is also the production site of ketone bodies.

- Endocrine/anabolic function: The liver secretes four (pro)hormones in the bloodstream including angiotensinogen, thrombopoietin, hepcidin and insulin-like growth factor. In addition, hepatocytes synthesize almost all serum proteins, including apolipoproteins, albumin, coagulation factors, complement proteins and acute phase proteins. Moreover, they manufacture carrier proteins that transport ions, lipids, hormones and vitamins to

significantly increase their half-lives. Note that the liver indirectly maintains oncotic pressure through all these intravascular proteins. The liver is also a main distribution hub for lipids. Postprandially, hepatocytes remove chylomicrons from the bloodstream and repackage them together with freshly synthesized cholesterol and fatty acids into lipoproteins.

- Exocrine function: The liver secretes bile in the bile ducts which drain in the gall bladder. Bile is a liquid solution of conjugated bilirubin, bile salts, electrolytes, cholesterol and other lipids such as lecithin. Bile salts are indispensable for emulsifying dietary lipids in the gut.

- Catabolic function: Endogenous substances such as hormones and serum proteins can be broken down by the liver. When proteins are degraded, the resulting amino acids are deaminated to form ammonia, which the liver converts to urea. Better known is the liver's capacity to detoxify drugs and other foreign compounds.

- Storage function: The liver plays a major role as a warehouse for glycogen, triglycerides, iron, copper and lipid-soluble vitamins.

- Hematopoiesis: In mammals the liver is the principal hematopoietic organ until birth and even a short period thereafter (Tavassoli, 1991).

Given the extensive role of the liver, its importance cannot be underestimated. Experimental complete hepatectomy in dogs resulted in onset of coma and secondary bleeding within 24-36 hours, followed by death a few hours later. Average survival time of this procedure was a mere 30 hours (Starzl et al., 1959).

The liver does however display a unique capacity to regenerate after injury. This is most evident after partial hepatectomy when 2/3 of liver volume is resected. The remaining parenchyma undergoes compensatory hyperplasia until liver mass is functionally restored. This process, meticulously regulated by cell signaling circuitry (see 1.10.2: cell signaling in liver regeneration), is completed in mice and rats within 5 to 7 days of surgical procedure (Michalopoulos, 2007).

1.7.2 LIVER ARCHITECTURE

Hepatocytes are the principal cell type found in the liver. They form the functional parenchyma of the organ, and together they account for nearly 80 % of liver volume (Blouin et al., 1977). The other cell types of the liver include (i) cholangiocytes that form the epithelial cells of the bile ducts, (ii) endothelial cells of the hepatic vasculature, (iii) Kupffer cells, which are the resident macrophages in the liver, (iv) hepatic stellate cells (HSCs), which store vitamin A and can be activated with fibroblastic properties in response to liver injury, (v) fibroblasts in the portal areas providing connective tissue surrounding the ducts of the portal triad and (vi) pit cells, which are a liver-specific form of natural killer cells with cytotoxic activity (Nakatani et al., 2004; Ramadori and Saile, 2004; Si-Tayeb et al., 2010).

Unlike most other organs, histological coupes of the liver display a rather homogenous scenery of hepatocytes interspersed with vascular channels and bile ducts. This uniform appearance masks an intricate histological organization. The basic architectural entity is the hexagonally shaped liver lobule, or hepatic acinus (Fig. 9A). It consists of cuboid hepatocytes arranged in cords, radiated towards the center of the lobule where the central vein is located. Each of the six corners of the lobule harbors a small patch of connective tissue containing the portal triad: a branch of the portal vein, a branch of the hepatic artery and a bile duct. The blood enters the lobule in the portal area and travels through a network of sinusoidal capillaries before leaving the lobule in the central vein (Fig. 9B). The liver sinusoids are fenestrated with small openings which serve as portals to the perisinusoidal space, or space of Disse. This small space between the hepatocytes and the lining of the sinusoidal endothelial cells allow an intense exchange of components between the blood plasma and hepatocytes (Fig. 9C). In other words, hepatocytes display a double-sided interface which enables it to selectively secrete components into either the bloodstream or the bile canaliculus (Fig. 10).

For this selective vectorial transportation and secretion capacity a complex polarization is required. Regular polarized epithelial cells have one apical pole directed towards the lumen of the organ, and one basolateral pole oriented towards the basement membrane. A hepatocyte is peculiar because of its multipolarity. The basolateral surfaces interact directly with blood plasma, while the apical membrane lines up a narrow lumen between two adjacent hepatocytes where bile is continually secreted into. A chain of adjacent lumina forms the bile canaliculus, or canal of Hering, and every

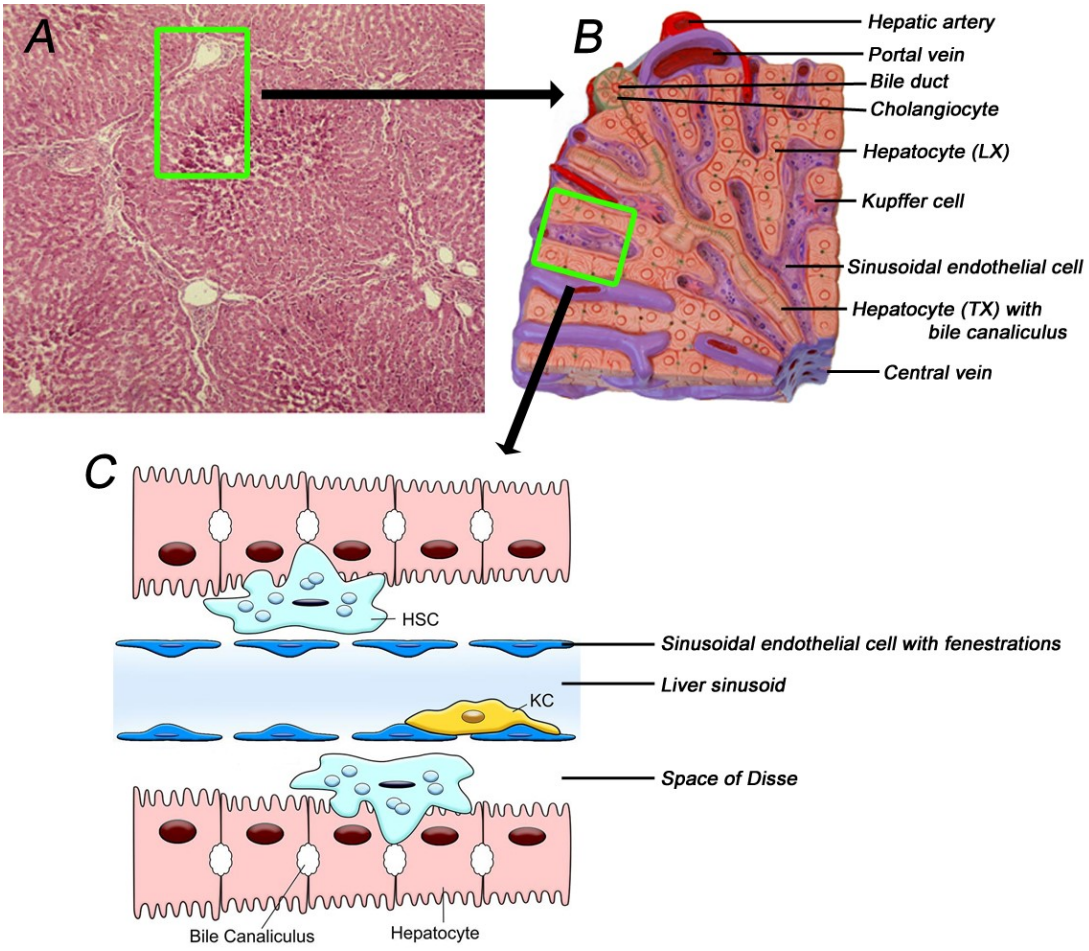

Fig. 9: (A) Close-up of hepatic tissue clearly shows the hexagonal organization of liver lobules. **(B)** Cartoon showing the structure of a part of the lobule at single-cell resolution. The bile canaliculi, formed by the apical membranes of hepatocytes are pictured in green and can be seen as long channels in transverse sections (TX) of hepatocytes, while in longitudinal sections (LX) they can be spotted as small pores. Adapted from the San Diego Mesa College Human Anatomy Website, 2015. **(C)** Detail of the microarchitecture of the liver. (KC) Kupffer cell; (HSC) Hepatic stellate cell. Blood cells inside the liver sinusoid are omitted for clarity reasons. Adapted from Xu et al, 2014.

canaliculus drains into the bile duct situated in the portal triad. Basolateral and apical membranes are efficiently segregated by a closed meshwork of tight junctions which tie the hepatocytes together (Treyer and Musch, 2013).

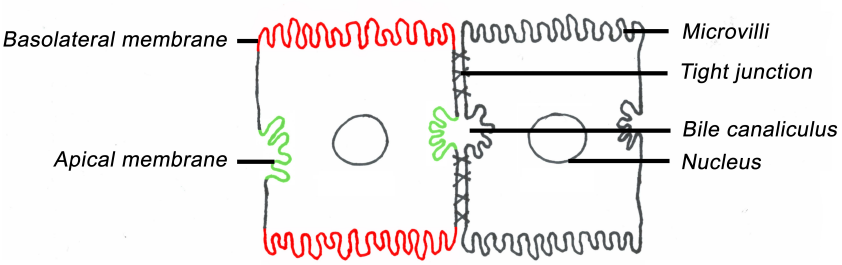

Fig. 10: Outline of the multipolar phenotype of hepatocytes, with basolateral membranes in red and apical membranes in green.

Even though the embryologic organogenesis of the liver is beyond the scope of this introduction, it is important to highlight that the liver is generated as a proliferation of endodermal cells in the embryonic foregut (Severn, 1971). These cells, known as hepatoblasts, are bipotent as they give rise to both hepatocytes and cholangiocytes. The blood vessels, Kupffer cells, fibroblasts and hepatic stellate cells are of mesodermal origin (Asahina et al., 2009; Si-Tayeb et al., 2010). The liver is thus a complex organ consisting of different cell types originating from different germinal layers.

1.7.3 HEPATIC MICROCIRCULATION

The liver receives about 25 % of the cardiac output, and it is unique among organs in that the blood is supplied by two different sources: first the systemic circulation and second, the hepatic portal system which drains the gastrointestinal capillary beds. Only 25 % of blood comes from the well-oxygenated *arteria hepatica*, while the remaining 75 % is venous blood received through the *vena portae*. Hepatic arterial pressure amounts to 90 mm Hg, while portal venous pressure approximates 10 mm Hg. There is therefore a dramatic drop in hepatic arteriole blood pressure before it is mixed with venous blood in the portal zones. When interprandial blood flow in the portal vein is reduced, the arterioles dilate, thereby maintaining blood flow (Despopoulos and Silbernagl, 2003; Levy et al., 2006).

After leaving the portal area, mixed arterial and venous blood enters the liver sinusoids, which are specialized fenestrated blood vessels. In the liver sinusoids, the blood flow velocity drops dramatically because of the increase in total cross-sectional area. This allows a wide range of molecules to sieve efficiently through the fenestrated endothelium lining the sinusoids. These

openings in the endothelium measure around 150-175 nm in size (Wisse et al., 1985) and give access to the space of Disse. In this micro-environment, plasma components come into contact with hepatocytes that are studded with microvili for surface enlargement, thus maximizing their interface with the blood plasma. Blood exits the sinusoids through the centrolobular vein of each lobule, which at the very end collects in the hepatic veins, where blood pressure has now dropped to only 5 mm Hg (Despopoulos and Silbernagl, 2003; Levy et al., 2006).

The hepatic vascular microarchitecture creates a physiological oxygen gradient, with periportal oxygen tension around 60-65 mm Hg, dropping to about 30-35 mm Hg when reaching the centrolubular vein. By comparison, PaO_2 in normal physiological circumstances is about 74-104 mm Hg in other organs, and only drops to 34-46 mm Hg (Jungermann and Kietzmann, 2000). The hepatic circulation therefore constitutes a slightly hypoxemic microenvironment which is considered normophysiological.

1.7.4 THE LIVER AS A HUB FOR LIPID DISTRIBUTION

A major function of the liver is fat redistribution throughout the body. Postprandially, enterocytes absorb non-esterified fatty acids (NEFA) and monoacylglycerol and recombine them as triglycerides. These triacylglycerols are then packaged with cholesterol-esters, phospholipids and ApoB isoform 48 into chylomicrons. Chylomicrons are one of the five types of lipoproteins, each having a different size and density. The higher the relative fat content, the lower the density. Chylomicrons are the largest in size, have the highest triglyceride content, and thus have the lowest density. They are secreted after synthesis into the mesenterial lymph, and subsequently enter the blood circulation. After fatty

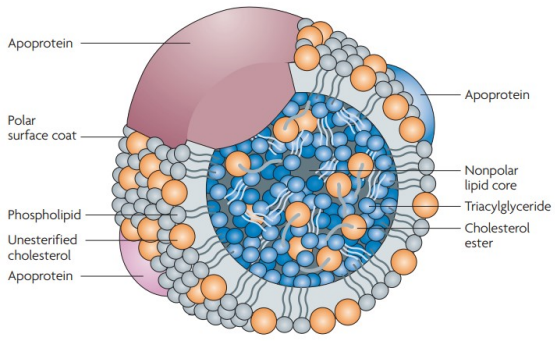

Fig. 11: General structure of a lipoprotein. Neutral lipids such as triglycerides and cholesterol esters are emulsified inside a phospholipid monolayer decorated with apoproteins. This structure enables the transport of hydrophic lipid content in the hydrophilic environment of the blood. Adapted from Wasan et al 2008.

acids are hydrolyzed by lipoprotein lipases (LPL) on the endothelial wall, released NEFAs can enter peripheral cells. The remaining chylomicron remnants travel to the liver where they are taken up by hepatocytes (Despopoulos and Silbernagl, 2003).

Inside hepatocytes, triglycerides are repackaged with cholesterol esters into very low density lipoproteins (VLDLs) together with ApoB and ApoE, and secreted in the bloodstream. Similar to chylomicrons, VLDLs lose triglyceride content by LPL-mediated hydrolysis. As a consequence, the VLDL particle progressively increases in density and becomes an intermediate density lipoprotein (IDL). Fifty percent of IDL lipoproteins are taken up again by the liver, while the remaining half keep on distributing triglycerides in the capillaries and evolve to low density lipoproteins (LDL), which are low in triglycerides but rich in cholesterol esters. LDLs are absorbed by peripheral cells as a major source of cholesterol for the body, but they can also be taken up by the hepatocytes. The last class of lipoproteins, high density lipoproteins (HDLs), transports excess cholesterol away from peripheral tissue towards the liver, where it is absorbed after interaction with SR-BI.

Table 2: Overview of the different types of lipoproteins. Adapted from Wasan et al., 2008.

Characteristics	Chylomicrons	Very low-density lipoproteins	Low density lipoproteins	High density lipoproteins
Density (g per mL)	< 0.95	0.95–1.006	1.019–1.063	1.063–1.210
Particle diameter (nm)	> 75	30–80	18–25	5–12
Protein composition (% dry weight)	1–2	8–10	20–25	52–60
Triacylglycerol composition (% dry weight)	80–88	45–53	5–9	2–3
Cholesterol composition (% dry weight)	2–4	17–27	43–50	12–25
Phospholipid composition (% dry weight)	7–9	17 – 19	19–21	17–24
Function	Transport of exogenous triacylglycerol and cholesterol	Transport of endogenous triacylglycerol	Cholesterol transport to all tissues	Reverse cholesterol transport

1.8 PATHOPHYSIOLOGY OF HCV-INDUCED LIVER DISEASE

Chronic HCV is a leading cause of liver disease, including progressive fibrosis, cirrhosis and eventually HCC. Disease originates both directly from HCV, through changes in cellular metabolism and signaling circuitry by viral proteins, and indirectly as a result of chronic inflammation. Here we outline the current knowledge relevant to HCV-associated pathogenesis in patients.

1.8.1 ACUTE VIRAL HEPATITIS

Initially, exposure to HCV causes an acute viral hepatitis: infected hepatocytes undergo necrosis and produce an inflammation of the liver. On a microscopic level, necrosis of single cells and small patches of hepatocytes can be seen dispersed in the liver lobules. They appear as small acidophilic bodies. Mononuclear inflammatory cells infiltrate the lobules and surround necrotic hepatocytes, and can accumulate around larger patches of necrotic hepatocytes. Also polymorphonuclear cells are not uncommon. As a result of hepatocyte cell death, liver enzymes are released in the bloodstream, and upon acute infection they can be detected within 1 to 3 months after infection. HCV RNA can be detected within two weeks of infection, but antibodies only appear 7 to 8 weeks after infection. The clinical signs of acute HCV are exceptionally mild, and in most cases infection is completely asymptomatic (Rubin and Reisner, 2009). Only 10 % of patients become jaundiced in the acute stage. Other symptoms of acute viral hepatitis include nausea and malaise (NIH Consensus panel, 1997). In extremely rare cases, entire lobules or groups of lobules may succumb to cell death. When this happens, the patient suffers from fulminant hepatitis which can lead to the life-threatening condition of liver failure (Farci et al., 1996). If the infection resolves spontaneously, which occurs in around 20 % of transmission events, the acute stage is followed by a complete remission without clinical backlash. All other 80 % of infections become persistent and cause chronic viral hepatitis (Lauer and Walker, 2001).

1.8.2 CHRONIC HCV: DYNAMICS OF HCV PERSISTENCE

In most patients the immune system is unable to clear the infection and HCV becomes chronic. This persistence is more on the hepatocyte population level than in individual hepatocytes. Healthy hepatocytes are normally long-lived, with a half-life estimated at 6 to 12 months (Seeger and Mason, 2000). But when infected with HCV, hepatocyte half-life drops to just a few days (Graw et al., 2014; Neumann et al., 1998). In other words, when HCV-infected hepatocytes do not manage to halt HCV replication, they are marked for death. HCV has thus developed a peculiar way of persistence. The virus only infects around 20 % (range 1 % - 50 %) (Wieland et al., 2014) of all hepatocytes, and has a timespan of a few days to produce progeny virus, spread through cell-cell transmission and secrete virions in the bloodstream. A few days later the infected hepatocytes die, but by then the secreted virions have initiated new cycles in naive hepatocytes, maintaining the number of colonies of

infected cells. Intriguingly, HCV mainly spreads through cell-cell transmission, while cell-free transmission mediated by secreted virions is rather inefficient (Xiao et al., 2014). As a consequence HCV infects only a limited percentage of all hepatocytes distributed in multiple clusters throughout the liver (Graw et al., 2014; Wieland et al., 2014), jumping from location to location over time. Since chronic HCV is thus a succession of *de novo* infections, it is no wonder entry inhibitors can efficiently clear infections and are a promising new avenue for treating patients that failed conventional antiviral treatment (Mailly et al., 2015; Xiao et al., 2015). Clinically speaking, chronic infection is typically characterized by a lack of symptoms over a long period.

1.8.3 HCV-INDUCED FIBROSIS AND CIRRHOSIS

In chronic HCV, viremia, hepatocyte necrosis and liver enzyme elevations endure. Over the course of years, liver regeneration is insufficient and a classical wound-healing mechanism occurs: the replacement of functional parenchyma by fibrillar matrix to ensure tissue continuity. This is called liver fibrosis. Clinical morbidity remains mostly absent in the first decade of infection, and many patients do not show liver disease for 20 years or more. In the long run however, fibrogenesis becomes a pathogenic process that distorts the liver architecture and eventually results in organ dysfunction.

1.8.3.1 HISTOLOGICAL CHANGES ASSOCIATED WITH LIVER FIBROSIS

Chronic hepatitis is characterized by a variable infiltration by lymphocytes, plasma cells and macrophages, extending variably from the portal areas into the lobules. Inside the lobules, focal necrosis with scattered acidophilic bodies –necrotic hepatocytes- and enlarged Kupffer cells can be seen (Fig. 12A). The process of infected hepatocytes undergoing necrosis never stops, and the turnover of healthy hepatocytes drastically increases as a compensatory measure to restore liver parenchyma. In the course of years, the progressive erosion of periportal hepatocytes leads to the deposition of collagen which gives the portal tract an oversized appearance (Fig. 12B). This pathological feature is called early fibrosis.

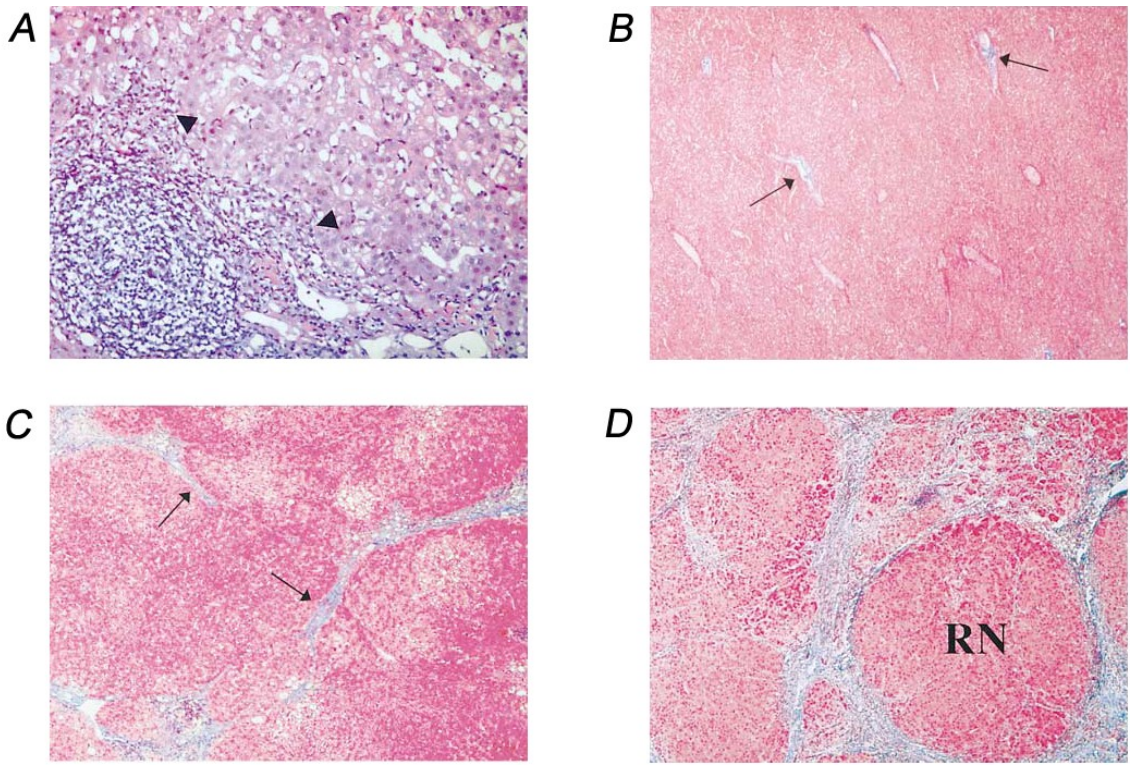

Fig. 12: Histological presentations of chronic HCV infection. (A) Dense infiltration of lymphocytes (arrowheads) extending from the portal areas into the lobules. Hematoxylin and eosin staining. (B) Masson's trichrome staining of early fibrosis. Discrete collagen deposits in the portal areas are stained in blue (arrows). (C) When fibrotic areas expand to adjacent portal zones, bridging fibrosis occurs. (D) Cirrhosis is the precarcinogenic end-stage of liver fibrosis. Liver architecture is lost and parenchyma is pushed aside into regenerative nodules surrounded by fibrous bands. RN: regenerative nodule. Taken from Lauer and Walker, 2001.

Over time the deposition of connective tissue may continue and extend from portal to portal zone: bridging fibrosis (Fig. 12C). Fibrotic septa can also extend towards the centrolobular veins. As a consequence, normal liver architecture is lost and parenchyma is pushed aside into regenerative nodules of hepatocytes. This stage is known as cirrhosis (Fig. 12D). Fibrosis progression is a slow process, and progression rates are extremely variable in HCV patients and accelerate with age and duration of infection. Only 16 % of patients reach the cirrhosis stage after 20 years of infection, a number that rises to 40 % after 30 years (Thein et al., 2008).

1.8.3.3 CLINICAL SEQUELAE OF CIRRHOSIS

Concomitant with the progressive loss of liver structure in cirrhotic livers, the hepatic microcirculation is increasingly compromised. Hepatic blood flow becomes obstructed causing blood pressure in the *vena portae* to rise significantly. This clinical condition is referred to as portal hypertension and it causes serious systemic complications. Portosystemic anastomoses arise as an adaptation to lower the pressure in the portal venous system. The increased hydrostatic pressure opens collateral circulations eventually draining in the *vena cava superior* or *inferior*. The collateral veins implicated in this process become varices: distorted and distended veins prone to bleeding. They include esophageal, anorectal and gastric varices. The increase in hydrostatic pressure is further exacerbated by the decrease in oncotic pressure caused by the drop in serum protein production in a cirrhotic liver. The result is an effusion of transudate in the peritoneal cavity: ascites. When over 80 % of liver parenchyma is functionally lost, the liver can no longer fulfill its functions ('liver decompensation') and a life-threatening complication arises: liver failure (Rubin and Reisner, 2009).

1.8.3.4 MECHANISMS UNDERLYING HEPATIC FIBROGENESIS

Apart from HCV, hepatitis B virus (HBV), sustained alcohol abuse and non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH) are the main causes of cirrhosis and these liver diseases share a comparable clinical and histopathological presentation (Schuppan and Afdhal, 2008). It is therefore believed that –at least in part– similar pathogenic processes are implicated in hepatic fibrosis and cirrhosis irrespective of the underlying etiology.

The primary cells responsible for extracellular matrix (ECM) production are HSCs and perivascular and portal fibroblasts that become activated into myofibroblasts (Friedman, 2000; Knittel et al., 1999). This transdifferentiation is driven by fibrogenic cytokines released by injured hepatocytes, Kupffer cells and infiltrating lymphocytes. The most potent of these pro-fibrinogenic factors activating HSCs is transforming growth factor beta (TGF-β) (Bissell et al., 2001; Friedman, 2000).

As a result of continual replacement of hepatocytes by excessive ECM, the vascular microarchitecture is lost and tissue oxygenation, which is already lower than in other capillary beds, becomes increasingly compromised (Moreau et al., 1988). Hypoxia is a primary activator of fibroblasts, and in the liver HSCs react to hypoxia by increasing collagen deposits (Copple et al., 2011) which deteriorates oxygenation even further. This creates a vicious cycle which results in liver parenchyma

ultimately being pushed aside into regenerative nodules of hepatocytes. This degree of fibrosis (namely, cirrhosis) is regarded as the end-stage of liver disease, and constitutes the procarcinogenous field on which most HCCs grow.

1.8.4 HEPATOCELLULAR CARCINOMA (HCC)

HCC is the unrestrained proliferation of hepatocytes into neoplastic masses in the liver. It is the most common form of liver cancer, and a major cause of cancer-related death (El-Serag, 2011). First this chapter provides general insight into the principles underlying cancerogenesis before applying these concepts to HCV-induced HCC.

1.8.4.1 FUNDAMENTALS OF CANCER BIOLOGY

In metazoan life, single cells evolved to differentiate into specialized cell types allowing a higher order of organization into diverse tissues and organs. Most individual cells preserve their skill to undergo mitosis long after the organism reached developmental maturity. This ability allows them to replenish cells that have succumbed to injury or attrition. Individual cells thus display a remarkable plasticity with a limited degree of autonomy. This format can pose grave danger if genetic information is corrupted and brings forth novel phenotypic traits. Alternatively, if a cell is allowed access to genetic information that it is normally denied, it may execute biological programs outside its physiological scope. Among these programs and newly acquired functions is cellular proliferation, and when performed inappropriately, large clusters of cells that no longer fall under the rules of normal tissue organization can appear. This is the conceptual basis of cancer (Weinberg, 2013).

As a consequence, all neoplasms arise from normal tissue and are monoclonal in origin, i.e. derived from one cell. They are broadly categorized into two classes based on their aggressiveness of growth. Locally growing non-invasive neoplasms are termed benign, while those that invade adjacent tissue and give rise to metastases are known as malignant. Benign tumors hardly ever cause life-threatening problems. Malignant transformations on the other hand causes the overwhelming majority of cancer-related deaths (Weinberg, 2013).

Tumorigenesis is now viewed as a multi-step accumulation of randomly occurring genomic and epigenomic changes, leading to alterations in cellular gene expression and proteomic architecture. Every single one of those changes can confer a disadvantageous or advantageous proliferation

phenotype to all subclonal cells derived from it. As tumoral cells with beneficial growth characteristics will outgrow all others, cancer development resembles an intra-organismic Darwinian process, eventually culminating in cells with an aggressive neoplastic phenotype that threaten the integrity and survival of the organism itself (Chaffer and Weinberg, 2015).

In the classical view, genetic changes involved in tumor formation affect either oncogenes, which stimulate growth, and tumor suppressors, which keep growth in check (Marx, 1994). As genome-wide approaches of cancer research reached maturity, it became evident that a seemingly endless combination of (epi)genetic alterations can lead to malignant transformation (Wood et al., 2007). In many cases, these changes take place in other genes than the traditional oncogenes or tumor suppressors. So even if the roles of oncogenes and tumor suppressors are still relevant, cancer development is now seen more as a complex phenomenon which can be summarized in a handful of principles. In general, tumor cells have acquired a certain set of distinctive and complementary traits ('hallmarks of cancer') that allow cell proliferation and metastasis. They encompass (i) perturbations in signaling circuitry promoting cell growth and proliferation, (ii) evasion of growth suppressors, (iii) defying cell death, (iv) acquiring replicative immortality, (v) induction of angiogenesis, (vi) activation of invasion and metastasis, (vii) recalibration of energy metabolism to favor glycolysis ('Warburg effect') and finally (viii) evasion of destruction by the immune system (Hanahan and Weinberg, 2011).

For such aggressive cancer cells to come into existence, two specific driver mechanisms are identified. First, mutability and genomic instability that allow selective advantage of subclones. Secondly, inflammation. It may sound counterintuitive, but inflammation does enhance malignant progression in a remarkable *pas de deux* between infiltrating immune cells and tumor cells. Every neoplastic cell mass attracts a variable number of inflammation cells that actively secrete bioactive molecules in the tumor microenvironment. Cancer cells will undergo Darwinian selection and only those that can revert these signals to their own proliferative advantage will thrive. As a consequence, immune cells normally aimed to contain transformed cells actually secrete molecules that foster them, such as growth factors to maintain cell growth signaling, survival factors to evade cell death activation, ECM modifying enzymes or other factors promoting angiogenesis, invasion or metastasis and all other signals that co-operate in giving tumor cells cancer hallmark capabilities. It is not uncommon that transformed cells themselves start secreting molecules that attract inflammation

cells and stimulate them to provide a microenvironment that is even more advantageous to their proliferation (Hanahan and Weinberg, 2011).

1.8.4.2 HEPATOCELLULAR CARCINOMA IN HCV PATIENTS

HCC is the most common type of liver cancer and affects people on all continents of the planet. The primary etiologies associated with HCC are chronic viral infections such as HBV and HCV, alcohol abuse, aflatoxin B1 contaminated food, as well as practically all dispositions leading to cirrhosis (Farazi and DePinho, 2006). Indeed, cirrhosis promotes hepatocarcinogenesis and is now recognized as a procarcinogenic condition, but chemopreventive agents that obstruct progression of cirrhosis to HCC are unavailable. Patients that develop HCV-associated cirrhosis are thus at high risk of developing HCC, with an incidence ranging from 1 % to 4 % per year (NIH Consensus panel, 2002).

In the decade that passed our comprehension of the molecular pathogenesis of HCC has increased dramatically. The emergence of high-throughput analyses of HCC samples has provided a unique insight into the scenery of genetic alterations implicated in HCC (Guichard et al., 2012; Nahon and Zucman-Rossi, 2012; Schulze et al., 2015). It is becoming increasingly evident that telomeres and telomerase enzymes are frequently implicated in this process (Farazi et al., 2003; Satyanarayana et al., 2004). The telomerase complex, which is normally not expressed in mature hepatocytes, was found to be reactivated in over 90 % of HCCs (Totoki et al., 2014). The promotion of telomerase

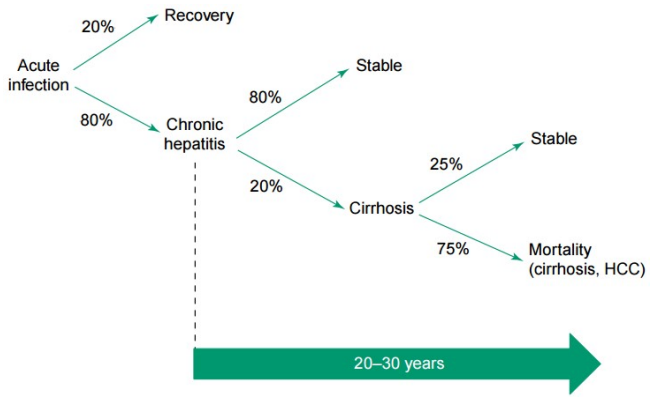

Fig. 12: Liver disease progression in HCV patients. 80 % of cases become chronically viremic upon exposure. Of all patients with persistent HCV, only around 1 in 5 progresses to liver cirrhosis. Adapted from Pawlotsky, 2004.

expression is now seen as a key event that happens early in the multi-step sequence of events leading to HCC. Other (epi)genetic changes then further stimulate the transformation process. Apart from telomere maintenance, the pathways and biological processes recurrently implicated in hepatocarcinogenesis include (i) Wnt/β-catenin pathways, (ii) p53 cell cycle pathways, (iii) epigenetic modification machinery, (iv) oxidative stress pathways and (v) PI3K/Akt/mTOR and mitogen activated protein kinase (MAPK) pathways (Zucman-Rossi et al., 2015).

When looking closer in detail for HCV-associated HCCs, it is believed HCV persistence foments hepatocarcinogenesis directly through viral proteins, but also indirectly. The latter originates in first place from the regeneration of liver parenchyma. The dramatic increase in hepatocyte turnover is a source of mutations which, over the course of twenty or thirty years, can accumulate and permit individual cells to escape growth controls. *In vivo* experiments with genetically modified mice elegantly support this idea, namely that liver regeneration alone is sufficient for HCC to arise (Yamaji et al., 2010). In second place, the interplay between increasingly genetically derailed hepatocytes and infiltrating inflammatory cells bolsters the process of malignant transformation (see above). Together, mutation and inflammation compose the two enabling characteristics of cancerogenesis that are only indirectly caused by HCV.

Over the years it became clear that HCC is also directly caused by HCV. In a transgenic mouse model, hepatic expression of HCV Core was found to elicit a dramatic overproduction of reactive oxygen species that induce genetic damage, and eventually result in HCC (Fujinaga et al., 2011). Interestingly, in this model the transgene is not recognized by the immune system because of immunotolerance and therefore no inflammatory response is developed. HCV Core is thus a HCV factor that can produce HCC independent from inflammation.

