Mechanisms of Osteopontin-mediated effects on chronic hepatic inflammation and cancer

Dissertation

Zur Erlangung der Würde des Doktors der Naturwissenschaften des Fachbereichs Biologie, der Fakultät für Mathematik, Informatik und Naturwissenschaften, der Universität Hamburg

vorgelegt von

Christine Loscher, geb. Kranig

aus Schwerin

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Sehr geehrte Damen und Herrn,

hiermit bestätige ich, dass die von Frau Christine Loscher mit dem Titel "Mechanisms of osteopontin-mediated effects on chronic hepatic inflammation and cancer " vorgelegte Doktorarbeit in korrektem Englisch geschrieben ist.

Mit freundlichen Grüßen,

Carol Stocking

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1 Abstract

Chronic liver inflammation can be induced by hepatitis C virus (HCV), which often persists, leading to chronic hepatitis with progression to cirrhosis and hepatocellular carcinoma (HCC). HCC has a high rate of mortality and poorly understood mechanisms lead to very limited therapeutic options. Osteopontin (OPN) has been found over-expressed in patients with HCV infection and in patients with HCC, where it is associated with poor prognosis. Moreover, it is not clearly understood, how OPN is involved in the different mechanisms of hepatitis. In my thesis, I investigated the Wnt signaling pathway because it is also associated with high HCV replication as well as tumor development.

To investigate the influences of OPN on the Wnt pathway and subsequent regulation of HCV protein expression or tumor cell survival, different human hepatoma cell lines were used as an easy to handle *in vitro* model. HepG2 and Huh7 cells were used, as well as cells expressing HCV nonstructural protein-3 (NS3) to-5 (NS5B) alone (Huh5-15) or in combination with firefly luciferase, as a reporter of HCV protein expression (LucUbiNeo-ET). Furthermore liver tissue of humanized, HCV infected Urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mice and tumor tissue of multi drug resistant transporter 2 knock-out (Mdr2ko) mice was used to analyze OPN expression in HCV infection or HCC. Knock-down of OPN expression was achieved by transfection of small interfering RNA (siRNA), up-regulation was achieved by transfection of an OPN coding plasmid. Gene expression was measured by real time reverse transcription-PCR (RT-PCR) and Western Blot analysis. To promote Wnt pathway activity the glycogen synthase kinase 3β (GSK3β) inhibitors Kenpaullone (KPLN) or SB216763 were used. Wnt signaling pathway activity was measured by a reporter assay for β-catenin related transcription (CRT) and by real time RT-PCR. Cell viability was measured by using 3-4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and to determine total cell numbers and percentages of dead cells, cells were counted.

OPN was found to be highly expressed in HCV infected uPA/SCID mice and HCV replicon cell lines and this up-regulation was mediated by HCV. In addition, OPN was shown to be increased in murine HCC and human hepatom cell lines. Furthermore, OPN manipulation affected the tumor relevant Wnt signaling pathway activity, which was shown to influence HCV protein expression, mediated by cell proliferation. Surprisingly, OPN was found not to directly regulate HCV protein expression. This may be explained by the fact, that in this *in vitro* system, OPN mediated effects on the Wnt signaling pathway are still below the threshold needed to

induce cell proliferation. Therefore, sufficient down-regulation of OPN or one of its effector molecules in the Wnt signaling pathway might not only represent a target for tumor growth interference, but might also become a novel approach for HCV therapy.

2 Zusammenfassung

Eine akute Leberentzündung kann durch verschiedene Auslöser induziert werden und wenn sie nicht ausheilt, kann sie chronisch werden. Hepatitis C Viren (HCV), zum Beispiel, persistieren in der Leber und können zu einer chronischen Hepatitis führen, welche im weiteren Verlauf über Leberzirrhose zum hepatozellulärem Karzinom (HCC) führen kann. HCC Patienten weisen eine hohe Sterblichkeitsrate auf und die zur Entstehung des HCC beitragenden Mechanismen sind nicht ausreichend bekannt, wodurch nur sehr geringe Therapieoptionen zur Verfügung stehen. Das Glycoprotein Osteopontin (OPN) ist in Patienten mit HCV Infektion und in Patienten mit HCC erhöht. In der HCC Diagnostik wird OPN als Tumormarker mit schlechten Überlebenschancen für den Patienten in Verbindung gebracht, jedoch ist nicht ausreichend verstanden, auf welche Weise OPN in die verschiedenen Mechanismen der Hepatitis und Tumorentwicklung involviert ist. In dieser Arbeit wird der Wnt Signalweg untersucht, dessen erhöhte Aktivität, ebenso wie der erhöhte OPN Spiegel, mit erhöhter HCV Replikation und Tumorentwicklung in Verbindung gebracht wird.

Für die Untersuchung der Einflüsse von OPN auf den Wnt Signalweg und daraus resultierende Veränderungen der HCV-Proteinexpression oder der Tumorzellproliferation, wurden verschiedene humane Hepatomzelllinien genutzt. Neben HepG2 und Huh7 Zellen, wurden auch Zelllinien genutzt welche die HCV Gene der Nicht-strukturproteine 3 (NS3) bis 5 (NS5) exprimieren (Huh5-15) und Zellen, welche zusätzlich zu diesen HCV Proteinen auch Firefly Luziferase, als Reporter für HCV Proteinexpression, exprimieren (LucUbiNeo-ET). Des Weiteren wurde Lebergewebe von humanisierten, HCV infizierten Urokinase-type plasminogen activator/severe combined immunodeficience (uPA/SCID) Mäusen und Tumorgewebe von multi drug resistance transporter 2 knock-out (Mdr2ko) Mäusen genutzt, um die entsprechende Höhe der OPN Expression zu untersuchen. Eine Reduktion der OPN Expression wurde durch Transfektion der entsprechenden Zellen mit siRNA erreicht und eine verstärkte OPN Expression durch Transfektion der entsprechenden Zellen mit einem OPN kodierenden Plasmid, dabei wurde die Genexpression mittels real time RT-PCR und Westernblot bestimmt. Zur Steigerung der Aktivität des Wnt Signalweges, wurden die Inhibitoren der Glycogen Synthase Kinase 3β (GSK3β), Kenpaullone (KPLN) und SB216763, verwendet. Gemessen wurde die Aktivität des Wnt Signalweges mit einem Reportertestverfahren für die β-catenin regulierte Transktiption (CRT) und mittels real time RT-PCR. Zelllebensfähigkeit wurde unter Verwendung von (3-4, 5-Dimethylthiazol-2-yl)-2, 5-

diphenyltetrazolium Bromide (MTT) ermittelt und zur Bestimmung der absoluten Zellzahlen sowie des Anteils lebendiger Zellen an der gesamtzellzahl, wurden diese gezählt.
In dieser Arbeit wurde eine erhöhte, durch HCV vermittelte OPN Expression in humanisierten, HCV infizierten uPA/SCID Mäusen und HCV Replikonzellen detektiert. Des Weiteren zeigte sich eine erhöhte OPN Expression in murinen HCC Probenund in humanen Hepatomzelllinien. Die Regulierung von OPN führte zu Änderungen der Aktivität des Wnt Signalweges, welcher die HCV Proteinexpression mittels Proliferation beeinflussen kann. Jedoch konnte nicht gezeigt werden, dass die OPN Manipulation einen direkten Einfluss auf HCV Expression hat. Das könnte damit erklärt werden, dass in diesem *in vitro* System die durch OPN vermittelten Effekte auf den Wnt Signalweg zu gering sind, um die Proliferation zu verändern, die dann die HCV Replikation beeinflussen kann. Diese Ergebnisse zeigen, dass eine Regulierung von OPN oder nachfolgender Signalwege ein guter Ansatz für die Weiterentwicklung der HCV und der HCC Therapie darstellt.

3 Introduction

3.1 Liver anatomy and function

The liver is the biggest gland organ in man and responsible for many metabolic processes. It is divided into four lobes; two main lobes, the left *Lobus sinister* and the right *Lobus dexter*, and two additional lobes, *Lobus caudatus* and *Lobus quadratus* (Figure 1 A). Furthermore, the liver lobes are subdivided into several small lobules (Figure 1 B). These lobules are composed of liver parenchymal cells, so called hepatocytes, which are embedded in cords next to the blood vessels. Single branches of, the hepatic artery, the portal vein, and the bile duct jointly build the portal triad.[1]

A **B**

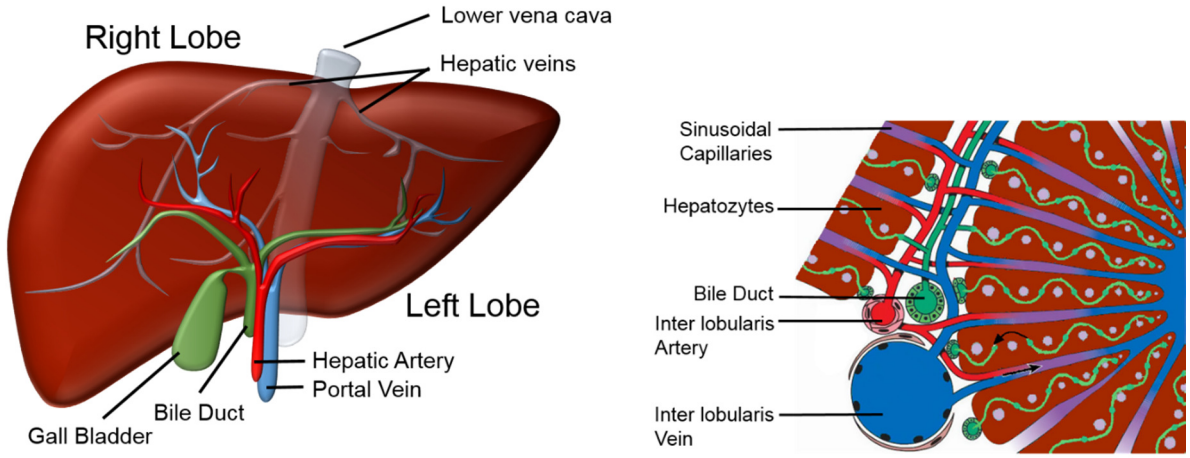

Figure 1: The liver, schematic
A: Overview of the liver and its vessels; B: Overview of a lobule, modified[1]

Hepatocytes have high metabolic activity; they are involved in several metabolic functions including detoxification, protein synthesis, hormone production, and glycogen storage.[2] This is enabled by highly developed cell organelles like the Golgi apparatus, endoplasmic reticulum, and mitochondria, with a wide variety of enzymes.[3] The hepatocytes produce bile salts and excrete metabolites into bile or blood.[4] The liver is supplied with 20% oxygen rich blood via the hepatic artery and 80% oxygen low blood via the portal vein from the gut. The contact between hepatocytes and the blood is facilitated by the liver sinusoidal capillaries. These capillaries are slightly lined with the liver sinusoidal endothelial cells (LSEC) that are fenestrated, thus allowing the extension of hepatic stellate cells (HSC) and liver resident macrophages, the Kupffer cells. HSCs can detach from the outer sinusoidal wall and can circulate in the gap between hepatocytes and sinusoidal wall (space of Disse). Kupffer cells can

detach from the internal site of the sinusoidal wall and circulate in the blood. They are able to absorb cell debris, bacteria and viruses.[3] Due to the fact that the liver receives most of its blood supply from the digestive tract, it is continuously exposed to bacterial products, environmental toxins and food antigens. Therefore the liver has to tolerate harmless antigen[5]; however, in case of an infection, the liver initiates an immune response[2].