Furthermore, Core and NS5A trigger MAPK signaling (see chapter 1.10.3) which hampers apoptosis induction of infected cells. NS5A may further aid anti-apoptosis programs by activating NFκB (Jiang et al., 2011). Other studies show that NS5A hijacks Wnt pathways by stabilizing β-catenin and enhancing its transcriptional activity (Milward et al., 2010; Park et al., 2009).

Various *in vitro* studies also show that Core (Kao et al., 2004), NS3 (Kwun et al., 2001) and NS5 (Majumder et al., 2001) interact with tumor suppressor p53 and may modulate its function. Another tumor suppressor affected by HCV is the retinoblastoma protein (Rb). The NS5B polymerase

associates with Rb through a specific motif and recruits ubiquitin ligases that flags Rb for proteasomal degradation (Munakata et al., 2007; Munakata et al., 2005).

Taken together, it appears to be a recurrent theme that HCV proteins tweak regulatory grids commonly implicated in HCC development and establish pathophysiological conditions identified as universal hallmarks of cancerogenesis. As a conclusion, HCV proteins may actively facilitate cancer development via bypassing some of the crucial steps in the multi-stage process that is hepatocarcinogenesis.

1.8.2.2 OTHER PATHOLOGIES ASSOCIATED WITH HCV

Even if HCV is a leading cause of cirrhosis and HCC worldwide, a significant fraction of HCV-induced disease and mortality is due to HCV-associated comorbidities. There is evidence supporting an intimate relationship between HCV and steatosis, insulin resistance and type 2 diabetes. A clear link is also defined between HCV and atherosclerosis, leading to an increase in cardiovascular disease. Chronic HCV infection is also associated with mixed cryoglobulinemia and neurological disorders such as fatigue and cognitive impairment. In general however, the mechanisms driving these extrahepatic manifestations of HCV disease are incompletely understood (Negro et al., 2015).

Unexpectedly, chronic HCV patients also have a higher incidence for cancer types other than HCC, such as pancreas cancer, rectum cancer, lung cancer and non-Hodgkin lymphoma. In addition, mortality of these cancers is higher in HCV patients when compared to non-HCV patients (Allison et al., 2015).

1.9 HEPATITIS C TREATMENT

The main goal of chronic HCV treatment is simple: to eradicate the virus from the patient. In clinical terms, this is defined as a sustained virological response (SVR) which amounts to having no detectable HCV RNA for 12 or 24 weeks after the end of therapy. In HCV patients with HCC, more drastic interventions are needed.

1.9.1 CHRONIC HCV THERAPY

1.9.1.1 INTERFERON-BASED REGIMENS

Until 1998, there was no other therapy available than subcutaneous injection of IFN-α three times per week. Treatment was protracted with up to 48 weeks and resulted in disappointing cure rates of only 15 % to 20 % of patients (Carithers and Emerson, 1997; Hoofnagle and di Bisceglie, 1997; Poynard et al., 1996; Tine et al., 1991). Combination therapy of IFN-α and orally administrated ribavirin during 24 to 48 weeks increased SVR rates to 30 to 40 % (McHutchison et al., 1998). Pegylating IFN-α (i.e. the addition of polyethylene glycol) improved the biological half-life of IFN-α, and allowed a more convenient once a week dosing. The combination therapy of pegylated IFN-α and ribavirin was found to be more effective (Manns et al., 2001) and constituted the standard of care for over a decade. By using this regimen, SVR rates increased to ~45 % for genotype 1 and up to 80 % for genotypes 2, 3, 5 and 6 (Antaki et al., 2010). Unfortunately IFN-α therapy came with many side-effects including flu-like symptoms, gastritis/gastroenteritis, pruritus, and even severe depression and suicidal ideation. The main adverse effect of ribavirin is hemolytic anemia. Taken together, these side-effects led 10 % to 20 % of all patients to withdraw prematurely from IFN-α and ribavirin combination therapy (Manns et al., 2006).

1.9.1.2 DIRECT-ACTING ANTIVIRALS

2011 marked the beginning of a new era in HCV therapy with the approval of NS3-4A-targeting direct-acting antivirals (DAAs) for genotype 1 patients. The orally administrated NS3-4A protease inhibitors boceprevir or telaprevir were combined with IFN-α/ribavirin into “triple therapy” which increased viral cure for genotype 1 from 45 % to around 70 %. Triple therapy had serious limitations however. It was only available for genotype 1 patients, it was presented with a high pill-burden and complex dosing schedule, and moreover, side-effects were worse than IFN-α/ribavirin alone (Bacon et al., 2011; Jacobson et al., 2011; Poordad et al., 2011; Zeuzem et al., 2011). In 2014-2015 the second generation of oral DAAs completely revolutionized HCV therapy. Sofosbuvir (NS5B nucleotide analogue) and daclatasvir or ledipasvir (NS5A inhibitors) with or without ribavirin for 12 or 24 weeks achieved SVRs of 90 % and beyond. These new interferon-free all-oral regimens are well tolerated and present very few side effects (Afdhal et al., 2014a; Afdhal et al., 2014b; Sulkowski et al., 2014).

On the downside, pricing of these novel antivirals is so exorbitant in affluent industrialized countries (€50,000 – €100,000 for 1 patient) that availability is restricted. (Kamal-Yanni, 2015). Moreover, viral cure reduces but does not eliminate a heightened risk for HCC development (van der Meer et al., 2012). Finally, primo-infection does not prevent reinfection, which highlights the unmet need for an effective vaccine if HCV is ever to be eliminated from the human population.

1.9.2 TREATMENT OF CIRRHOSIS-ASSOCIATED LIVER FAILURE

In case of decompensated cirrhosis, treatment of the underlying cause (e.g. HCV antiviral therapy) can improve the patient's condition. If treatment fails, the only treatment option left is liver transplantation (Lauer and Walker, 2001). Until the arrival of DAAs, reinfection of the liver graft was certain. With DAA treatment before or after transplantation however, reinfection can be halted (Webster et al., 2015).

1.9.3 TREATMENT OF HEPATOCELLULAR CARCINOMA

For HCC, curative approaches are limited: early stage HCC can be treated by surgical resection, but only for patients with a single tumor mass and without portal hypertension. If surgical resection is not possible, liver transplantation is the most appropriate choice for early HCC patients. If transplantation is no longer an option, radiofrequency ablation is the next best strategy. Unfortunately, tumor recurrence is common following such procedures. For intermediate or advanced HCC (multinodular, large masses) chemoembolization or chemotherapy is the primary therapy of choice but in this case patient survival times are abysmal with or without therapy (El-Serag, 2011). Chemotherapeutic strategies targeting signaling pathways involve the multi-kinase inhibitor sorafenib which blocks multiple signaling cascades including the MAPK pathway. It was shown in an extensive study to prolong median survival time of advanced HCC patients to only 11 months, a mere 3 months more than the control group (Llovet et al., 2008). Other strategies or compounds with increased efficacy against advanced HCC are thus urgently needed.

1.10 CELL SIGNALING CIRCUITS IN THE LIVER

1.10.1 CELL SIGNALING: A BRIEF INTRODUCTION

Cell signaling is the mechanism by which the cell communicates, either with other cells or with the molecular machinery inside its own microenvironment. When the cell senses a stimulus, it instigates a tightly regulated biochemical signaling cascade to a well-defined set of effector molecules, allowing the cell to mount an integrated real-time response. These co-operative pathways form a regulatory grid that coordinate all physiological processes including metabolism, cell growth, proliferation, differentiation, immune response and even cell death.

The molecular underpinnings of this network are complex, as signaling molecules belong to a wide range of chemical classes including lipids, amino acids, peptides, proteins, monoamines, carbohydrates, cyclic nucleotides, ions, or even gases. The basic concept however is straightforward: one molecule can transmit, distribute, enhance or dampen a signal which results in the required effect. Signaling thus coordinates the activity of different enzymes, creates action potentials by opening ion channels, modifies protein interfaces and protein-protein interactions, instigates transcriptional programs, triggers intracellular trafficking etc.

Aberrant functioning of this communication system can seriously harm the cell and organism, and ultimately contribute in the development of diseases such as diabetes (Cohen, 2006), cancer (Brognard and Hunter, 2011; Julien et al., 2011), autoimmune diseases (Cho and Feldman, 2015) etc.

One of the major pillars of cell signaling networks in eukaryotic cells is protein phosphorylation. Protein kinases add phosphate residues on amino acids tyrosine, serine and threonine (phosphorylation), while protein phosphatases remove them (dephosphorylation). The human genome boasts 518 protein kinases (Manning et al., 2002) and only 147 protein phosphatases (Moorhead et al., 2007). The phosphorylation status of a protein is sometimes explained as an on- and off-switch, where phosphorylation activates the protein machinery, while a dephosphorylated protein acts as its idle counterpart (Fig. 13). This ‘on and off’ phosphodynamics is tightly regulated by kinases and phosphatases.

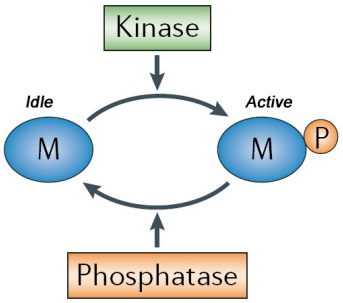

Fig. 13: Cartoon showing 'on and off' dynamics of protein phosphorylation. Adapted from Kholodenko, 2006.

1.10.2 CELL SIGNALING IN LIVER REGENERATION

Given its role in metabolizing toxic substances, the liver is exceptionally prone to cell death. As a countermeasure, evolution has granted liver tissue with an exceptional, yet tightly regulated compensatory growth capacity. However, liver parenchyma is unable to maintain repair in cases of ever-enduring injury such as viral hepatitis or prolonged alcohol abuse. Restoration of liver mass depends on hepatocyte proliferation, but mature hepatocytes are quiescent cells in G0 phase that hardly ever divide in response to growth factors. For efficient proliferation, hepatocytes need to be primed by cytokines tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) (Webber et al., 1998; Zimmers et al., 2003). Upon liver damage, Kupffer cells secrete TNF-α which stimulates their own TNF-α receptors in an autocrine fashion. This leads to NFκB activation and subsequent IL-6 transcription and secretion by Kupffer cells (Libermann and Baltimore, 1990). IL-6 plays a key role and is absolutely crucial in the regenerative process (Cressman et al., 1996). IL-6 docks on IL-6 receptors on the basolateral membranes of hepatocytes. This leads to Janus kinase (JAK) activation, which in turn phosphorylates transcription factor signal transducer and activator of transcription 3 (STAT3). Phospho-STAT3 proteins dimerize and translocate to the nucleus where they start transcription of proliferation genes such as Cyclin D1, thereby allowing transition from G1 to S phase. STAT3 also induces the expression of anti-apoptotic and anti-oxidation proteins, and may therefore also exert hepatoprotective effects (Fujiyoshi and Ozaki, 2011).

Next, hepatocyte growth factor (HGF) is released from HSCs and stimulates hepatocytes to proliferate (Huh et al., 2004). In fact, HGF is secreted as an inactive precursor pro-HGF that is stored

in the ECM. Activation of pro-HGF into HGF requires proteolytic processing by uPA (Kim et al., 1997). Upon release, HGF binds to its hepatocyte receptor c-Met and activates downstream MAPK pathways and Akt signaling (Borowiak et al., 2004), stimulating proliferation. Transforming growth factor alpha (TGF-α) is another important growth factor for hepatocyte proliferation. It is synthesized and released by hepatocytes, binds to EGFR on hepatocyte in an autocrine and paracrine way, and further inceases proliferation (Scheving et al., 2002). TGF-α production by hepatocytes is dependent on prior HGF activity (Tomiya et al., 2000). Apart from TGF-α, other EGFR ligands such as EGF, amphiregulin and heparin-binding EGF-like growth factor (HB-EGF) are also expected to play a role in this process (Fausto et al., 2006).

1.10.3 HCV-INDUCED CELL SIGNALING

HCV ingeniously tweaks the host cell signaling machinery -including kinases and phosphatases- in order to adapt its intracellular habitat to its own needs. But this also has a profound effect on cell physiology including survival, proliferation and transformation capacity (Llovet and Bruix, 2008; McGivern and Lemon, 2011; Tsai and Chung, 2010). Some of these HCV-induced changes in signaling circuitry are well known, such as an activation of EGFR and the MAPK pathways (see below). However, many of these signaling perturbations, especially those pertaining to protein phosphatases, are thus far poorly studied.

1.10.3.1 EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) SIGNALING

EGFR was originally identified by the Baumert lab as an essential host factor for HCV entry through a siRNA screen targeting host cell kinases (Lupberger et al., 2011). EGFR is one of many receptor tyrosine kinases (RTKs), transmembrane proteins that receive extracellular signals from cytokines. They transmit the message to the intracellular milieu by activating their intracellular kinase domains which trigger downstream signaling events. Even if HCV does not bind directly to EGFR, inhibiting its expression or kinase activity impairs HCV co-receptor complex formation. Conversely, treatment with EGF, the main ligand of EGFR, increases HCV entry (Lupberger et al., 2011). EGFR signaling predominantly activates the MAPK pathway in hepatocytes, but only the membrane-associated components and scaffolding proteins are important for entry. The downstream MAPK signaling is unimportant for HCV entry, but crucial for its long-term persistence by hindering IFN-α induced antiviral gene expression (Lupberger et al., 2013; Zona et al., 2013). Interferons, especially type I and

type III interferons are signaling molecules produced as a first line of defense of the immune system. They bind to different receptors but provoke similar downstream signaling events, namely the activation of the JAK-STAT axis. STAT phosphorylation then enables STAT homo- or heterodimerization which triggers their relocalization to the nucleus and leads to the execution of a complete transcriptional program involving scores of genes counteracting viral infection (Makowska and Heim, 2012). The nuance of the IFN-α induced transcriptional effect in hepatocytes depends on the type of STAT proteins involved. STAT1 triggers a predominantly antiviral and pro-apoptotic state, while STAT3 functions as a counterbalance promoting cell proliferation and survival (Regis et al., 2008). EGFR signaling represses suppressor of cytokine signaling 3 (SOCS3) which normally keeps STAT3 at bay. In other words, virus-induced EGFR activation inhibits this negative feedback loop, and tips the STAT1-3 balance in favor of STAT3, thereby severely hampering the antiviral impact of STAT1. This antagonistic effect of STAT3 is so important that it is considered a host-factor for HCV (Fig. 14) (Lupberger et al., 2013).

1.10.3.2 MAPK SIGNALING PATHWAYS

The Ras/Raf/MEK/ERK MAPK signaling pathway (also known as the ERK signaling pathway or MAPK signaling pathway) is an evolutionary conserved pathway involved in many vital physiological processes. It was originally identified as a linear cascade of proteins transducing signals coming from cell surface receptors. Many of these receptors activate Ras, a cell-membrane bound GTPase that upon activation recruits its downstream partner Raf. Raf then phosphorylates MEK, and MEK in turn phosphorylates and activates ERK (also known as MAPK). ERK is the main effector kinase in the pathway, with substrates in the nucleus, cytosol, membranes and cytoskeleton. These substrates consist of multiple kinases and transcription factors that execute biological programs including cell proliferation, cell survival, differentiation, anti-apoptosis, motility and metabolism (Kolch, 2005; Samatar and Poulikakos, 2014).

HCV sparks MAPK signaling through virions binding CD81 (Diao et al., 2012) and LDLR (Zhao et al., 2005). Moreover, HCV Core and NS5A enhance MAPK signaling events even further (Burckstummer et al., 2006; Hayashi et al., 2000) (Fig. 15). Apart from its impact on attenuating the antiviral response, ERK activation boosts HCV replication by regulating phospholipase A2 activity (Menzel et al., 2012). This ERK phosphorylation can be clearly discerned in biopsies of chronic HCV-patients, showing once more their importance for HCV persistence (Mailly et al., 2015).

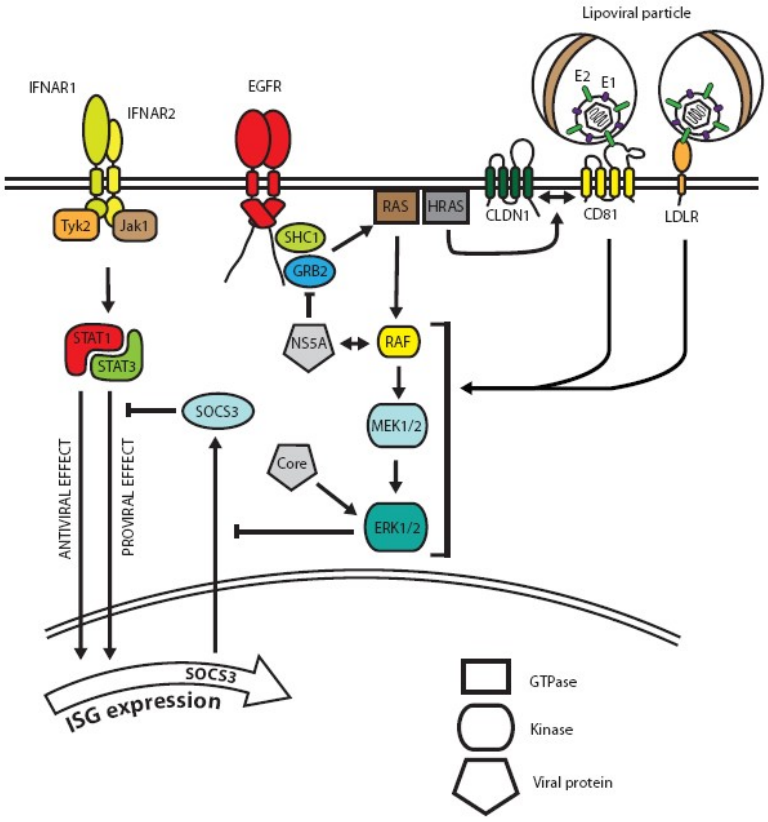

Fig. 14: HCV-induced MAPK signaling is necessary for HCV entry and aids in immune evasion of the interferon-mediated immune response. Arrow: activation; blunted arrow: inhibition; two-sided arrow: interaction (Lupberger and Van Renne, 2015).

1.11 ADVANCED ANALYSIS OF TRANSCRIPTOMIC DATA

In the final chapter of this introduction, we will very briefly touch on two of the novel approaches that have been developed in the field of big data analysis and that were used in this thesis: gene set enrichment analysis (GSEA) and supervised learning.

11.1 GENE SET ENRICHMENT ANALYSIS (GSEA)

In biomedical research in the 1990s, technical limitations made it hard to quantify more than just a handful of mRNA transcripts at a time. At the turn of the century however, the scene changed with

the development of microarrays, mapping thousands of transcripts in a sample in one go (Bumgarner, 2013). Initial attempts to gain biologically meaningful insight from such large data sets had only marginal success. A common approach was to look for those genes that are significantly up- or downregulated between two conditions (e.g. treated vs non-treated). After correcting for multiple hypothesis testing this approach yields a list of genes, yet without a unifying biological theme. The lack of coherence on the genes makes interpretation difficult. Moreover, it generally selects the genes with the highest fold change in expression, which is not necessarily relevant. Biological circuits work in co-opted networks of gene products. When a single gene in such a grid is upregulated 600 % it is not necessarily relevant, but when all the transcripts of a specific biological program (e.g. all genes implicated in glycolysis, cell division, interferon response…) are increased by 20 % it may have a profound effect on the cellular household. Previous conventional analysis techniques would detect the irrelevant 6 fold change, but neglect the relevant small change in an entire circuit.

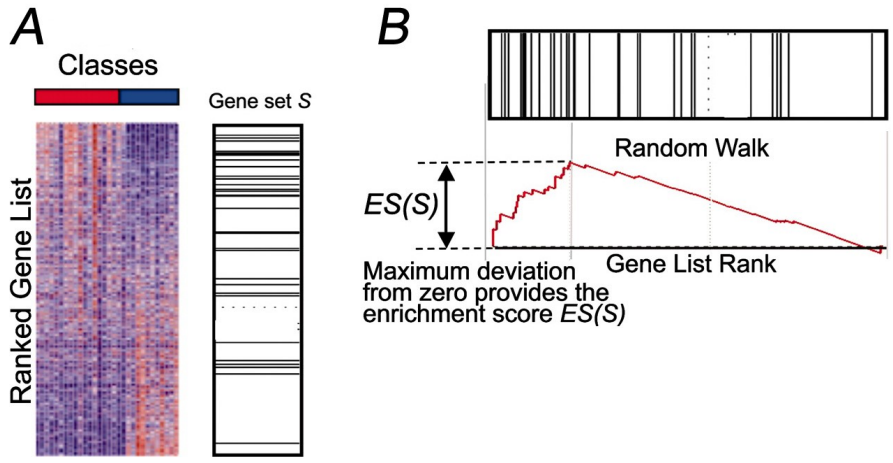

Fig. 15: Outline of the GSEA procedure. (A) A transcriptomic dataset is ranked based on average gene expression differences between two classes, exemplified by a heatmap. The adjacent bar shows where all the genes belonging to a certain gene set 'S' are situated in the sorted list. (B) The plot visualizes the running sum for gene set 'S' in the data set. The maximum deviation is the enrichment score (ES). The more genes of a gene set are clustered at the top of the list, the higher the enrichment score will be. Figure adapted from Subramanian et al., 2005.

A novel procedure to handle transcriptomic data as a collection of gene circuits, rather than single gene products, is gene set enrichment analysis (GSEA). Gene sets are assembled *a priori*, based on data such as biochemical pathways or co-expression in previous experiments. After ranking all genes on expression difference between two classes (e.g. treated vs non-treated), a Kolmogorov-Smirnov running-sum statistic is calculated to determine if a particular gene set is enriched among the genes ranked at the top. It basically runs from the top of the list to the bottom, and if a gene is part of the gene set ('hit'), the enrichment score increases, while if the gene is not part of the list ('miss') the score decreases. The resulting enrichment score represents the maximal deviation from zero encountered in the walk from top to bottom of the gene list. A set that is highly enriched in the top will have a high score (Fig. 15). This outcome is then mathematically adjusted to a normalized enrichment score (NES), and further corrected for multiple hypothesis testing into a false discovery rate (FDR). A FDR smaller than 0.25 is generally considered a good cut-off. Focusing on gene sets with FDRs smaller than 0.25 allows the identification of transcriptional networks that are significantly altered between two conditions (Mootha et al., 2003; Subramanian et al., 2005).

11.2 SUPERVISED LEARNING TO PREDICT PATIENT SURVIVAL

Another approach to make sense of transcriptomic data is linking gene expression data to survival times of patients (Bair and Tibshirani, 2004). The arrival of big data in the biomedical research scene prompted the development of many novel mathematical and statistical techniques such as supervised learning. In this technique, training data sets that are labeled (i.e. have a known outcome) can be used to mathematically infer a function that can call the outcome. This function can then be used on new data to predict an unknown outcome.

In this way, Hoshida et al. gathered transcriptomic data from formalin-fixed paraffin-embedded liver tissue samples of HCC patients that had undergone surgical resection up to 25 years earlier. This allowed them to have gene expression profiles at the moment of surgery (training data), while at the same time the outcome (still alive or already deceased) was known. They obtained Cox scores by quantifying the correlation between gene expression of each gene for every patient and his or her survival time. The higher the Cox score for a gene, the more it is correlated with death. On the other hand, the lower the score, the higher the correlation with long-time survival. By selecting 73 genes with a high score ('poor prognosis genes') and 113 genes with a low score ('good prognosis genes') they could obtain a transcriptomic profile that could efficiently predict survival and tumor recurrence

in HCC patients (Hoshida et al., 2008). Interestingly, these genes can also prophesize whether HCV patients with cirrhosis will progress towards HCC (Hoshida et al., 2013).

Why a precarious RNA profile eventually leads to genomic instability is still not understood, but it is an intriguing observation that one of the most prominent genes (i.e. highest Cox score) in this list of HCC high-risk genes is EGF (Hoshida et al., 2008). EGF signaling networks activating MAPK signaling appear to be a recurring theme in not only HCV infection (Lupberger et al., 2011) but also in liver regeneration (Scheving et al., 2002) and HCC development (Fuchs et al., 2014; Zucman-Rossi et al., 2015).

2. THESIS GOALS

In the last 25 years the study of HCV biology has advanced at an incredible pace, culminating in a well-tolerated antiviral treatment that can cure most of patients. Notwithstanding the breakthroughs, knowledge about the mechanisms underlying HCV-associated disease is still in its infancy. The narrow host range of HCV prohibits the use of a conventional animal model, and therefore *in vitro* models are a welcome alternative. However, traditional hepatic cell lines consist of dedifferentiated cells whose exponential growth is not inhibited by cell-cell contacts. They are therefore limited as a model for long-term infection studies recapitulating viral liver disease in patients. Moreover, regular HCVcc contain cell-derived contaminants and stress factors that may cloud over relevant discoveries. The lack of a suitable model system to study persistent HCV infection is thus a major hurdle for pathogenesis studies.

The first aim of this thesis therefore consisted in setting up a long-term *in vitro* model amenable to the study of HCV-induced liver disease. The immediate goals were to redifferentiate liver cell lines into hepatocyte-like cells, and infect them with affinity purified contaminant-free HCVcc inoculum to obtain persistent infection. In the next stage, we focused on mapping the transcriptomic fabric of this model over time to increase our understanding of the progression from acute to chronic infection.

In a second independent approach we focused on the cell signaling networks involved in HCV-associated disease. It is known that HCV recalibrates the signaling circuitry of their host cells, with disease development as a potential adverse effect. These regulatory grids are meticulously regulated by phosphatases, and disturbances in these proteins are implicated in many syndromes and diseases. Until this day, knowledge on how HCV influences protein phosphatases remains scarce. Exploring its impact thus holds the potential to provide new insight into altered signaling pathways, and as a consequence, also in the molecular underpinnings of HCV-associated morbidities.

Hence the second goal of this thesis: uncovering HCV-specific changes in protein phosphatase expression to gather a more profound understanding on how HCV impacts signaling cascades, and investigate how such alterations may foster disease development.

3. RESULTS

The work that was performed during my fellowship can mainly be summarized in two different parts. First, establishing a long-term *in vitro* model for HCV using affinity-purified virus, and secondly, studying altered phosphatase expression in patients with persistent HCV infection. This work led to two co-authored scientific papers. For clarity reasons, my personal contributions to each project will first be highlighted separately. For detailed materials and methods, I refer to the manuscripts themselves that are added at the end of this chapter.

3.1 ESTABLISHMENT OF A LONG TERM *IN VITRO* HCV INFECTION SYSTEM AMENABLE TO THE STUDY OF HCV PATHOGENESIS

3.1.1 INTRODUCTION

Cell culture-derived HCV for experimental research is classically obtained by harvesting cell-culture medium containing freshly produced viral particles after electroporating HCV RNA into Huh7-derived hepatoma cells (Lohmann and Bartenschlager, 2014). However, this harsh procedure causes the release of stress-induced cellular factors in the culture medium which may, when used as inoculum, prompt virus-unrelated cell signaling. Obtaining contaminant-free virus preparations are required to ensure that all registered biological events, such as HCV-induced signaling, are virus-specific. It was demonstrated that adding a FLAG-tag at the N-terminus of the E2 protein of HCV strain Jc1 (Jc1-$E2^{FLAG}$) allowed the affinity purification of infectious HCVcc with anti-FLAG antibodies (Merz et al., 2011).

The Huh7 cell line was originally isolated from a patient with HCC and together with its subclones such as Huh7.5.1 it proved to be an essential tool in HCV research as it is the only cell line highly permissive to HCV (see 1.5.4). However, a disadvantage of the Huh7-based HCV model is the unrestrained cell proliferation of Huh7 cells. Cell-cell contact does not inhibit cell growth, and as a result, cultured cells will multiply until massive cell death occurs. This makes long-term experiments impossible. Trypsinization and reseeding is a practical solution, but proteolytic enzymes dismantle at least part of the protein machinery on the cell-surface and may reset some of the regulatory cell circuits. The addition of DMSO to cell culture medium was described by Sainz and Chisari (2006) and

Bauhofer et al. (2012) to redifferentiate Huh7.5 cells into growth-arrested hepatocyte-like cells (Huh7.5^{dif}) that are physiologically closer to *in vivo* hepatocytes. This cell culture model provides the possibility of long-term infection and may be used as a model system to study the reprogramming of hepatocytes upon persistent infection, mimicking the viral perturbations that occur in chronic HCV patients.

3.1.2 SPECIFIC AIMS

The specific aims of this project were therefore (i) to establish Jc1-E2^{FLAG} affinity purification in the lab, (ii) to set up a robust infection model system using DMSO-induced differentiation and finally (iii) to combine these two technologies to study HCV-induced signaling events.

3.1.3 RESULTS

3.1.3.1 AFFINTY PURIFICATION YIELDS PURE HIGH-TITER HCV PARTICLES

The plasmid coding for the Jc1-E2^{FLAG} HCV construct was obtained as a gift from Dr. Bartenschlager (University of Heidelberg). The vector was amplified by growing transformed *E. coli*, yielding a high copy number. The bacteria were lysed and plasmids were purified using a commercial extraction kit. Next, the plasmids were electroporated into naive Huh7.5.1 cells yielding high-level replication and secretion of infectious virus in the cell culture medium. Cell culture medium containing viral particles was harvested daily starting at day 2 post electroporation. The obtained supernatant –up to 100 ml- was concentrated to 10 ml by centrifugal ultrafiltration at 3200 x g. 10 ml of concentrated virus was then affinity purified on anti-FLAG raisin, which contains agarose covalently linked to monoclonal anti-FLAG antibodies. After two hours of incubation on a rotating laboratory mixer, the raisin underwent another centrifugation step at 800 x g and was repeatedly washed with phosphate buffered saline (PBS). The virus was then eluted from the raisin with 2 ml of FLAG peptide solution which competes with the binding sites of the antibodies and releases the HCV particles. After another centrifugation step at 2300 x g, the approximate 2 ml of pure Jc1-E2^{FLAG} virus preparation can be transferred to a reaction tube and used for infection.

In a next step I studied the characteristics of pure Jc1-E2^{FLAG}. I observed that HCVcc particles in cell culture medium maintain their infectivity for weeks when kept at 4 °C. Highly purified HCVcc particles on the other hand are highly unstable at 4 °C and lose most of their infective capacity in a matter of

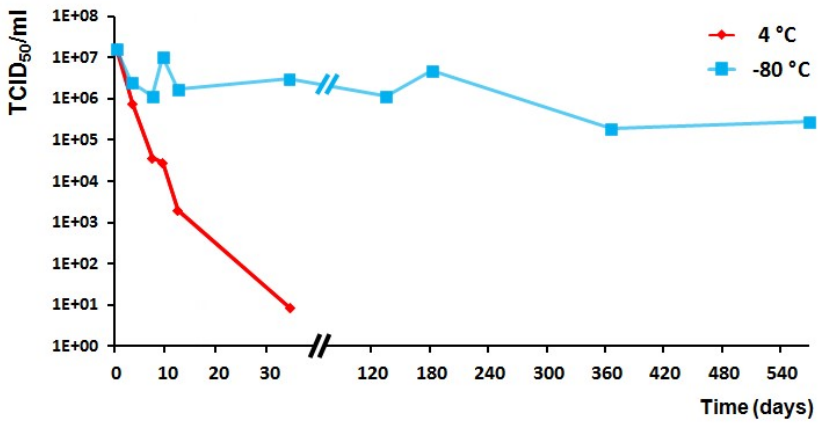

Fig. 16: Long-term stability of purified HCVcc. Affinity-purified HCVcc displays a dramatic drop in infectivity when kept at 4 °C in the first 48 hours. Freezing at -80 °C loses 1 logarithmic stage of infectivity, but then the virus preparations are highly stable for at least 6 months.

days. Freezing at -80 °C causes a loss of 1 log of $TCID_{50}$ upon thawing but maintains stability for at least 6 months. After that time a further drop in infectivity was observed (Fig. 16).

The production process of only a small volume of pure virus is extremely laborious. For 1 preparation of 10 ml it takes approximately 30-35 man-hours. However, only around 1 production out of 2 yields sufficient infectivity (i.e. higher than 10^6 $TCID_{50}$/ml) needed for proper cell-culture infection, which doubles the average work load. An attempt was therefore made to dilute pure high-titer Jc1-$E2^{FLAG}$ and accept a trade-off between volume and infectivity. Much to our regret, diluting pure virus 1:10 with PBS results in a 100 fold drop in infectivity as measured by $TCID_{50}$ (data not shown). In other words, such a procedure *de facto* inactivates HCV and makes it unsuitable for obtaining large quantities of high-titer pure HCV. This emphasizes once more the instability of pure HCVcc. We hypothesized serum components could account for HCV stability of classical HCVcc in cell culture medium. However, diluting pure HCV particles in neither fresh medium nor spent medium (i.e. recycled from Huh7.5.1 cell culture) increased infectivity compared to dilution in PBS. Also exogenous addition of ApoE or albumin to the diluent did not stabilize pure virus (data not shown). Why pure Jc1-$E2^{FLAG}$ is so unstable remains unclear. To avoid overly loss of infectivity and allow versatility in usage, pure virus preparations were generally divided in aliquots and stored at -80 °C.

Despite the limitations set by the poor stability and unpredictable yield of the preparations, I was able to establish affinity-purified HCVcc it in the lab for experimental infection procedures. This achievement provides the lab with a truly unique platform for studying HCV-specific virus-host interactions.

3.1.3.2 DMSO SUPPLEMENTATION DIFFERENTIATES HUH7.5.1 CELLS INTO HCV-PERMISSIVE HEPATOCYTE-LIKE CELLS (HUH7.5.1^{DIF})

Adding 1 % of DMSO to culture medium provokes profound changes in the molecular fabric of Huh7 or Huh7.5 cells, leading to redifferentiation into growth-arrested Huh7 or Huh7.5 cells (Huh7.5^{dif}). These hepatocyte-like cells were described to maintain HCV permissivity and allow long-term infection (Bauhofer et al., 2012; Sainz and Chisari, 2006). We took advantage of this strategy and assessed its applicability on Huh7.5.1 cells, which are more permissive than Huh7 or Huh7.5 cells for HCV replication. Indeed, when cultured with 1 % DMSO, the Huh7.5.1 proliferation rate slows down

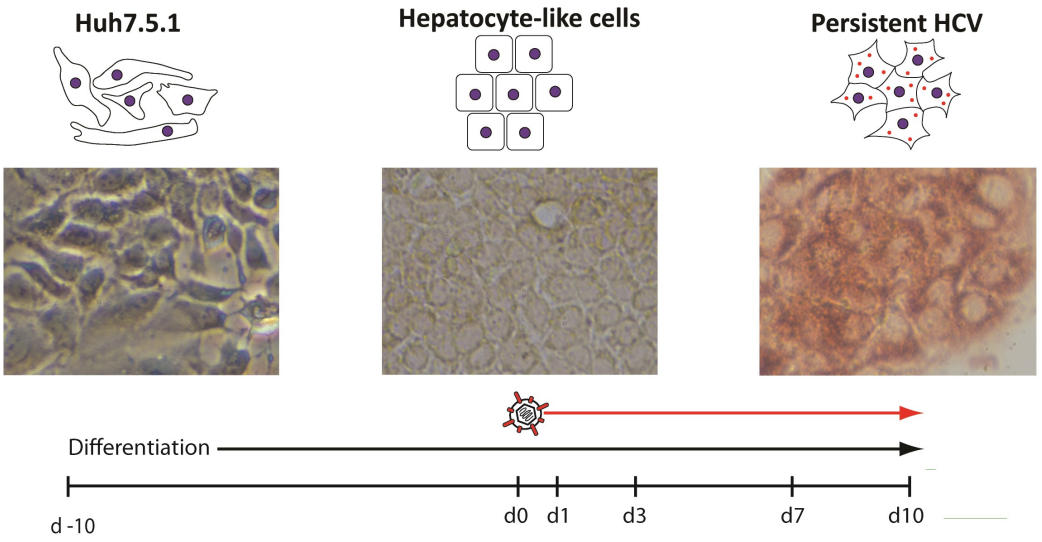

Fig. 17: Adding 1 % of DMSO causes Huh7.5.1 cells (left panel) to redifferentiate into hepatocyte-like cells (also referred to as Huh7.5.1^{dif} - middle panel) within 10 days. Infection after 10 days of differentiation yields persistent high-level HCV replication *in vitro* mimicking chronic HCV (right panel; immunohistochemistry staining with a goat anti-NS5A polyclonal antibody, gift from M. Harris, Leeds University, UK (Harris et al., 2008)).

significantly, and the cells eventually grow into confluent monolayers with growth inhibition upon cell-cell contact. They can be efficiently infected with HCV at day 7 or day 10, representing a robust and reproducible infection model (Fig. 17). The model can be upscaled from a 6 well plate to a 75 cm² culture flask format to increase biological sample quantity.

3.1.3.3 PERSISTENTLY HCV-INFECTED HEPATOCYTE-LIKE CELLS DISPLAY A TRANSCRIPTOMIC GENE SIGNATURE THAT PREDICTS CLINICAL OUTCOME IN PATIENTS WITH ADVANCED LIVER DISEASE

By combining the long-term Huh7.5.1^{dif} cell culture system with pure-virus inoculum, the hepatocyte-specific microenvironment of chronic infection *in vivo* could be simulated *in vitro.* In an effort to study altered signaling networks in persistently HCV-infected hepatocyte-like cells, a commercial phosphoarray was instrumental in assessing phosphorylation changes of 49 different RTKs. I uncovered a marked EGFR phosphorylation upregulation in these cells (see 3.1.4: Bandiera et al. Fig 4A and 4B). Analysis of microarray data generated from our advanced cell culture system demonstrated transcriptomic upregulation of an entire EGFR axis consisting of not only EGFR but also the EGFR ligand EGF (Fig. 18).

These findings were validated *in vitro* by quantifying EGF and EGFR mRNA in infected cells by RT-qPCR (Bandiera et al. Fig. 4C). Moreover, gene set enrichment analysis of microarray-derived transcriptomic data demonstrated an upregulation of genes associated with the EGF pathway in persistently infected hepatocyte-like cells (Bandiera et al. Fig. 4F).

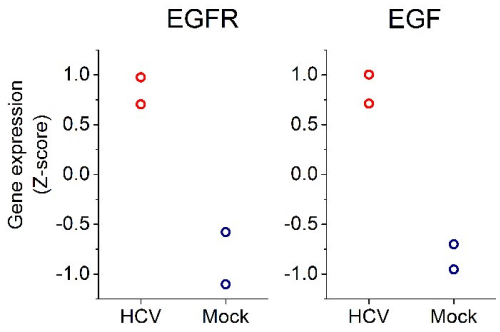

Fig. 18: mRNA expression of hepatocyte-like cells infected with purified HCVcc, measured by microarray (material and method see Bandiera et al. method section). The EGF/EGFR axis was upregulated after 7 days of infection. Graph represents a single experiment performed in duplicate.

In the framework of a collaboration with Dr. Hoshida at Mount Sinai Hospital (New York City, NY, USA) we found that we can induce a prognostic transcriptomic signature in our long-term *in vitro* infection system mimicking the mRNA profile of chronic HCV patients at high risk of developing HCC (Hoshida et al., 2008; Hoshida et al., 2013). In other words, we have developed a simple and robust liver cell-based system that closely recapitulates the transcriptional reprogramming in patients with carcinogenic liver disease caused by HCV (Bandiera et al. Fig. 1C-D). Moreover, this mRNA signature was also present in *in vitro* cell cultures infected by hepatitis B virus (HBV) or supplemented by ethanol (alcohol) (Bandiera et al. Fig. 3C), two other major HCC etiologies. Furthermore, the HCC risk signature was reversed by treating cells with DAAs, or by blocking EGFR signaling by the specific kinase inhibitor erlotinib (Bandiera et al. Fig. 5C). Since this inhibitor has recently been demonstrated to effectively prevent the progression of cirrhosis towards HCC in mouse and rat animal models (Fuchs et al., 2014), it validates our *in vitro* system as a model to test compounds for chemoprevention of HCC development.

3.1.4 PUBLICATION OF RESULTS

These results were integrated in the manuscript of Bandiera et al.: *"A clinical gene signature-based human cell culture model unravels drivers of hepatocarcinogenesis and compounds for cancer chemoprevention"* submitted to Nature Medicine.

A clinical gene signature-based human cell culture model unravels drivers of hepatocarcinogenesis and compounds for cancer chemoprevention

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Running title: A cell-based model for liver cancer chemoprevention

Keywords: Biomarker, cell circuits, cirrhosis, viral hepatitis, hepatocellular carcinoma, epidermal growth factor receptor signaling

Word count: 3316.

Hepatocellular carcinoma (HCC) is the second leading cause of cancer death. Curative approaches are limited and chemoprevention is not available. A 186-gene signature in liver tissue robustly predicts HCC risk in cirrhotic patients of various etiologies. However, the investigation of the drivers of hepatocarcinogenesis and the discovery of compounds for HCC chemoprevention has been hampered by the absence of tractable model systems. Here, we develop a human liver cell-based system, in which persistent hepatitis B and C virus infection or ethanol exposure induces the clinical HCC risk signature in a reversible manner. Using single cell profiling and perturbation studies, we identify EGFR/MAPK signaling as pan-etiology driver of the HCC high-risk signature and discover targets and compounds for cancer chemoprevention. By modeling the cell circuits of clinical liver disease progression and enabling fast-track drug discovery for chemoprevention, this system will improve the dismal prognosis of patients at risk of HCC. (150 words)

INTRODUCTION

HCC is the second leading cause of cancer death and develops from diseased livers chronically affected by a variety of etiological agents including hepatitis B and C viruses (HBV, HCV), alcohol abuse, and non-alcoholic fatty liver diseases/non-alcoholic steatohepatitis (NAFLD/NASH)[1]. Although the risk for HCC can be reduced by antiviral treatment or discontinuation of alcohol abuse, a marked and significant risk of HCC persists for decades once advanced liver fibrosis/cirrhosis is present[2,3]. Given that approximately 1-2% of the population is already affected by cirrhosis attributable to either of the etiologies, we urgently need measures to prevent HCC development by targeting cirrhotic patients[3,4]. Molecular biomarkers of HCC risk in cirrhosis could facilitate the identification of HCC chemopreventive drugs. Indeed, we previously defined and validated a 186-gene stromal liver signature predictive of HCC development and poor prognosis in multiple independent cohorts of patients with cirrhosis and HCC caused by HCV and other etiologies including HBV, alcohol and non-alcoholic fatty liver disease (HCC risk signature)[5-7]. This gene signature, which comprises 73 HCC high-risk and 113 HCC low-risk genes, was also present in cirrhosis-driven HCC rodent models[8] confirming its functional relevance for liver disease progression and hepatocarcinogenesis *in vivo*. Pharmacological inhibition of the EGF pathway resulted in chemoprevention of HCC[8] with subsequent initiation of a clinical trial (ClinicalTrials.gov NCT02273362). While our previous studies have validated that the HCC risk signature provides clues to new HCC chemoprevention targets, therapies, and biomarkers[5-8], the investigation of the HCC risk signature drivers as well the discovery of novel biomarkers and candidate compounds for HCC chemoprevention has been hampered by the absence of tractable model systems. Aiming to address these roadblocks, we developed a simple and robust clinical signature-based human cell culture system that models the clinical cell circuits of liver disease progression in a reversible manner. We show that this simple and robust system enables rapid

identification of candidate drivers of the HCC risk signature and uncovers compounds for HCC chemoprevention.

RESULTS

Induction of the HCC risk signature in a human liver cell-based system by HCV infection. Since chronic hepatitis C has been described as a major inducer of the HCC risk signature in patients[5-7], we first explored HCV infectious cell culture systems to model the clinically-derived signature. Taking advantage of a previous observation that DMSO differentiation of Huh7-derived liver cells results in induction of a hepatocyte-like phenotype in long-term culture[9], we established a model system allowing us to differentiate Huh7.5.1 cells into hepatocyte-like cells within 10 days (**Fig. 1a**). We then infected the hepatocyte-like Huh7.5.1^{dif} cells with HCV strain Jc1[10,11] (**Fig. 1a-b**). Strikingly, we discovered that persistent HCV infection of the differentiated liver cells resulted in robust induction of the HCC risk signature using an FDA-approved diagnostic platform, nCounter assay (NanoString)[7] (**Fig. 1c**). Induction of the signature was dose-dependent (with a threshold viral load of 10^4 tissue culture infectious dose 50/mL; **Fig. 1d**) and time-dependent (with full development from day 7 post-infection; **Supplementary Fig. 1**). Infection using highly purified FLAG-tagged virus (HCV Jc1E2^{FLAG};[12]) showed a similar induction of the HCC risk signature (**Fig. 1d**), confirming that the clinical signature was triggered by HCV. Interferon-alpha 2a (IFNα-2a) or direct-acting antiviral (DAA) treatment for HCV, which decreased viral load about 100-fold (**Fig. 5c**), resulted in partial suppression of the HCC high-risk gene expression and restoration of HCC low-risk gene expression (**Fig. 1d**), corroborating the causal link between persistent viral infection and induction of the HCC risk signature.

Genome-wide transcriptome profiling of HCV non-infected cells confirmed hepatocyte-like differentiation of Huh7.5.1 cells by DMSO: we observed a striking induction of a liver tissue-specific gene signature and suppression of HCC tissue-specific gene signature using Gene Set Enrichment Analysis (GSEA)[13] (**Supplementary Fig. 2a,** Method). These findings demonstrate

that cellular differentiation in non-infected cells results in a shift of the global transcriptome pattern from a malignant to a non-malignant hepatocyte-like profile (**Supplementary Fig. 2b**). Collectively, we have established a human liver cell system, mimicking HCV-infected human liver cells and allowing to model patient's liver stroma-derived HCC risk signature within a timeframe of days.

To further investigate virus-host interactions and the biogenesis of the signature, we performed single cell RNA-Seq profiling of 24 HCV Jc1E2^{FLAG}-infected and 18 non-infected Huh7.5.1^{dif} cells at day 7 post-infection. We calculated the Pearson correlation of all human genes to the normalized HCV copy number, which we defined as the percentage of viral read pairs mapped relative to all human and viral read pairs mapped (**Fig. 2**). We then used the pre-ranked GSEA analysis[13,14] to test enrichment of the HCC risk signature. We found that HCC high-risk genes are significantly correlated (false discovery rate [FDR] = 0.04) while HCC low-risk genes are significantly anti-correlated (FDR < 0.0001) with normalized HCV copy number, respectively (**Fig. 2**). These data show that induction of the HCC risk signature is viral load-dependent at the single cell level.

HBV infection and ethanol exposure induce an HCC risk signature in the liver cell-based system similar to clinical cohorts. Since the existence of shared mechanisms of hepatocarcinogenesis across distinct HCC etiologies has been suggested[15], we investigated whether the gene signature was similarly induced in liver cell-based models of other hepatocarcinogenic agents, including HBV and alcohol. To address this question, we used liver cell lines over-expressing NTCP, a recently identified cell entry factor that confers susceptibility for HBV (HepG2-NTCP cells) and hepatitis D virus (HDV) infection (Huh7.5.1^{dif}-NTCP cells)[16], as well as Huh7.5.1^{dif} cells chronically exposed to ethanol. Indeed, persistent HBV infection (**Fig. 3a-b**) as well as chronic ethanol incubation at higher dose resulted in a similar induction of the HCC high-risk genes in cell culture models (**Fig. 3c**). In contrast, HDV, another hepatotropic

virus that requires co-infection with HBV for persistent infection and disease manifestation, did not induce the HCC risk signature by itself (**Fig. 3a-c**). Most importantly, the induction of the HCC high-risk genes mirrored closely the transcriptional reprogramming in patients with progressive liver disease caused by HBV and alcohol based on the analysis of previously published genome-wide transcriptome profiles. The gene signature observed in cell-based models was similarly induced in diseased human liver in association with poorer prognosis of HCV-related cirrhosis as shown in our recent study[7] and HBV-related HCC[17], presence of alcoholic hepatitis[18], and more fibrotic NASH[19] as shown in **Fig. 3d**. Direct comparison of the global transcriptome between the cell-based systems and the clinical cohorts also showed significant similarity within each etiology (**Supplementary Fig. 3**). However, the resemblance of the HCC low-risk signature genes in the cell-based system with healthier clinical liver was less pronounced in the HBV infection model using HepG2-NTCP cells, currently the only available cell line allowing robust HBV infection (**Supplementary Fig. 3**, **Fig. 3a-b**). This is consistent with the less differentiated phenotype and morphology of HepG2 cells, which were originally derived from hepatoblastoma, not HCC[20].

Collectively, our results suggest that our liver cell-based system reflects transcriptional reprogramming common to the etiological agents capable of promoting HCC development, thereby offering unique opportunities to interrogate the mechanisms of hepatocarcinogenesis and test cancer preventive strategies for each of the major HCC etiologies.

Co-culture with non-parenchymal liver-resident pericytes models the liver microenvironment for the induction of HCC risk signature. The liver microenvironment has been suggested to play an important role in liver disease progression and HCC development[21]. The hepatic stellate cell is a non-parenchymal liver-resident pericyte well known for driving liver fibrogenesis and supporting carcinogenesis[21]. We had previously observed evidence of a hepatic stellate cell activation trait encoded in the HCC risk signature[6]. Interestingly, in our

Huh7.5.1^{dif} system, a subset of HCC high-risk genes, which were preferentially induced in the non-hepatocyte fraction in fibrotic rat liver tissue, were induced in the cells despite a lack of a non-hepatocyte component (**Supplementary Fig. 4**). To investigate the impact of the non-hepatocyte fraction, particularly hepatic stellate cells, on the biogenesis of the signature, we performed co-culture of Huh7.5.1^{dif} cells with immortalized hepatic LX-2 stellate cells[22]. Indeed, co-culture with stellate cells further enhanced induction of the gene signature in a cell- and dose-dependent manner (**Fig. 1d**, **Supplementary Fig. 4**). These findings suggest that hepatocytes alone are sufficient for generating the HCC high-risk gene signature, but this can be amplified through cross-talk with non-parenchymal cells. Our findings establish a simple and robust co-culture system for the study of hepatocyte-hepatic stellate cell interactions that mimic a cancer-permissive cirrhotic tissue microenvironment.

Identification of candidate drivers of HCC high-risk genes common to viral and metabolic HCC etiologies. To gain first insights into the cell circuits driving the HCC risk signature, we performed a time-course transcriptomic profiling of HCV-infected hepatocyte-like Huh7.5.1^{dif} cells. Genome-wide profiling of the cells at later time points after HCV infection revealed gradual modulation of pathways involved in a carcinogenesis-supporting tissue microenvironment (**Supplementary Fig. 1; Supplementary Table 1**) analogous to that seen in clinical cohorts of virus-infected patients[3]. Persistent HCV infection in hepatocyte-like Huh7.5.1^{dif} cells induced immune response-related pathways, growth factor signaling pathways, and enhanced cell cycle, DNA replication, and anti-apoptotic pathways. Target genes of the transcription factor E2F1, involved in liver fibrogenesis and carcinogenesis[3,23] were also up-regulated (**Supplementary Table 1**). Interestingly, persistent viral infection suppressed a range of metabolic pathways involved in physiological functions of hepatocytes, including cytochrome P450-based xenobiotic metabolism, amino acid biosynthesis, fatty acid and lipid metabolism, and steroid biosynthesis (**Supplementary Fig. 1; Supplementary Table 1**). The impact of HCV infection on the cellular

metabolic circuitry was examined directly by using mass spectrometry-based metabolomic profiling[24]. Analyses of the metabolic phenotype revealed alterations in steady-state metabolites in virus-infected cells, including pronounced increases in lactate and glutamine levels in parallel to elevated influx of glucose and intracellular glucose consumption (**Supplementary Fig. 5**) – a Warburg-like metabolic shift associated with increased cancer risk[25,26]. This is also consistent with the enrichment of a carbohydrate metabolism gene expression signature, which was the only metabolic pathway significantly activated by HCV infection (**Supplementary Fig. 1**).

The gene signature induced by HCV or HBV infection, or by ethanol treatment reflects modulation of carcinogenic pathways common to the etiological agents capable of promoting HCC development. Nine out of 73 HCC high-risk genes most prominently induced across HCV, HBV, and ethanol in our cell-based models represented oxidative stress response (e.g. *glutathione peroxidase 2, GPX2*), extracellular matrix remodeling (e.g. *lysyl oxidase-like 2, LOXL2*), and growth factor signaling (e.g. *epidermal growth factor, EGF; dual specificity protein phosphatase 5, DUSP5*) (**Supplementary Table 2**). Accordingly, there was strong enrichment of cancer-related pathways (**Supplementary Table 3**) and two co-regulated gene networks involved in inflammation/fibrogenesis (containing EGF) and oxidative stress/carcinogenesis (containing p53/Myc) (**Fig. 3e-f**).

EGFR signaling is a pan-etiology driver of the HCC high-risk gene signature in human liver cells. We have previously shown that HCV uses receptor tyrosine kinases including EGFR and activation of downstream signaling pathways to enter hepatocytes in cell culture and *in vivo*[27,28]. To assess whether HCV exploits receptor tyrosine kinases not only for entry, but also to trigger intracellular signaling cascades relevant for induction of the HCC risk signature, we investigated virus-induced signaling in infected liver cells. Taking advantage of our cell-based model system (**Fig. 1**), we screened the activation state of canonical signaling pathways using phospho-receptor tyrosine kinase arrays and gene expression analyses**.** We observed that HCV

infection triggers activation of specific host signaling networks, including the EGFR pathway as shown by virus-induced EGFR phosphorylation (**Fig. 4a-b**), enhanced *EGF* and *EGFR* expression and significant induction of experimentally defined EGF target gene signatures[29,30] (**Fig. 4c and f**). Interestingly, induction of the EGF/EGFR pathway was also observed in HBV-infected cells, and in ethanol-treated cells (**Fig. 4d-e**), confirming EGF as a driver of the HCC risk signature common to the major HCC etiologies. Moreover, enhanced induction of EGFR pathway was observed in the single cell RNA-Seq samples in association with increasing normalized HCV copy number (REACTOME signaling by EGFR in cancer, pre-ranked GSEA FDR = 0.04) (**Fig. 2**). Pharmacological inhibition of the EGF/EGFR pathway with erlotinib at concentrations that did not modify HCV viral load suppressed the HCC high-risk genes as observed in our previous *in vivo* HCC chemoprevention study[8] (**Fig. 5a, c-** and **d**). Transcriptome-based network analysis indicated that this drug impacts on a fibrosis network involving the EGFR/MAPK signaling pathway and a p53/Myc-related carcinogenesis network (**Fig. 5e-f**). The functional impact of the EGFR/MAPK signaling pathway was corroborated by perturbation studies using the Ras inhibitor tipifarnib and the ERK1/2 inhibitor Fr180204 (**Fig. 5a-d**). Inhibition of virus-induced MAPK signaling as shown by reduced ERK1/2 phosphorylation (**Fig. 5b**) at low inhibitor concentrations with absent effects on viral load (**Fig. 5c**) suppressed the HCC high-risk gene signature in differentiated liver cells (**Fig. 5d**). Thus, it is likely that the pharmacological inhibitors reverse the gene signature through direct suppression of EGF-induced oncogenic pathways independently of any antiviral effect. Collectively, these data demonstrate that the model system combined with perturbation studies enables to investigate drivers of the cell circuits associated with liver disease progression and hepatocarcinogenesis.

We next assessed the utility of the gene signature as a companion biomarker in the HCC chemoprevention trial of erlotinib (**Fig. 6a-c**). To this end, we used erlotinib to treat organotypic *ex vivo* cultures of surgically resected human fibrotic liver tissue slices from two HBV-infected HCC patients. Notably, erlotinib effectively suppressed the HCC high-risk genes in

these human tissues (**Fig. 6b**). Collectively, our data show that EGFR/Ras/MAPK signaling is an etiology-independent driver of the HCC high-risk signature in the human liver. Furthermore, these results indicate that the effect of pharmacological interventions on the carcinogenic milieu-related cellular circuits can be evaluated in a tractable cell-based system.

Computational prediction combined with perturbation studies identifies candidate compounds for HCC chemoprevention. Molecular signature-based *in silico* drug screening has been shown to be an effective way to identify or repurpose existing drugs for new indications. However, the functional relevance of candidates identified through these screens must be confirmed in subsequent usually laborious experimental verification[31,32]. A molecular signature-inducible cell-based system could allow more efficient secondary screening following an informatics-based primary screening or even direct high-throughput functional primary drug screening[33]. However, such cellular models are rarely available for specific disease contexts. Our results clearly indicate that the HCV Jc1-Huh7.5.1^{dif} cells could serve as a platform specialized for HCC chemoprevention drug discovery and assessment. To test this hypothesis, we first informatically interrogated a chemogenomics database of transcriptome profiles of 1,309 bioactive compounds using the HCC risk signature as a query. The analysis yielded 36 hits including several agents such as genestein, resveratrol, and captopril, which have been already tested as potential HCC chemoprevention therapies in preclinical and clinical settings[34] (**Supplementary Table 4**). Pioglitazone, currently indicated for the treatment of type 2 diabetes and NASH was among the top hits. Indeed, by exploring the LINCS database (www.lincscloud.org) we discovered that pioglitazone modulates EGF and MAPK signaling, which is a hallmark of the HCC risk signature (**Supplementary Table 5** and **6**). As predicted from the computational screen and pathway analyses, pioglitazone markedly and significantly reversed the HCC high-risk genes in our cell-based system (FDR = 0.04, **Fig. 6a**) independently on any antiviral effect (**Fig. 6c**). This was also confirmed in organotypic *ex vivo* culture of fibrotic

human liver tissue (FDR = 0.046, **Fig. 6b**). Furthermore, genes targeted by pioglitazone treatment were also significantly modulated by increasing HCV copy number in the single cell RNA-Seq samples (pre-ranked GSEA FDR<0.0001, **Fig. 2**). Transcriptome-based network analyses revealed that pioglitazone perturbed the two gene networks, i.e., EGF-containing fibrogenesis network and p53/Myc-containing carcinogenesis network) common to erlotinib (**Fig. 5e-f**). Overall, these data support the validity of the system to efficiently bridge preclinical to clinical evaluation of novel HCC chemoprevention agents, ensuring clinical relevance by relying on the clinically well validated HCC risk signature.

DISCUSSION

There is an unmet need for experimental systems modeling human disease-specific gene expression to understand disease biology and enable disease-specific drug discovery. Here we have developed a simple and robust liver cell-based system that closely recapitulates the transcriptional reprogramming in patients with carcinogenic liver disease caused by HCV, HBV and alcohol (**Fig. 1-4**). Notably, the workflow presented here permits robust and rapid modeling of gene expression patterns predictive of long-term HCC risk in patients with decades of chronic disease (**Fig. 1-4**). This cell-based system further enables modeling of inter-cell type cross-talk in a cancer-permissive cirrhotic tissue microenvironment as shown by co-culture of hepatocytes and hepatic stellate cells, liver-resident pericytes that drive fibrogenesis (**Fig. 1d, Supplementary Fig. 4**). Overall, our model offers unique opportunities to investigate the molecular mechanisms and cell circuits that drive hepatocarcinogenesis across the distinct HCC etiologies.

Indeed, using this system we have uncovered the functional role of EGFR as a pan-etiology driver of the HCC high-risk signature (**Fig. 4, 5**). Perturbation studies using small molecule inhibitors (**Fig. 5**) confirmed the functional impact of EGFR/MAPK signaling for the biogenesis of the HCC risk signature. Furthermore, computational analyses revealed previously undiscovered candidate drivers of this gene signature and hepatocarcinogenesis (**Fig. 4, Supplementary Table 2** and **3**). These include oxidative stress response (e.g. *GPX2*), extracellular matrix remodeling (e.g. *LOXL2*), and growth factor signaling (e.g. *EGF, DUSP5*), which are now linked to long-term clinical HCC risk via a patient-derived gene signature. Since oxidative stress response-related genes such as *KEAP1* and *NFE2L2* are frequently inactivated by somatic DNA mutations in HCC[35], *GPX2* may be a useful marker to monitor the pathway and/or serve as a point of intervention by antioxidants to rescue this pathway. Monoclonal antibody (simtuzumab) and small molecule inhibitors targeting LOXL2 protein are currently under clinical evaluation as anti-liver fibrosis agents (ClinicalTrials.gov NCT01672879) and most

recent studies suggest also a role of LOXL2 for hepatocarcinogenesis, which may be prevented by simtuzumab[36]. Assessment of our HCC risk signature in these trials may provide insight into the potential role of these drugs in HCC prevention. EGFR, MAPK and DUSP5 are closely linked within the same pathway as phosphatase DUSP5, whose expression is induced by MAPK signaling, regulates the nuclear phosphorylation of ERK[37]. Importantly, similar pathways and networks were present in liver tissues from HBV-related or alcoholic liver disease and NASH, supporting the clinical relevance of the findings (**Supplementary Fig. 6**). Thus, our results uncover *EGF*, *GPX2*, and *LOXL2* as targetable candidate drivers for liver disease and HCC progression common to viral and metabolic etiologies associated with chronic inflammation, fibrogenesis, and hepatocyte turnover/proliferation that collectively incite a cancer-permissive milieu. Moreover, our results suggest that the HCC risk signature can be used as a companion biomarker universally applicable to HCC chemoprevention therapies targeting these pathways. Collectively, these findings highlight the impact of our cell-based system for the discovery of clinically relevant targets of hepatocarcinogenesis.

One reason for the absence of HCC chemopreventive strategies has been the lack of robust and convenient experimental systems to model and investigate clinically-relevant mechanisms and targets in HCC development. Moreover, current animal models for HCC only partially recapitulate human disease and preclude fast-track drug discovery and development. The opportunity to perform perturbation studies (**Fig. 5, 6**) using small molecules in our clinical signature-inducible cell-based system not only permits the discovery of the cell circuits of hepatocarcinogenesis, but also paves the way for systematic and high-throughput drug screening to discover novel therapeutic strategies that block the progression of fibrosis/cirrhosis to HCC (**Fig. 6d**). As a proof-of-concept, the therapeutic efficacy of erlotinib in alleviating HCC risk *in vivo*[8] could be similarly validated by assessing the HCC risk signature in our liver cell-based system as well as in *ex vivo* patient liver tissues (**Fig. 6**). Interestingly, metformin also

showed reversal of the gene signature (**Fig. 6a**), echoing clinical studies that suggest its HCC chemopreventive effect[38].

Our findings indicate that the cell-based system provides a specialized platform for drug discovery and assessment following *in silico* computational screening (**Fig. 6d**). Indeed, we discovered that pioglitazone, which is currently indicated for the treatment of type 2 diabetes and NASH, significantly reversed the HCC risk signature (**Fig. 6**). This ability to efficiently assess candidate HCC chemopreventive agents in our cell-based system will greatly expedite the process of HCC chemoprevention drug development and could substantially improve the dismal prognosis of patients with cirrhosis at risk of HCC.

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Acknowledgments: This work was supported by ARC, Paris and Institut Hospitalo-Universitaire, Strasbourg (TheraHCC IHUARC IHU201301187 to T.F.B.), the European Union (INTERREG-IV-Rhin Supérieur-FEDER-Hepato-Regio-Net 2012 to T.F.B. and M.B.Z) and FP7 HepaMab to T.F.B., ANRS, Paris (2013/108 to T.F.B), NIH (DK099558 to Y.H.) and Irma T. Hirschl/Monique Weill-Caulier Trust. This work has been published under the framework of the LABEX ANR-10-LABX-0028_HEPSYS and benefits from funding from the state managed by the French National Research Agency as part of the Investments for the future program. The nCounter assay was performed at Mount Sinai qPCR Shared Resource Facility. Bioinformatics analysis was performed by using High Power Computing facility at Mount Sinai Genomics Core and Department of Scientific Computing. De-identified surgical liver tissues were provided by Mount Sinai Biorepository. RNA-Seq for single-cell profiling was performed at the Broad Technology Labs, Broad Institute of MIT and Harvard, Cambridge, MA. Transcriptomic data reported in this paper have been deposited with the Gene Expression Omnibus database (GEO). We thank Dr. R. Bartenschlager (University of Heidelberg, Germany) for providing plasmids for production of HCVcc Jc1 strains, Dr. F. Chisari (The Scripps Research Institute, La Jolla, CA) for the gift of Huh7.5.1 cells, Dr. A. Patel (MRC Virology Unit, Glasgow, UK) for E2-specific mAb AP33, Dr. J. Taylor (Fox Chase Cancer Center, Philadelphia, CA) for HDV expression plasmids and Dr. F. Habersetzer (Strasbourg University Hospitals) for patient samples for isolation of anti-HDV antibodies and infectious HBV. We acknowledge M. Oudot, C. Bach and L. Heydmann (Inserm U1110, Strasbourg, France) for excellent technical assistance, Drs. M. Jovanovic, C. Nusbaum, I. Tirosh (Broad Institute of MIT and Harvard) and Dr. S. L. Friedman (Mount Sinai Hospital) for helpful discussions.

Author Contributions

Y.H., N.B., M.B.Z. and T.F.B. designed experiments and analyzed data. S.B., A.V., N.V.R., J.L., C.T., H.E.S., E.R.V., S.C.D., S.P., S.N., N.G., L.W., M.S., A.K., V.D.N., and M.M. performed experiments, and analyzed data. A.K.S, O.R. and A.R. designed single cell RNA-Seq experiments. N.P. and K.K. performed computational analyses of single cell RNA-Seq profiling. X.S. performed liver tissue experiments and bioinformatic analyses. B.N.N. performed metabolic analyses. B.C.F., K.K.T., M.E.S., R.T.C. and G.G. provided key reagents. S.B., Y.H., M.B.Z., and T.F.B. wrote the manuscript. Y.H. supervised the bioinformatic analyses and human liver tissue experiments. T.F.B. initiated and coordinated the study.

Competing financial interests

Y.H. is named investigator on a pending patent application entitled “Compositions, kits, and methods for detecting, characterizing, preventing, and treating hepatic disorders (USPTO #20110263441). NanoString has secured the option to an exclusive worldwide license. NanoString has no role in conduction of the current study. There is no other relevant declaration relating to employment, consultancy, patents, products in development, or modified products.

FIGURE LEGENDS

Fig. 1. Modeling the transcriptional profiling of cirrhotic patients progressing to HCC in a simple and robust liver cell-based system. a. Approach. Huh7.5.1 cells were differentiated into Huh7.5.1^{dif} cells, persistently infected using HCV Jc1 and subject to molecular analyses at different time points post-infection. **b.** Top panel: analysis of HCV infection by qRT-PCR of HCV RNA (mean ± s.d.; *n* = 3). Given very low s.d., error bars are not visible on the graph. Dashed line indicates limit of detection (top panel). Bottom panel: immunodetection of HCV E2 protein at day 10 post-infection. Nuclei are counterstained with DAPI. Scale bar, 50 µm. Results are representative of one out of two experiments. **c.** 186-gene HCC risk signature assay in Huh7.5.1^{dif} cells. Heatmap shows high (red) and low (blue) gene expression across samples. HCV Jc1-infected (HCV Jc1) and non-infected (Control) cells were predicted as HCC high- or low-risk as previously described[39]. Results are representative of one out of three independent experiments performed in triplicate. **d.** Heatmap (top) and plots (bottom) showing the significance of high-/low-risk genes induction/suppression under persistent infection of cells using increasing titers of HCV Jc1; highly purified HCV particles (HCV Jc1E2^{FLAG}); following antiviral treatment using IFNα-2a or DAAs; or following co-culture with increasing percentages of hepatic stellate LX-2 cells. In scale bar, dense red indicates significant induction, blue indicates significant suppression. For each condition, results are representative of one experiment performed in triplicate. FDR: false discovery rate; NES: normalized enrichment score.

Fig. 2. Single cell RNA-Seq profiling reveals virus-dependent induction of the HCC high-risk gene signature in HCV-infected liver cells. Huh7.5.1^{dif} cells were HCV Jc1E2^{FLAG}-infected as described in **Fig. 1a**. Following isolation of single cells on day 7, total cellular RNA was purified, subjected to RNA-Seq and analyzed as described in Methods. The heatmap shows on top gene sets that are significantly associated with the normalized HCV copy number

(EGFR pathway, false discovery rate [FDR] = 0.04; HCC high-risk genes, FDR = 0.04; HCC low-risk genes, FDR<0.0001; pioglitazone treatment targets, FDR<0.0001), and below HCC high- and low-risk genes that are associated with the normalized HCV copy number (Pearson correlation >0.1 or <-0.1). For each gene set (rows on top), the mean expression of the 'leading-edge' genes (which supported the association with the normalized HCV copy number) in each of the single cells (columns) is shown. The single cells are ordered by infection status (white: non-infected cells, *n* = 18; black: infected cells, *n* = 24), and by increasing normalized HCV copy number (purple bar plot on top). Genes (rows below) are ordered by the correlation of their expression levels with the normalized HCV copy number (blue bar plot on the right). For each gene, its membership to one of the HCC risk signature subgroups is shown (color bar on the right; high-risk genes: orange, low-risk genes: green). Only genes that are detected (FPKM >1) in at least 10% of the samples are visualized. Gene set and gene expression is row normalized for visualization.

Fig. 3. HBV infection and ethanol exposure induces HCC risk signature in the liver cell-based system similar to clinical cohorts. Liver cells were infected with HBV, HDV or incubated with ethanol as described in Methods. **a.** Immunodetection of viral antigens in HBV-infected HepG2-NTCP or HDV-infected Huh7.5.1-NTCP^{dif} cells using antigen-specific antibodies as described in Methods. Nuclei are counterstained with DAPI. Scale bar, 50 µm. Results are representative of one out of three experiments. **b.** Relative HBV pregenomic (pg) RNA and HDV RNA expression in cell-based models as assessed by qRT-PCR (mean ± s.d.; *n* = 3). **c.** 186-gene HCC risk signature in cells shown in **a** as well as in Huh7.5.1^{dif} cells incubated with ethanol. For each condition, results are representative of one experiment performed in triplicate. **d.** HCC risk signature in clinical liver tissues from HBV-related liver disease and HCC (HBV), alcoholic hepatitis (Alcohol) and NASH cohorts. In **c** and **d**, heatmaps show the significance of HCC high-/low-risk gene signature induction (red) or suppression (blue). FDR: false discovery

rate. **e-f.** Co-regulated gene networks in HCC high-risk genes commonly modulated by HCV, HBV and ethanol in cell-based models (Ingenuity Pathway Analysis). A fibrosis network involving the EGFR/MAPK signaling pathway (**e**) and a p53/Myc/GPX2-including carcinogenesis network (**f**) are shown. The HCC high-risk genes included in networks are in red. Solid and dashed lines indicate direct and indirect interactions, respectively.