3.2 Liver inflammation and cancer

Liver tumors are often based on chronic hepatitis, which can be initiated by various factors like antigens, toxins, bacteria, or viruses. In the beginning of an infection, Kupffer cells absorb pathogenic particles via phagocytosis and initiate the adaptive immune response.[1] Furthermore, cells of the innate immune response, like neutrophilic granulocytes, release cytokines, which in turn can stimulate hepatocytes to produce pattern-recognition receptors (PRRs). These PRRs recognize pathogen-associated molecular patterns (PAMPs) of infectious microorganisms[2]. If, in some cases the infection cannot be cleared, the inflammation becomes chronic, which is accompanied by an excessive wound healing response.[6] Over time, continuous fibrotic tissue remodeling results in cirrhosis and loss of liver function. Chronic inflammation and fibrosis initiate regeneration processes, that may result in accumulation of mutations, hence supporting the development of hepatocellular carcinoma (HCC).[7]

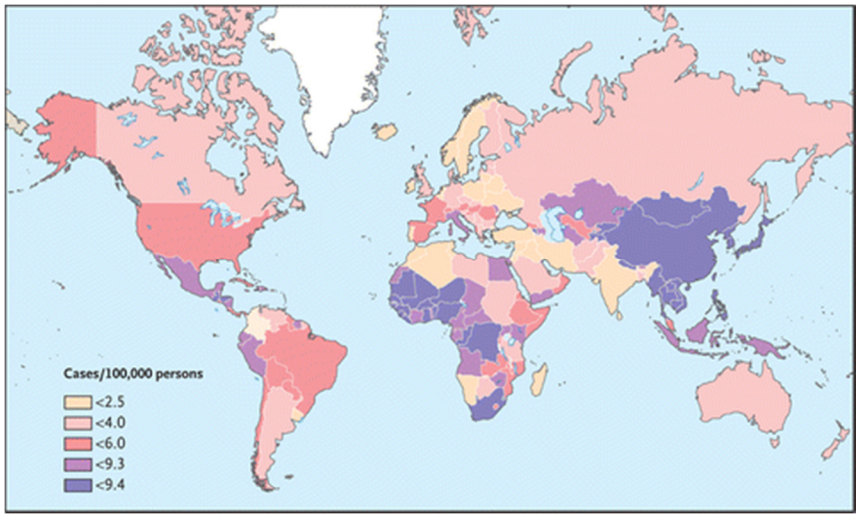

Figure 2: Regional variation in the estimated age-standardized incidence rates of liver cancer 2008
Modified from the World Health Organization[8]

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the ninth in women worldwide (Figure 2). Liver cancer has a high rate of mortality, which is the reason why it is the second most common cause of death from cancer worldwide, thereof 83% of all

HCC occur in developing countries.[9] The specific mechanisms of HCC development are poorly understood and therapy options are still limited. Early-stage HCC is often asymptomatic, therefore many are diagnosed at advanced stages and therapies are less effective. Furthermore, HCC show a high heterogeneity in gene expression and no specific therapy is known to slow tumor growth.[10] Therefore, a better understanding of gene and pathway regulation might result in successful tumor suppression by the use of personalized therapy. A small step to increase survival of HCC patients was achieved in 2008. Henceforward the therapy with Sorafenib was applied, which extends live time of responder patients for three to four month.[11]

3.3 Hepatitis C Virus

Hepatitis can be induced by viral infection, which often persists in liver cells. Hepatitis C Virus (HCV) belongs to the family of *Flaviviridae,* which is classified in the *Hepacivirus* genus. Caused by its high variability, HCV can be distinguished into 7 genotypes and several subtypes.[12] The HCV is enveloped by a membrane that consists of glycoproteins and includes a core-capsid with the viral genome. The genome (Figure 3) is a positive-stranded RNA, that consists of a 5' untranslated region (UTR) with an internal ribosome entry site (IRES), an open reading frame, which contains gene information for structural proteins (core, E1, E2, p7) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B), and a 3'-UTR. The virus exclusively infects humans and chimpanzees and the main target cells are hepatocytes. During its life cycle the virus binds to receptors such as the surface protein cluster of differentiation (CD) 81 or the LDL receptor, which initiates the internalization of the virus into the hepatocytes. Once in the cell, the viral particles are released and uncoated, translation and protein processing is mediated by the IRES. For virus multiplication RNA replication is performed, viral particles are assembled, packaged and released. The HCV genome does not translocate in the nucleus or integrate in the host genome.[13]

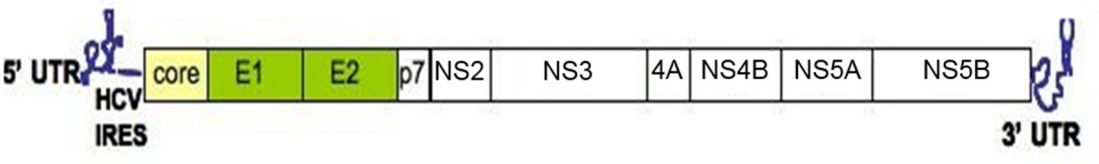

Figure 3: Schematic hepatitis C virus (HCV) RNA genome
Modified from Pawlotsky *et al.*[14]; untranslated regions (UTR), capsid protein (core), envelope glycoproteins (E1, E2), ion channel (p7), cysteinprotease (NS2), serine protease RNA helicase/NTPase (NS3, 4A), membranous web formation (NS4B), phosphoprotein (NS5A), RNA-dependent RNA polymerase (NS5B); NS = nonstructural

HCV induces an acute inflammation, which may become chronic, especially under poor health care conditions. The acute infection can be cleared within 6 month but in 55-85% of the cases the infection becomes chronic. This leads to development of liver cirrhosis within 20 years in 15-30% of all patients. HCV infection is a blood-born disease and can be transmitted in case of contaminated transfusions, from an infected mother to her baby during birth or the reuse or inadequate sterilization of medical equipment, especially needles.[15] The development of a vaccination is difficult because of the high variability of the virus and the lack of an adequate animal model.[16] Typically an HCV infection is treated with a combination of Peg-Ala-Interferons and Ribavirin and supplemented, in dependence of the genotype, by HCV-protease inhibitors. This is a lengthy process with a lot of adverse effects and its success depends on the genotype of the virus. Therapeutic approaches are permanently optimized. In December of 2013 Sofosbuvir was approved. This NS5B-inhibitor increases cure rate up to 90% and tolerability.[17]

3.4 Wnt signaling pathway

Regeneration processes trigger several independent pathway regulations, which support tumor development; the Wnt signaling pathway (Figure 4) is one of them. Activation of this pathway is important for embryonic development, however, if activated in adults it contributes to tumor development.[18] Wnt ligand proteins[19] activate the pathway by binding to the Frizzled receptor and a low-density lipoprotein-receptor-related protein (LRP) co-receptor. This inhibits the formation of a complex, which consists of Axin, Adenomatosis-Polyposis-Coli-Protein (APC), Casein-Kinase 1α (CK1α), and Glykogen-Synthase-Kinase-3β (GSK3β). Under normal conditions this complex phosphorylates and degrades β-catenin. If this complex is not formed, β-catenin is not degraded and can translocate into the nucleus. It further binds to a regulatory protein of the T-Cell Factor/Lymphocyte-Enhancer-Factor (TCF/LEF) family to form a transcription factor and hence activates the Wnt signaling pathway target gene expression (Figure 4). These target genes have various functions, c-myc as well as cyclinD1 can regulate cell cycle progression and the Matrix-Metalloprotease Matrilysin (MMP-7) or CD44 are involved in cell invasion and metastasis. In addition, target genes like growth factors or cyclic-aspartic protease (caspase) inhibitors influence proliferation or apoptosis.[20]

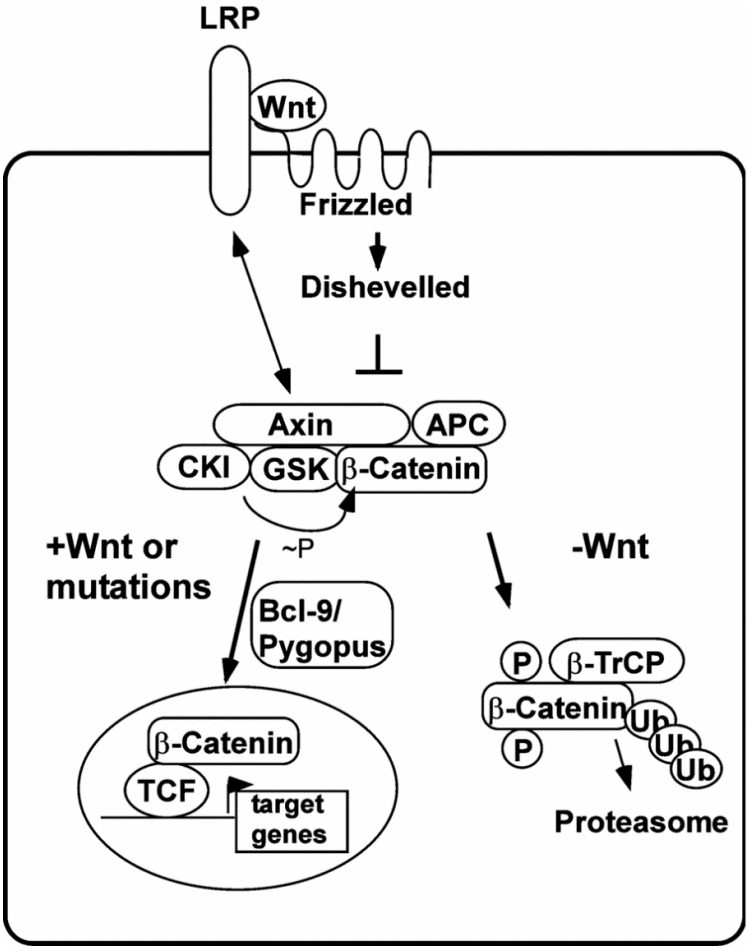

Figure 4: Wnt signaling pathway
APC = adenomatosis-polyposis-coli-protein, CK1α = casein-kinase 1α, GSK-3β = glykogen-synthase-kinase-3β, TCF = T-cell-factor, LRP = low-density lipoprotein-receptor-related protein[20]

3.5 Cell proliferation and activity

The cell division process, so called cell proliferation, is necessary to increase cell numbers. If the cells are continuously passing through the cell cycle, it results in an exponential proliferation rate. The cell cycle has to be tightly controlled, because many a time, this permanent growth is unwanted and may lay the ground for tumor development. The homeostasis in normal tissue is presented by a balance between apoptosis and replacement of cells via proliferation.[21] To achieve this homeostasis, the cells are still active and in the G0-phase until they are triggered, for instance by growth factors, to re-enter the cell cycle. The cell cycle is divided in G1-, S-, G2-, and Metaphase. During the G1 phase cells are growing and proteins and nucleotides are synthesized, which are necessary for the following S phase. In this S phase DNA is replicated in the nucleus. During the G2 phase RNA and proteins for the division are synthesized. In the following Metaphase the cell is dividing into two daughter cells.[22] During the G1 phase, the cell can transit to the G0 phase if no further proliferation is needed (Figure 5).