Fig. 4. EGFR signaling is a pan-etiology driver of the HCC high-risk gene signature in human liver cells. Huh7.5.1^{dif} cells were infected with HCV Jc1E2^{FLAG} and harvested for proteomic analyses as shown in **Fig. 1**. **a.** Receptor tyrosine kinase (RTK) phosphorylation was assessed in cell lysates using the Human Phospho-RTK Array Kit (R&D Systems). Infection with HCV Jc1E2^{FLAG} results in increased EGFR phosphorylation. One representative experiment out of three is shown. **b.** Quantification of dot blot intensities of phosphorylated proteins (in arbitrary units, AU) using the Image J software. Results show the mean ± s.e.m. of integrated dot blot densities from three independent experiments performed in duplicate. **c-e.** *EGFR and EGF* mRNA expression (relative to *GAPDH* mRNA) in non-infected (Control) and HCV Jc1-infected Huh7.5.1^{dif} cells (**c**; n = 9); non-infected (Control) and HBV-infected HepG2-NTCP cells (**d**; n = 12); Huh7.5.1^{dif} cells incubated in absence (Control) or presence of 40 mM ethanol (**e**; n = 12). Mean percentage ± s.e.m. is shown. **f.** Virus-mediated signaling induces an EGF signature in HCV Jc1-infected Huh7.5.1^{dif} cells. Panels show presence of previously reported EGF-related gene signatures derived from other cell lines[29,30] assessed by GSEA in virus-infected Huh7.5.1^{dif} cells. FDR: false discovery rate; NES: normalized enrichment score. * Two-tailed Mann-Whitney U-test (p-value < 0.01) in **b-e**.

Fig. 5. Pharmacological inhibition of EGF/MAPK signaling partially reverses the HCC risk signature in HCV-infected Huh7.5.1^{dif} cells. a. Scheme of the canonical EGFR/MAPK signaling cascade. Inhibitors targeting members of this pathway are indicated. **b.** Treatment with low dose erlotinib (0.1 µM), tipifarnib (10 µM) and Fr180204 (10 µM) reverses the enhancement

of ERK1/2 phosphorylation in HCV Jc1E2^{FLAG}-infected cells. Phospo-ERK1/2 was assessed in cell lysates using the Proteome Profiler Human Phospho-kinase Array (Methods). Results are shown as mean ± s.e.m. of integrated dot blot densities from one experiment performed in duplicate. **c**. Treatments using small molecule MAPK inhibitors do not modulate viral load in HCV Jc1-infected Huh7.5.1^{dif} cells. A DAA treatment leading to a significant decrease of HCV load was performed as a control. Relative HCV RNA expression (normalized to *GAPDH*) was analyzed as described in Methods. Results are shown as mean ± s.e.m. (n = 3)**. d.** Small molecule inhibitor treatments reverse the HCC high-risk genes as shown by GSEA. Heatmaps show the significance of induction (red) or suppression (blue) of HCC high-/low-risk genes in the signature. For each condition, results are representative of one experiment performed in triplicate. **e, f.** Co-regulated gene networks in HCC high-risk genes commonly modulated by erlotinib and pioglitazone in cell-based models (Ingenuity Pathway Analysis). A fibrosis network involving the EGFR/MAPK signaling pathway (**e**) and a p53/Myc-including carcinogenesis network (**f**) are shown. The HCC high-risk genes included in networks are in red. Solid and dashed lines indicate direct and indirect interactions, respectively.

Fig. 6. Perturbation studies based on chemogenomics identify candidate compounds for HCC chemoprevention. a. Treatment with low dose erlotinib (0.1 μM), pioglitazone (1 μM) and metformin (0.1 mM) reverses the HCC high-risk genes as shown by GSEA. Heatmaps show the significance of induction (red) or suppression (blue) of HCC high-/low-risk genes in the signature. For each condition, results are representative of one experiment performed in triplicate. **b.** Liver tissue sections from surgically resected fibrotic livers (patients #1 and #2: HBV-infected liver tissue) express the HCC risk signature, which is reverted by treatment with erlotinib (5 μM for 48 hours) or pioglitazone (10 μM for 48 hours). Modulation of the HCC high-risk genes in treated liver tissues was quantitatively assessed by GSEA as in a. **c.** Small molecule inhibitors treatment does not impact on hepatitis C and B viruses load in HCV Jc1-

infected Huh7.5.1^{dif} cells nor in HBV-infected patients. A DAA treatment leading to a significant decrease of HCV load in the cell-based system was performed as a control. Relative HCV RNA expression (normalized to *GAPDH*) and HBV pgRNA expression (normalized to *RPL13A*) were analyzed as described in Methods. HCV load in cell-based model, mean ± s.e.m., n = 3; HBV load in patient liver tissue, weighted mean ± s.e.m., n = 2. **d.** Suggested workflow that displays the steps of high-throughput target and drug discovery using the liver cell-based system with inducible patient-derived HCC risk signature. This liver cell-based system innovates target and drug discovery for HCC chemoprevention by using a patient-derived functional gene signature as readout. A high-throughput format and processing time of less than 4 weeks enables fast-track drug discovery.

ONLINE METHODS

Human subjects. Human material including serum from patients with chronic HBV or HDV infection and followed at the Strasbourg University Hospitals, Strasbourg, France was obtained with informed consent from all subjects (CPP 10-17). Human liver tissues were obtained from HCC patients undergoing liver resection with informed consent from the all the patients for de-identified use at Mount Sinai Hospital, New York City, NY (HS13-00159). The protocols were approved by the local Ethics Committee of the University of Strasbourg Hospitals and Mount Sinai Hospital, respectively.

Research experiments on live vertebrates. *In vivo* experiments were performed at the Massachusetts General Hospital Simches Research Center animal facility according to local laws and ethical committee approval (protocol approval number 2007N000113). Rats (n = 3 per group) were randomly assigned to receive diethylnitrosamine (DEN) in PBS or PBS alone as a vehicle control by a blinded technician. Our sample size estimate was based on a p-value of 0.01 at 95% power assuming that the frequency of fibrosis is 100% in DEN-injured animals at 12 weeks whereas PBS-treated animals have normal livers. All animals were used for liver cell isolation as described below and no animals were excluded from the study. All animals were housed in accordance with the guidelines of the Massachusetts General Hospital Institutional Animal Care and Use Committee and received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" of the National Academy of Sciences.

Reagents and Antibodies. Absolute ethanol was purchased from Thermo Fisher Scientific (Waltham, Massachusetts); erlotinib from LC Laboratories (Woburn, Massachusetts); interferon-alpha 2a from Roche (Penzberg, Germany); pioglitazone, metformin and dimethyl sulfoxide (DMSO) from Sigma-Aldrich (St. Louis, Missouri); Fr180204 from Merk (Billerica, Massachusetts); and tipifarnib from Selleckchem (Houston, Texas). Daclatasvir and sofosbuvir

were synthesized by Acme Biosciences (Palo Alto, California). The Human Phospho-RTK Array kit and the Proteome Profiler Human Phospho-kinase Array kit were obtained from R&D Systems (Minneapolis, Minnesota). The ECL reagent and Hyperfilms were purchased from GE Healthcare (Cleveland, Ohio). HCV E2-specific AP33 antibody (mouse) has been described[40]. Human IgG containing antibodies targeting the hepatitis delta Ag (HDAg) were purified from the serum of a HDV infected patient using MAbTrap Kit (GE Healthcare, Cleveland, Ohio) according to manufacturer's instructions. Hepatitis B surface antigen (HBsAg)-specific monoclonal antibody (NCL-HBsAg-2, clone 1044/341) was obtained from Leica Biosystems (Wetzlar, Germany) Alexa Fluor® 647 anti-mouse IgG (goat) and Alexa Fluor® 647 anti-human IgG (goat) from Jackson ImmunoResearch (West Grove, Pennsylvania), DAPI from Life Technologies (Carlsbad, California).

Cell Lines. Hepatocellular carcinoma-derived Huh7.5.1[41] and HepAD38[42], hepatoblastoma-derived HepG2[27] and human stellate LX-2 cells[22] have been described. All cell lines were certified mycoplasma-free. For HDV and HBV infection, Huh7.5.1 and HepG2 cells were stably transduced using human NTCP-expressing vesicular stomatitis virus pseudoparticles (VSVpp) (GeneCopoeia, Rockville, Maryland). NTCP-overexpressing Huh7.5.1-NTCP or HepG2-NTCP cells were selected using puromycin as described previously[16,43,44]. For proliferation arrest and differentiation (Huh7.5.1^{dif} cells), 2.5 to 3 x 10^4 Huh7.5.1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 1% DMSO. For co-culture experiments, 2.5 x 10^4 Huh7.5.1 cells were seeded in a P6-well format together with different percentages of LX-2 hepatic stellate cells (0, 5, 10 and 20 % of total cells), and maintained in 1% DMSO-complemented medium.

HCV infection of Huh7.5.1^{dif} cells. Cell culture-derived HCVcc Jc1 (genotype 2a/2a)[10] were generated in Huh7.5.1 cells as described[45]. HCV Jc1E2^{FLAG} was purified using anti-FLAG M2

affinity gel (Sigma-Aldrich, St.Louis, Missouri) as described[12]. HCVcc infectivity was determined by calculating the 50% tissue culture infectious doses ($TCID_{50}$) as described[46]. Huh7.5.1^{dif} cells were infected with HCV Jc1 or HCV Jc1E2^{FLAG}. Cell culture supernatants from mock-electroporated cells or 100 µg/mL of FLAG peptide were used for control experiments. HCV infection was assessed by qRT-PCR of intracellular HCV RNA[47] as well as immunostaining using HCV E2-specific AP33 antibody as described[48]. Seven days after HCV Jc1 infection, Huh7.5.1^{dif} cells were incubated for three days with either 10 IU/mL interferon-alpha 2a, a combination of 1 nM daclatasvir and 1 µM sofosbuvir, erlotinib (0.1 µM), tipifarnib (10 µM), Fr180204 (10 µM), pioglitazone (1 µM) or metformin (0.1 mM).

Single cell RNA-Seq profiling. Huh7.5.1^{dif} cells were infected with HCV Jc1E2^{FLAG} ($TCID_{50}$= 7 x 10^5/mL) as described above. On day 7, single-cells were sorted into 96-well plates using a well-established limiting dilution assay[49]. Single-cells were lysed in 5 µl TCL buffer (Qiagen, Hilden, Germany) supplemented with 1% 2-mercaptoethanol. Cellular mRNA was isolated and analyzed as described[50,51]. HCV RNA was co-amplified with cellular mRNA using a SMART-compatible primer (sequence: 5'-Biotin-AAGCAGTGGTATCAACGCAGAGTACTCTGCGGAAC CGGTGAGTA-3') derived from[47].

HDV infection of Huh7.5.1^{dif}-NTCP cells. Recombinant HDV was produced as described[52]. NTCP-overexpressing Huh7.5.1^{dif} cells were infected with recombinant HDV in presence of 4% polyethylene glycol and cultured for 10 days in 2% DMSO-complemented medium as described by our laboratory[43] and[16,44]. HDV infection was assessed by qRT-PCR of HDV RNA[53] and immunostaining using serum anti-HDVAg as described[52].

HBV infection of HepG2-NTCP cells. HBV (genotype D) was purified from the serum of a HBV carrier[54]. Viral particles were concentrated through ultracentrifugation by pelleting over a 30%

sucrose cushion with subsequent gradient centrifugation using a 10-45% iodixanol density gradient similar as described[55]. Recombinant HBV was purified from the supernatant of HepAD38 cells that constantly replicate HBV genome (genotype D strain adw)[42]. HepG2-NTCP cells were then infected with purified HBV or control preparation in the presence of 4% polyethylene glycol and maintained in culture for additional 10 days in 2% DMSO-complemented medium as described[43]. HBV infection was assessed by qRT-PCR quantification of HBV pregenomic RNA in cell lysates[56] as well as by immunostaining of HBsAg using HBsAg-specific monoclonal antibody (NCL-HBsAg-2, clone 1044/341)[16].

Ethanol treatment of Huh7.5.1^{dif}cells. Huh7.5.1^{dif} cells were incubated in presence or absence of ethanol (20 or 40 mM) as described[57]. Fresh medium containing ethanol was replenished every day[57].

Rat liver cell isolation. 8 week old male Wistar rats received weekly intraperitoneal injections of DEN (50 mg/kg) or PBS control for 12 weeks. After a one-week washout period to eliminate acute effects of DEN, rats were sacrificed and primary cells were isolated according to established procedures[58]. Briefly, livers were perfused with a buffer containing collagenase A, the digested liver was filtered through a 100 µm nylon membrane and hepatocytes and non-parenchymal cells were separated by three rounds of density-based centrifugation. Total RNA was extracted from these enriched cell populations using TRIzol (Life Technologies, Carlsbad, California) according to the manufacturer's instructions and subsequently treated with DNase I (Promega, Madison, Wisconsin). RNA quality was assessed using a Bioanalyzer (Agilent Technologies, Santa Clara, California) with a quality cut-off of RNA Integrity Number >8. Using 200 ng of total RNA, whole-genome gene-expression profiling was performed using RatRef-12 BeadChip microarrays (Illumina, San Diego, California) as described[7,8].

Liver slices. Fresh liver tissue sections (300 μm-thick) were made from surgically resected fibrotic livers from HCC patients using Krumdieck Tissue Slicer MD6000 (Alabama Research and Development, Munford, Alabama). The tissues were cultured with erlotinib (5 μM) or pioglitazone (10 μM) for 48 hours and harvested for gene expression analysis.

Transcriptional analyses. Liver cells were lysed in TRI-reagent (Molecular Research Center; Cincinnati, Ohio), and RNA was purified using Direct-zol RNA MiniPrep (Zymo Research, Irvine, California) according to manufacturer's instructions. RNA quantity and quality was assessed using NanoDrop (Thermo Scientific, Waltham, Massachusetts) and Bioanalyzer 2100 (Illumina, San Diego, California) with a quality cut-off of RNA Integrity Number >8. RNA from HDV- and HBV-infected liver cells as well as from human liver tissue was purified using the RNeasy kit (Qiagen, Hilden, Germany). Gene expression profiling was performed using 250-500 ng total RNA by using either nCounter Digital Analyzer system (NanoString) or the HumanHT-12 beadarray (Illumina, San Diego, California). *EGF* and *EGFR* mRNA expression was analyzed by qRT-PCR using specific TaqMan Gene Expression Assays (Life Technologies, Carlsbad, California; #Hs01099999_m1 and #Hs01076078_m1, respectively) according to the manufacturer's instructions. The expression of *GAPDH* mRNA was analyzed as a reference gene using a specific TaqMan Gene Expression Assays (Life Technologies, Carlsbad, California; #4333764). Relative gene expression level was calculated by the ΔΔCt method as described[59]. Single cell RNA-Seq paired-end reads were aligned to the human hg19 UCSC reference as well as the HCV Jc1 reference using TopHat[60-64]. Gene expression levels for 21948 human genes were estimated using Cufflinks[64-68].

Proteomic analyses. For proteomic analyses, Huh7.5.1^{dif} cells that had undergone DMSO-mediated differentiation, were infected with HCV Jc1E2^{FLAG} ($TCID_{50}$=10^7/mL) as described above. On day 7, receptor tyrosine kinase (RTK) phosphorylation was assessed in cell lysates

using the Human Phospho-RTK Array Kit as previously described[27,28]. The effect of MAPK inhibitors on signaling was confirmed using the Proteome Profiler Human Phospho-kinase Array according to manufacturer's instructions (R&D Systems). Amounts of phospho-proteins were assessed using a horseradish peroxidase–conjugated pan–phospho-tyrosine–specific antibody (R&D Systems, Minneapolis, Minnesota) followed by chemiluminescence detection (GE Healthcare, Cleveland, Ohio) according to manufacturer's instructions. The relative dot-blot density of the phosphorylated proteins in HCV Jc1E2^{FLAG} infected samples compared to non-infected controls was quantified using Image J software (NIH) by elliptical selection of individual dots and measuring standard deviation and integrated density.

Metabolomics. Analysis of polar metabolites was performed in Huh7.5.1^{dif} cells infected with HCV Jc1E2^{FLAG} ($TCID_{50}$=10^5/mL). On day 7, intra- and extra-cellular metabolites were extracted from cell lysates and supernatants, respectively, and further analyzed by mass spectrometry similar as described[24,69].

Bioinformatics. Induction or suppression of HCC risk signature in HumanHT-12 beadarray (Illumina, San Diego, California) data was determined as previously reported by using Nearest Template Prediction algorithm and Gene Set Enrichment Analysis (GSEA), implemented in GenePattern genomic analysis toolkits[13,39,70]. Molecular pathway deregulations were determined in Molecular Signature Database (MSigDB, ver.4.0)[71] using GSEA[13]. Liver- or HCC-specific gene signatures were defined by comparing global transcriptome profiles of 82 human liver tissues and 118 HCC tumor tissues from our previous studies[5,72] using random permutation-based t-test implemented in GenePattern. Genome-wide transcriptome profiles in Huh7.5.1^{dif} cells were compared to previously published transcriptomic data in naïve Huh7.5 cells (NCBI Gene Expression Omnibus database, accession number: GSE62546). Induction or repression of HCC high- and low-risk genes, the EGFR pathway, and pioglitazone target genes in

association with the normalized HCV copy number in single cell RNA-Seq data was determined using the pre-ranked GSEA module implemented in GenePattern[13,14,70]. Transcriptome-based *in silico* drug screening was performed using the HCC risk signature as a query in the chemogenomics database connectivity map (www.broadinstitute.org/cmap) as previously described[31,33,73-75]. Comparison between the cell-based systems and clinical datasets was performed using Subclass Mapping algorithm[76] implemented in GenePattern. Co-regulated gene networks and enriched molecular pathways in the HCC high-risk genes common to multiple HCC etiologies were determined using Ingenuity Pathway Analysis (www.ingenuity.com/). False discovery rate (FDR) <0.05 or Bonferroni-corrected p <0.05 were regarded as statistically significant. All genomic datasets used for this study are available at NCBI Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo, accession number: GSE66843).

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Figure 1

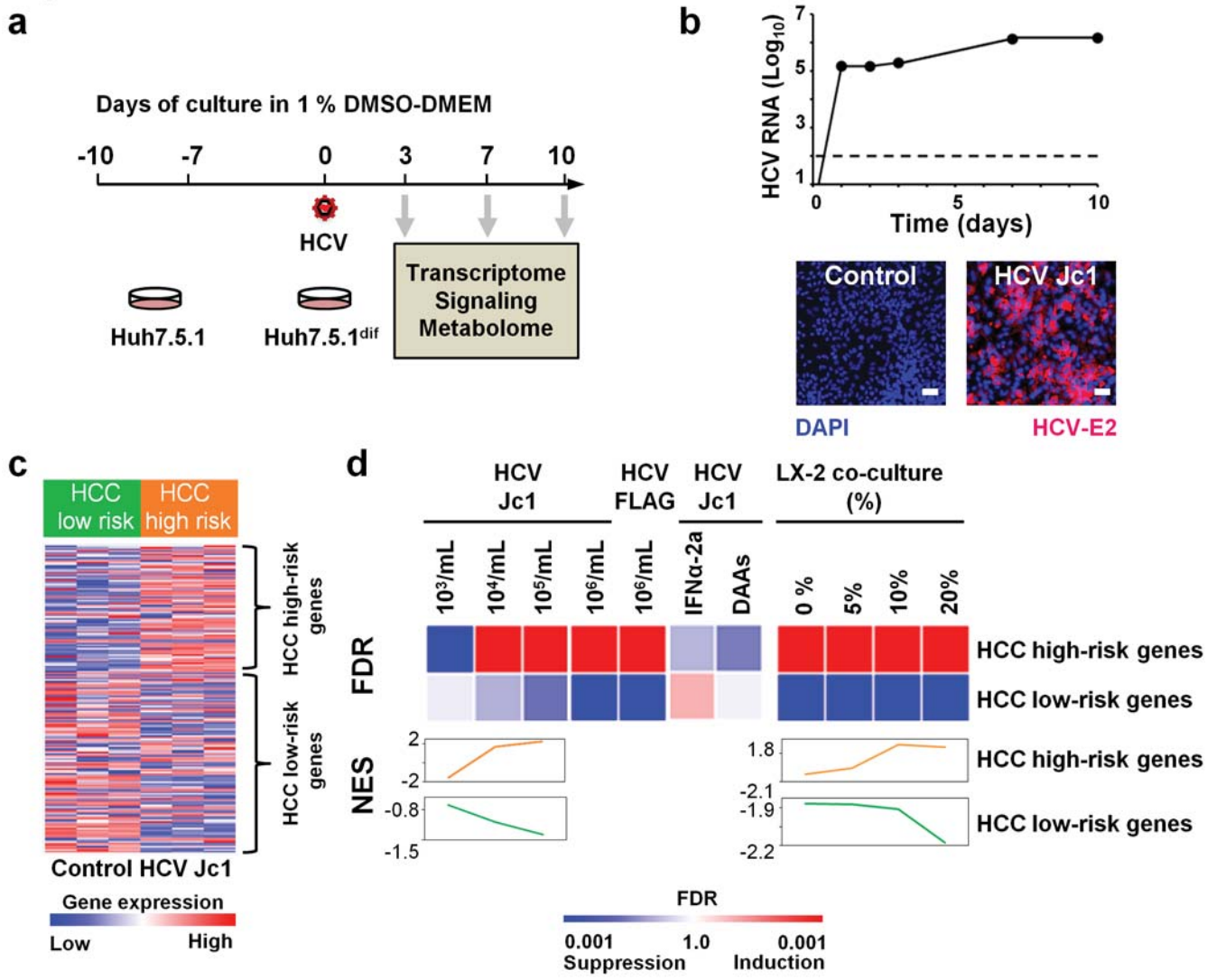

a
Days of culture in 1 % DMSO-DMEM
-10
-7
0
3
7
10
HCV
Huh7.5.1
Huh7.5.1dif
Transcriptome
Signaling
Metabolome
b
HCV RNA (Log10)
7
5
3
1
0
5
10
Time (days)
Control
HCV Jc1
DAPI
HCV-E2
c
HCC low risk
HCC high risk
HCC high-risk genes
HCC low-risk genes
Control HCV Jc1
Gene expression
Low
High
d
HCV Jc1
HCV FLAG
HCV Jc1
LX-2 co-culture (%)
10³/mL
10⁴/mL
10⁵/mL
10⁶/mL
10⁶/mL
IFNα-2a
DAAs
0 %
5%
10%
20%
FDR
NES
HCC high-risk genes
HCC low-risk genes
2
-2
-0.8
-1.5
1.8
-2.1
-1.9
-2.2
FDR
0.001
Suppression
1.0
0.001
Induction

Figure 2

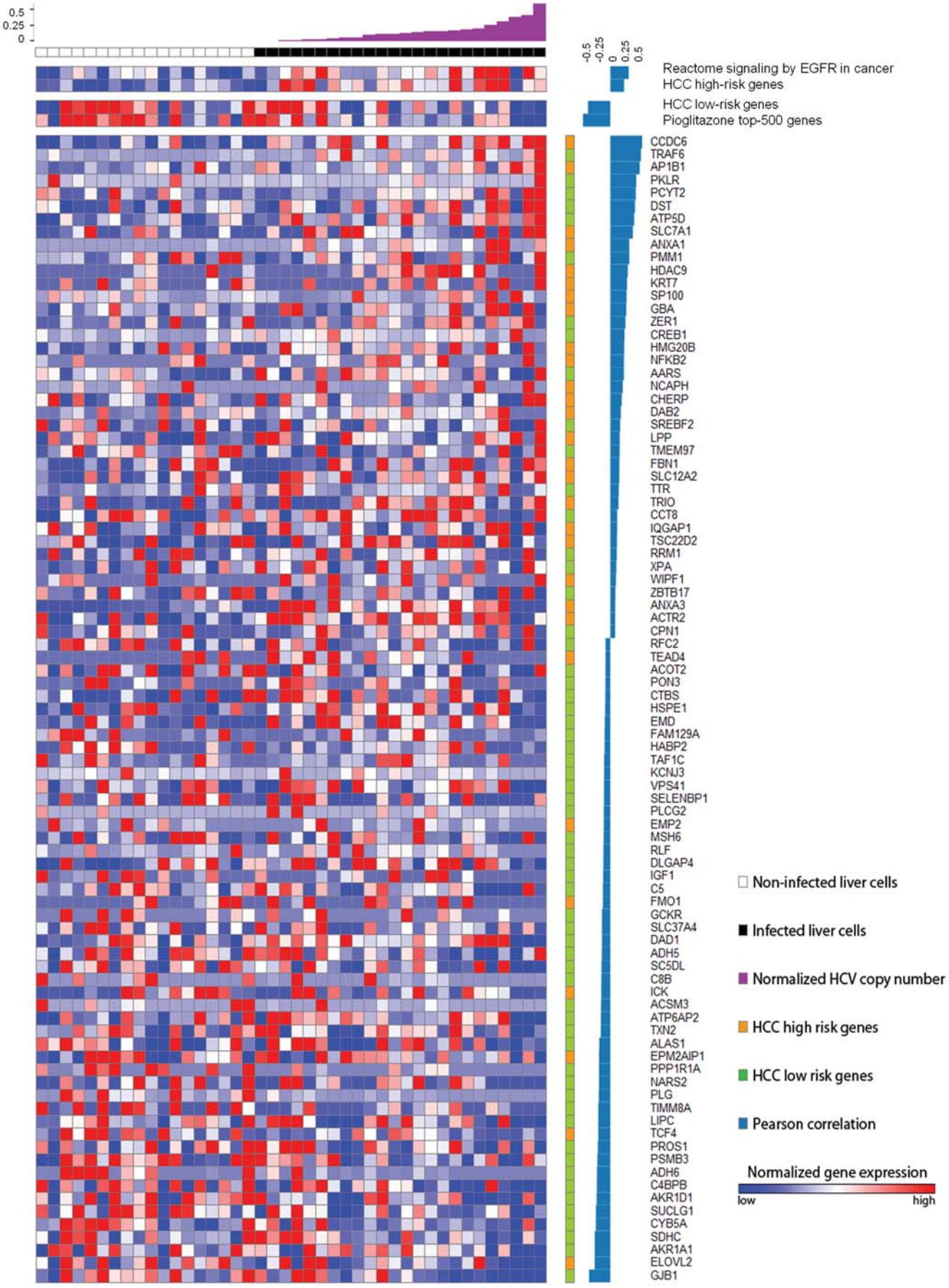

0.5
0.25
0
-0.5
-0.25
0
0.25
0.5
Reactome signaling by EGFR in cancer
HCC high-risk genes
HCC low-risk genes
Pioglitazone top-500 genes
CCDC6
TRAF6
AP1B1
PKLR
PCYT2
DST
ATP5D
SLC7A1
ANXA1
PMM1
HDAC9
KRT7
SP100
GBA
ZER1
CREB1
HMG20B
NFKB2
AARS
NCAPH
CHERP
DAB2
SREBF2
LPP
TMEM97
FBN1
SLC12A2
TTR
TRIO
CCT8
IQGAP1
TSC22D2
RRM1
XPA
WIPF1
ZBTB17
ANXA3
ACTR2
CPN1
RFC2
TEAD4
ACOT2
PON3
CTBS
HSPE1
EMD
FAM129A
HABP2
TAF1C
KCNJ3
VPS41
SELENBP1
PLCG2
EMP2
MSH6
RLF
DLGAP4
IGF1
C5
FMO1
GCKR
SLC37A4
DAD1
ADH5
SC5DL
C8B
ICK
ACSM3
ATP6AP2
TXN2
ALAS1
EPM2AIP1
PPP1R1A
NARS2
PLG
TIMM8A
LIPC
TCF4
PROS1
PSMB3
ADH6
C4BPB
AKR1D1
SUCLG1
CYB5A
SDHC
AKR1A1
ELOVL2
GJB1
Non-infected liver cells
Infected liver cells
Normalized HCV copy number
HCC high risk genes
HCC low risk genes
Pearson correlation
Normalized gene expression
low
high

Figure 3

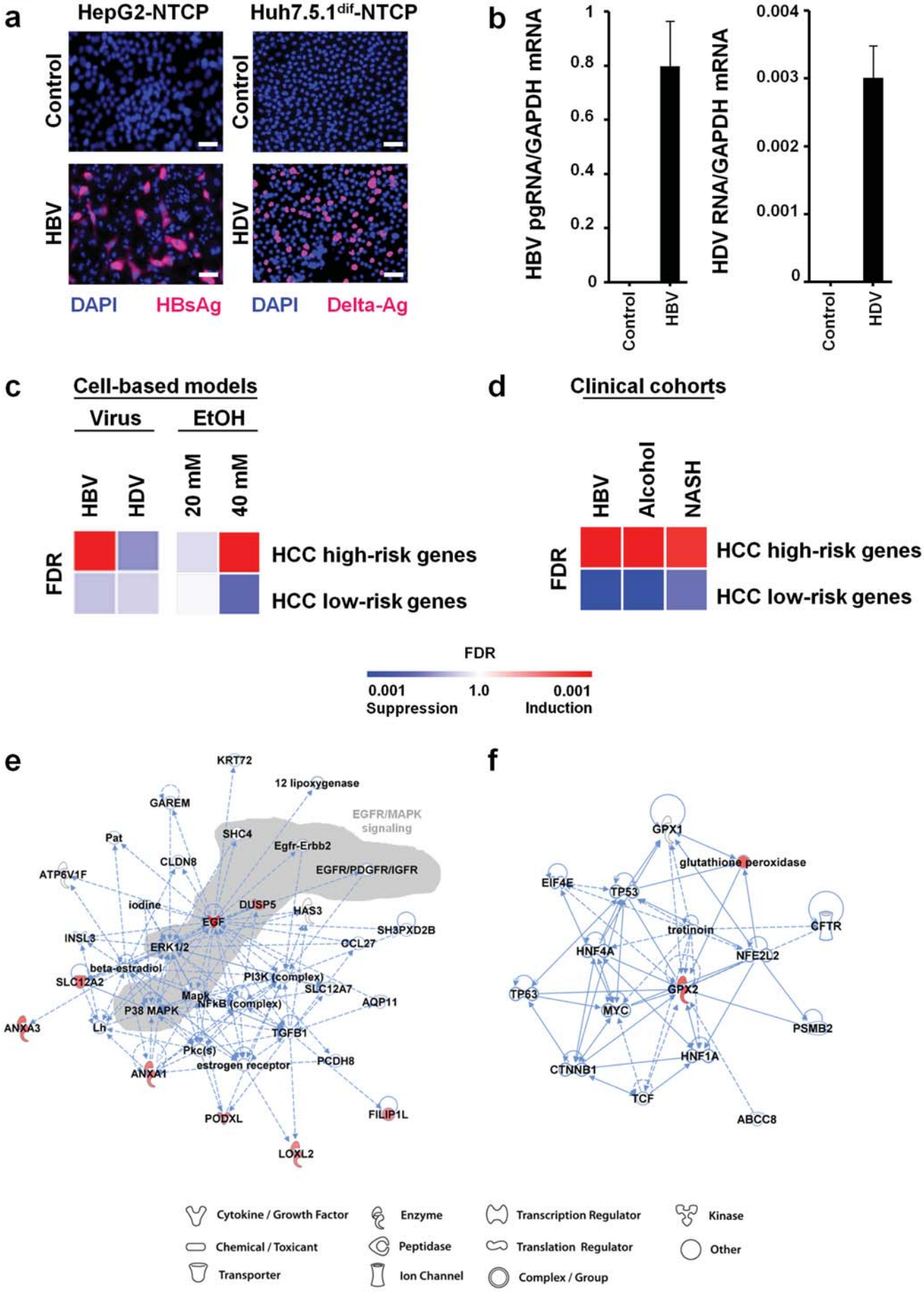
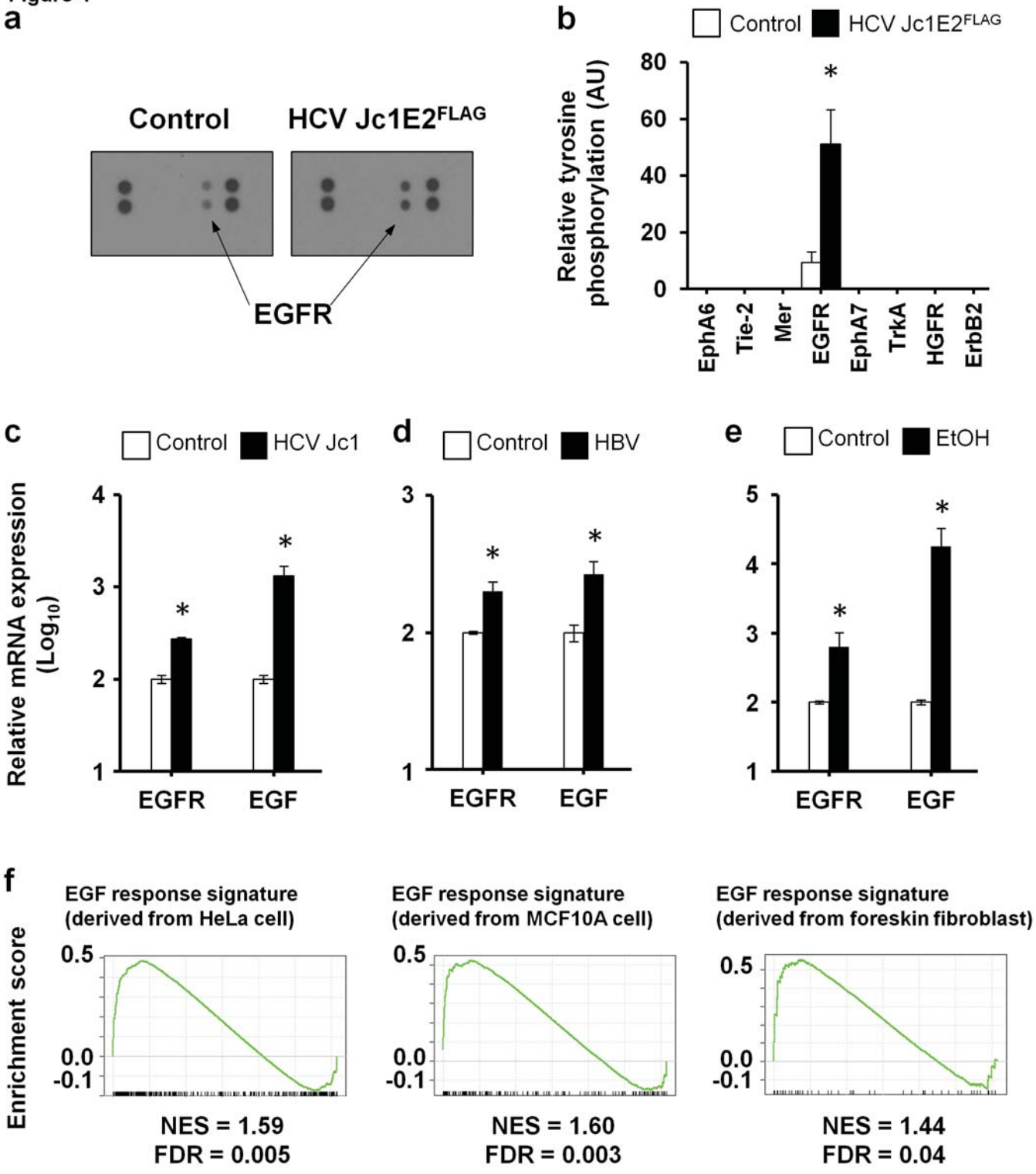

a
HepG2-NTCP
Huh7.5.1dif-NTCP
Control
HBV
HDV
DAPI
HBsAg
Delta-Ag
b
HBV pgRNA/GAPDH mRNA
HDV RNA/GAPDH mRNA
Control
HBV
HDV
c
Cell-based models
Virus
EtOH
HBV
HDV
20 mM
40 mM
FDR
HCC high-risk genes
HCC low-risk genes
d
Clinical cohorts
HBV
Alcohol
NASH
FDR
HCC high-risk genes
HCC low-risk genes
FDR
0.001
Suppression
1.0
0.001
Induction
e
KRT72
12 lipoxygenase
GAREM
EGFR/MAPK signaling
Pat
SHC4
Egfr-Erbb2
CLDN8
EGFR/PDGFR/IGFR
ATP6V1F
iodine
DUSP5
HAS3
EGF
SH3PXD2B
INSL3
ERK1/2
OCL27
beta-estradiol
PI3K (complex)
SLC12A2
SLC12A7
Mapk
NFkB (complex)
AQP11
P38 MAPK
ANXA3
Lh
TGFB1
Pkc(s)
estrogen receptor
PCDH8
ANXA1
PODXL
FILIP1L
LOXL2
f
GPX1
glutathione peroxidase
EIF4E
TP53
tretinoin
CFTR
HNF4A
NFE2L2
TP63
GPX2
MYC
PSMB2
HNF1A
CTNNB1
TCF
ABCC8
Cytokine / Growth Factor
Enzyme
Transcription Regulator
Kinase
Chemical / Toxicant
Peptidase
Translation Regulator
Other
Transporter
Ion Channel
Complex / Group

Figure 4


a
Control
HCV Jc1E2FLAG
EGFR
b
Control
HCV Jc1E2FLAG
Relative tyrosine phosphorylation (AU)
80
60
40
20
0
*
EphA6
Tie-2
Mer
EGFR
EphA7
TrkA
HGFR
ErbB2
c
Control
HCV Jc1
Relative mRNA expression (Log10)
4
3
2
1
*
*
EGFR
EGF
d
Control
HBV
3
2
1
*
*
EGFR
EGF
e
Control
EtOH
5
4
3
2
1
*
*
EGFR
EGF
f
EGF response signature (derived from HeLa cell)
EGF response signature (derived from MCF10A cell)
EGF response signature (derived from foreskin fibroblast)
Enrichment score
0.5
0.0
-0.1
NES = 1.59
FDR = 0.005
NES = 1.60
FDR = 0.003
NES = 1.44
FDR = 0.04

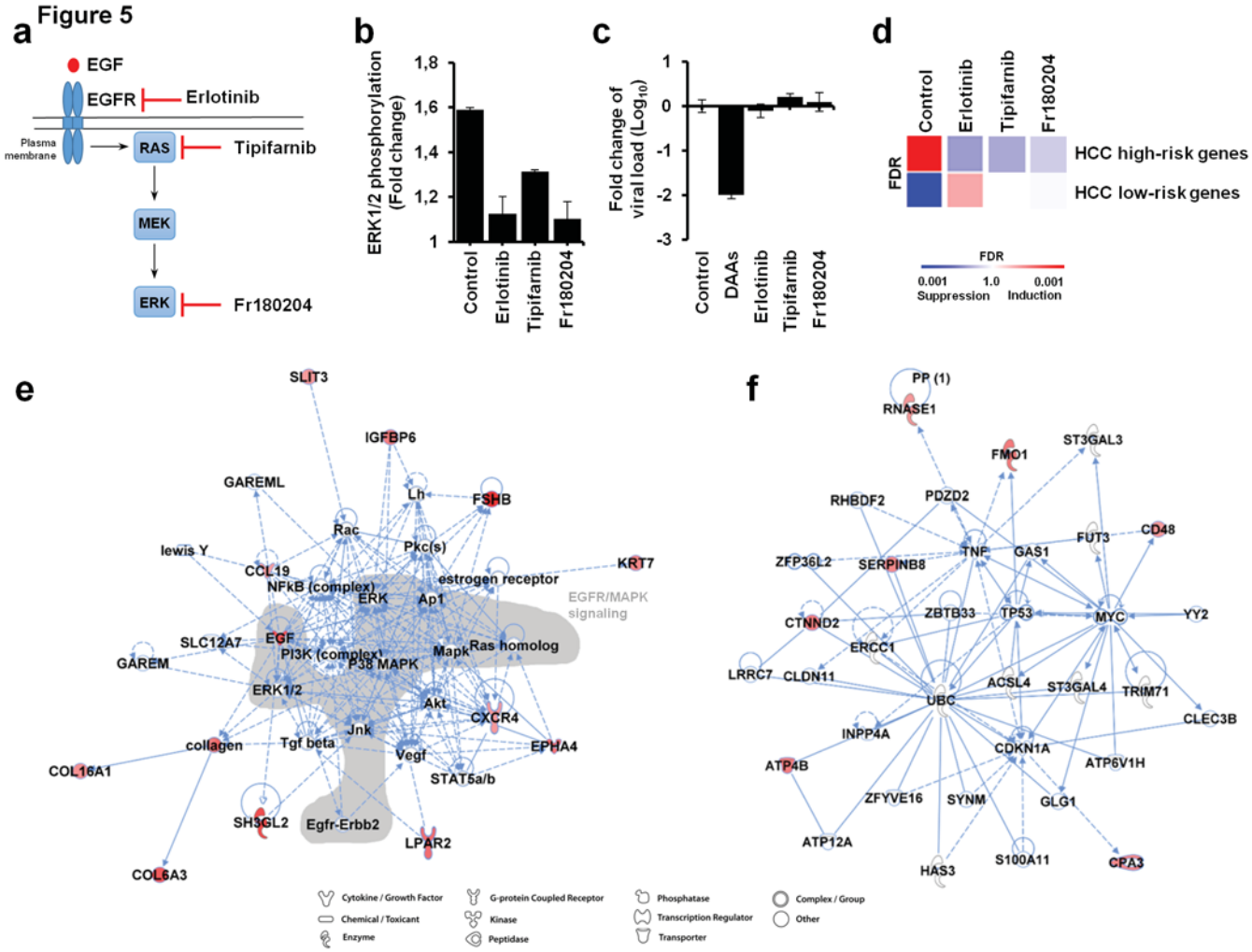
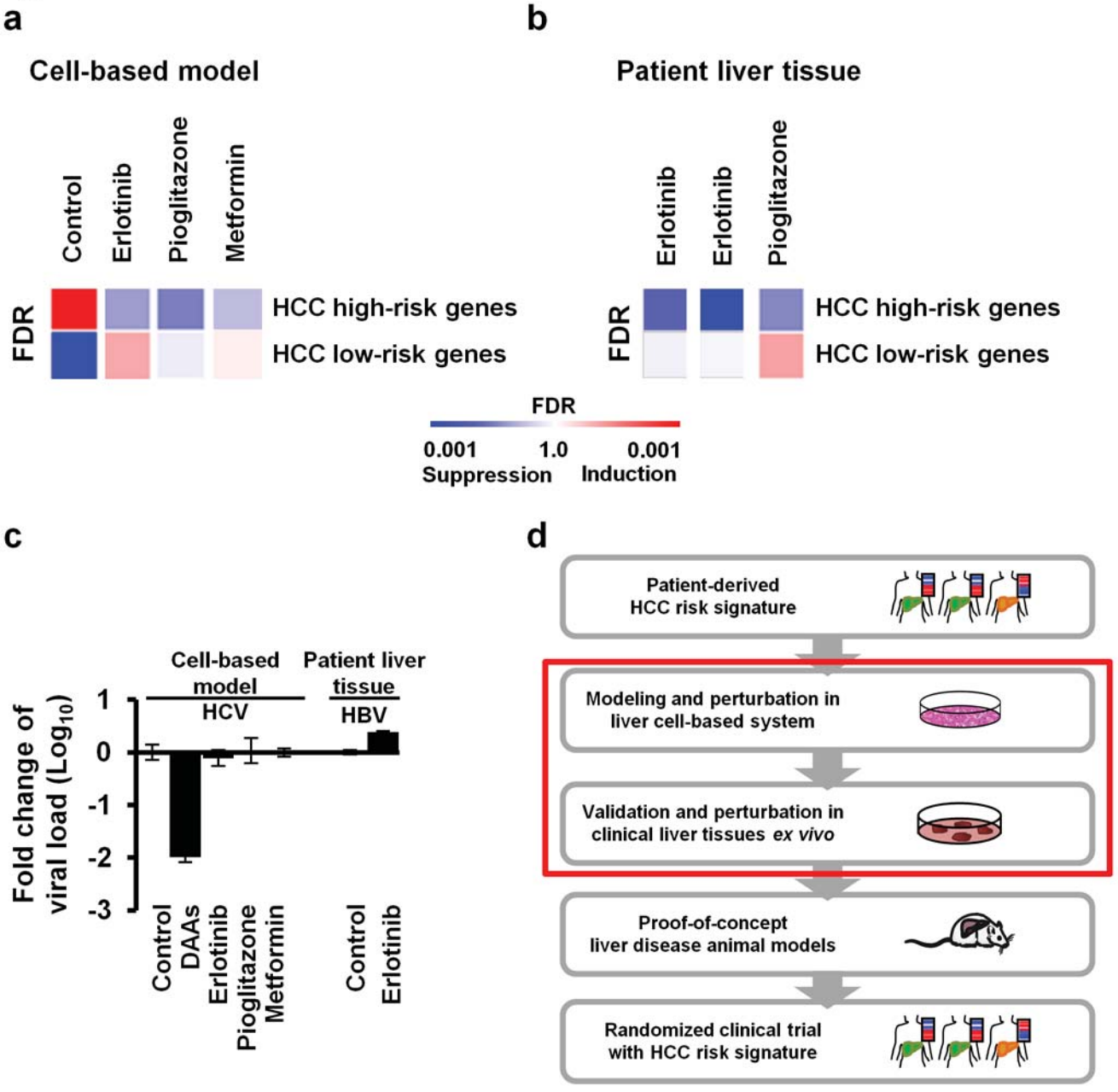


Figure 5
a
EGF
EGFR
Erlotinib
Plasma membrane
RAS
Tipifarnib
MEK
ERK
Fr180204
b
ERK1/2 phosphorylation (Fold change)
1,8
1,6
1,4
1,2
1
Control
Erlotinib
Tipifarnib
Fr180204
c
Fold change of viral load (Log10)
1
0
-1
-2
-3
Control
DAAs
Erlotinib
Tipifarnib
Fr180204
d
Control
Erlotinib
Tipifarnib
Fr180204
FDR
HCC high-risk genes
HCC low-risk genes
FDR
0.001
1.0
0.001
Suppression
Induction
e
SLIT3
IGFBP6
GAREML
Lh
FSHB
Rac
Pkc(s)
lewis Y
CCL19
NFkB (complex)
ERK
Ap1
estrogen receptor
KRT7
EGFR/MAPK signaling
SLC12A7
EGF
PI3K (complex)
P38 MAPK
Mapk
Ras homolog
GAREM
ERK1/2
Akt
CXCR4
Jnk
collagen
Tgf beta
Vegf
EPHA4
COL16A1
STAT5a/b
SH3GL2
Egfr-Erbb2
LPAR2
COL6A3
f
PP (1)
RNASE1
FMO1
ST3GAL3
RHBDF2
PDZD2
FUT3
CD48
ZFP36L2
SERPINB8
TNF
GAS1
CTNND2
ZBTB33
TP53
MYC
YY2
ERCC1
LRRC7
CLDN11
ACSL4
ST3GAL4
TRIM71
UBC
CLEC3B
INPP4A
CDKN1A
ATP4B
ATP6V1H
ZFYVE16
SYNM
GLG1
ATP12A
S100A11
CPA3
HAS3
Cytokine / Growth Factor
Chemical / Toxicant
Enzyme
G-protein Coupled Receptor
Kinase
Peptidase
Phosphatase
Transcription Regulator
Transporter
Complex / Group
Other

Figure 6


a
Cell-based model
Control
Erlotinib
Pioglitazone
Metformin
FDR
HCC high-risk genes
HCC low-risk genes
b
Patient liver tissue
Erlotinib
Erlotinib
Pioglitazone
FDR
HCC high-risk genes
HCC low-risk genes
FDR
0.001
1.0
0.001
Suppression
Induction
c
Cell-based model
Patient liver tissue
HCV
HBV
Fold change of viral load (Log10)
1
0
-1
-2
-3
Control
DAAs
Erlotinib
Pioglitazone
Metformin
Control
Erlotinib
d
Patient-derived HCC risk signature
Modeling and perturbation in liver cell-based system
Validation and perturbation in clinical liver tissues ex vivo
Proof-of-concept liver disease animal models
Randomized clinical trial with HCC risk signature

A clinical gene signature-based human cell culture model unravels drivers of hepatocarcinogenesis and compounds for cancer chemoprevention

Simonetta Bandiera, Anu Venkatesh, Nicolaas Van Renne, Xiaochen Sun, Joachim Lupberger, Christine Thumann, Hussein El Saghire, Eloi R. Verrier, Sarah C. Durand, Sophie Pernot, Shigeki Nakagawa, Lan Wei, Karun Kiani, Magali Soumillon, Bryan C. Fuchs, Raymond T. Chung, Nicolas Goossens, Anna Koh, Milind Mahajan, Venugopalan D. Nair, Ganesh Gunasekaran, Myron E. Schwartz, Kenneth K. Tanabe, Brandon N. Nicolay, Nabeel Bardeesy, Alex K. Shalek, Orit Rozenblatt-Rosen, Aviv Regev, Nathalie Pochet, Mirjam B. Zeisel, Yujin Hoshida, Thomas F. Baumert

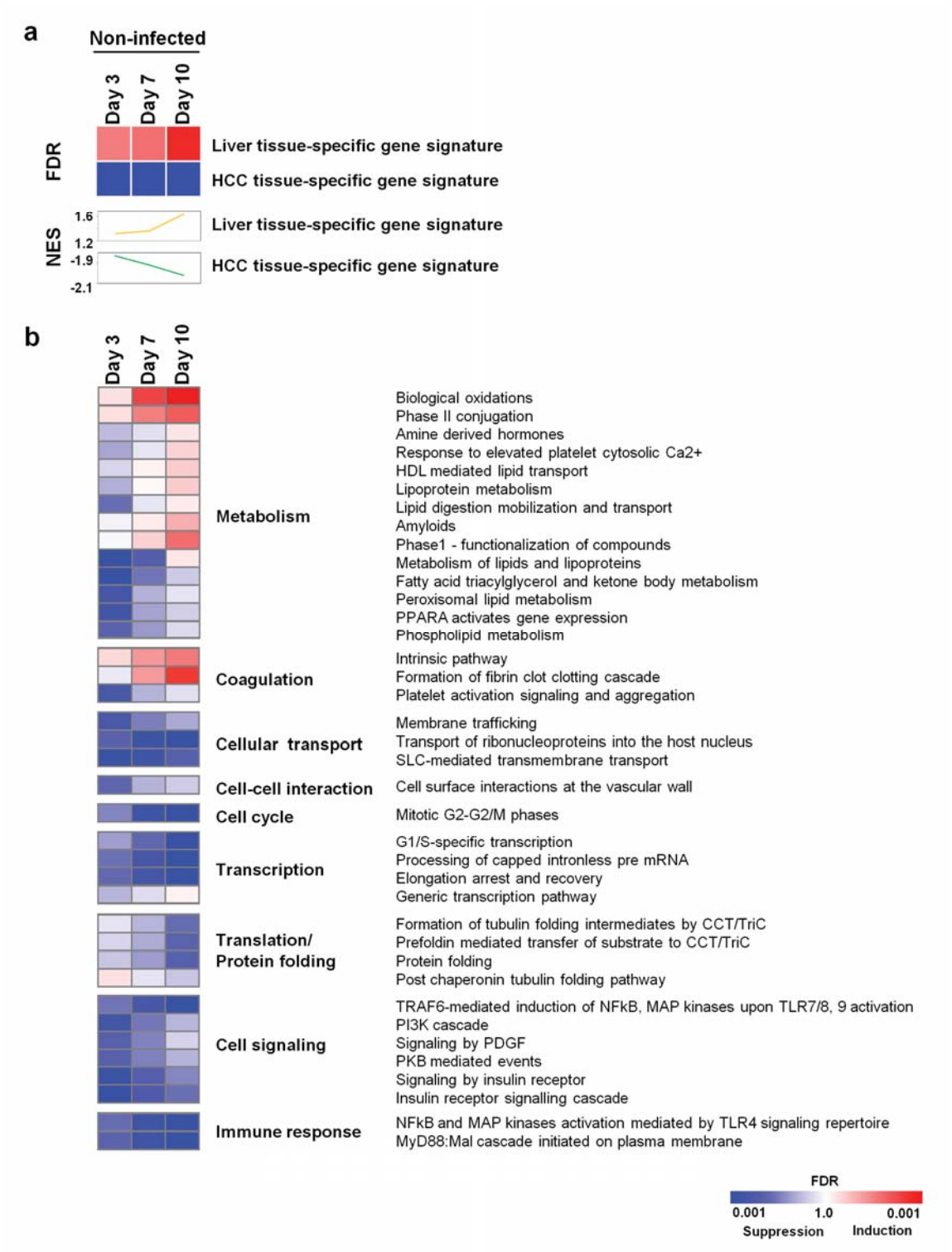
Supplementary Information

SUPPLEMENTARY FIGURES

Supplementary Figure 1. Time-course of molecular pathway modulation by HCV infection (Jc1 clone) in Huh7.5.1^{dif} cells.

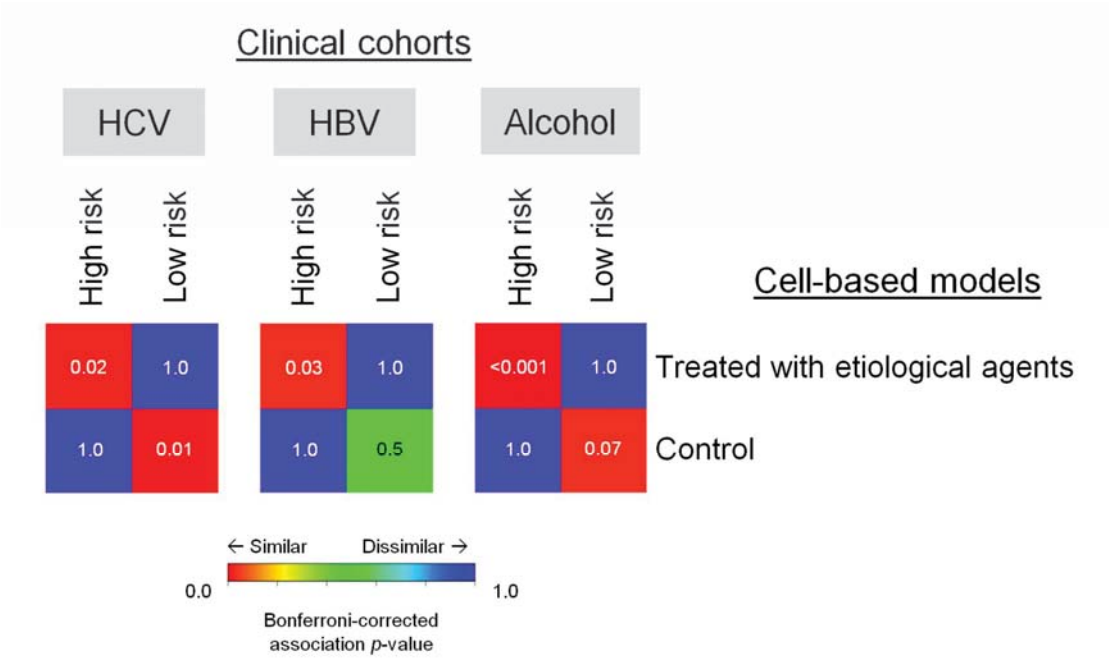
Differentiated hepatocyte-like Huh7.5.1^{dif} cells were infected with HCV Jc1 and RNA was isolated and analyzed as described in **Fig. 1a**. Induction or suppression of molecular pathways was assessed using Gene Set Enrichment Analysis (GSEA;[1]) on a comprehensive curated pathway database, Reactome (www.reactome.org). Significance of induction or suppression of each pathway is shown by false discovery rate (FDR) of gene set enrichment. In the heatmap, dense red indicates significant induction, blue indicates significant suppression. Gradually induced or suppressed gene sets overtime were selected based on absolute correlation coefficient with a vector (1, 2, 3) >0.95 and maximum index – minimum index > 1. Results are representative of one experiment performed in triplicate. Metabolism- and coagulation-related pathways, except carbohydrate metabolism pathway, were suppressed by HCV Jc1 infection overtime. Genome maintenance-related pathways were also suppressed. Cell proliferation-, signaling-, and immune response-related pathways were gradually induced.

Supplementary Figure 2. Time-course of molecular pathway modulation during DMSO-induced differentiation of Huh7.5.1 into hepatocyte-like cells.



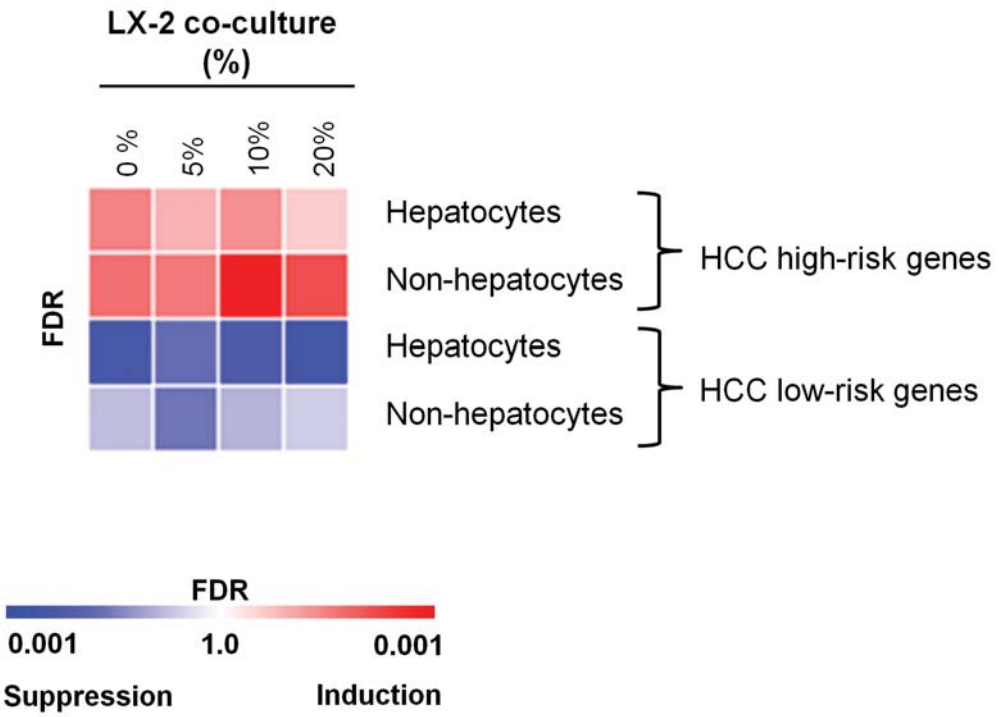
Naïve Huh7.5.1 cells were differentiated with DMSO and RNA was isolated and analyzed as described in **Fig. 1a. a.** DMSO-differentiation induces a hepatocyte-like phenotype in Huh7.5.1^{dif} cells as shown by a gradual shift of an HCC tissue-specific gene signature to a non-cancer liver tissue-specific gene signature over time. Modulation of 482 genes specifically over-expressed in human non-malignant liver tissue (liver tissue-specific gene signature) or 1,106 genes specifically over-expressed in HCC tissues (HCC tissue-specific gene signature) defined in genome-wide transcriptome profiles of 200 clinical specimens (Methods) was assessed in Huh7.5.1^{dif} cells. Genome-wide transcriptome profiles in Huh7.5.1^{dif} cells were compared to previously published transcriptomic data in naïve Huh7.5 cells (see Methods for details). In the heatmap, dense red indicates significant induction, blue indicates significant suppression. FDR: false discovery rate; NES: normalized enrichment score. Results are representative of one experiment performed in triplicate. **b.** Induction or suppression of molecular pathways was assessed using Gene Set Enrichment Analysis (GSEA;[1]) on a comprehensive curated pathway database, Reactome (www.reactome.org). Significance of induction or suppression of each pathway is shown by FDR of gene set enrichment. Gradually induced or suppressed gene sets overtime were selected based on absolute correlation coefficient with a vector (1, 2, 3) >0.95 and maximum index – minimum index > 1. Results are representative of one experiment performed in triplicate. Metabolism- and coagulation-related pathways, the major functions of matured hepatocytes, were gradually induced. In contrast, cell proliferation-, signaling-, and immune response-related pathways were suppressed overtime.

Supplementary Figure 3. Similarity of global transcriptome between clinical cohorts and the cell-based models for each major HCC etiology.



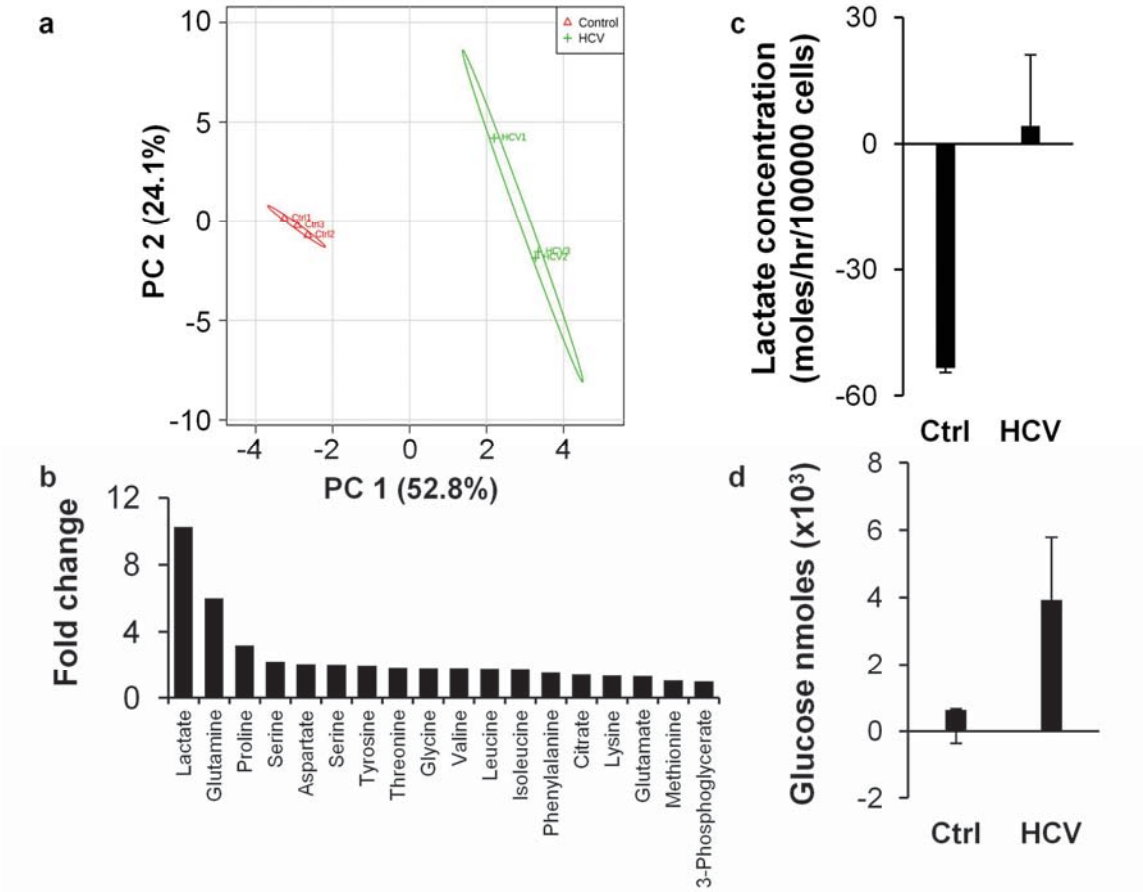
Similarity of transcriptome patterns in our cell-based systems (HCV- or HBV-infected, or ethanol-treated, n = 3) were compared to liver transcriptome profiles from published clinical cohorts of HCV-related cirrhosis (n = 145, NCBI, Gene Expression Omnibus, accession number GSE54100)[2], HBV-related HCC (n = 199, GSE14520)[3], and alcoholic hepatitis (n = 22, GSE28619)[4] using a bi-directional gene signature-based similarity determination method, Subclass Mapping[5]. Statistically significant similarity was observed between the cells treated with the etiological agents and a subset of patients with poorer prognosis or severer disease manifestation (Bonferroni-corrected p<0.05). The resemblance of the HCC low-risk signature genes in the cell-based system with healthier clinical liver was less pronounced in the HBV infection model using HepG2-NTCP cells, currently the only available cell line allowing robust HBV infection (**Fig. 3a-b**). This is consistent with the less differentiated phenotype and morphology of HepG2 cells, which were originally derived from hepatoblastoma, not HCC (**Fig. 3a-b**)[6].

Supplementary Figure 4. Induction of HCC risk signature genes preferentially expressed in hepatocyte or non-hepatocyte fraction from cirrhotic liver tissue in co-culture of Huh7.5.1^{dif} cells with LX-2 stellate cell line.



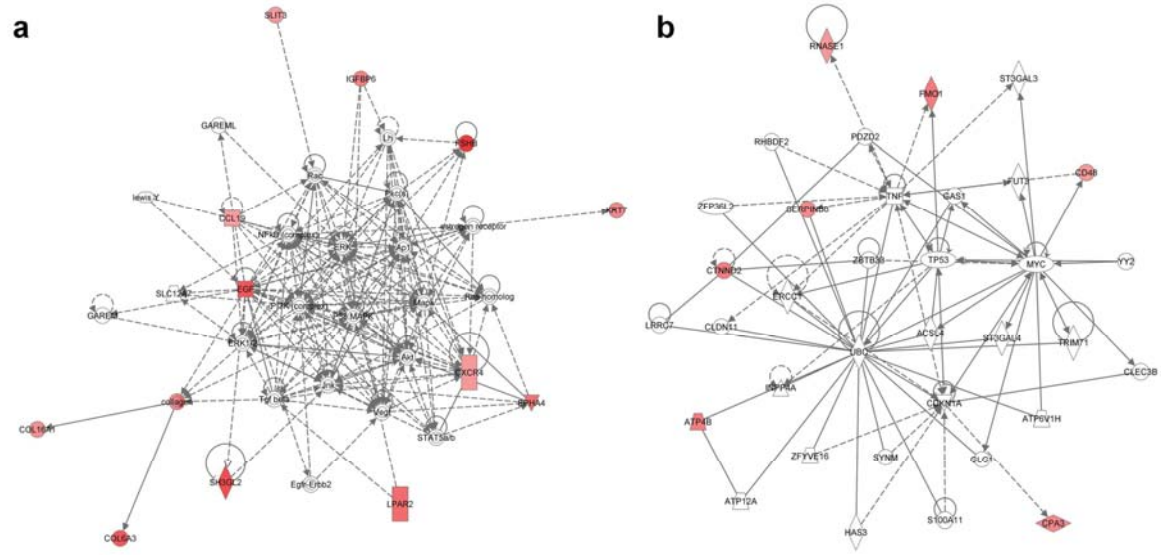
Huh7.5.1^{dif} cells were infected with HCV Jc1 ($TCID_{50}$ = 10^6/mL) and co-cultured with hepatic stellate cell line LX-2 cells at different proportions (0%, 5%, 10%, and 20%) as described in **Fig. 1a** and **d**. On day 10 post-infection, cellular RNA was purified and the HCC risk signature was profiled. HCC high- and low-risk genes were classified into either hepatocyte or non-hepatocyte origin based on global transcriptome profiles of isolated hepatocyte and non-hepatocyte (hepatic stellate cell-enriched) fractions from cirrhotic livers of low-dose diethylnitrosamine-treated rat model of cirrhosis-driven HCC[7]. Heatmap displays the induction/suppression of each group of signature genes assessed by GSEA in red/blue, respectively. FDR: false discovery rate. Results are representative of one experiment performed in triplicate.

Supplementary Figure 5. Persistent HCV infection perturbs glucose consumption and lactate metabolism in differentiated Huh7.5.1^{dif} liver cells.



Differentiated Huh7.5.1^{dif} cells were infected using HCV Jc1E2^{FLAG} ($TCID_{50}$ = 10^5/mL) as described in **Fig. 1a**. On day 7, metabolites were extracted followed by mass spectrometry[8], and data analyzed as described[9]. **a.** Principle Component Analysis (PCA) shows clustering of metabolites in control and HCV Jc1E2^{FLAG}-infected cells. **b.** Fold changes of detected metabolites indicated on the x-axis comparing infected to uninfected metabolite concentrations (Ctrl). **c.** Liver cell lactate flux. Negative values: accumulation outside the cells, positive values: accumulation inside the cells. **d.** Glucose consumption. Persistent HCV Jc1E2^{FLAG} infection in a differentiated liver cell line induces metabolic changes similar to profiles observed in tumor cells[10]. Mean ± s.e.m. of one experiment in triplicate is shown.

Supplementary Figure 6. Co-regulated gene networks in HCC high-risk genes common to HBV-related liver disease, alcoholic liver disease and NASH (Ingenuity Pathway Analysis).



186-gene HCC risk signature was analyzed as described in **Fig.1** in clinical liver tissues from HBV-related liver disease, alcoholic liver disease, and NASH. The HCC high-risk correlated genes that were commonly modulated in clinical samples as core enriched genes in GSEA were then analyzed by Ingenuity Pathway Analysis tools as described in Methods. A fibrosis-related network including the EGFR/MAPK signaling pathway (**a**) and an oncogenic network including the p53/Myc pathway (**b**) are shown. The HCC high-risk genes included in networks are in red. Solid and dashed lines indicate direct and indirect interactions, respectively.

SUPPLEMENTARY TABLES

Supplementary Table 1. Gene sets modulated by persistent HCV Jc1 infection in Huh7.5.1^{dif} cells (Gene Set Enrichment Analysis). Gene expression profiles of uninfected (Control) and HCV Jc1-infected Huh7.5.1^{dif} cells were analyzed by GSEA as described in Methods. The molecular pathway gene sets were retrieved from Molecular Signature Database (MSigDB, www.broadinstitute.org/msigdb). Gene sets with significant enrichment (FDR <0.25) or top 20 are shown. NES: normalized enrichment score, FDR: false discovery rate. Available as datasheet (.xls).