The cell possesses an endogenous cell cycle control system at different points of the cell cycle. At each check point the cell verifies if, the cell size is sufficient for dividing, the number of

nucleotides is adequate or if the DNA is synthesized correctly. This control system depends on cyclin-dependent kinases (CDK). Each cell cycle phase requires a defined amount of different cyclins, which can be regulated by phosphorylation/dephosphorylation, inhibitory enzymes or transcription[21].

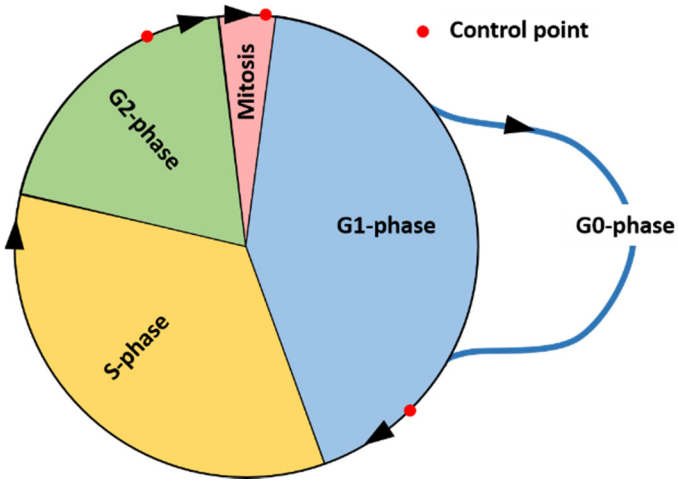

Figure 5: Phases of cell cycle with subset control points
Modified, Löffler/Petrides Biochemie und Pathobiochemie[4]

Impaired cell cycle progression can result in an increased cell cycle arrest on the one hand, and in uncontrolled proliferation on the other hand. In any case, aberrations may be pathogenic. Among others, the cell cycle is regulated by the tumor suppressor p53, which is mutated in 50% of all types of cancer.

Under physiological conditions, p53 expression is maintained on a low level. If DNA damage occurs, this is recognized by the Serin/Threoninkinase ataxia telangiectasia mutated (ATM), which in turn activates p53. Higher concentration of p53 results in a higher activation of itself and in the induction of the transcription of p21, a cyclin inhibitor. This leads to cell cycle arrest, to repair the DNA damage. Permanent activity of p21 triggers apoptosis, whereas the inactivation of p21 may lead to uncontrolled proliferation and tumor development.[4]

3.6 Human hepatoma cell lines as an in vitro model

To investigate influences of signaling pathways on mechanisms that are involved in tumor cell growth, human hepatoma cell lines are an easy to handle *in vitro* model. Human hepatoma cell lines Huh7[23] and HepG2[24] were isolated from human liver tumor tissue. HepG2 cells express normal amounts of p53, whereas Huh7 cells contain significantly higher amounts, due to a block in p53 degradation.[25] The p53 gene of Huh7 cells caries a mutation that leads to a p53 protein, that lacks an epitope and in consequence it binds to heat shock protein 70 instead of binding on

its typical promoter regions, like the promoter for p21. This results in a longer half-life of p53[26] and abrogates its tumor suppressive function[27].

To investigate effects on HCV protein expression, a replicon system was developed by Prof. Ralf Bartenschlager. To generate the replicon cells, Huh7 cells were transfected with a part of viral RNA and a gene for a phosphotransferase, which inhibits Geneticin to generate a resistance to this antibiotic. Geneticin inhibits protein biosynthesis and is used for selection of cells, transfected with viral RNA combined with a gene for phosphotransferase. These replicon cells, named Huh5-15[28], are expressing the non-structural proteins from NS3 to NS5b of HCV genotype 1b. In order to analyze viral protein expression by using a luciferase reporter assay, another replicon cell line was generated, LucUbiNeoET[29]. This cell line is identical to Huh5-15, but includes an additional HCV luciferase reporter gene, that expresses luciferase as a function of HCV protein expression.

3.7 Osteopontin

Osteopontin (OPN; Synonym: secreted phosphoprotein, SPP1) was first identified in 1986 in the bone matrix of rats; today it is known to be ubiquitously expressed.[30] It occurs as a soluble protein or as an extracellular matrix (ECM) protein.[31] Among other functions, it is involved in bone remodeling, immune regulation and tumor development.[32] OPN has been found to be over-expressed in HCV infected patients and it occurs in several tumors, including HCC. In tumors, a higher OPN level is associated with poor prognosis and metastasis. Due to its up-regulation in serum of tumor patients, it is used as a diagnostic tumor marker.[33]

The predicted molecular weight of OPN is around 33 kDa[30] and three different splice variants (Figure 6) are known (OPN-a, -b, -c)[34], whereupon functional differences are still under investigation. Additionally, OPN is posttranslational modified by N-and O-glycosylation, which leads to an increased weight up to 75kDa.[30]

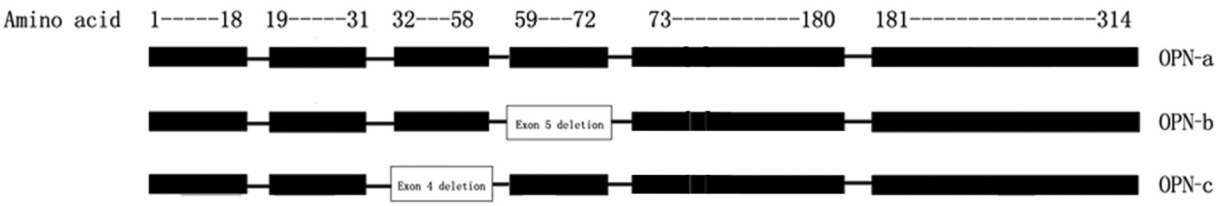

Figure 6: OPN splice variants
OPN splice variants; OPN-a is the full length protein, OPN-b lacks exon 5, OPN-c lacks exon 4[35]

OPN consists of an N-terminal and a C-terminal fragment (Figure 7). The N-terminal fragment has a specific amino acid sequence (RGD), which allows to bind cell surface receptors like integrins (αvβ1, αvβ3, αvβ5, α5β1, α8β1).[30] Integrins are transmembrane proteins, which are expressed by all cell types except erythrocytes and are involved, among other things, in cell adhesion.[36] The C-terminal fragment possesses a receptor region for isoforms of CD44, a cell surface protein, which is expressed in a large number of mammalian cell types, but has been found to be over-expressed in many human cancers.[37] OPN has a cleavage site for MMP-3, MMP-7, and Thrombin, thus enabling a fragmentation into a N- and a C-terminal domain.[38] Furthermore, this proteolytic cleavage enhances OPN adhesion efficiency by revealing an additional adhesion site, which is recognized by integrins α4β1, α4β1, and α9β1.[30]

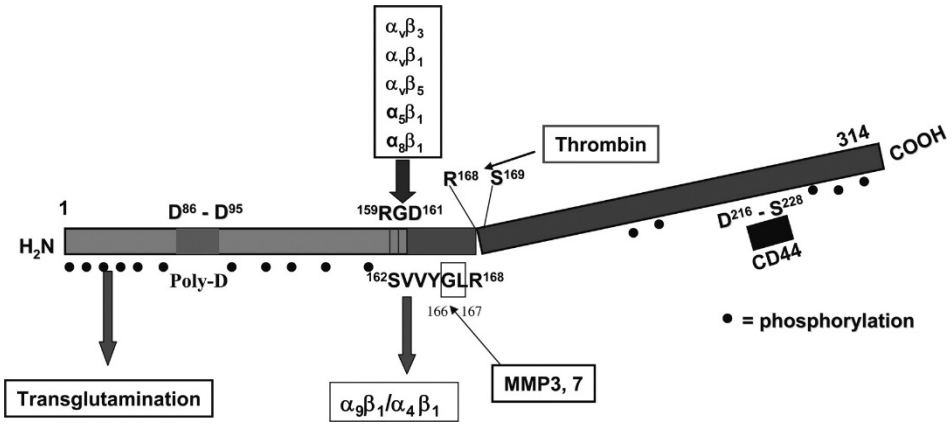

Figure 7: Osteopontin (OPN) with cleavage and integrin binding sites
MMP = matrix metalloprotease; CD44 = cluster of differentiation 44[39]

Mechanisms how OPN is regulated are not well understood. In healthy adults, OPN expression is low, whereas it is increased in patients with disease or injury[32]. The expression can be influenced by diverse binding sequences in its promoter[40] and in addition several hormones, cytokines, oncogenes, and growth factors have been described to modulate its expression[41]. Moreover, OPN can be a self-activating protein by induction of OPN regulators in target cells. Furthermore, the variability in post-translational modifications can influence the activity of OPN, which leads to its multiple functions.[30]

In detail, OPN is regulated in dependence of its tissue- and microenvironment. In bone, OPN is involved in bone remodeling. Via expression of OPN and collagen by osteoblasts, the bone matrix is formed and additionally OPN leads to the adhesion between bone matrix and osteoclasts, a bone resorbing cell.[32] This process is regulated by diverse signal molecules like cytokines, growth factors, hormones, or Vitamin D.[42] If OPN is expressed in endothelial cells of blood vessels, it can inhibit vascular calcification by binding the calcium of calcium phosphate crystals. Hence OPN prevents cardiovascular diseases.[43] OPN is additionally

involved in acute inflammatory responses, wound healing and regeneration. Acute inflammation is triggered by various stimuli including Lipopolysaccharide (LPS), NO, inter leukine-1β (IL-1β), and tumor necrosis factor-α (TNF-α). These inflammatory mediators induce the expression of OPN in macrophages and T-cells.[32] OPN acts further as a chemoattractant protein[31] and recruits more cells including macrophages or T-cells[42]. Regarding wound healing, OPN recruits and activates for instance HSC, which in turn express ECM molecules like collagen.[44] Additionally, OPN supports cell-cell contact or cell-matrix contact and thereby, wound sealing is promoted.[32] During the following regeneration processes, OPN stimulates downstream signaling cascades for cell proliferation and survival.[42] Chronic inflammation and fibrosis leads to excessive regeneration processes, that may result in accumulation of mutations, thus supporting the development of HCC.[7] In tumors, the transcription of OPN is increased by oncogenes like Ras or Src or by proliferation regulators like p53 or activator protein-1 (AP-1). Hence, tumor development is supported due to the fact that OPN can inhibit apoptosis or stimulates proliferation. For tumors, exceeding a certain size, the blood supply becomes a critical requirement. By stimulating cells to migrate or to express growth factors, OPN can support angiogenesis in tumors.[42] Furthermore, formation of metastasis is promoted by OPN. To metastasize, the ECM must be degraded in order to liberate the tumor cells. OPN is able to regulate ECM-degrading proteins. It is therefore involved in the formation of metastasis and due to its ability to regulate migration, it is influencing tumor spreading.[42] Additionally, there were found more inactive macrophages in OPN expressing tumors than in OPN negative tumors, this implies, that OPN supports tumors to escape immune surveillance.[45] Taken together, OPN is involved in migration, adhesion, anchoring, proliferation, and survival of cells, it activates cells or regulates their signaling pathways by binding with its several binding domains, which leads to diverse outcome. These functions are essential for several physiological activities, however if dysregulated, they may contribute to the development of various diseases, including cancer.

3.8 Aim of the study

During an infection, the liver initiates an immune response[2] that, if it cannot be cleared, turns into a chronic inflammation, which is often the base for tumor formation. For instance, HCV infection often persists, leading to chronic hepatitis with progression to cirrhosis and HCC. Patients with HCC bear a high rate of mortality and the therapeutic options are very limited due to a lack of knowledge about the mechanisms of HCC development and progression. OPN, as well as Wnt signaling pathway, are associated with HCV[33, 46] and tumor development[47, 18]. Recently it was shown that OPN analogs are able to increase Wnt signaling pathway activity.[48] Aim of this study was to analyze the role of OPN in the Wnt signaling pathway and subsequent regulation of HCV protein expression or tumor cell survival. Given that an active Wnt signaling pathway is known to promote proliferation[18] and is associated with HCV infection[49], OPN might be a promising regulator of this pathway to improve HCV therapy or to interfere with tumor cell growth.

4 Material and Methods

4.1 Material

4.1.1 Technical equipment

Equipment	Supplier
CasyTT	Roche, Basel, Switzerland
Centrifuge 5417	Eppendorf, Hamburg, Germany
Centrifuge 5810R	Eppendorf, Hamburg, Germany
Clean Bench Hera Safe	Heraeus Instruments, Hanau, Germany
HandyStep® electronic	BRAND, Wertheim, Germany
Incubator Heracell 240	Thermo Fisher Scientific, Waltham, USA
Magnetic stirrer IKAMAG® RCT	Janke und Kunkel, Staufen, Germany
Microscope CK40	Olympus, Center Valley, USA
Mini Cell XCell Sure Lock	Invitrogen, Darmstadt, Germany
Mini Trans-Blot® Cell	Biorad, Hercules, USA
MyCycler™ Thermal Cycler	Biorad, Hercules, USA
NanoDrop ND-1000	Peqlab, Erlangen, Germany
Neubauer Improved Chamber	Roth, Karlsruhe, Germany
Photometer Tecan® infinite M200	Tecan, Crailsheim, Germany
Pipetboy Integra	INTEGRA Biosciences, Fernwald, Germany
Pipettes Eppendorf Research® Plus	Eppendorf, Hamburg, Germany
Pipettes Eppendorf Research® Plus	Eppendorf, Hamburg, Germany
PowerPac™ HC Power Supply	Biorad, Hercules, USA
Scale ATLION ATL-423-I	Acculab Satorius, Göttingen, Germany
Scale TE124S	Satorius, Göttingen, Germany
Sonorex RK 102H	Bandelin electronics, Berlin, Germany
Thermal Cycler C1000 + CFX 96	Biorad, Hercules, USA
Thermoleader Dry Block Heat Bath	Uniequip, Martinsried, Germany
Versadoc™ Imaging Systhem 4000 MP	Biorad, Hercules, USA
ViiA™ 7 System	Life Technologies, Darmstadt, Germany
Vortexer	Heidolph, Schwabach, Germany

4.1.2 Consumables

Consumables	**Supplier**
Cell culture flask (25 m², 75 m², 175 m²)	Sarstedt, Nümbrecht, Germany
Cell culture plates	Thermo Fisher Scientific Waltham, USA
MicroWell™ 96-Wel, optical-Bottom Plates	Nunc, Roskilde, Denmark
MicroWell™ 96-Wel, white	Nunc A/S, Roskilde, Denmark
Protran Whatman™ membran	GE Healthcare L. S., Little Chalfont, UK
Parafilm M	American National Can. USA
PCR tubes	Thermo Fisher Scientific, Waltham, USA
PCR tubes	Kisker Biotech, Steinfurt, Germany
PCR tubes	Kisker Biotech, Steinfurt
Pipette tips (10 µl, 200 µl, 1000 µl)	Sarstedt, Nümbrecht, Germany
Pipette tips, sterile and RNAse free (10 µl, 20 µl, 200 µl, 1000 µl)	Sarstedt, Nümbrecht, Germany
Pipettes (2 ml, 5 ml, 10 ml, 25 ml)	Sarstedt, Nümbrecht, Germany
Polyacrylamide gel, gradient (4-12%) SDS	Invitrogen, Darmstadt, Germany
Reaction tubes (1.5 ml, 2 ml)	Sarstedt, Nümbrecht, Germany
Reaction tubes (15 ml, 50 ml)	Sarstedt, Nümbrecht, Germany
Reaction tubes, sterile and RNAse free (1.5 ml, 2 ml)	Sarstedt, Nümbrecht, Germany
Tips, positive displacement (500 µl, 2.5 mL, 5 mL, 12.5 mL)	BRAND, Wertheim, Germany

4.1.3 Reagents and Kits

Reagents and Kits	**Supplier**
Bradford assay	Bio-Rad, Hercules, USA
$C_2H_4O_2$ (acetic acid)	Roth, Karlsruhe, Germany
Chloroform	Roth, Karlsruhe, Germany
Distilled water, RNase free	Thermo Fisher Scientific, Waltham, USA
DMEM (Dulbecco's modified Eagles Medium)	Gibco® by Life Technologies, Darmstadt, Germany
DMSO (Dimethyl sulfoxide)	Sigma Aldrich, St. Louis, USA
Dry milk	Bio-Rad, Hercules, USA
EDTA (Ethylenediaminetetraacetic acid)	Roth, Karlsruhe, Germany
Ethanol	Roth, Karlsruhe, Germany
FCS (fetal calf serum)	Gibco® by Life Technologies, Darmstadt, Germany
Flavopiridol (FLPD)	Sigma Aldrich, St. Louis, USA
Geneticin solution (G418) [50 mg/ml]	Gibco® by Life Technologies, Darmstadt; Germany
Glycerol	Roth, Karlsruhe, Germany

Glycine	Roth, Karlsruhe, Germany
H_2O_2 (Hydrogen peroxide)	Roth, Karlsruhe, Germany
HCl (Hydrochloric acid)	Roth, Karlsruhe, Germany
Isopropanol	Roth, Karlsruhe, Germany
KCl (potassium chloride)	Roth, Karlsruhe, Germany
Kenpaullone (KPLN)	Sigma Aldrich, St. Louis, USA
KH_2PO_4 (potassium dihydrogen phosphate)	Sigma-Aldrich, St Louis, USA
Lipofectamine™ 2000	Invitrogen, Karlsruhe, Germany
Luciferase Assay System	Promega, Mannheim, Germany
Luminol sodium salt	Sigma-Aldrich, St Louis, USA
Maxima SYBR™ Green/ROX qPCR Master Mix	ABgene®, Epsom, UK
methanol	Roth, Karlsruhe, Germany
MTT (3-4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide	Sigma Aldrich, St. Louis, USA
Na_2HPO_4 x 2 H_2O (di-sodiumhydrogenphosphat di-hydrat)	Roth, Karlsruhe, Germany
NaCl (sodium chlorid)	AppliChem, Darmstadt, Germany
NaOH (sodium hydroxide)	Roth, Karlsruhe, Germany
NP 40	Sigma-Aldrich, St Louis, USA
Opti-MEM	Gibco® by Life Technologies, Darmstadt, Germany
Para-hydroxy-Coumarinacid	Sigma-Aldrich, St Louis, USA
Penicillin/streptomycin [10.000 U/ml]	Gibco® by Life Technologies, Darmstadt, Germany
Ponceau S	Sigma-Aldrich, St Louis, USA
Precesion Plus Protein ™ Streptactin Conjugate	Bio-Rad, Hercules, USA
Precesion Plus Protein™ WesternC™ Standard	Bio-Rad, Hercules, USA
SB216763	Sigma Aldrich, St. Louis, USA
SDS (Sodium dodecyl sulfate)	AppliChem, Darmstadt, Germany
Telaprevir (TLPV)	Janssen-Cilag Pharma, Wien, Austria
Tris HCl	Roth, Karlsruhe, Germany
Tris-Base	Sigma-Aldrich, St Louis, USA
TRIzol® reagent	Invitrogen, Karlsruhe, Germany
Trypan blue	Sigma-Aldrich, St Louis, USA
Tween-20	Roth, Karlsruhe, Germany
Verso™ cDNA Kit	Thermo Fisher Scientific, Waltham, USA
NucleoSpin®RNA II Kit	Macherey-Nagel, Düren, Germany

4.1.4 Antibodies for Western Blot

Protein name and origin	Dilution	Supplier
Anti-mouse from goat HRP	1:5000	Jackson ImmunoResearch Laboratories Inc., Suffolk, UK
Anti-rabbit from goat HRP	1:5000	Jackson ImmunoResearch Laboratories Inc., Suffolk, UK
GAPDH human from goat	1:100	Santa Cruz Biotechnology, Texas, USA
SPP1(OPN) monoclonal from rabbit	1:1000	Epitomoics, Burlingame, USA
SPP1(OPN) polyclonal from goat	1:500	R&D Systems

4.1.5 siRNA and plasmid DNA

siRNA gene target	5'-3' sequence or order number	Supplier
siControl	CGAAUCCUACAAGCGCGC55	Eurogentec, Köln, Germany
siOPN1_7 (siOPN)	SI03055409	QIAGEN, Hilden, Germany
siOPN1_4 (siOPN-a)	SI00012222	QIAGEN, Hilden, Germany
siOPN1_6 (siOPN-b)	SI02757615	QIAGEN, Hilden, Germany

Plasmid	order number	Supplier
M50 Super 8x TOPFlash (reporter)	12456	addgene, Cambridge, USA
M51 Super 8x FOPFlash (control)	12457	addgene, Cambridge, USA
pCDNA3.1	K4800-01	Invitrogen, Karlsruhe, Germany
pCMV-Tag1-NS3 (pNS3)	7645	addgene, Cambridge, USA
pCMV-Tag1-NS5A (pNS5A)	17646	addgene, Cambridge, USA
pOPN	11617	addgene, Cambridge, USA
pOPNLuc	11996	addgene, Cambridge, USA

4.1.6 Oligonucleotides

All oligonucleotides are direct against the human gene sequence, or indicated otherwise as (mus musculus). Oligonucleotides were obtained from Metabion International, Martinsried, Germany. Probes for TAQMan were obtained from Life Technologies, Darmstadt, Germany

Target gene	Forward primer 5'-3'	Reverse primer 5'-3'	Reference
OPN mus	CTCTGATCAGGACAACAAC	CCTCAGAAGATGAACTCTC	AF515708
OPN	TGAAACGAGTCAGCTGGATG	TGAAATTCATGGCTGTGGAA	J 04765.1
HCV	TGCGGAACCGGTGAGTACA	AGGTTTAGGATTCGTGCTCAT	NC_004102.1