Supplementary Table 2. HCC high-risk signature genes commonly induced by HCV, HBV and ethanol in liver cell-based systems. Genes contributing to the GSEA enrichment (core enrichment genes) that were common to HCV/HBV infection and alcohol treatment are shown.

Gene symbol	Gene title	Entrez Gene ID
ANXA1	annexin A1	301
ANXA3	annexin A3	306
DUSP5	dual specificity phosphatase 5	1847
EGF	epidermal growth factor	1950
FILIP1L	filamin A interacting protein 1-like	11259
GPX2	glutathione peroxidase 2 (gastrointestinal)	2877
LOXL2	lysyl oxidase-like 2	4017
PODXL	podocalyxin-like	5420
SLC12A2	solute carrier family 12 (sodium/potassium/chloride transporter), member 2	6558

Supplementary Table 3. Enriched canonical pathways in HCC high-risk genes common to HCV, HBV and ethanol-treated liver cell-based models (Ingenuity Pathway Analysis). Pathways with enrichment *p*-value less than 0.05 are shown.

Pathway	*p*-value
Glutathione Redox Reactions I	0.008
EGF Signaling	0.025
Regulation of Cellular Mechanics by Calpain Protease	0.025
ERK5 Signaling	0.028
Non-Small Cell Lung Cancer Signaling	0.029
Macropinocytosis Signaling	0.030
Caveolar-mediated Endocytosis Signaling	0.032
HER-2 Signaling in Breast Cancer	0.034
ErbB Signaling	0.038
Bladder Cancer Signaling	0.038
FAK Signaling	0.038
Neuregulin Signaling	0.039
Glioma Signaling	0.042
Telomerase Signaling	0.044
Pancreatic Adenocarcinoma Signaling	0.047

Supplementary Table 4. Drugs predicted to reverse the HCC risk gene signature (connectivity map analysis). Compounds with significant negative enrichment (p<0.05) are shown.

Compound name	No. of instances	Enrichment score	p-value	Specificity score
CP-690334-01	8	-0.620	0.002	0.034
Genistein	17	-0.418	0.004	0.121
Cefazolin	5	-0.708	0.005	0.000
Pioglitazone	11	-0.500	0.005	0.091
Bretylium tosilate	4	-0.770	0.006	0.006
Triamcinolone	5	-0.692	0.006	0.000
Isocarboxazid	5	-0.689	0.007	0.007
Semustine	4	-0.746	0.008	0.044
Melatonin	4	-0.745	0.008	0.032
Primidone	4	-0.744	0.008	0.011
3-acetamidocoumarin	4	-0.740	0.009	0.136
2-aminobenzenesulfonamide	4	-0.738	0.009	0.035
Bacampicillin	4	-0.717	0.013	0.015
Resveratrol	9	-0.494	0.015	0.241
Ethosuximide	4	-0.706	0.015	0.023
Daunorubicin	4	-0.704	0.016	0.114
Bacitracin	3	-0.792	0.018	0.030
Chlorambucil	4	-0.694	0.019	0.058
Methotrexate	8	-0.509	0.019	0.096
Coralyne	4	-0.690	0.020	0.007
Verapamil	6	-0.576	0.020	0.000
SR-95531	4	-0.684	0.022	0.037
PNU-0251126	6	-0.571	0.022	0.080
Trimethobenzamide	5	-0.612	0.024	0.101
Tetraethylenepentamine	6	-0.566	0.025	0.017
Ramipril	4	-0.667	0.028	0.011
PHA-00745360	8	-0.488	0.028	0.295
Oxetacaine	5	-0.592	0.033	0.034
Captopril	5	-0.586	0.036	0.048
Cyclic adenosine monophosphate	4	-0.647	0.037	0.110
Demeclocycline	6	-0.539	0.038	0.111
Nizatidine	4	-0.641	0.040	0.079
Hydroxyachillin	4	-0.633	0.044	0.024
Pralidoxime	4	-0.631	0.045	0.047
Tolnaftate	5	-0.567	0.046	0.144
Cefixime	4	-0.625	0.049	0.034

For details about the metrics in the table, see www.broadinstitute.org/cmap.

Supplementary Table 5. Global transcriptome-based predicted pioglitazone target genes in Library of Integrated Cellular Signatures (LINCS) database (www.lincscloud.org). This table summarizes genes whose knockdown by shRNA showed similar transcriptome profiles with pioglitazone treatment.

Rank	cmap_ID	Gene symbol	Enrichment score
1	CGS001-4194	MDM4	92.1
3	CGS001-5427	POLE2	90.9
4	CGS001-1017	CDK2	89.7
5	CGS001-83439	TCF7L1	89.3
6	CGS001-7082	TJP1	87.6
7	CGS001-1459	CSNK2A2	87.2
8	CGS001-4149	MAX	86.8
9	CGS001-375449	MAST4	86.3
10	CGS001-4677	NARS	85.5
11	CGS001-5347	PLK1	85.4
12	CGS001-23387	SIK3	84.9
13	CGS001-10165	SLC25A13	83.8
14	CGS001-966	CD59	82.1
17	CGS001-23528	ZNF281	81.0
18	CGS001-23678	SGK3	80.6
20	CGS001-1869	E2F1	79.4
21	CGS001-2625	GATA3	79.0
22	CGS001-1737	DLAT	78.7
23	CGS001-84844	PHF5A	78.7
24	CGS001-2547	XRCC6	77.4
25	CGS001-4254	KITLG	77.3
26	CGS001-5013	OTX1	77.3
27	CGS001-7048	TGFBR2	76.8
28	CGS001-874	CBR3	76.7
29	CGS001-253430	IPMK	76.4
31	CGS001-89765	RSPH1	76.1
32	CGS001-3281	HSBP1	75.6
33	CGS001-51564	HDAC7	75.5
35	CGS001-84547	PGBD1	75.1
40	CGS001-10451	VAV3	72.6
41	CGS001-599	BCL2L2	72.5
42	CGS001-10252	SPRY1	72.3
43	CGS001-54957	TXNL4B	72.2
44	CGS001-3706	ITPKA	72.2

45	CGS001-57147	SCYL3	72.1
46	CGS001-223	ALDH9A1	72.1
47	CGS001-136	ADORA2B	72.1
48	CGS001-818	CAMK2G	71.6
49	CGS001-2639	GCDH	71.4
50	CGS001-5252	PHF1	71.4
51	CGS001-440435	GPR179	71.4
52	CGS001-4233	MET	71.4
53	CGS001-7849	PAX8	71.4
54	CGS001-4088	SMAD3	70.9
55	CGS001-23534	TNPO3	70.8
56	CGS001-2592	GALT	70.8
57	CGS001-5999	RGS4	70.3
58	CGS001-81539	SLC38A1	70.2
59	CGS001-2152	F3	70.2
60	CGS001-10600	USP16	70.2
62	CGS001-7976	FZD3	69.9
63	CGS001-1111	CHEK1	69.9
64	CGS001-558	AXL	69.8
65	CGS001-6773	STAT2	69.8
67	CGS001-11243	PMF1	69.6
68	CGS001-10607	TBL3	69.6
70	CGS001-1646	AKR1C2	69.4
71	CGS001-10128	LRPPRC	69.2
73	CGS001-5522	PPP2R2C	68.9
74	CGS001-64077	LHPP	68.8
75	CGS001-84923	FAM104A	68.8
76	CGS001-891	CCNB1	68.8
77	CGS001-1956	EGFR	68.8
78	CGS001-10038	PARP2	68.7
79	CGS001-115201	ATG4A	68.7
80	CGS001-64223	MLST8	68.4
81	CGS001-509	ATP5C1	68.4
82	CGS001-8737	RIPK1	68.2
83	CGS001-1407	CRY1	68.2
86	CGS001-8780	RIOK3	67.5
88	CGS001-8536	CAMK1	67.4
89	CGS001-5696	PSMB8	67.1
90	CGS001-1345	COX6C	67.0
91	CGS001-7343	UBTF	66.9
92	CGS001-8942	KYNU	66.5
93	CGS001-5708	PSMD2	66.4
94	CGS001-10365	KLF2	66.3
95	CGS001-6885	MAP3K7	66.2

96	CGS001-5538	PPT1	66.1
97	CGS001-672	BRCA1	66.0
99	CGS001-10919	EHMT2	65.7
100	CGS001-6240	RRM1	65.7

For details about the metrics in the table, see www.lincscloud.org.

Supplementary Table 6. Predicted upstream regulators of pioglitazone target genes (Supplementary Table 5) (Ingenuity Pathway Analysis).

Upstream regulator	Molecule type
TBX2	transcription regulator
SP1	transcription regulator
SIM1	transcription regulator
MYC	transcription regulator
MED1	transcription regulator
E2F1	transcription regulator
ARNT2	transcription regulator
AHR	ligand-dependent nuclear receptor
MAP2K1	kinase
ERBB2	kinase
EGFR	kinase
IGF1	growth factor
HGF	growth factor
FGF2	growth factor
NF-kB (complex)	complex

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3.2 PROTEIN PHOSPHATASE EXPRESSION PATTERNS IN HCV-INFECTED PATIENTS, AND ITS IMPACT ON CELL SIGNALING AND DISEASE DEVELOPMENT.

3.2.1 INTRODUCTION

Chronic HCV infection modulates host signaling patterns that may contribute to the development of virus-induced liver disease (see 1.9: Hepatitis C treatment). These signaling processes are tightly regulated by protein phosphatases and aberrant phosphatase expression is involved in various syndromes and diseases (Tonks, 2006). As a consequence, HCV-induced alterations in phosphatase expression may contribute to liver disease. However, how HCV affects protein phosphatases in liver tissue is not very well characterized. In order to gain more insight into this matter, we initiated a screening for phosphatase expression in liver biopsies of chronic HCV patients.

Liver biopsies have the advantage that the derived data reflect the true situation *in vivo* and therefore represent an authentic snapshot of liver biology and disease in patients. Insight gained from cell culture models on the other hand always have to be verified in a real-life setting to avoid foregone conclusions based on artefacts proper to the *in vitro* system itself. The big disadvantage of liver specimens is that they are precious and moreover, they cannot be used for experimental procedures. For such studies, cell culture systems are more suitable. Both, *in vitro* systems and liver biopsies, thus have their advantages which are complementary to each other's drawbacks. For our phosphatase screen we opted to start with *in vivo*-derived material to ensure relevance for patients, and resort to *in vitro* systems for validation.

3.2.2 SPECIFIC AIMS

Our aims in this project were (i) to assess expression patterns of disease-relevant protein phosphatases in HCV-infected liver tissue and (ii) to explore potential mechanisms leading to deregulated phosphatase expression during HCV infection.

3.2.3 RESULTS

3.1.3.1 CHRONIC HCV INFECTION IMPAIRS PTPRD EXPRESSION

In the framework of an inter-laboratory collaboration with Basel University Hospital, we were granted access to patient-derived liver specimens. We extracted RNA from 6 biopsies from chronic

HCV-infected patients and 6 non-infected biopsies and performed RT-qPCR to quantify the mRNA expression of 84 disease-relevant protein phosphatases. As a result, I identified a total of 24 protein phosphatases that are significantly deregulated in livers of chronic HCV-infected patients (see 3.2.4: Van Renne et al. Fig. 1A). Among the most derailed phosphatases was protein tyrosine phosphatase receptor type D (PTPRD), a well-known tumor suppressor that dephosphorylates STAT3 (Veeriah et al., 2009). Moreover, STAT3 is a HCV co-factor whose activation is important for HCV replication. STAT3 is also a key player in liver regeneration signaling networks mediated by IL-6. Since aberrant activation of STAT3 through impaired PTPRD expression in HCV-infected hepatocytes may impact the development of liver disease, we decided to follow up on PTPRD. We further validated PTPRD downregulation in chronic HCV patients in an additional set of biopsies (Van Renne et al. Fig. 2B). It is important to highlight that these results may simply reflect the continual inflammatory setting, more than being a specific effect of PTPRD. Moreover, the outcome of these measurements, expressed as a global fold change, may overlook individual differences. We therefore had a closer look at every single biopsy. To find out if PTPRD would be differentially expressed in livers with severe levels of inflammation or liver scarring, mRNA levels were correlated with the metavir score, a measure for liver inflammation and fibrosis. No correlation between PTPRD expression and metavir scores could be discerned, demonstrating that impaired PTPRD transcription was independent from either inflammation or fibrosis (Van Renne et al. Fig. 1C and 1D, respectively).

Since PTPRD protein is not expressed in all the liver-derived immortal cell lines we tested, including Huh7, Huh7.5.1, Huh7.5.1^{dif}, HepG2, and HepaRG, we resorted to PHH for further *in vitro* studies. We acquired infected PHH lysates through a collaboration with INSERM U1183 in Montpellier, and demonstrated that PTPRD is downregulated upon HCVcc infection *in vitro,* both on the mRNA and the protein level (Van Renne et al. Fig. 2A-B). PTPRD was not upregulated in PHH by IFN-α treatment, confirming that a hampered PTPRD expression was not dependent on the innate immune response (Van Renne et al. Fig. 2C-D).

The absence of PTPRD protein in transformed cell lines suggested this protein also plays a role in HCC. Indeed, when we looked in paired liver biopsies of HCC patients, PTPRD protein expression was markedly reduced in tumor tissue compared to adjacent normal liver tissue in 4 out of 6 patients (Van Renne et al. Fig. 3A). A similar conclusion could also be drawn from the analysis of a publicly available cDNA microarray database derived from paired liver biopsies. In 30 out of 46 samples from chronic HCV patients with HCC was PTPRD mRNA expression in tumor lesions lower than in the

adjacent non-tumoral tissue (Van Renne et al. Fig. 3B). Moreover, high levels of PTPRD in adjacent liver tissue of HCC patients –independent of etiology- correlate with survival and reduced tumor recurrence after surgical resection (Van Renne et al. Fig. 3C-E). Taken together, these findings firmly establish PTPRD as a candidate suppressor of HCV-associated hepatocarcinogenesis.

3.2.3.2 MIR-135A-5P MEDIATES PTPRD DOWNREGULATION IN HCV

In an attempt to mechanistically explain this downregulation, we employed computational microRNA (miRNA) target site prediction programs and identified multiple miRNAs that potentially repress PTPRD. This list was further refined with miRNA sequencing data in HCV infected Huh7.5.1 cells from a single experiment. We ended up with a set of 8 miRNAs potentially targeting PTPRD that are putatively upregulated in HCV-infected cells (Van Renne et al. Suppl. Table S2). We screened for their expression in liver biopsy samples and revealed a dramatic upregulation of miR-135a-5p (Van Renne et al. Fig. 4A-B). We validated miR-135a-5p-mediated repression of PTPRD expression by cloning the 3' UTR of PTPRD mRNA in a luciferase reporter construct, and showing a dramatic drop of luciferase activity when treated with miR-135-5p (Van Renne et al. Fig. 4E). Intriguingly, miR-135-5p promotes HCVcc replication as shown by a HCV luciferase reporter assay (Van Renne et al. Fig. 4D). Taken together, this data demonstrates that miR-135a-5p is a HCV-orchestrated regulatory element for PTPRD expression.

3.2.4 PUBLICATION OF RESULTS

These results were integrated in the manuscript: "*miR-135a-5p-mediated downregulation of protein-tyrosine phosphatase delta is a candidate driver of HCV-associated hepatocarcinogenesis*" which was submitted to Journal of Hepatology.

miR-135a-5p-mediated downregulation of protein-tyrosine phosphatase delta is a candidate driver of HCV-associated hepatocarcinogenesis

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Word Count : 5584

Number of figures and tables: Four figures and two supplementary tables

List of abbreviations (in the order of appearance):

HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
miRNA	MicroRNA
PTPRD	Protein tyrosine phosphatase delta
RTK	Receptor tyrosine kinase
PHH	Primary human hepatocytes
FISH	Fluorescence *in situ* hybridization
OCT	Optimal cutting temperature compound
CHC	Chronic hepatitis C
HCVcc	Cell culture-derived HCV
IFN-α	Interferon-alpha
3'UTR	Three prime untranslated region of mRNA
fc	Fold change
NI	Non infected

Key words: HCC; pathogenesis; tumor suppressor; miRNA

Conflicts of interest: The authors disclose no conflicts.

Financial support: This work was supported by a grant to T.F.B. from the Wilhelm Sander-Stiftung (Förderantrag 2010.023.1), the European Union (EU-Interreg IV-Hepato-Regio-Net) and the French Cancer Agency (ARC IHU201301187) and a grant to J.L. from the University of Strasbourg (IDEX, Projet Attractivité 2014; ANR). This work has been published under the framework of the LABEX ANR-10-LAB-28 and benefits from a funding from the state managed by the French National Research Agency as part of the Investments for the future program.

Author's contributions: J.L. initiated and supervised the study. J.L. and T.F.B. obtained funding. N.V.R., F.H.T.D., J.L., C.G., D.C., A.A.R.S, N.F., and A.A. designed and conducted experiments and analyzed data. P.P., M.H.H., provided patient liver tissue. N.V.R and J.L. wrote the manuscript, M.H.H. and T.F.B. critically reviewed the manuscript.

ABSTRACT

Background and Aims: Hepatitis C Virus (HCV) infection is a main cause of hepatocellular carcinoma (HCC). Even though novel antivirals can efficiently eradicate HCV infection, HCC risk remains elevated after viral clearance. HCV contributes to HCC development by perturbing signaling circuitry, and these signaling events are tightly regulated by protein phosphatases. However, the impact of HCV on phosphatases is largely unknown. Thus we aimed to identify phosphatases that are potentially relevant for HCC development and that respond to HCV infection *in vivo*.

Methods: Using RT-qPCR and FISH we screened and validated protein phosphatase and microRNA (miRNA) expression in liver biopsies of HCV patients and in primary human hepatocytes. Phosphatase expression in paired liver biopsies of HCC patients was correlated to patient survival and tumor recurrence.

Results: We show that tumor suppressor PTPRD is consistently downregulated upon HCV infection *in vivo*. Moreover, we demonstrate that PTPRD expression is impaired in tumor lesions of paired liver biopsies and that high levels of PTPRD in adjacent liver tissue of HCC patients correlate with survival and reduced tumor recurrence after surgical resection. We identified miR-135a-5p as a mechanistic regulator of hepatic PTPRD expression in HCV patients.

Conclusions: Our results demonstrate impaired PTPRD levels in infected hepatocytes and HCCs potentially represent a hallmark of liver disease progression. PTPRD is a tumor suppressor and phosphatase of STAT3, a HCV co-factor. HCV may maintain STAT3 activity via miR-135a-5p-mediated downregulation of its negative regulator PTPRD, leaving the liver more prone to malignant transformation as a side-effect.

INTRODUCTION

More than 150 million people worldwide are infected by Hepatitis C Virus (HCV) [1], which is a leading cause of liver cirrhosis and hepatocellular carcinoma (HCC) [2]. The arrival of highly effective new therapies consisting of direct-acting antivirals can cure the vast majority of patients [3], but those with advanced liver disease remain at risk for developing HCC even after viral clearance [4]. Moreover, the exact mechanisms responsible for this increased susceptibility remain only partly understood. Traditionally, carcinogenesis has been attributed to a multi-step accumulation of genetic damage resulting in gain of function by proto-oncogenes, and inactivation of tumor suppressor genes [5]. Only a minority of these driver genes is frequently involved in cell transformation, while the majority is only occasionally affected [6]. In other words, a myriad of combinations in genetic and epigenetic alterations can lead to cancer, and as a consequence, every tumor displays a heterogeneous molecular profile, further clouding understanding of cancer development. In chronic hepatotropic virus infections such as HCV and hepatitis B virus the picture is even more complicated as both viral and non-viral factors are drivers of hepatocarcinogenesis [7, 8]. Nonetheless, the complexity of malignant transformation can be reduced to a handful of logical underlying principles, of which derailed signaling circuitry is a key component [9]. We have previously demonstrated that cellular receptor tyrosine kinase (RTK) signaling is involved in regulating HCV entry into hepatocytes and that virus binding to hepatocytes triggers RTK activation [10-12]. This indicates that chronic HCV infection modulates host signaling patterns that may contribute to the development of virus-induced liver disease. Since these signaling processes are tightly regulated by protein phosphatases and aberrant phosphatase expression is involved in various syndromes and diseases [13], we screened for disease-relevant phosphatase expression in liver biopsies of chronic HCV patients.

MATERIALS AND METHODS

Liver tissue, cells and virus

Human needle liver biopsies were collected at Gastroenterology and Hepatology outpatient clinic of the Basel University Hospital, Switzerland. Protocol for patient tissue collection was reviewed and approved by the Ethics Committee of the University Hospital of Basel. Written informed consent was obtained from all patients. Eligible patients were identified by a systematic review of patient charts at the Hepatology center of the University Hospital of Basel, Switzerland. Histopathological grading and staging of the HCV liver biopsies, according to the METAVIR classification system, was performed at the Pathology Institute of the University Hospital Basel. All the patients that donated liver tissue are summarized in supplementary table S1. Liver biopsy tissues were analyzed as described [14]. Liver tissue was lysed and subjected to RT-qPCR and immunoblot analysis (described below). Primary human hepatocytes (PHHs) were isolated and cultured as previously described [11]. Huh7.5.1, HEK293T cells and HCVcc strain Luc-Jc1 have been described [11, 12].

Antibodies and Western blotting

P-STAT1 mAb (58D6) was obtained from Cell Signaling Technology, β-Actin mAb (AC-15) was obtained from Invitrogen and PTPRD pAb (C-18) from Santa Cruz Biotechnology. Western blots were performed using Hybond–P membranes (GE Healthcare), visualized using ECF substrate and quantified with a fluorescence scanner (Typhoon Trio, GE Healthcare).

Analysis of mRNA and miRNA expression

mRNA expression of 84 disease-relevant protein phosphatases was assessed in 6 liver biopsies from patients with chronic HCV infection and in 6 non-infected biopsies using RT-qPCR (Human Protein Phosphatases RT² Profiler PCR Array, Qiagen). Total RNA from Huh7.5.1 cells, PHHs and liver biopsies was extracted using RNeasy Mini Kit (Qiagen) or Trizol (Life Technologies). Gene expression in the total RNA extracts was assessed using two-step RTqPCR. The reverse transcription on total RNA extract was made using MAXIMA reverse

transcriptase (Thermo Scientific). qPCR for detecting PTPRD, RSAD2, USP18 and GAPDH was performed with RT² qPCR Primer Assays (Qiagen) using Real-time PCR ABI Prism 7500 (Thermo scientific) or Corbett Rotor Gene 6000 (Qiagen). All qPCRs were performed following manufacturers' instructions. Specificities of phosphatase PCR products were validated by melting curve analysis and PCR product sequencing. Differential gene expression of patient biopsies was calculated after $2^{-\Delta C_T}$ transformation into individual data points. miRNA expression was measured using miScript (Qiagen) with forward DNA primers derived from the miRNA MIMAT sequence (supplementary table S2). miRNA target sites were predicted using miRSystem [15]. Synthetic miRNAs (mimics) of hsa-miR-135a-5p, has-miR-122 and the non-targeting negative control miRNA cel-miR-67 (miR-CTRL) were obtained from GE Healthcare.

Luciferase reporter assay

3'UTR of PTPRD was amplified with 5'-TTT CTC GAG CTT TGA CCA CTA TGC AAC GTA G-3' and 5'-TTT CTC GAG CTG TCC TCG CCG TTT TCT AA-3' and subcloned in siCheck2 (Promega) using *Xho*I. 150 ng luciferase reporter plasmid was co-transfected with 10 ng miRNA in HEK293T cells using Lipofectamine 2000 (Thermo Fisher Scientific). *Renilla* and *firefly* luciferase activity was assessed 24h post transfection using Dual-Luciferase Reporter assay (Promega) and a Mithras LB940 plate reader (Berthold Technologies).

FISH analysis

Human needle liver biopsies were collected, immediately embedded in optimal cutting temperature compound (OCT) and frozen in liquid nitrogen chilled 2-methylbutane. Tissues were then stored at -80 °C until use. Sections (10 µm) were cut at cryostat (Leica) and mounted onto Superfrost Plus Gold glass slides (K5800AMNZ72, Thermo-Scientific, Waltham, MA, USA), fixed overnight in 4 % formaldehyde at 4 °C and hybridized, as previously described [16], with the following modifications: tissue sections were pre-treated by boiling (90-95 °C) in pre-treatment solution (Panomics, Affymetrix) for 1 min, followed by a protease QF (Panomics, Affymetrix) digestion for 10 min at 40 °C. Hybridization was performed using probe sets against patient-specific HCV RNA sequence (type 1) and against human PTPRD mRNA (NM_002839,

target region 4754-5835). Pre-amplification, amplification and detection were performed according to provider's protocol. Images were acquired with a laser scanning confocal microscope (LSM710, Carl Zeiss Microscopy, Göttingen, Germany) and Zen2 software, using same settings for all the tissues analyzed. Five random fields were acquired from each section. Image analysis was performed using ImageJ and CellProfiler software, with a customized pipeline. Total number of cells, frequency of HCV positive and PTPRD positive cells, and signal intensity were then evaluated and exported for statistical analysis (two-way ANOVA, $p \leq 0.05$).

Bioinformatics of gene expression database

Paired gene expression of 60 formalin-fixed paraffin embedded tumor and adjacent liver tissues were explored from a publicly accessible database (National Centre for Biotechnology Information Gene Expression Omnibus GSE10143 [17]). Downregulation of PTPRD expression in tumor tissue was analyzed *in silico* for 46 patients with chronic HCV. P-values were calculated with Wilcoxon signed-rank test (two-tailed). Probability of survival and tumor recurrence was evaluated by the log-rank test available on the GenePattern Survival analysis module (http://www.broad.mit.edu/cancer/software/genepattern).

RESULTS

Chronic HCV infection impairs PTPRD expression *in vivo*

To study the impact of chronic HCV infection on phosphatase expression in patient liver tissue, we extracted RNA from 6 biopsies of patients with chronic hepatitis C (CHC) and 6 non-infected biopsies (supplementary table S1) and quantified expression of 84 disease-relevant protein phosphatases using RT-qPCR. We identified 24 phosphatases that were significantly ($p<0.01$, U-test) deregulated in HCV-infected tissue compared to non-infected biopsies (Fig. 1A). Interestingly, among the phosphatases with most deregulated expression levels in HCV biopsies we observed an enrichment of candidates with potential relevance for the development of HCC. More particularly, some of these phosphatases act as tumor

suppressors in various cancers including protein tyrosine phosphatase receptor type delta (PTPRD). PTPRD is frequently inactivated and mutated in human cancers [18, 19] including HCC [20]. In order to validate HCV-induced PTPRD downregulation, we measured mRNA expression in a second sample series of 24 liver biopsies of patients with chronic HCV and validated that PTPRD mRNA expression is significantly ($p=0.0003$, U-test) downregulated compared with 11 additional non-viral control biopsies (Fig. 1B).

PTPRD expression in HCV patients is independent from fibrosis or inflammation

Since biological circuits in liver tissues from CHC patients reflect a complex interaction of different cell types integrating an inflammatory immune response, we first compared phosphatase expression levels with METAVIR grading and staging data (supplementary table S1). No significant relationship between METAVIR score and PTPRD expression was observed in 30 liver biopsies (Fig. 1C-D), suggesting that PTPRD expression is independent from the degree of inflammation and fibrosis of the studied biopsies.

PTPRD expression is impaired in HCV-infected hepatocytes

To identify the cell type displaying impaired PTPRD expression levels upon HCV infection, we applied an established protocol for multiplex fluorescent in situ hybridization (FISH) analysis of liver biopsies at single cell resolution [16]. This strategy enabled us to distinguish HCV-infected cells from uninfected cells in liver biopsies. Simultaneous hybridization with specific probe set for PTPRD mRNA demonstrated that PTPRD was significantly and specifically ($p<0.05$, two-way ANOVA) impaired in infected cells compared to uninfected cells in three biopsies from different patients infected with HCV genotype 3a (supplementary table S1, Fig. 1E). Differential PTPRD expression was detected in cells with hepatocyte morphology. The analyzed fields did not show an abnormal level of infiltrating non-parenchymal cells and the level of PTPRD expression in those cells was marginal compared to hepatocytes (data not shown). This indicates that HCV impairs PTPRD expression directly in hepatocytes. To validate this observation, we measured PTPRD expression levels in isolated primary human hepatocytes (PHH) infected *in vitro* with HCV. We observed that PHH robustly express PTPRD

both at the mRNA and the protein level, providing a competent model system to study PTPRD. Two days after isolation, PHH were infected for five days with cell culture-derived HCV (HCVcc) strain JFH1, after which cells were lysed and both mRNA and protein was isolated. With this established protocol up to 20-30 % of hepatocytes can be infected by HCVcc (strain JFH1) as shown [21] using a reporter RFP-nuclear localization sequence-interferon-β promoter stimulator (RFP-NLS-IPS) [22]. HCV-infected PHH from different donors showed a significant decrease ($p=0.015$, U-test) in PTPRD mRNA expression (Fig. 2A), which also translated in a decreased PTPRD protein level as detected by immunoblot ($p=0.016$, U-test) (Fig. 2B). To exclude that PTPRD downregulation was caused by an antiviral response mounted by immunocompetent PHH, we treated PHH for five days with interferon-alpha (IFN-α) (Fig. 2C). No change in PTPRD protein levels was observed by Western blot for different concentrations of IFN-α, while STAT1 phosphorylation was properly induced. In parallel, interferon response genes USP18 and RSAD2 displayed an increase in mRNA transcription after IFN-α stimulus, while PTPRD remained at baseline levels (Fig. 2D). These data confirm that PTPRD downregulation is independent from the innate immune response.

PTPRD is impaired in tumor tissue and high PTPRD expression correlates with survival of HCC patients and absence of recurrence

Intriguingly, PTPRD mRNA is only marginally expressed in hepatoma cell lines such as Huh7.5.1. Moreover, it does not translate into detectable protein levels in Western blot of all hepatic cell lines tested including HepG2, Huh7, Huh7.5.1, differentiated HepaRG and non-differentiated HepaRG (data not shown), which suggests that PTPRD knockout is a hallmark of cell transformation. To ascertain whether PTPRD is also downregulated in hepatocellular carcinoma, we assessed PTPRD protein expression in six paired biopsies of infected and uninfected HCC patients (supplementary table S1). In four out of six donors, PTPRD expression was downregulated or even completely absent in tumor lesions compared to paired adjacent tissue samples (Fig. 3A). To verify whether this downregulation was also specifically present in HCV-infected HCC patients, we analyzed a publicly accessible gene expression

database of paired tumor and adjacent liver tissue specimens [17]. In this data set of patients undergoing surgical resection of liver tumors, information for 46 patients with HCV-associated HCCs could be retrieved. In 30 out of 46 paired biopsies was PTPRD expression downregulated in tumor lesions compared to adjacent non-tumoral tissue (Fig. 3B). This 30/46 ratio echoes the Western blot results of paired HCC biopsies (Fig. 3A), and the observed downregulation in tumor tissue was statistically significant (p=0.01, two-sided Wilcoxon signed-rank test) (Fig. 3B). Next, we took advantage of the long-term clinical follow-up information provided in the database. Strikingly, in HCC patients with the 25% highest levels of PTPRD expression in adjacent tissue (Fig. 3C) had a higher long-term survival rate (p=0.03, log-rank test) (Fig. 3D) and had less chance of recurrent liver cancer (p=0.01, log-rank test) (Fig. 3E), irrespective of cancer etiology. This adds further evidence of a potential involvement of PTPRD in the exacerbation of chronic liver disease.

miR-135a-5p is a mechanistic regulator of PTPRD expression in HCV-infected hepatocytes

The microRNA (miRNA) machinery is a regulator of gene expression and is exploited by HCV to maintain its replication [23, 24]. We applied multiple bioinformatical algorithms [15] scanning for human miRNAs potentially targeting PTPRD mRNA. Combining computational miRNA prediction with observations in HCV-infected Huh7.5.1 cells (data not shown) we defined a panel of 8 miRNAs with two essential characteristics: they potentially target PTPRD and they are upregulated by HCVcc in Huh7.5.1 cells (data not shown) (supplementary table S2). To validate the relevance of these 8 miRNAs in a real life setting we screened for their expression in liver biopsies of HCV-infected patients (Fig. 4A). This uncovered a striking two fold upregulation of miR-135a-5p in HCV patient liver specimens (p=0.0006, U-test) and establishes it as a possible PTPRD regulator (Fig. 4B). Moreover, similar to PTPRD, miR-135a-5p expression is independent from the host antiviral response as shown by a kinetics study measuring miR-135a-5p in IFN-α stimulated PHH. miR-135a-5p expression remained level (p=0.5, U-test) while the interferon response gene RSAD2 was significantly (p=0.02, U-

test) upregulated (Fig. 4C). This suggests that miR-135a-5p does not take part in the innate immune response, but is rather relevant to the HCV life cycle itself. Indeed, transfection of Huh7.5.1 cells with miR-135a-5p increased HCVcc infection (strain Luc-Jc1) up to six fold (p=0.02, U-test) (Fig. 4D) suggesting that HCV-induced miR-135a-5p expression in patients promotes HCV infection similar to miR-122, a known co-factor for HCV infection. To prove that miR-135a-5p targets PTPRD mRNA, we subcloned the 3'-untranslated region (3'UTR) of PTPRD mRNA in the *renilla* luciferase expression cassette (Luc-3'UTR) of a bicistronic *renilla*/*firefly* luciferase reporter construct (siCheck2, Promega). The 3'UTR of PTPRD harbors two predicted highly conserved miR-135a-5p target sites (supplementary table S2). Co-transfection of a miR-135a-5p mimic but not a non-targeting miR-CTRL with the Luc-3'UTR reporter significantly ($p=1x10^{-8}$, U-test) impaired normalized luciferase activity compared to empty vector ($p=2x10^{-7}$, U-test) (Fig. 4E), demonstrating that miR-135a-5p is able to silence PTPRD mRNA. This is also reflected *in vivo* where a significant (p=0.04, r=-0.03, one-tailed Spearman's correlation test) inverse correlation of PTPRD mRNA and miR-135a-5p levels could be discerned in liver biopsies studied in Fig. 4B. Remarkably, high levels of miR-135a-5p always corresponded with low PTPRD expression, while low amounts of miR-135a-5p give PTPRD expression more leeway to vary. Interestingly, some of the studied liver biopsies did neither exhibit high PTPRD mRNA nor miR-135a-5p levels (Fig. 4F, grey population) suggesting the presence of an additional more general regulatory mechanism of PTPRD expression that is independent from HCV. Taken together, these data establish miR-135a-5p as a potent HCV-driven regulatory element for PTPRD expression.