GAPDH	ACCCAGAAGACTGTGGATGG	TTCTAGACGGCAGGTCAGGT	M33197
mATPsy mus	ATTGCCATCTTGGGTATGGA	AATGGGTCCCACCATGTAGA	NM_016774
mATPsy	GCCCACTTCTTACCACAA GG	GCGACAGCGATTTCTAGGAT	AF368271
Conductin	AGGGAGAAATGCGTGGATAC	TGGAATCAATCTGCTGCTTC	NM004655

Target gene	**ID number**
GAPDH TAQMan	Hs99999905_m1
OPN TAQMan	Hs00959010_01

4.1.7 Buffers and solutions

5% milk powder solution	5% dry milk (w/v) 1x TBST
lysis buffer for protein isolation	137 mM NaCl 0.5% NP 40 (v/v) 2 mM EDTA 50 mM Tris HCl pH 8.0 10% Glycerol (v/v)
PBS	137.9 mM NaCl 6.5 mM Na_2HPO_4 x 2 H_2O 1.5 mM KH_2PO_4 2.7 mM KCl pH 7.4 NaOH
Ponceau S solution	1% Ponceau S (w/v) 5% acetic acid (v/v)
SDS Lysispuffer	20% SDS (w/v) in PBS + 100 µl 10 M HCl
self-made ECL buffer	1.25 mM Luminol in 0.1M Tris-HCl pH 8.6 + 15 mM Para-hydroxy- Coumarinacid in DMSO activated with 0.01% H_2O_2
TBS (10x)	1.5 M NaCl 1 M Tris-Base
TBST 1x	TBS 0.1% Tween-20 (v/v)
transfer buffer	25 mM Tris-base 200 mM glycine 20% methanol (v/v), freshly added
5% MTT solution	5% (3-4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) in PBS

4.1.8 Software

Bio-Rad CFX Manager 2.0	Bio-Rad, Hercules, USA
GraphPad Prism5	GraphPad Software, San Diego, USA
iControl 5.0	Tecan, Crailshaim, Germany
Image Lab™ 2.0	Bio-Rad, Hercules, USA
MS Office 2013	Microsoft, Redmond, USA
VersaDoc™ Imaging Systhem 4000 MP	Bio-Rad, Hercules, USA
Windows XP	Microsoft, Redmond, USA

4.2 Methods

4.2.1 Murine samples

Samples of mouse tissue were provided as purified complementary DNA (cDNA) samples of liver and tumor tissue. Used cDNA liver samples of FVB background control mice, as well as tumor samples of FVB/Mdr2ko (FVB.129P2-Abcb4$_{tm1Bor}$) (age ≥ 65 weeks) were kindly provided by Dr. Roja Barikbin (our group). FVB/Mdr2ko mice have chronically inflamed livers and develop HCC after one year. Samples were snap frozen, RNA was isolated using the NucleoSpin®RNA II Kit and reversely transcribed into cDNA using the Verso^{TM} cDNA Kit. Used cDNA liver samples of humanized uPA/SCID mice were kindly provided by Dr. Janine Kah (group of Prof. Maura Dandri; Virushepatitis, UKE). These mice were generated by crossing Alb-uPA transgenic mice with SCID/beige mice, followed by transplantation of cyroconserved primary human hepatocytes via injection into the spleen at the age of 3-4 weeks. If the murine liver was stabile repopulated with human hepatocytes after around 9 weeks, mice were infected with HCV serum or left uninfected. Livers were sampled > 3 weeks after infection (Titers of uPA/SCID mice (n=4): 6.5E+5; 3.2E+5; 1.2E+7; 1.0E+6). RNA was isolated using the NucleoSpin®RNA II Kit and reversely transcribed into cDNA using the Verso^{TM} cDNA Kit. All mice received human care according to the guidelines of the National Institute of Health as well as to the legal requirements in Germany. They were maintained under controlled conditions (22°C, 55% humidity and 12-hour day/night rhythm) and fed with standard laboratory chow and water ad libitum.

4.2.2 Cell culture

All cell lines were cultivated in 20% O_2 and 5% CO_2 humidified atmosphere at 37°C. Human hepatoma cell line HepG2[24], the human hepatoma replicon cell lines Huh-5-15[28] and LucUbiNeo-ET[29] as well as their parental cell line Huh7[23] were cultured in Dulbecco's modified Eagle medium (DMEM), containing 10% fetal calf serum (FCS) and penicillin [c=100 U/ml]/streptomycin [c=100 µg/ml]. For selection, medium for replicon cell lines contained Geniticin [c=0.5 mg/ml]. These replicon cells express HCV nonstructural proteins NS3 to NS5B of HCV genotype 1b and the resistance for Geniticin. Cells were passaged at a ratio of 1:5 two times a week. For experiments, cells were counted via hemocytometer and seeded in culture plates with complete medium. In case of treatment, cells were treated 24 h after seeding.

4.2.3 Transfection of siRNA and plasmid DNA

Transfection of siRNA or plasmid DNA was performed using Lipofectamine™ 2000 according to the manufacturer's instructions. siRNA [c=25 nM] or plasmid DNA [c=8 µg/ml] was solved in Opti-MEM + 5% FCS. As a control, siControl (siC) directed against Green fluorescent protein (GFP) as an irrelevant control gene or an empty pCDNA3.1 vector was used. Plasmids and siRNAs are listed above (4.1.5)

4.2.4 Detection of mRNA by real time reverse-transcription-PCR (real time RT-PCR)

Isolation of total RNA from cultivated cells was performed using the TRIzol® reagent according to manufacturer's instruction, isolated RNA was diluted in RNase-free, distilled water and stored at -80°C. RNA concentration was measured with the NanoDrop ND-1000. To analyze gene expression, RNA was reversely transcribed into cDNA using the Verso™ cDNA Kit. For amplification of target cDNA, real time reverse-transcription polymerase chain reaction (RT-PCR) was performed with specific oligonucleotide pairs (listed above 4.1.6) and Maxima SYBR™ Green/ROX qPCR Master Mix, using the C1000 Thermal Cycler + CFX™ 96 Real-Time System. Amplification specificity of PCR products were confirmed by melting curve analysis. Gene expression analysis of humanized uPA/SCID mice was performed via the ViiA™ 7 System, using TaqMan probes (listed above 4.1.6) for human specific transcripts. The gene expression was normalized to mitochondrial adenosine triphosphate synthase β subunit (mATPsy) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as indicated.

4.2.5 Protein isolation and Western Blot analysis

Proteins were isolated from cultured cells. Cells were washed with PBS, lysed with 'lysis buffer for protein isolation', incubated in an ultrasonic bath for 2 min and rest on ice for 10 min. After centrifugation (1 min, 20000 rcf), supernatants were split and stored at -80°C. For Western Blot analysis, protein amount was determined via the Bradford assay according to manufacturer's instruction. Indicated amount of total protein was fractionated by gradient (4-12%) SDS-polyacrylamide gel electrophoresis according to NuPAGE® Technical Guide. Separated proteins were blotted onto nitrocellulose membrane using the wet blot method with transfer buffer on ice, in a Mini Trans-Blot® Cell blotting tank (300 mA, 70 min). Afterwards membrane was washed with TBST, incubated for 1 min in Ponceau S and washed with water to control successful protein transfer. The membrane was washed with TBST, blocked in 5% milk powder solution, and incubated over night at 4°C with the primary antibody. The membrane was washed 3 times for 10 min with TBST, incubated for 1 h at room temperature

with secondary antibody and Precision Plus Protein TM Streptactin Conjugate (1:10000) to visualize the Precision Plus Protein™ WesternC™ Standard. Western Blots were developed, using self-made enhanced chemiluminescence (ECL) buffer and the VersaDoc™ Imaging System 4000 MP. Image editing and quantification was performed with the Image Lab™ Software.

4.2.6 Luciferase reporter assay

Luciferase activity was measured, using the Luciferase Assay System according to manufacturer's instruction, via the Tecan® infinite M200. The results were normalized to the total protein content of the individual sample via the Bradford assay. Luciferase activity monitors either HCV protein expression in HCV replicon cell lines or expression of specific reporter plasmids. To monitor OPN expression, pOPNLuc, a reporter plasmid containing the OPN promoter region, was transfected. As a control, pCDNA3.1 vector was transfected. To analyze the Wnt signaling pathway activity, β-catenin-regulated transcription (CRT) was monitored by using the reporter plasmid M50 Super 8x TOPFlash (reporter) or M51 Super 8x FOPFlash (control). The TOPFlash plasmid contains 6 TCF/LEF binding sites enhancing firefly luciferase expression. The control plasmid FOPFlash carries a mutant TCF/LEF binding sites.

4.2.7 Analysis of cell vitality and viability

Cell viability was measured by using (3-4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). Cultivated cells of interest, growing in 100µl medium, were incubated with 20 µl of 5% MTT solution, thus the cells absorbed the yellow tetrazolium salt. Viable and proliferating cells metabolized it into formazan, a purple product. When cells achieved adequate staining (30 min to 2 h), 50 µl of SDS-lysis buffer were added for an overnight incubation at 37°C to lyse the cells and dissolve the formazan salt product into a purple colored solution. For measurement, 100 µl of the colored solution were transferred into a 96-well plate and analyzed with the Tecan® infinite M200 at a wavelength of λ = 570 nm. For analysis, values of untreated cells were compared to treated cells. Cell vitality was analyzed by determination of total cell numbers and percentages of dead cells with the Casy TT cell counter.

4.2.8 Statistical analysis

Statistical analyses were performed with the GraphPad Prism5 software. Two-tailed Student's *t*-test was used, if two groups were compared. If variances were inhomogeneous, the Welsh correction was additionally used. All data in this study are expressed as a mean ± standard error of the mean (SEM). $P \leq 0.05$ denotes significance with following ranges: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

5 Results

5.1 OPN expression is up-regulated in mice with HCV infection or HCC

OPN is known to be induced in HCV patients[33] and up-regulated in human HCC, therefore it is used as a diagnostic tumor marker[47]. Increased OPN levels are related to higher metastatic potential and poor prognosis.[50] OPN messenger RNA (mRNA) expression levels were determined in livers of uPA/SCID mice, which were repopulated with human hepatocytes and infected with HCV (n=4; titers shown in 4.2.1), in comparison to uninfected control mice (n=3). OPN mRNA expression was found to be up-regulated in livers of HCV infected mice in comparison to uninfected control animals. Expression levels of OPN mRNA were measured by real time RT-PCR (normalized to GAPDH) via the ViiA™ 7 System with TaqMan primers to recognize human specific transcripts (Figure 8).

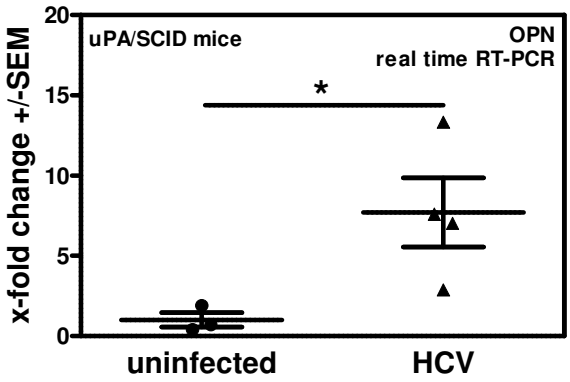

Figure 8: OPN mRNA expression is up-regulated in livers of HCV infected uPA/SCID mice
Livers of uPA/SCID mice, which were repopulated with human hepatocytes and infected with HCV, were analyzed for mRNA expression levels of human OPN by real time RT-PCR (normalized to human GAPDH). * $P \leq 0.05$

In order to investigate, whether OPN mRNA expression levels are elevated in murine liver tumors, tumor tissue of FVB/Mdr2ko mice (n=23) and liver tissue of FVB background control mice (n=5) (age ≥ 65 weeks) were analyzed. OPN (normalized to mATPsy) was found to be more than 2 fold over-expressed in about 50% of all analyzed murine HCC in comparison to healthy liver tissue of background control mice (Figure 9).

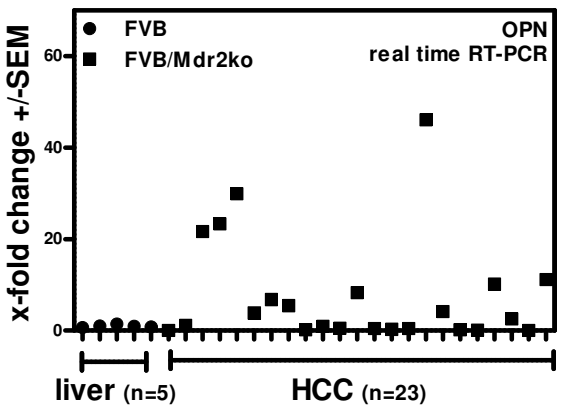

Figure 9: OPN mRNA expression is up-regulated in HCC of FVB/Mdr2ko mice
Liver tumors from FVB/Mdr2ko mice or livers from FVB mice (age ≥65 weeks) were isolated. mRNA expression level of OPN (normalized to mATPsy) was determined by real time RT-PCR in tumor tissue (n=23) compared to healthy liver tissue (n=5).

5.2 OPN expression levels in human hepatoma cell lines and human hepatoma HCV replicon cell lines

To analyze effects of OPN expression on tumor development, human *in vitro* cell culture systems were used. OPN expression levels were determined in different human hepatoma cell lines and human hepatoma HCV replicon cell lines. OPN expression levels of human hepatoma cell line Huh7 and HepG2 were determined. The results of the real time RT-PCR (normalized to mATPsy) (Figure 10 A) and the Western Blot (10 µg total protein, monoclonal OPN antibody; (Figure 10 B) show, that OPN expression was found to be clearly elevated in HepG2 cells compared to Huh7 cells. Taken together, OPN is expressed differently in hepatoma cell lines (Figure 10) as well as in various HCC of the FVB/Mdr2ko mice (Figure 9).

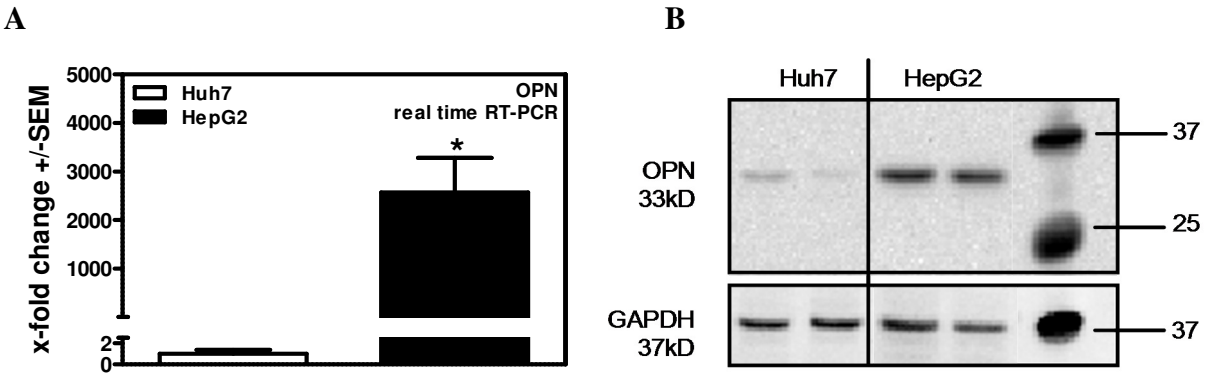

Figure 10: Higher OPN expression level in HepG2 than in Huh7 cells
Huh7 and HepG2 cells were cultivated for 24 h. Expression levels of OPN mRNA were determined by real time RT-PCR (normalized to mATPsy) (A) or by Western Blot (10 µg total protein) (B). Expression of GAPDH is shown as a housekeeping gene (B). * $P \leq 0.05$

The OPN expression levels were additionally analyzed in human hepatoma HCV replicon cell lines Huh5-15, LucUbiNeo-ET, and compared to their parental cell line Huh7. We previously showed (*Loscher, Keller et al.*, unpublished), that OPN protein expression levels in HCV replicon cell lines were up-regulated in comparison to their parental cell line. To underline these results, the higher OPN mRNA expression (normalized to GAPDH) was shown by real time RT-PCR (Figure 11 A, B) and the higher activity of the OPN promotor by a luciferase reporter assay for a transfected plasmid containing the OPN promoter region (Figure 11 C).

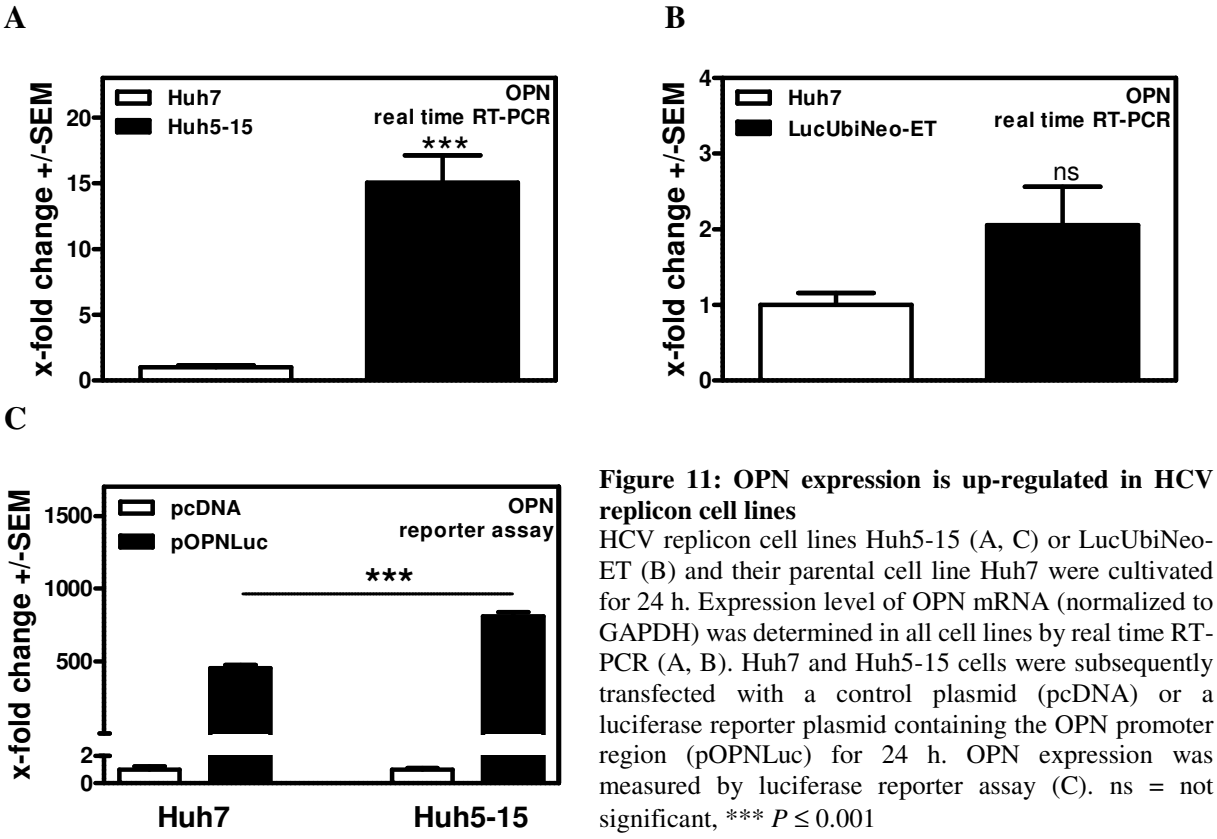

Figure 11: OPN expression is up-regulated in HCV replicon cell lines
HCV replicon cell lines Huh5-15 (A, C) or LucUbiNeo-ET (B) and their parental cell line Huh7 were cultivated for 24 h. Expression level of OPN mRNA (normalized to GAPDH) was determined in all cell lines by real time RT-PCR (A, B). Huh7 and Huh5-15 cells were subsequently transfected with a control plasmid (pcDNA) or a luciferase reporter plasmid containing the OPN promoter region (pOPNLuc) for 24 h. OPN expression was measured by luciferase reporter assay (C). ns = not significant, *** $P \leq 0.001$

Since OPN was shown to be up-regulated in HCV infected mice (Figure 8) and in cell lines that express viral proteins (Figure 11), HCV replication might be involved in this up-regulation. Thus, the HCV protein expression in HCV replicon cell line LucUbiNeo-ET was down-regulated with Telaprevir (TLPV) [250 nM] for 3 days. The successful down-regulation of HCV protein expression was shown by real time RT-PCR (normalized to GAPDH) (Figure 12 A). Subsequently, OPN mRNA expression level (normalized to GAPDH) was measured and found to be decreased (Figure 12 B). To test whether TLPV is toxic, an MTT viability assay was performed, but no changes were found (Figure 12 C).