DISCUSSION

HCC is the second largest cause of death from cancer world-wide and poses an increasing burden on global health [25]. Chronic Hepatitis C Virus infection is a main cause of HCC and even though recent pharmacological breakthroughs can efficiently eradicate HCV infection, the risk of developing HCC in patients after sustained virological response remains elevated. Moreover, given the epidemiological history of the virus, HCV-associated complications are set to peak over the next decade, and novel preventive strategies are urgently needed. By studying the impact of HCV infection on protein phosphatase expression patterns in liver biopsies we aimed to identify drivers for HCV-associated disease development. Here we show that tumor suppressor PTPRD is consistently downregulated upon HCV infection, both *in vivo,* in chronically infected patient biopsies, and *in vitro* in infected primary human hepatocytes. PTPRD is a well-established tumor suppressor [19] whose chromosomal locus on 9p23–24.1 is regularly subject to genetic deletion or epigenetic inactivation in a broad spectrum of human malignancies including neuroblastoma, glioblastoma [19], lung cancer, cutaneous squamous cell carcinoma [26], laryngeal sqamous cell carcinoma [27], melanoma [28] and also hepatocellular carcinoma [20]. In addition, PTPRD copy number loss associates with a poor prognosis in breast cancer, colon cancer [29], and gastric adenocarcinoma [30]. Here we demonstrate for the first time that PTPRD protein expression is down-regulated in chronically HCV-infected patients. Moreover, we show that this downregulation is even more pronounced in tumor lesions of paired liver biopsies compared to adjacent non-tumor tissue of non-infected patients. Taken together, the evidence suggests that a gradual loss of PTPRD expression is a common event in liver disease progression, and it highlights the role of PTPRD as a potential suppressor of hepatocarcinogenesis, irrespective of etiology.

In this study we identified a HCV-specific regulatory mechanism that silences PTPRD expression *in vivo*. We demonstrate that PTPRD mRNA is targeted by miR-135a-5p, a miRNA that is markedly elevated in sera of CHC patients [31]. We demonstrate that miR-135a-5p expression is also induced in liver biopsies of HCV patients (Fig. 4A-B). Moreover we show

that miR-135a-5p is markedly increasing HCV infection (Fig. 4D). Indeed, miR-135a has been recently suggested as a co-factor for HCV replication by interacting with the viral genome [32]. Our data demonstrate HCV-induced upregulation of miR-135a-5p expression is a cause of PTPRD silencing (Fig. 4E-F) and that high miR-135a-5p levels in liver tissues significantly ($p=0.02$, one-tailed Spearman's correlation test) correlate with low PTPRD levels and *vice versa*. The existence of a population of samples where both miR-135a-5p and PTPRD are weakly expressed (Fig. 4F, highlighted in grey), suggests the existence of an additional regulatory mechanism PTPRD potentially independent from HCV. These findings support the view that miR-135a-5p controls PTPRD especially in HCV-infected biopsies, while the other so far unidentified mechanism for PTPRD mitigation is predominant in non-infected liver tissue.

Interestingly, the transcription factor STAT3 is a confirmed target for PTPRD and a key molecule during liver regeneration. Loss of PTPRD function leads to aberrant STAT3 phosphorylation [19, 33]. We have previously demonstrated that STAT3 is an indispensable host factor for HCV infection, and that the viral infection is promoted by STAT3 activation [10]. Thus, it is conceivable that HCV downregulates PTPRD in order to benefit from a STAT3-driven transcriptional program. Since STAT3 activity also plays a role in cancer progression, this may contribute to the pro-tumorigenic environment during chronic HCV infection. In other words, our model suggests the existence of a perturbed PTPRD-STAT3 axis driving malignant progression of liver disease. This finding may also be of further clinical relevance since it provides a target for HCC chemoprevention. Indeed, therapeutic intervention on signaling events constitutes a new chemopreventive strategy as proof of concept has been demonstrated using the clinical EGFR-inhibitor erlotinib to attenuate liver fibrosis and the development of HCC in an animal model [34]. Given that PTPRD expression is suppressed by chronic HCV-infection and associated with HCC and patient survival, our data suggest a PTPRD-centered signaling network as a potential target for novel chemo-preventive strategies for HCV-induced HCC.

ACKNOWLEDGEMENT

This work was supported by a grant to T.F.B. from the Wilhelm Sander-Stiftung (Förderantrag 2010.023.1), the European Union (EU-Interreg IV-Hepato-Regio-Net) and the French Cancer Agency (ARC IHU201301187) and a grant to J.L. from the University of Strasbourg (IDEX, Projet Attractivité 2014; ANR). This work has been published under the framework of the LABEX ANR-10-LAB-28 and benefits from a funding from the state managed by the French National Research Agency as part of the Investments for the future program. The authors thank Dr. Michel Tremblay (McGill University, Montreal, Canada), Dr. Mirjam Zeisel, Dr. Simonetta Bandiera and Dr. Catherine Schuster (Inserm U1110, University of Strasbourg, France) for helpful discussions, Sarah Durand (Inserm U1110, University of Strasbourg, France) for isolation of primary human hepatocytes, Dr. Frank Chisari (The Scripps Research Institute, La Jolla, CA, USA) for the Huh7.5.1 cells, and Dr. Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan) for the HCV JFH1 strain.

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FIGURE LEGENDS

Fig. 1 PTPRD expression is significantly impaired in livers of patients with chronic Hepatitis C. Expression of 84 protein phosphatases associated to diseases and syndromes was quantified in needle stick biopsies using qPCR and *in situ* hybridization of RNA probes. (**A**) mRNA expression of 11 phosphatases was significantly ($p<0.01$, U-test) increased (red) and expression of 13 phosphatases was significantly ($p<0.01$, U-test) decreased. Data are expressed as fold change (fc) phosphatase expression non-HCV (n=6) and HCV-infected biopsies (n=6) relative to the average expression levels of five housekeeping genes (actin, B2M, GAPDH, HPRT1, RPLP0). Arrow indicate tumor suppressor PTPRD. (**B**) Expression of PTPRD is significantly impaired in liver biopsies of patients with chronic HCV ($p=0.0003$, U-test). Validation of HCV-induced downregulation of PTPRD compared to non-infected biopsies (NI) observed in (A) in additional liver biopsies from HCV-infected patients (supplementary table S1). Data are expressed as PTPRD mRNA expression relative to GAPDH and visualized as raw data box plot and median (line). (**C-D**) Expression of PTPRD is independent from fibrosis and inflammation in liver tissues. PTPRD expression was correlated with METAVIR score of the HCV-infected liver biopsies but no significant correlation ($p>0.5$, U-test) between METAVIR grading (C) or staging (D) of the studies biopsies in panel A-B (supplementary table S1) was observed. Data are expressed as PTPRD mRNA expression relative to GAPDH and visualized as raw data box plot and median (line). (**E**) PTPRD expression is impaired in HCV-infected hepatocytes in liver biopsies. FISH analysis of liver biopsies infected with HCV genotype 3 by simultaneous hybridization with HCV-specific and PTPRD-specific probe sets. PTPRD labeling was assessed in five random fields from each section. Data are expressed as mean of PTPRD fluorescence intensities in HCV positive and HCV negative cells per patient (±SD, $p<0.05$, two-way ANOVA).

Fig. 2 PTPRD is significantly impaired by HCV in hepatocytes independent from the innate immune response. (**A**) PTPRD mRNA expression is significantly impaired in primary human hepatocytes (PHH) after 5 d infection with HCVcc (strain JFH1). Data are expressed

as mean PTPRD expression relative to GAPDH ±SEM (p=0.015, U-test; n=6 from three donors). (**B**) PTPRD protein expression is significantly impaired by HCV in PHH. PTPRD and actin expression were assessed by Western blotting 5 d after infection of PHH with HCVcc (strain JFH1) compared to non-infected PHH (NI). Band intensities were quantified using Image Quant and are expressed as mean PTPRD expression relative to actin ±SEM (p=0.016, U-test, n=14 from two different donors). (**C**) PTPRD protein expression is independent from innate immune response. Host antiviral response was simulated by incubation of PHH with interferon-alpha (IFN-α). Cell culture medium with IFN-α was replaced every day. Cells were lysed after 5 d incubation and PTPRD, phospho-STAT1 (P-STAT1) and actin were measured by Western blotting. (**D**) PTPRD is not an IFN-response gene. Primary human hepatocytes were incubated for 10 min with 10^3 IU/mL IFN-α and mRNA expression of PTPRD and the known interferon-response genes USP18 and RSAD2 were assessed by RT-qPCR relative to GAPDH 6h and 16h post IFN stimulation.

Fig. 3 Low PTPRD expression is associated to HCCs and correlates to a decreased patient survival from HCC and increased tumor recurrence after surgical resection. (**A**) PTPRD is impaired in tumor lesions of HCC patients. Expression of PTPRD and actin was assessed HCC lesions (*T*) and the corresponding paired adjacent tissue (*A*) of 6 HCC patients by Western blotting using specific antibodies. A random PHH lysate served as positive control. (**B**) PTPRD is significantly (p=0.01, two-sided Wilcoxon signed-rank test) impaired in HCV-associated HCCs. A gene expression database was analyzed for PTPRD expression in paired liver biopsies from 46 HCV-infected patients. Pairs of tumor lesions (blue) with the corresponding adjacent non-tumor tissue (blue) are connected by a dashed line. Same data is summarized side-by-side as box-and-whisker plot (box= 50% of biopsies, line=median, whiskers=minimal and maximal values) in the same panel. (**C-E**) Patients with high PTPRD expression in adjacent non-tumor tissues exhibit a significantly (p=0.03, log-rank test) higher survival rate from HCC and a significantly (p=0.01, log-rank test) decreased tumor recurrence after surgical resection of the tumors. Product-limit estimation of PTPRD expression in

adjacent non-tumor tissue of 82 patients compared with disease progression data from patients. Patient survival from HCC (D) and tumor recurrence after surgical resection (E) were compared between biopsies with highest PTPRD expression (C) (top quartile, blue) and biopsies exhibiting intermediate to low PTPRD expression (other quartiles, red) using Kaplan-Meyer estimator (Software OriginPro 9).

Fig. 4 HCV-induced expression of miR-135a-p stimulate HCV infection and mediates HCV-specific degradation of PTPRD mRNA. (**A**) miRNA expression screening of potential regulators of PTPRD mRNA in livers of CHC patients. Predicted miRNA target sites on the PTPRD mRNA (supplementary table S2) were screened in liver biopsies using qPCR. Data are expressed as fold change (fc) miRNA expression relative to SNORD61 and visualized as box-and-whisker plot (box= 50% of biopsies, line=median, whiskers=minimal and maximal values) centered to the median expression levels of the miRNA in the corresponding non-infected liver biopsies. Grey bar highlights miR-135a-5p that is >2fold upregulated in HCV-patients. (**B**) miR-135a-5p expression is significantly (p=0.0006, U-test) upregulated in livers of HCV patients (dark grey circles) compared to non-infected (NI) tissues (open circles). (**C**) miR-135a-5p is not induced by the antiviral response to infection. Isolated PHH were treated over 16 h with 1000 IU/mL IFN-α prior RT-qPCR measuring miR-135a-5p expression and the abundance of the interferon-stimulated gene RSAD2. While interferon-response gene RSAD2 is significantly (p=0.02, U-test) induced by IFN-α, miR-135a-5p remains level (p=0.5, U-test) (**D**) miR-135a-5p expression significantly (p=0.02, U-test) stimulate HCV infection. Huh7.5.1 cells were transfected with a miR-135a-5p mimic, a miR-122 mimic or a non-targeting miRNA derived from *C. elegans* miRNA (miR-CTRL) three days prior infection with HCVcc (strain Luc-Jc1). Data are expressed as fold change HCVcc infection ±SD relative to miR-CTRL. (**E**) miR-135a-5p induce silencing of PTPRD mRNA. 3'UTR of PTPRD harboring two miR-135a-5p target sites were subcloned in the *renilla* luciferase expression cassette of a bicistronic *renilla*/*firefly* luciferase reporter plasmid (Luc-3'UTR). Co-transfection of Luc-3'UTR with a miR-135a-5p mimic but not with a non-targeting miRNA (miR-CTRL) significantly ($p=1x10^{-8}$, U-test)

impairs *renilla* luciferase expression in HEK293T compared to empty vector (Luc) ($p=2x10^{-7}$, U-test). Data are expressed as mean *renilla* luciferase activity ±SEM normalized to firefly luciferase (five independent experiments, n=15). (**F**) PTPRD mRNA levels significantly and inversely correlate (p=0.04, r=-0.03, one-tailed Spearman's correlation test) with miR-135a-5p levels in liver biopsies. PTPRD mRNA and miR-135a-5p expression in non-infected and HCV-infected liver biopsies analyzed in (B) were compared. All liver biopsies with high miR-135a-5p levels exhibited low PTPRD mRNA expression and *vice verca*. Liver biopsies with neither high PTPRD mRNA nor high miR-135a-5p levels were highlighted in grey.

Figure 1

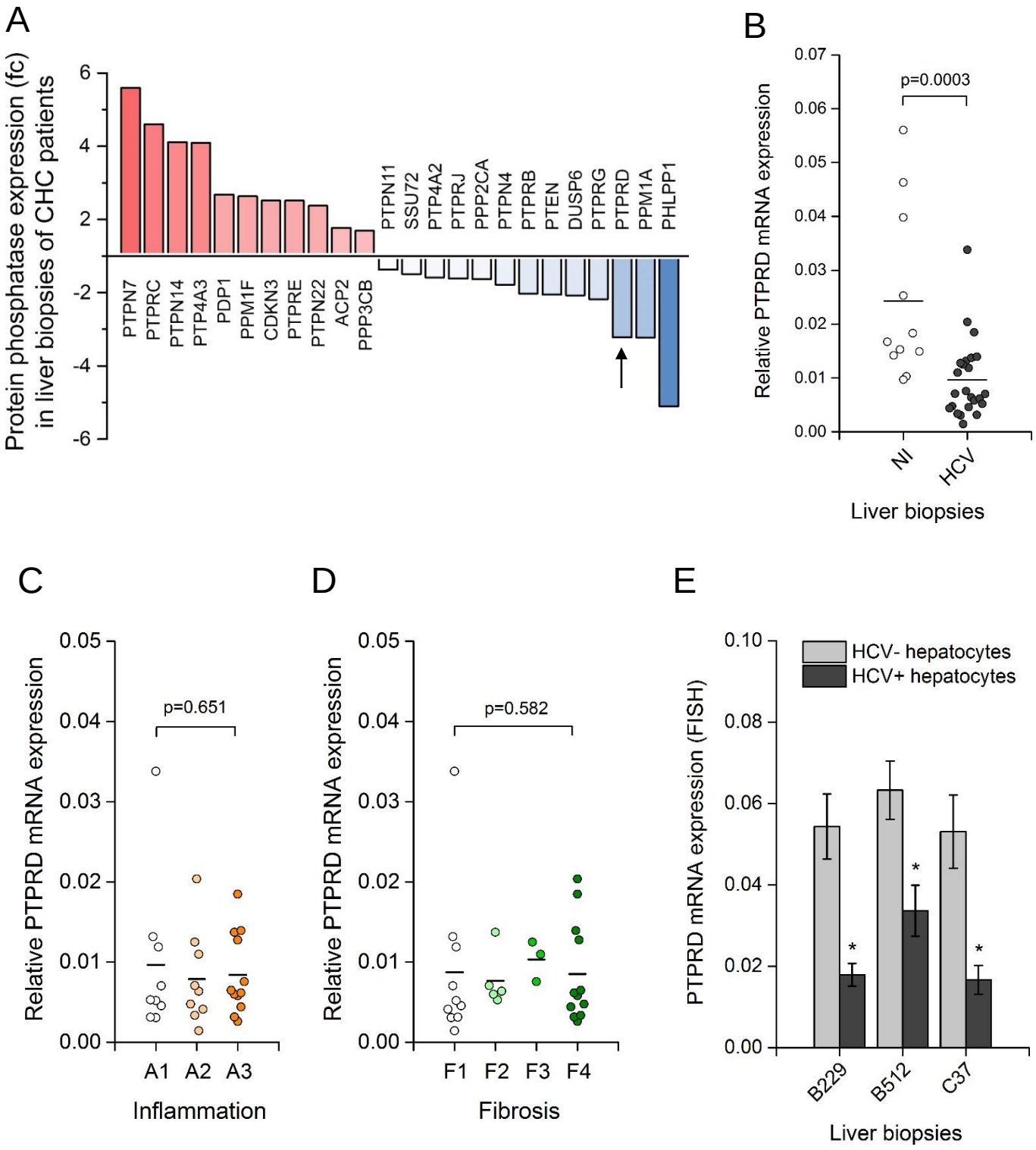

A
Protein phosphatase expression (fc) in liver biopsies of CHC patients
6
4
2
-2
-4
-6
PTPN7
PTPRC
PTPN14
PTP4A3
PDP1
PPM1F
CDKN3
PTPRE
PTPN22
ACP2
PPP3CB
PTPN11
SSU72
PTP4A2
PTPRJ
PPP2CA
PTPN4
PTPRB
PTEN
DUSP6
PTPRG
PTPRD
PPM1A
PHLPP1
B
Relative PTPRD mRNA expression
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
p=0.0003
NI
HCV
Liver biopsies
C
Relative PTPRD mRNA expression
0.05
0.04
0.03
0.02
0.01
0.00
p=0.651
A1
A2
A3
Inflammation
D
Relative PTPRD mRNA expression
0.05
0.04
0.03
0.02
0.01
0.00
p=0.582
F1
F2
F3
F4
Fibrosis
E
PTPRD mRNA expression (FISH)
0.10
0.08
0.06
0.04
0.02
0.00
HCV- hepatocytes
HCV+ hepatocytes
*
*
*
B229
B512
C37
Liver biopsies

Figure 2

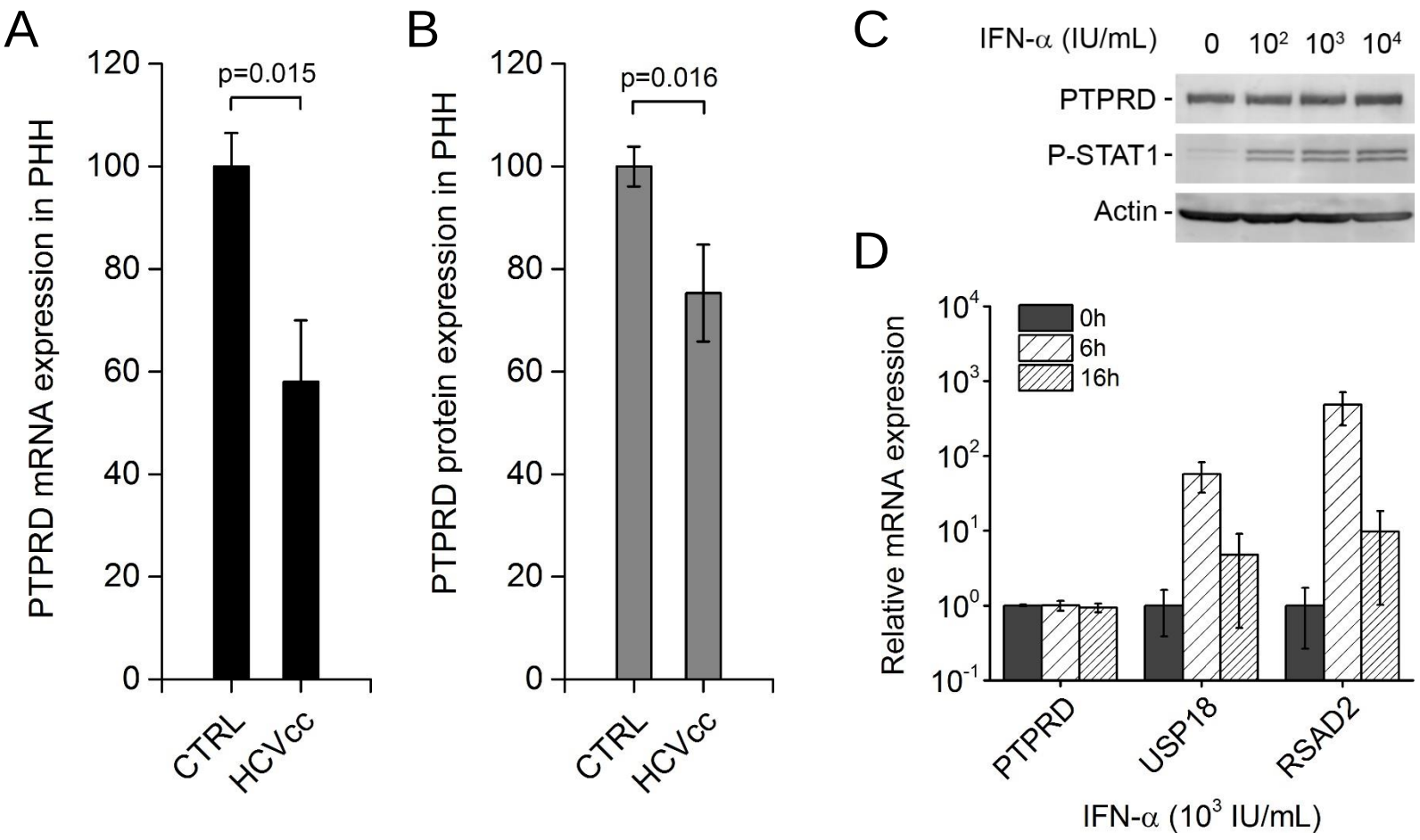

A
PTPRD mRNA expression in PHH
p=0.015
120
100
80
60
40
20
0
CTRL
HCVcc
B
PTPRD protein expression in PHH
p=0.016
120
100
80
60
40
20
0
CTRL
HCVcc
C
IFN-α (IU/mL)
0
10²
10³
10⁴
PTPRD
P-STAT1
Actin
D
Relative mRNA expression
10⁴
10³
10²
10¹
10⁰
10⁻¹
0h
6h
16h
PTPRD
USP18
RSAD2
IFN-α (10³ IU/mL)

Figure 3

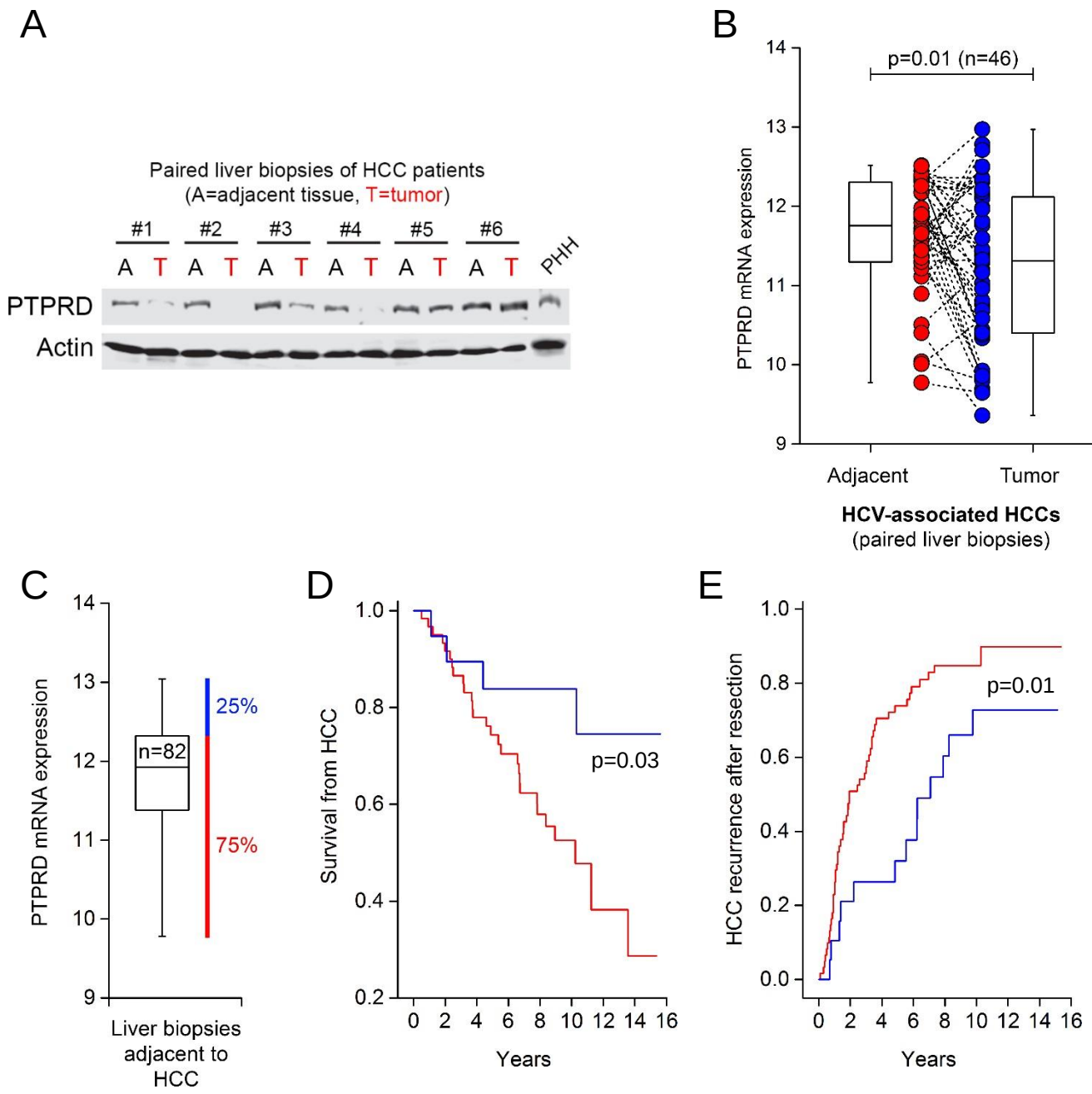
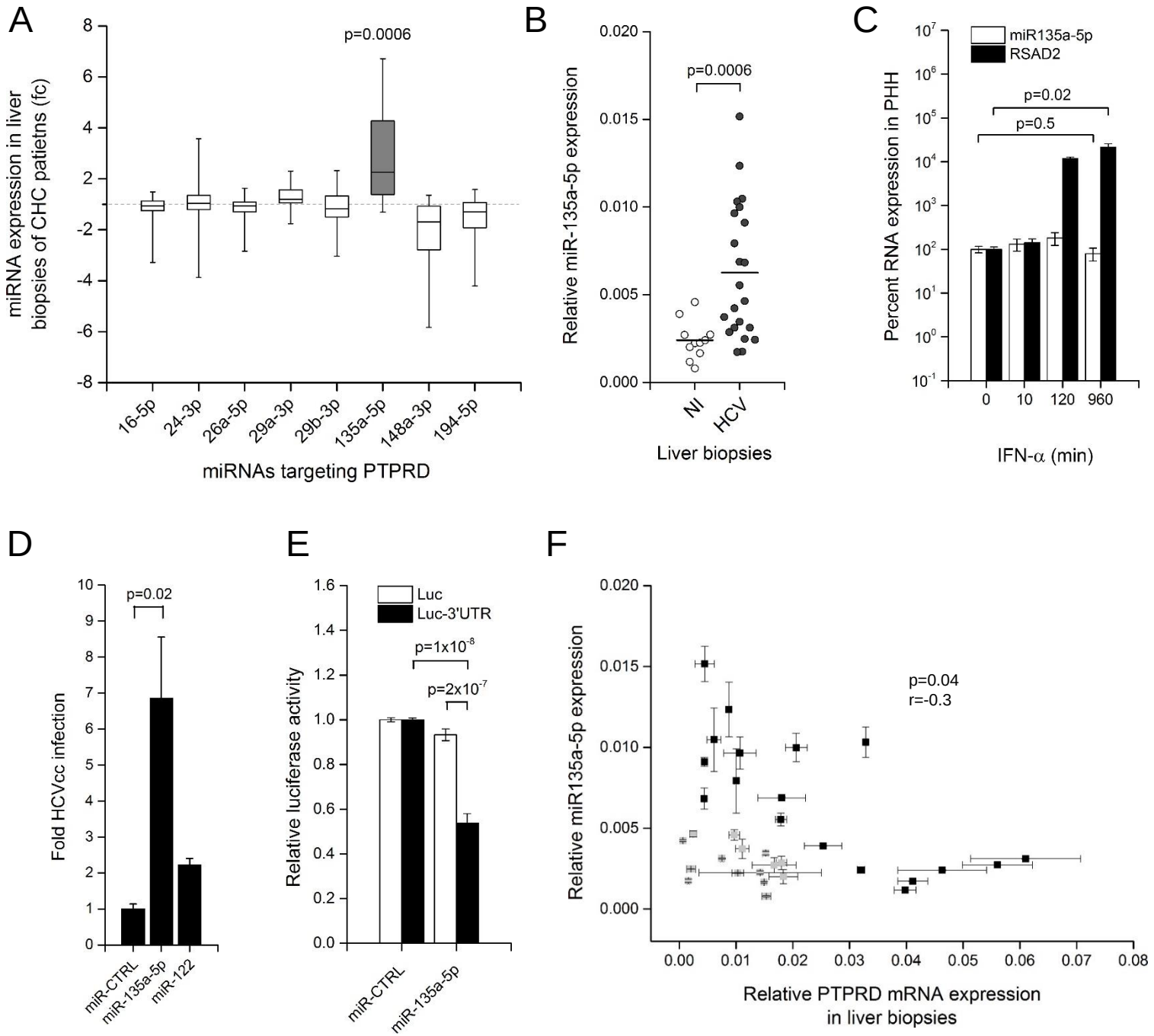

A
Paired liver biopsies of HCC patients
(A=adjacent tissue, T=tumor)
#1
#2
#3
#4
#5
#6
A T A T A T A T A T A T
PHH
PTPRD
Actin
B
p=0.01 (n=46)
PTPRD mRNA expression
14
13
12
11
10
9
Adjacent
Tumor
HCV-associated HCCs
(paired liver biopsies)
C
PTPRD mRNA expression
n=82
25%
75%
Liver biopsies
adjacent to
HCC
D
Survival from HCC
1.0
0.8
0.6
0.4
0.2
p=0.03
0 2 4 6 8 10 12 14 16
Years
E
HCC recurrence after resection
1.0
0.8
0.6
0.4
0.2
0.0
p=0.01
0 2 4 6 8 10 12 14 16
Years

Figure 4


A
p=0.0006
miRNA expression in liver biopsies of CHC patietns (fc)
16-5p
24-3p
26a-5p
29a-3p
29b-3p
135a-5p
148a-3p
194-5p
miRNAs targeting PTPRD
B
p=0.0006
Relative miR-135a-5p expression
NI
HCV
Liver biopsies
C
miR135a-5p
RSAD2
p=0.02
p=0.5
Percent RNA expression in PHH
IFN-α (min)
D
p=0.02
Fold HCVcc infection
miR-CTRL
miR-135a-5p
miR-122
E
Luc
Luc-3'UTR
p=1x10-8
p=2x10-7
Relative luciferase activity
miR-CTRL
miR-135a-5p
F
p=0.04
r=-0.3
Relative miR135a-5p expression
Relative PTPRD mRNA expression in liver biopsies

SUPPLEMENTARY INFORMATION

miR-135a-5p-mediated downregulation of protein-tyrosine phosphatase delta is a candidate driver of HCV-associated hepatocarcinogenesis

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SUPPLEMENTARY TABLES

Table S1 Human liver biopsies used in this study. NET= neuroendocrine tumor, gGT= gamma-glutamyl transferase, HCC= hepatocellular carcinoma, ASH= alcoholic steatohepatitis, FNH= focal nodular hyperplasia, f= female, m= male, NA= not assessed

Figure	Code	Sex	Age	HCV	Viral load	METAVIR	Diagnosis
				Genotype	*IU/mL*		
1A	B318	f	78				NET, sample shows normal histology
1A	B724	m	66				Transaminase elevations
1A	B748	f	53				gGT and transaminase elevations
1A	B803a	m	69				Adenocarcinoma, sample shows normal histology
1A	B831	f	46				gGT elevations
1A	B895a	f	29				FNH, sample shows normal histology
1A,C-D	C32	m	59	1b	8.57E+05	A3/F2	HCV
1A,C-D	C12	m	53	4	3.31E+06	A2/F1	HCV
1A,C-D	C20	f	51	1a	NA	A1/F1	HCV
1A,C-D	C21	m	40	1a	NA	A1/F2	HCV
1A,C-D	C4	m	58	1b	3.21E+05	A3/F4	HCV
1A,C-D	C7	m	46	1a	4.33E+06	A3/F4	HCV
1B-D	C114	m	45	1a	1.17E+06	A1/F1	HCV
1B-D, 4	C121	m	65	2	5.89E+06	A2/F2	HCV
1B-D, 4	C124	f	48	3a	1.51E+06	A3/F4	HCV
1B-D, 4	C146	m	42	1a	NA	A2/F4	HCV
1B-D, 4	C149	m	34	3a	2.29E+04	A3/F4	HCV
1B-D, 4	C172	m	51	3a	3.47E+06	A2/F3	HCV
1B-D, 4	C221	m	34	3c	5.28E+05	A2/F4	HCV
1B-D, 4	C238	f	54	1b	NA	A1/F1	HCV
1B-D, 4	C239	m	52	NA	2.79E+05	A3/F4	HCV
1B-D, 4	C257	m	30	1a	1.08E+05	A1/F1	HCV
1B-D, 4	C263	f	62	3a	1.93E+05	A2/F1	HCV
1B-D, 4	C269	m	46	1a	1.62E+06	A2/F2	HCV
1B-D, 4	C270	f	76	1b	1.75E+06	A3/F3	HCV
1B-D, 4	C281	f	40	3a	NA	A1/F1	HCV
1B-D	C285	f	42	1a	2.80E+06	A1/F1	HCV
1B-D, 4	C291	m	53	3a	1.42E+05	A3/F2	HCV
1B-D, 4	C293	m	57	NA	NA	A3/F4	HCV
1B-D, 4	C300	m	55	3a	1.95E+06	A3/F4	HCV
1B-D, 4	C304	f	38	1a	1.55E+06	A2/F3	HCV
1B-D, 4	C44	f	41	1b	2.91E+05	A3/F4	HCV
1B-D, 4	C53	m	55	1b	2.17E+06	A2/F4	HCV
1B-D, 4	C58	m	54	4c/d	1.31E+06	A1/F1	HCV
1B-D, 4	C73	m	55	1b	2.62E+06	A3/F4	HCV

1B-D, 4	C89	m	29	3a	3.22E+04	A1/F1	HCV
1B-D, 4	C145	f	43				Normal
1B-D, 4	C187	f	31				Normal
1B-D, 4	C28A	f	51				Normal
1B-D, 4	C29	m	62				Normal
1B-D, 4	C305	m	46				Minimal unspecific hepatitis
1B-D, 4	C330	m	47				Minimal steatosis
1B-D, 4	C366A	f	56				Normal
1B-D, 4	C369	m	37				Normal
1B-D, 4	C442A	m	69				Minimal reactive hepatitis, 10% steatosis
1B-D, 4	C445	m	33				Normal
1B-D, 4	C51A	f	55				Normal
1E	B229	m	37	3a	8.3E+05	A2/F3	HCV
1E	B512	m	44	3a	2.4E+04	A2/F2	HCV
1E	C37	f	51	3a	8.5E+06	A2/F1	HCV
3A	C126	m	81				HCC, ASH, cirrhosis
3A	C127	m	66				HCC, ASH, cirrhosis
3A	C133	m	67				HCC
3A	C65	m	77				HCC, ASH, cirrhosis
3A	C66	m	72				HCC, ASH, cirrhosis
3A	C78	f	57	3a	2.8E+04	A2/F4	HCV, HCC, cirrhosis

Table S2 miRNA expression in human liver biopsies. miRNAs that are upregulated by HCVcc (Jc1) in undifferentiated huh7.5.1 cells (data not shown) and that are predicted to target the 3' untranslated region (3'UTR) of the PTPRD mRNA. Online prediction tools for miRNA target sites: a= DIANA, b= MIRANDA, c= MIRBRIDGE, d= PICTAR, e= PITA, f= TARGETSCAN. P-values correspond to miRNA expression levels in liver biopsies.

miRNA	ID *(MIMAT)*	Target sites on 3'UTR	Predicted by *(tools)*	Expression in HCV+ liver biopsies *(median fc)*	U-test *(p)*
16-5p	0000069	1	a,b,c,e,f	1.03	0.90
24-3p	0000080	1	a,b,c,d,e,f	1.33	0.59
26-5p	0000082	1	a,b,c,e,f	1.03	0.64
29a-3p	0000086	1	a,c,f	1.28	0.19
29b-3p	0000100	1	a,c,f	1.13	0.94
135a-5p	0000428	2	a,b,d,e,f	2.51	6E-04
148a-3p	0000243	1	a,b,c,e,f	-1.34	0.04
194-5p	0000460	1	a,b,c,e,f	-1.05	0.40

4. DISCUSSION AND PERSPECTIVES

4.1 CONSIDERATIONS ON THE ADVANCED IN *VITRO* SYSTEM AMENABLE TO THE STUDY OF HCV PATHOGENESIS

In the framework of this thesis I successfully built an advanced cell culture model allowing persistent infection over a protracted time frame. This state-of-the-art *in vitro* model permits robust and rapid modeling of gene expression patterns predictive of long-term HCC risk in patients with decades of chronic disease. It is widely accepted that viruses reprogram host cells to their own advantage (Alvisi and Palu, 2013) and it is thus conceivable that HCV benefits from the transcriptomic recalibration of hepatocytes that was described in this thesis. To comprehend why HCV may thrive more efficiently in such an altered milieu, it has to be regarded in the context of viral evolution. By reconstructing its past, we can better understand how HCV currently operates. So let us go back in time before the phylogenetic divergence between *flavivirus* and *hepacivirus*.