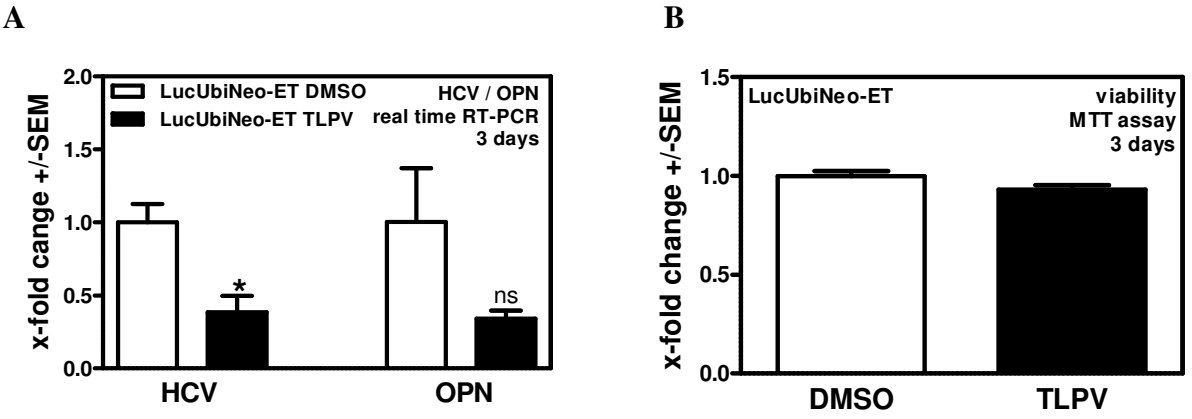

Figure 12: Down-regulation of HCV leads to decreased OPN mRNA expression level
LucUbiNeo-ET cells were cultivated for 24 h. Subsequently, expression levels of viral proteins were down-regulated by treatment with the polymerase inhibitor Telaprevir (TLPV) [250 nM], DMSO was used as a solvent control. **A:** HCV and OPN mRNA expression levels (normalized to GAPDH) were measured on day 4 by real time RT-PCR. **B:** viability of cells was measured with a MTT assay. ns = not significant, * $P \leq 0.05$

5.3 Manipulation of OPN expression

To analyze effects of OPN on cancer development, OPN expression level was modified in different ways. On the one hand, down-regulation of OPN was achieved by using siRNA against OPN (siOPN) or, as a control, control siRNA (siC), on the other hand, up-regulation was realized by inserting an OPN coding plasmid (pOPN) or, as a control, an empty plasmid (pcDNA). To show the down-regulation of OPN on the protein level, OPN expression was analyzed in HepG2 cells and Huh7 cells, 24 h and 48 h after transfection, by Western Blot (8 µg total protein) with a polyclonal antibody against OPN (Figure 13 A, B). The successful down-regulation of OPN was shown for HepG2 cells 24 h and 48 h after transfection (Figure 13 B), while basic OPN expression level in Huh7 was not detectable in this experimental set-up. In an additional experiment, mRNA expression levels of OPN (normalized to GAPDH) were determined in Huh7, Huh5-15 and HepG2 cells after transfection with siOPN or siC, respectively. A significant down-regulation of OPN was shown in Huh7 and Huh5-15 cells, while HepG2 cells indicate just a tendency to down-regulation (Figure 13 C). This can be justified by the very high level of OPN expression in HepG2 cells (Figure 10). For OPN up-regulation, Huh7 cells were transfected with pOPN and increased OPN expression was measured with real time RT-PCR (normalized to GAPDH) (Figure 13 D).

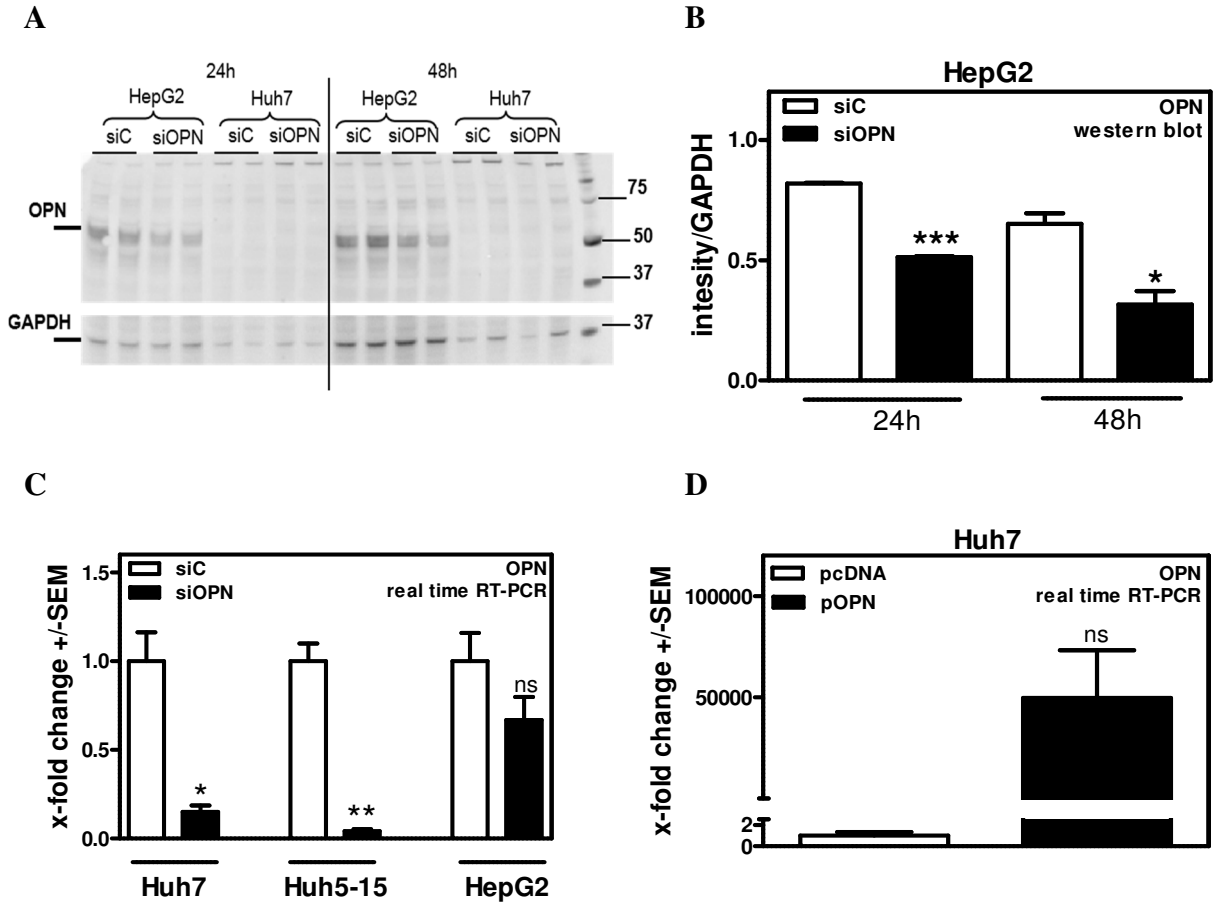

Figure 13: OPN regulation via siOPN and pOPN
A, B: Huh7 and HepG2 cells were cultivated for 24 h and subsequently transfected with siRNA directed against OPN (siOPN) or control siRNA (siC) for 24 h or 48 h. Expression levels of OPN and GAPDH, as a housekeeping gene, were measured by Western Blot (8 µg total protein) (A) and quantified for HepG2 cells (B). **C:** Huh7, Huh5-15 and HepG2 cells were cultivated for 24 h and subsequently transfected with siOPN or siC for 24 h. Expression level of OPN mRNA (normalized to GAPDH) was measured by real time RT-PCR. **D:** Huh7 cells were cultivated for 24 h and subsequently transfected with an OPN coding plasmid (pOPN) or control plasmid (pcDNA). Expression level of OPN mRNA (normalized to GAPDH) was measured by real time RT-PCR. ns = not significant, * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

5.4 OPN regulates the Wnt signaling pathway

OPN is up-regulated in murine HCC (Figure 9), HCV replicon cell lines (Figure 11) and HCV infected mice (Figure 8); likewise, the Wnt signaling pathway is up-regulated in HCV infection and HCC[46]. In the context of analyzing the mechanism of OPN-mediated effects on chronic hepatic inflammation and cancer, it is interesting to investigate the influences of OPN on the Wnt signaling pathway. The Wnt signaling pathway activity was analyzed by monitoring the β-catenin-regulated transcription (CRT) with a luciferase reporter assay. Huh7 cells were either transfected with a control plasmid (pcDNA) or with an OPN coding plasmid (pOPN). The up-regulation of OPN expression increases Wnt signaling pathway activity (Figure 14 A). HepG2 cells were transfected with a control siRNA (siC) or with siRNA directed against

OPN (siOPN). Knockdown of OPN expression interferes with the Wnt signaling pathway activity (Figure 14 B). OPN up or down regulation leads to similar regulation of the Wnt signaling pathway (Figure 14).

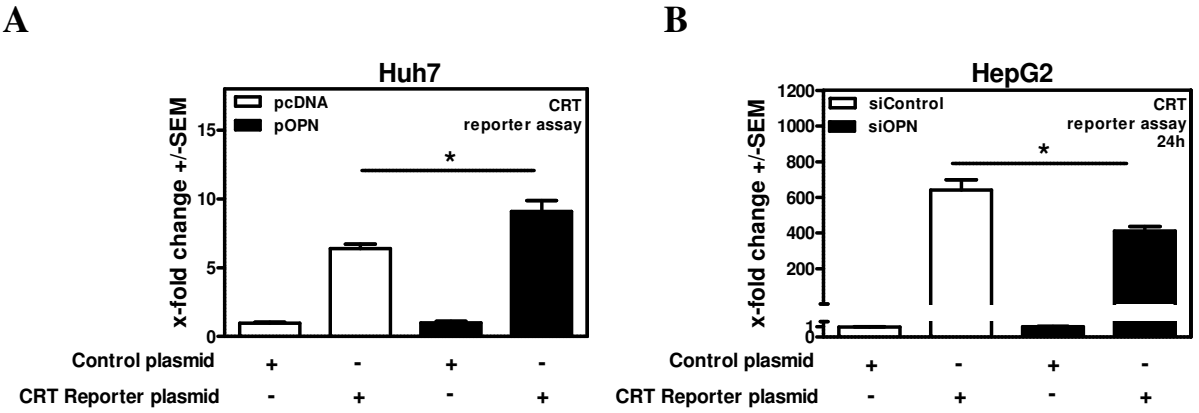

Figure 14: OPN regulates Wnt signaling pathway activity
A: Huh7 cells were cultivated for 24 h and subsequently transfected with a control plasmid (pcDNA) or with an OPN coding plasmid (pOPN), to up-regulate OPN expression. Cells were additionally transfected with a β-catenin regulated transcription (CRT) reporter plasmid or control plasmid. **B:** HepG2 cells were cultivated for 24 h and subsequently transfected with a control siRNA (siC) or with siRNA directed against OPN (siOPN), to down-regulate OPN expression. Cells were additionally transfected with a CRT reporter plasmid or control plasmid. Wnt signaling pathway activity was measured by monitoring the CRT with a luciferase reporter assay. * $P \leq 0.05$

5.5 Wnt pathway activity affects expression of HCV proteins

We could show, that OPN influences the Wnt signaling pathway activity (Figure 14) and due to the fact, that the Wnt signaling pathway is known to be up-regulated in HCV infected patients[46], we analyzed the consequences of Wnt signaling pathway regulation on HCV protein expression. An up-regulated activity of the Wnt signaling pathway was shown in HCV replicon cell lines Huh5-15 and LucUbiNeo-ET, compared to their background cell line Huh7 (*Loscher, Keller et al.*, unpublished). To identify the HCV protein, which might be responsible for the activation of the Wnt signaling pathway, Huh7 cells were subsequently transfected with plasmids coding for NS3B (pNS3B), NS5A (pNS5A) or a control plasmid (pcDNA). An increase of the Wnt signaling pathway activity was detected by the luciferase reporter assay for CRT after induction of each single protein (Figure 15). It was found, that the Wnt pathway is activated in HCV replicon cell lines as well as in the background cell line, if these cells were transfected with viral proteins NS3B or NS5A.