Initially, the proto-*hepacivirus* was a canonical *flavivirus* consisting of a capsid inside an envelope decorated with glycoproteins. As it made its entrance in humans, it penetrated and replicated inside hepatocytes, causing hepatocyte necrosis and triggering an inflammatory reaction and concomitant immune response. This initial stage in evolution of HCV might even have caused a fulminant hepatitis with widespread hepatocyte necrosis and liver failure. As a countermeasure, the virus evolved different ways of immune evasion which went hand in hand with adaptation to the hepatocyte micromilieu and less aggressive disease presentation. The most striking of these adaptive features is without doubt the association of the viral glycoproteins with lipoproteins, an absolutely crucial step in the transformation of *flavivirus* into *hepacivirus*. By sticking to lipoproteins, the pathogen could now simply piggyback its way into hepatocytes. At the same time, it did not matter anymore whether or not the humoral immune response could neutralize the virus, the lipoprotein moiety of the primordial lipoviral particle would always authorize access to hepatocytes no matter how potent the neutralizing antibodies. In a next step, the HCV glycoproteins adapted to interact with the lipoprotein receptors, in co-operative harmony with the lipoproteins themselves. The lipoproteins, in turn, were used by the virus to shield important epitopes from neutralizing antibodies. Later, the assembly even got completely intertwined with lipoprotein synthesis, and HCV matured into the lipoviral particle it is today. Obviously, this metamorphosis tightened its relationship with hepatocytes and explains for its intrinsic hepatotropism from an evolutionary perspective.

It is of paramount importance to emphasize that the HCV forerunner evolved -and to this day continues- to replicate in the microenvironment of an inflamed liver, causing continual cell death and increased turnover of hepatocytes. This milieu is thus perennially infused with regenerative growth factors and cytokines such as EGF, IL-6, TGF-α and HGF. These molecules trigger signaling through different axes such as EGFR-MAPK pathways and the IL6-STAT3 cascade promoting cell survival and proliferation (see 1.10.2: cell signaling in liver regeneration). If at the beginning HCV would have found it difficult to survive in this environment, over time it would surely adapt to feel comfortable in cellular systems where these pathways are activated. It is only logical that in a next step of virus-host evolution, HCV would undergo selection for progeny that could activate these networks on its own.

If the question thus arises to why HCV infection can so dramatically induce the transcriptional profile of liver disease patients with poor prognosis ("high-risk signature"), the answer is that it reflects HCV's capacity to recalibrate its host cell circuitry into an inflammatory-regeneratory state in which it has evolved to thrive. Likewise, the patients in whose livers this transcriptomic *state of emergency* is pronounced most strongly will succumb first. And therefore their profile correlates with reduced overall survival chances.

What is truly innovative about our model is that we can investigate *in vitro* the factors that propel liver disease *in vivo*. HCV-associated morbidity is driven by a complex pattern of proteogenomic changes in hepatocytes that we can manipulate in our model: we cannot only induce, but also perturb it. This makes our system a highly valuable tool to test potential therapeutic agents against liver disease progression. In other words, our system enables fast-track drug discovery for chemoprevention of hepatocarcinogenesis. In future work, we envisage scaling down the system to a 96-well format and perform an unbiased screen with complete drug libraries. Moreover, we can manipulate our current model system with the complete biotech toolbox currently available and examine what causes, relieves or exacerbates the profile, and clarify the relationship between gene expression and patient prognosis/survival. New technologies including gene editing by CRISPR-Cas9 technology could demonstrate the effect of perturbing single genes on the generation of the HCC risk signature. Co-cultures with stellate cells or Kupffer cells could provide clues on how other cell types of complete liver tissue influence HCV-induced reprogramming of hepatocytes. Last but not least, i*n vitro* modeling these cell circuits grants a unique opportunity to probe the changes that occur in hepatocytes when an acute infection progresses into chronicity. In that respect, it would be interesting to see if the predictive transcriptomic gene signature would also present itself in the

proteome of our model. Indeed, protein abundance represents a level further downstream in the multilayered cascade of gene expression. Proteins are subject to their own regulatory mechanisms such as translation and degradation that eventually determine their abundance, which is not necessarily correlated with mRNA expression. In this way proteins are more relevant to the cellular state of affairs than mRNA. It is after all the proteome that defines the structural architecture of the cellular biological system. Advanced proteomic approaches using high-throughput mass spectrometry could demonstrate the ability of our system to induce the high-risk signature on the protein level, providing further evidence of its relevance. Moreover, it would allow mathematical modeling of the proteogenomic data and introduce HCV into the rapidly developing field of systems biology. Such an endeavor could elucidate how HCV regulates altered abundance of gene expression products through individual synthesis and degradation rates. Further study of the temporal transcriptomic and proteomic fabric upon viral infection could then increase comprehension of how HCV evades immune responses, reprograms host cells for viral persistence and results in chronic inflammation and eventually cancer.

4.2 CONSIDERATIONS ON PTPRD AS A CANDIDATE FOR HEPATOCARCINOGENESIS

We and others demonstrated that chronic HCV infection rearranges cellular signaling cascades. Among these modulators of signal transduction is protein phosphatase PTPRD. PTPRD is an established tumor suppressor with STAT3 phosphatase activity. The lab has previously demonstrated that STAT3 activity is a necessary co-factor for HCV infection (Lupberger et al., 2013). Our results imply that HCV impairs PTPRD to maintain elevated levels of phospho-STAT3, however up to now we failed to demonstrate this in hepatocytes. In 293T cells PTPRD is readily expressed after transfection with a plasmid or transduction with a lentiviral vector, however when transfecting Huh7.5.1 cells we could detect only mRNA and no protein. Blocking protein degradation by employing proteasome inhibitor MG132 did not yield protein expression (data not shown), which excludes that PTPRD is rapidly degraded after translation. Taken together, it appears that translation is seriously hampered in Huh7.5.1 cells. It may be that the 3'UTR of PTPRD is required for translation and the construction of a plasmid vector containing the PTPRD coding region with its 3'UTR are currently underway.

A potential system for studying PTPRD biology in hepatocytes are PHH. They have plenty of PTPRD expression, easily detectable at both RNA and protein level. However, we were unable to dislodge

PTPRD protein expression in PHH in our lab using RNA silencing techniques, which is not surprising as PHH are notoriously intractable as a model system. A possible solution would be the isolation of primary mouse hepatocytes from PTPRD knockout mice and use those as an *in vitro* model to study the effect of PTPRD on cell signaling (see below).

Intriguingly, the apparent size of PTPRD in immunoblots of PHH lysates (around 150 kDa) is a lot smaller than the full length canonical PTPRD isoform expressed in neurons (215 kDa), suggesting post-translational processing or a hepatocyte-specific isoform smaller in size. Preliminary sequencing data of PHH from one donor suggests that PTPRD in hepatocytes may be a novel isoform (data not shown), but full sequencing still has to confirm whether this novel isoform is smaller in size. Further sequencing efforts are currently under way, it is hoped that uncovering the hepatocyte-specific form of PTPRD will allow expressing it successfully in Huh7.5.1 of 293T cell lines. This would enable much desired functional studies proving that STAT3 is indeed a functional target of hepatocyte-specific PTPRD isoforms.

Another perspective of this work is the generation of $PTPRD^{-/-}$ knockout mice. This model was first described in 2002, and it was shown that even though mice without PTPRD are viable, they have severe learning impairment (Uetani et al., 2000). Tumor development in these mice has so far not been described. Eliciting HCC in these animals and healthy control mice by diethylnitrosamine (DEN) or carbon tetrachloride (CCl_4) administration could confirm whether PTPRD is a true tumor suppressor in the liver. The $PTPRD^{-/-}$ knockout mouse model is currently established in the Mouse Clinical Institute, a platform for mouse models in Strasbourg. *In vivo* experiments are planned to be performed in the course of 2016 to 2017. If knockout mice produce more advanced HCC (more and larger neoplastic lesions) after liver cancer inducing treatment, it would constitute the ultimate proof that PTPRD activity counteracts tumorigenesis. Furthermore, it would identify signaling pathways downstream of PTPRD as potential targets for chemoprevention of HCC.

4.3 FINAL CONCLUDING REMARKS

As a final remark, it is important to view this thesis in the context of a changing field in HCV research. Novel efficient antiviral treatments have shifted attention from curing HCV to preventing HCC. This is self-evident since HCV generally becomes only problematic in chronic patients where continual cell

death and regeneration spanning decades culminates in liver cirrhosis and HCC. In addition, patients with advanced fibrosis that clear the virus remain at high risk for HCC. The perceived triumph of viral cure therefore does not solve all problems. All in all, new strategies and therapies preventing HCC are urgently needed and constitute an unmet medical need. HCV research priorities have thus shifted over the course of my PhD fellowship from 'what we can do to the virus' to 'what the virus does to us'. I believe the work presented here reflects that change in priority.

Taken together, the research carried out in the framework of this thesis sheds new light on the molecular underpinnings of chronic hepatopathology and enables fast-track drug discovery for chemoprevention of hepatocarcinogenesis. Collectively, these findings will help to improve the dismal prognosis of patients with cirrhosis at risk of HCC.

5. RÉSUMÉ DE LA THÈSE DE DOCTORAT

IDENTIFICATION DES CIRCUITS BIOLOGIQUES INDUITS PAR LE VIRUS DE L'HÉPATITE C ET LEURS IMPLICATIONS DANS LE DÉVELOPPEMENT DU CARCINOME HEPATOCELLULAIRE

5.1 CONTEXTE

Le virus de l'hépatite C (HCV) est une cause majeure de maladie chronique du foie et de carcinome hépatocellulaire (CHC) dans le monde (Mohd Hanafiah et al., 2013). Plus de 150 millions de personnes sont infectées, la plupart d'entre elles ne sont pas conscientes qu'elles portent le virus car une infection aigue est généralement asymptomatique et peu diagnostiquée. Dans environ 80% des cas l'infection devient chronique, ce qui cause une cicatrisation extensive du foie, menant à une cirrhose, qui peut résulter en un cancer du foie 20 ans ou plus après avoir le début de l'infection. Il n'y pas de vaccin disponible, mais une nouvelle génération d'antiviraux permet maintenant d'éliminer efficacement le virus (Chung and Baumert, 2014). Par contre, même dans les pays industrialisés les plus riches, l'accès à ces nouveaux traitements est limité à cause de leurs coûts élevés(Kamal-Yanni, 2015). De plus, même après éradication du virus, un risque augmenté de développer le cancer du foie persiste (van der Meer et al., 2012), et les options thérapeutiques pour le CHC sont limitées (Forner et al., 2012). Par conséquent, le CHC induit par le HCV restera une indication majeure pour la transplantation hépatique au cours des prochaines années.

Malheureusement, les mécanismes responsables de cette susceptibilité accrue des patients atteints d'hépatite C de développer des cancers du foie ne sont pas encore bien compris. La recherche a été ralentie pendent des années car il n'existait pas de système de culture cellulaire robuste, et ce n'est qu'en 2005 que le cycle viral complet du HCV a pu être récapitulé *in vitro* (modèle du HCV dérivé de culture cellulaire (HCVcc)). Depuis l'arrivé des « big data » dans la recherche biomédicale, qui ont permis des approches informatiques de grande envergure, la pathogenèse du HCV peut être caractérisée plus en détails. Ainsi, le Dr. Hoshida et ses collaborateurs ont isolé de l'ARN du foie de

patients ayant subi une résection chirurgicale de CHC et ont identifié une signature transcriptomique du tissu hépatique qui peut prédire la survie des patients à long terme (Hoshida et al., 2008). Ce profil transcriptomique est aussi capable d'identifier quels patients avec une cirrhose ont un risque élevé de développer un CHC (Hoshida et al., 2013). Autrement dit, cette signature ne peut pas seulement servir de biomarqueur polyvalent pour identifier les patients qui auront le plus besoin d'un traitement, mais également nous aider à comprendre les mécanismes moléculaires qui favorisent la progression des maladies hépatiques.

Afin de mieux comprendre les interactions virus-hôte, le laboratoire a précédemment identifié le récepteur du facteur de croissance épidermique (EGFR) comme facteur de l'hôte essentiel pour l'entrée et l'infection du HCV, à la fois *in vitro* et *in vivo (Lupberger et al., 2011)*. De plus, le laboratoire a montré que les récepteurs à activité tyrosine kinase (RTK) modulent l'entrée du virus via la protéine Hras qui régule la formation du complexe de co-récepteurs constitué de claudin-1 et CD81 (Zona et al., 2013). Par ailleurs, l'attachement des virions à ses récepteurs suffit pour déclencher l'activation des RTK (Diao et al., 2012). Ceci suggère qu'en modulant des voies de signalisation des cellules hôtes, le HCV module leur transcriptome et leur protéome ce qui peut contribuer à la progression des maladies hépatiques. A ce jour, il est difficile d'étudier les voies de signalisation induites par le HCV à l'aide des modèles disponibles, étant donné que les cellules produisant les HCVcc peuvent sécréter des cytokines et des facteurs de croissance qui peuvent interférer avec les effets induits par le virus. Il est ainsi indispensable de mettre au point de nouveaux modèles pour étudier l'impact du virus sur sa cellule hôte.

5.2 OBJECTIFS

Le but de mon travail de thèse a ainsi été (i) d'établir un système de culture *in vitro* à long terme en utilisant des HCVcc purifiés pour étudier la pathogenèse du HCV et (ii) d'identifier des changements dans les circuits biologiques spécifiquement induits par le HCV, à la fois *in vitro* et *in vivo*, qui pourraient contribuer au développement des maladies du foie. Je me suis plus particulièrement intéressé aux phosphatases et leur impact sur les voies de signalisation.

5.3 RÉSULTATS

5.3.1 ETABLISSEMENT D'UN SYSTÈME D'INFECTION À LONG TERME PAR LE HCV PERMETTANT L'ÉTUDE DE LA PATHOGENÈSE DU HCV

Publication n°1 : Bandiera S, Venkatesh A, **Van Renne N**, Sun X, Lupberger J, Thumann C, El Saghire H, Verrier E.R, Durand S.C, Pernot S, Nakagawa S, Wei L, Kiani K, Soumillon M, Fuchs B.C, Chung R.T, Goossens N, Koh A, Mahajan M, Nair V.D, Gunasekaran G, Schwartz M.E, Tanabe K.K, Nicolay B.N, Bardeesy N, Shalek A.K, Rozenblatt-Rosen O, Regev A, Pochet N, Zeisel M.B, Hoshida Y, Baumert T.F. A clinical gene-signature based human cell culture model unravels drivers of hepatocacinogenesis and compounds for cancer chemoprevention. ***Soumis à Nature Medicine***

Le HCV dérivé de culture cellulaire (HCVcc) utilisé pour la recherche expérimentale est classiquement obtenu en récoltant le milieu de culture qui contient des particules virales après avoir électroporé l'ARN viral dans des cellules Huh7 ou des clones dérivés de ces cellules (Lohmann and Bartenschlager, 2014). Cette procédure cause la libération de facteurs de stress dans le milieu de culture, et, au moment de l'utilisation des virus comme inoculum dans des expériences *in vitro*, ceux-ci peuvent activer des voies de signalisation indépendamment du virus. Pour éviter cela, j'ai établi et optimisé un système de purification sur colonne d'affinité pour isoler des HCVcc (Merz et al., 2011) d'une grande pureté permettant d'effectuer des infections à large échelle. Ces particules purifiées sont stables à -80°C pendant au moins 6 mois. Grâce à ce modèle, nous pouvons spécifiquement étudier toutes les modifications dans les voies signalisation induites par le HCV en l'absence de contaminant.

La lignée cellulaire Huh7, qui fut originairement isolé d'un CHC d'un patient japonais en 1982 (Nakabayashi et al., 1982), et son sous-clone Huh7.5.1 représentent des outils essentiels pour la recherche sur le HCV, car ce sont les seules lignées cellulaires capables de conduire le cycle viral entier *in vitro.* Par contre, comme ces lignes cellulaires se divisent sans cesse, elles se distinguent des hépatocytes primaires du foie, qui sont plus différentiés et ne se divisent presque pas (Michalopoulos and DeFrances, 1997). Il a été montré que l'addition de 1% de diméthylsulfoxyde (DMSO) au milieu de culture pouvait induire l'arrêt de croissance des cellules Huh7 et l'expression de gènes spécifiques aux hépatocytes, tout en conservant en même temps leur permissivité pour le HCV(Sainz and Chisari, 2006). Ces cellules Huh7.5.1^{diff} représentent donc un modèle qui se rapproche plus de la physiologie

hépatique que les lignées hépatocytaires classiques. De plus, elles ont l'avantage de permettre l'étude de l'infection virale chronique *in vitro* (Bauhofer et al., 2012).

En combinant ce système de culture à long terme avec un inoculum viral purifié, j'ai montré que la phosphorylation de l'EGFR est régulée positivement au cours d'une infection chronique. Ensuite, en analysant le transcriptome (puces à ADN) des cellules chroniquement infectées, j'ai observé une augmentation de l'expression d'un axe EGFR, constitué non seulement de l'EGFR lui-même, mais également de l'EGF, le ligand de l'EGFR. Ces résultats ont été validés *in vitro* en quantifiant par RT-qPCR l'ARNm de l'EGF et de l'EGFR dans les cellules infectées. En outre, une analyse transcriptomique par "Gene Set Enrichment Analysis" (GSEA) démontre une régulation positive des gènes associés à la voie de l'EGF. Dans le cadre d'une collaboration avec le Dr. Hoshida au Mount Sinai Hospital (New York, NY, Etats Unis) nous avons constaté que nous pouvons induire la signature transcriptomique pronostique décrite ci-dessus dans notre système d'infection *in vitro,* c'est à dire nous récapitulons *in vitro* le profil des ARNm des patients chroniquement infectés par le HCV et présentant un risque élevé de développer un CHC (Hoshida et al., 2008; Hoshida et al., 2013). Nous avons donc développé un système simple et robuste basé sur des cellules hépatiques qui récapitule la reprogrammation transcriptionnelle des patients atteints de HCV à risque de développer un cancer du foie. En outre, cette signature d'ARNm est également présente *in vitro* dans des cultures de cellules infectées par le virus de l'hépatite B, ou incubées en présence d'alcool (éthanol), deux autres étiologies majeures de CHC. De plus, nous avons montré qu'il est possible de réverser cette signature de mauvais pronostic *in vitro* en traitant les cellules avec des antiviraux, ou en bloquant la voie de signalisation de l'EGFR par l'erlotinib, un inhibiteur spécifique de cette RTK. Étant donné que cet inhibiteur a récemment été démontré capable d'empêcher efficacement la progression de la cirrhose vers le CHC dans un modèle murin (Fuchs et al., 2014), ces expériences indiquent que notre système *in vitro* est un bon modèle pour tester des composés pour la prévention du développement de CHC.

5.3.2 PTPRD (PROTEIN TYROSINE PHOSPHATASE RECEPTOR TYPE D) EST UN SUPPRESSEUR DE TUMEURS POTENTIEL DANS LE FOIE

Publication n°2 : **Van Renne N**, Duong F.H.T, Gondeau C, Calabrese D, Fontaine N, Roca Suarez A.A, Ababsa A, Durand S.C, Pessaux P, Heim M.H, Baumert T.F, Lupberger J. miR-135a-5p-mediated downregulation of protein-tyrosine phosphatase delta (PTPRD) is a candidate suppressor of HCV-associated hepatocarcinogenesis. ***Soumis à Journal of Hepatology***

Dans la deuxième partie de ma thèse, je me suis intéressé aux modifications des voies de signalisation cellulaire induites par le virus qui pourraient contribuer au développement des maladies hépatiques. Étant donné que ces processus de signalisation sont étroitement régulés par des protéines phosphatases et que l'expression aberrante de phosphatases joue un rôle dans différents syndromes et maladies (Tonks, 2006), nous avons examiné dans des biopsies du foie de patients atteints d'hépatite C chronique l'expression de phosphatases qui avaient précédemment été associées à des maladies. De manière intéressante, parmi les phosphatases dont les niveaux d'expression étaient les plus dérégulés dans ces biopsies, nous avons observé un enrichissement de candidats pouvant jouer un rôle dans le développement du CHC. Plus particulièrement, certaines de ces phosphatases agissent comme suppresseur de tumeur dans divers cancers, y compris PTPRD. En effet, PTPRD est fréquemment inactivée et mutée dans des cancers humains (Julien et al., 2011; Veeriah et al., 2009), y compris le CHC (Acun et al., 2015). Afin de valider la modulation de l'expression de PTPRD par le HCV, nous avons analysé un plus grand nombre de biopsies et confirmé une diminution significative de l'expression de PTPRD dans les biopsies de patients atteints d'hépatite C chronique. Nous avons également montré que l'expression de PTPRD est diminuée après infection d'une culture d'hépatocytes primaires humains par le HCV *in vitro*, tandis que cette phosphatase n'est pas exprimée dans des lignées cellulaires hépatocytaires. De plus, nous avons démontré que l'expression de PTPRD est plus faible dans des lésions tumorales dans des biopsies de foie provenant de patients infectés par le HCV et non infectés. En outre, nous avons constaté que des niveaux élevés de PTPRD dans le tissu hépatique adjacent aux lésions cancéreuses sont corrélés avec la survie des patients ainsi qu'avec une réduction de la récidive de la tumeur après la résection chirurgicale. Finalement, nous avons démontré que la surexpression de miR-135a-5p, un micro-ARN surexprimé dans le foie des patients chroniquement infectés par le HCV, entraînait une diminution de l'expression de PTPRD. L'ensemble de ces résultats suggère que PTPRD régule de manière négative l'hépatocarcinogenèse associée au HCV.

5.4 CONCLUSIONS ET PERSPECTIVES

Notre nouveau modèle *in vitro* permet la modélisation robuste et rapide des profils d'expression de gènes prédictifs du risque de CHC chez les patients atteints de maladies hépatiques chroniques. En

outre, il offre des opportunités uniques pour étudier les mécanismes moléculaires et les circuits cellulaires qui conduisent au CHC. En effet, en utilisant ce système, nous avons découvert le rôle fonctionnel de l'EGFR dans la génération de la signature de risque du CHC indépendamment de l'étiologie, et nous avons révélé d'autres candidats potentiellement impliqués dans ce processus.

Par ailleurs, nous avons découvert que l'infection chronique par le HCV a un effet sur des voies de signalisation cellulaire en modulant la transduction du signal. Nous avons démontré que l'infection induit une diminution de l'expression de PTPRD. PTPRD est un suppresseur de tumeur qui est connu pour réguler la phosphorylation de STAT3. Le laboratoire avait démontré auparavant que l'activité de STAT3 est nécessaire à l'infection par le HCV (Lupberger et al., 2013). Nos résultats impliquent que l'infection prolongée par le HCV peut diminuer l'expression de PTPRD dans les hépatocytes infectés et ainsi rendre l'hépatocyte infecté plus sensibles à la transformation maligne.

L'ensemble des résultats obtenus dans le cadre de cette thèse apporte un nouveau regard sur les fondements moléculaires des maladies hépatiques chroniques. La modélisation des circuits biologiques impliqués dans la progression des maladies du foie permettra à court et moyen terme une découverte accélérée de médicaments pour prévenir cette progression. A plus long terme, ces résultats contribueront à améliorer le pronostic des patients atteints de cirrhose et à risque de développer un CHC.

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7. CURRICULUM VITAE

GENERAL INFORMATION:

Full Name:	Nicolaas Renée Cesar Van Renne
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PERSONALIA

Nicolaas Van Renne was born in Ghent, Belgium on October 18th, 1983. He completed his secondary education at the royal atheneum Voskenslaan in Ghent (Latin and Greek). He continued to study English and Spanish, obtaining his licenciate in translation studies in 2006 at Hogeschool Gent (nowadays incorporated as Master of translation in the Faculty of Arts and Philosophy, Ghent University). He then studied Veterinary Medicine at Ghent University, and in 2012 obtained his degree of Master of Veterinary Medicine with distinction (main subject: research). Captivated by the subject of virology, he spent a large part of his last year as a vet student in the veterinary virology lab at the faculty of veterinary medicine. Under the supervision of lab director Prof. Hans J. Nauwynck, he investigated *in vivo* diagnostics and *in vitro* replication of Porcine Circovirus Type 2. This work led to two orginal publications in peer-reviewed scientific journals.

In an effort to increase his knowledge and acquire international experience, he then moved to Strasbourg where he obtained a competitive IDEX scholarship of the University of Strasbourg for a PhD fellowship. At INSERM U1110, the institute of viral and liver disease of lab director Prof. Thomas F. Baumert, he established a state-of-the art *in vitro* model amenable to study hepatitis C virus pathogenesis. He further characterized changes in signaling patterns contributing to the development

of virus-induced hepatocellular carcinoma.

SCIENTIFIC OUTPUT

ORIGINAL PUBLICATIONS

2016 **Van Renne N**, Duong F.H.T, Gondeau C, Calabrese D, Fontaine N, Roca Suarez A.A, Ababsa A, Pessaux P, Heim M.H, Baumert T.F, Lupberger J. miR-135a-5p-mediated downregulation of protein-tyrosine phosphatase delta is a candidate driver of HCV-associated hepatocarcinogenesis. *Submitted to Journal of Hepatology.*

Bandiera S, Venkatesh A, **Van Renne N**, Sun X, Lupberger J, Thumann C, El Saghire H, Verrier E.R, Durand S.C, Pernot S, Nakagawa S, Wei L, Kiani K, Soumillon M, Fuchs B.C, Chung R.T, Goossens N, Koh A, Mahajan M, Nair V.D, Gunasekaran G, Schwartz M.E, Tanabe K.K, Nicolay B.N, Bardeesy N, Shalek A.K, Rozenblatt-Rosen O, Regev A, Pochet N, Zeisel M.B, Hoshida Y, Baumert T.F. A clinical gene-signature based human cell culture model unravels drivers of hepatocacinogenesis and compounds for cancer chemoprevention. *Submitted to Nature Medicine*

2015 Huang L*, **Van Renne N***, Liu C, Nauwynck H.J. (*contributed equally). A sequence of basic residues in the Porcine Circovirus Type 2 capsid protein is crucial for its co-expression and co-localization with the replication protein. *Journal of General Virology 2015 Dec; 96(12):3566-76.*

2014 Saha D, Del Pozo Sacristán R, **Van Renne N**, Huang L, Decaluwe R, Michiels A, Rodriguez A.L, Rodríguez M.J, Durán M.G, Declerk I, Maes D, Nauwynck H.J. Anti-porcine circovirus type 2 (PCV2) antibody placental barrier leakage from sow to fetus : impact on the diagnosis of intra-uterine PCV2 infection. *Virologica Sinica* 2014 Apr; 29(2):136-8.

REVIEW ARTICLE

2015 Lupberger J, **Van Renne N**, Baumert T.F. Chapter 37 - Signaling of hepatitis C virus. Signaling pathways in liver diseases, 3rd ed, Wiley-Blackwell publishing group, pp459-468.

ORAL COMMUNICATIONS

2015 **Van Renne N**[+], Duong F.H.T, Gondeau C, Calabrese D, Fontaine N, Ababsa A, Durand S.C, Pessaux P, Heim M.H, Baumert F.T, Lupberger J. ([+] presenter) The expression of tumor suppressor PTPRD is downregulated in HCV-infected primary human hepatocytes and liver tissues of HCC patients. *50th annual meeting of the European Association for the Study of the Liver, Vienna, Austria.*

Van Renne N, Duong F.H.T, Gondeau C, Calabrese D, Fontaine N, Ababsa A, Durand S.C, Pessaux P, Heim M.H, Baumert F.T, Lupberger J.[+] ([+] presenter) The expression of tumor suppressor PTPRD is down-regulated in the liver of patients with HCV infection and in tumor lesions of patients with hepatocellular carcinoma. ([+] presenter) *15e réunion du réseau national hépatites de l'ANRS, Paris, France.*

POSTERS

2015 **Van Renne N**, Duong F.H.T, Gondeau C, Calabrese D, Fontaine N, Durand S.C, Ababsa A, Pessaux P, Heim M.H, Baumert T.F, Lupberger J. Expression of PTPRD is downregulated in liver tissue of chronic HCV patients and in tumor lesions of HCC patients. *22nd International symposium on hepatitis C virus and related viruses, Strasbourg, France.*

Van Renne N, Duong F.H.T, Gondeau C, Calabrese D, Fontaine N, Durand S.C, Ababsa A, Pessaux P, Heim M.H, Baumert T.F, Lupberger J. Expression of PTPRD is downregulated in liver tissue of chronic HCV patients and in tumor lesions of HCC patients. *9e Forum du cancéropôle du Grand-Est, Strasbourg, France.*

Van Renne N, Duong F.H.T, Gondeau C, Calabrese D, Fontaine N, Durand S.C, Ababsa A, Pessaux P, Heim M.H, Baumert T.F, Lupberger J. Expression of PTPRD is downregulated in liver tissue of chronic HCV patients and in tumor lesions of HCC patients. *Doctoral school days, Université de Strasbourg, Strasbourg, France.*

Ababsa A, Duong F.H.T, **Van Renne N**, Gondeau C, Heim M.H, Baumert T.F, Lupberger J. Expression of tumor suppressor PTPRD is downregulated in HCV-infected PHH and liver tissue of patients with chronic HCV infection. *65th Annual meeting of the*

American Association for the Study of Liver Diseases, Boston MA, USA.

EDUCATION

SECONDARY EDUCATION

1996-2001: Koninklijk Atheneum Voskenslaan (Ghent, Belgium): Latin and Greek

HIGHER EDUCATION

- 2003-2006: Master of Arts in Translation, option English-Spanish (4 years) – Hogeschool Gent, Belgium, now integrated in Faculty of Arts & Philosophy, Ghent University, Belgium

- 2007-2012: Master of Veterinary Medicine (6 years, graduated with distinction) - Ghent University, Belgium

- 2013-2015: PhD student INSERM U1110, Institute of viral and liver disease, Strasbourg, France. Thesis title: "Unraveling HCV-induced biological circuits contributing to HCC development" (Defense scheduled April 19th 2016)

ADDITIONAL CREDITS OBTAINED AT GHENT UNIVERSITY

- 2004: Political and social history of Belgium (course from 1st Bachelor Political Sciences)
- 2005: Classical Arabic (course from 1st Bachelor Oriental languages and cultures)

OTHER RELEVANT QUALIFICATIONS

- Trained in lab animal science based on Felasa C requirements, recognized by the Belgian federal public service of health, food chain safety and environment.
- Experience with lab animals (pigs)

Nicolaas Van Renne

IDENTIFICATION DES CIRCUITS BIOLOGIQUES INDUITS PAR LE VIRUS DE L'HÉPATITE C ET LEURS IMPLICATIONS DANS LE DÉVELOPPEMENT DU CARCINOME HEPATOCELLULAIRE

Résumé

En combinant un nouveau système de culture cellulaire à partir d'hépatocytes différenciés avec du virus de l'hépatite C (VHC) purifié, nous pouvons induire un profil transcriptomique caractéristique des patients à risque élevé de développer un carcinome hépatocellulaire (CHC). En utilisant ce modèle, nous avons découvert le rôle fonctionnel de l'EGFR comme élément moteur de la signature du risque de développement d'un CHC. De plus, nous avons identifié des gènes candidats impliqués dans le développement du CHC. Pour étudier les maladies du foie *in vivo*, nous avons caractérisé l'expression des protéines phosphatases dans des biopsies hépatiques de patients infectés par le VHC. Nous avons observé une régulation négative de PTPRD, un suppresseur de tumeur, causé par une augmentation de miR-135a-5p qui cible l'ARNm de PTPRD. Par ailleurs, l'analyse *in silico* montre que l'expression de PTPRD dans le tissu hépatique est corrélée à la survie chez les patients atteints de CHC.

Mots clés : Virus de l'hépatite C, carcinome hépatocellulaire, maladie hépatique, signalisation cellulaire, phosphatase, PTPRD

Résumé en anglais

By combining a cell culture system of hepatocyte-like cells with purified hepatitis C virus (HCV), we effectively simulated chronic infection *in vitro*. We found this infection model induces a transcriptomic profile of chronic HCV patients at high risk of developing hepatocellular carcinoma (HCC). Using this model, we have uncovered the functional role of EGFR as a driver of the HCC risk signature and revealed candidate drivers of the molecular recalibration of hepatocytes leading to liver cancer. In an approach to study liver disease *in vivo*, we opted to screen for protein phosphatase expression in liver biopsies of chronic HCV patients. We observed a downregulation of PTPRD, a well-known tumor suppressor. We demonstrated that this effect is mediated by an increase in miR-135a-5p which targets PTPRD mRNA. Moreover, *in silico* analysis shows that PTPRD expression in adjacent liver tissue of HCC patients correlates with survival and reduced tumor recurrence after surgical resection.

Keywords: hepatitis C virus, hepatocellular carcinoma, liver disease, cell signalling, phosphatase, PTPRD