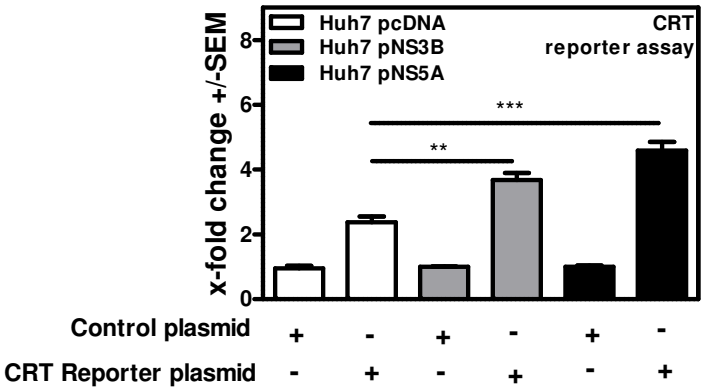

Figure 15: Wnt signaling patway activity is up-regulated by expression of HCV proteins
Huh7 cells were cultivated for 24 h and subsequently transfected with plasmids coding for NS3B (pNS3B), NS5A (pNS5A) or a control plasmid (pcDNA). Cells were additionally transfected with a β-catenin regulated transcription (CRT) reporter plasmid or control plasmid. Wnt signaling pathway activity was measured by monitoring the CRT with a Luciferase reporter assay. ** $P \leq 0.01$; *** $P \leq 0.001$

We further showed, that down-regulation of Wnt signaling pathway activity decreases HCV protein expression (*Loscher, Keller et al.*, unpublished). Thereupon, expression of HCV proteins was analyzed after activation of the Wnt pathway. To activate Wnt signaling, LucUbiNeo-ET and Huh5-15 cells were treated with the GSK3 inhibitors Kenpaullone (KPLN) and SB216763. Their ability to activate Wnt signaling was verified by measuring conductin expression level (normalized to mATPsy) via real time RT-PCR (Figure 16 A) and luciferase reporter assay for CRT (Figure 16 B). As a consequence of the activation of the Wnt signaling pathway, a significant increase of HCV protein expression was observed via luciferase reporter assay for HCV (Figure 16 C). An MTT test showed that the substances were not toxic (Figure 16 D).

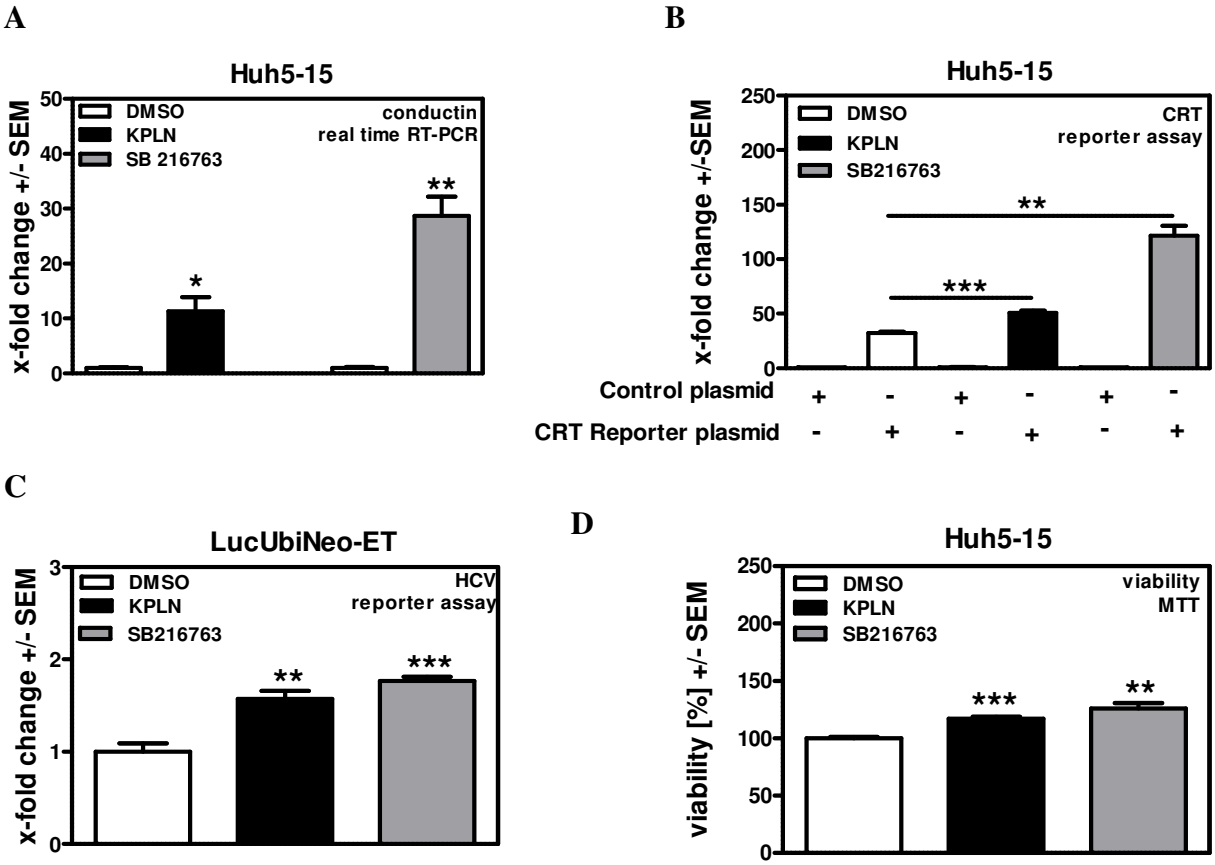

Figure 16: Activation of Wnt signaling pathway increased expression of HCV proteins
HCV replicon cell lines Huh5-15 (A, B, D) or LucUbiNeoET (C) were cultivated for 24 h and subsequently incubated with the Wnt signaling pathway activators Kenpaullone (KPLN) or SB216763 [c=10 µM] for 24 h. Wnt signaling pathway activity was measured by real time RT-PCR of conductin (normalization to mATPsy) (A). Cells were additionally transfected with a β-catenin regulated transcription (CRT) reporter plasmid or control plasmid. Wnt signaling pathway activity was measured by monitoring the CRT with a luciferase reporter assay (B). Expression of HCV in LucUbiNeo-ET cells was measured by luciferase reporter assay (C). Cell viability was measured by MTT assay (D). *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

5.6 OPN has no direct effect on expression of HCV proteins

OPN was found to regulate Wnt activity (5.4) and independent of that, Wnt signaling pathway was found to regulate expression of HCV proteins (5.5). Therefore it is of interest to investigate, whether OPN regulation directly affects HCV regulation. LucUbiNeo-ET cells were transfected with a control siRNA (siC) or with siRNA directed against OPN (siOPN), to down-regulate OPN expression. Effects on HCV regulation were either measured by luciferase reporter assay for HCV (Figure 17 A) or by real time RT-PCR (normalized to mATPsy) (Figure 17 B). Down-regulation of OPN had no direct effect on HCV mRNA expression. To confirm this result, the experiment was performed again with two others siRNA's against OPN (siOPN-a, siOPN-b).

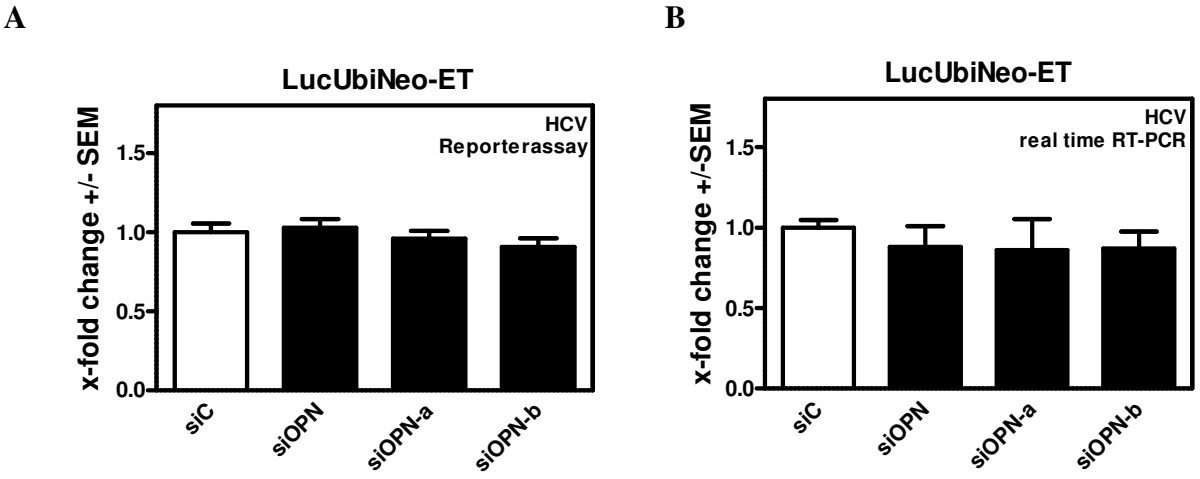

Figure 17: OPN has no effect on expression levels of HCV protein
LucUbiNeo-ET cells were cultivated for 24 h and subsequently transfected with a control siRNA (siC) or with 3 different siRNAs (siOPN, siOPN-a, siOPN-b), to down-regulate OPN expression. Expression of HCV proteins was measured by luciferase reporter assay (A) or real time RT-PCR (normalized to mATPsy) (B).

5.7 Expression of HCV proteins depends on cell viability

A higher Wnt pathway activity promotes cell proliferation and viability (Figure 16 D), which in turn could result in an elevated virus production. It is therefore plausible that the Wnt pathway supports HCV production via the enhancement of viability. In order to verify this assumption the interdependence of viral protein expression and cellular viability was analyzed. In a first experiment, HCV replicon cell line LucUbiNeo-ET was cultivated over a period of 10 days, medium was changed daily. Cells were counted with CasyTT on day 2, 4, 6, 8, and 10 (Figure 18 A, B) dots are illustrating mean values, showing an exponential growth until day 6 (Figure 18 B; interrupted line depicts standard exponential growing). The percentage of vital cells among all cells was determined via CasyTT (Figure 18 C). The results show that the cells grow in an exponential way until day 6 (Figure 18 A, B, C). In a second experiment HCV replicon cell lines LucUbiNeo-ET (Figure 18 D) or Huh5-15 (Figure 18 E) cells were cultivated for 8 days and medium was changed daily. Expression levels of HCV proteins were measured on day 1, 2, 4, and 8 by luciferase reporter assay (Figure 18 D) or real time RT-PCR (normalized to GAPDH) (Figure 18 E). The highest HCV expression level was detected on day 4 by luciferase reporter assay (Figure 18 D) or real time RT-PCR (Figure 18 E), a time point at which the cells are still growing exponentially (Figure 18 A, B, C). The expression of viral proteins is significantly reduced between day 6 through 8; this is the time point at which cells switch from the exponential growth to the stationary phase. To further analyze if HCV protein expression depends on proliferation, cell proliferation was reduced by Flavopiridol (FLPD) for 3 days. The successful down-regulation of proliferation is indicated on day 4 by cell counting with

CasyTT (Figure 18 F) or by determining the viability with a MTT assay (Figure 18 G). In addition, expression of HCV proteins was measured by the luciferase reporter assay for HCV, which revealed a down-regulated expression of HCV proteins (Figure 18 H).

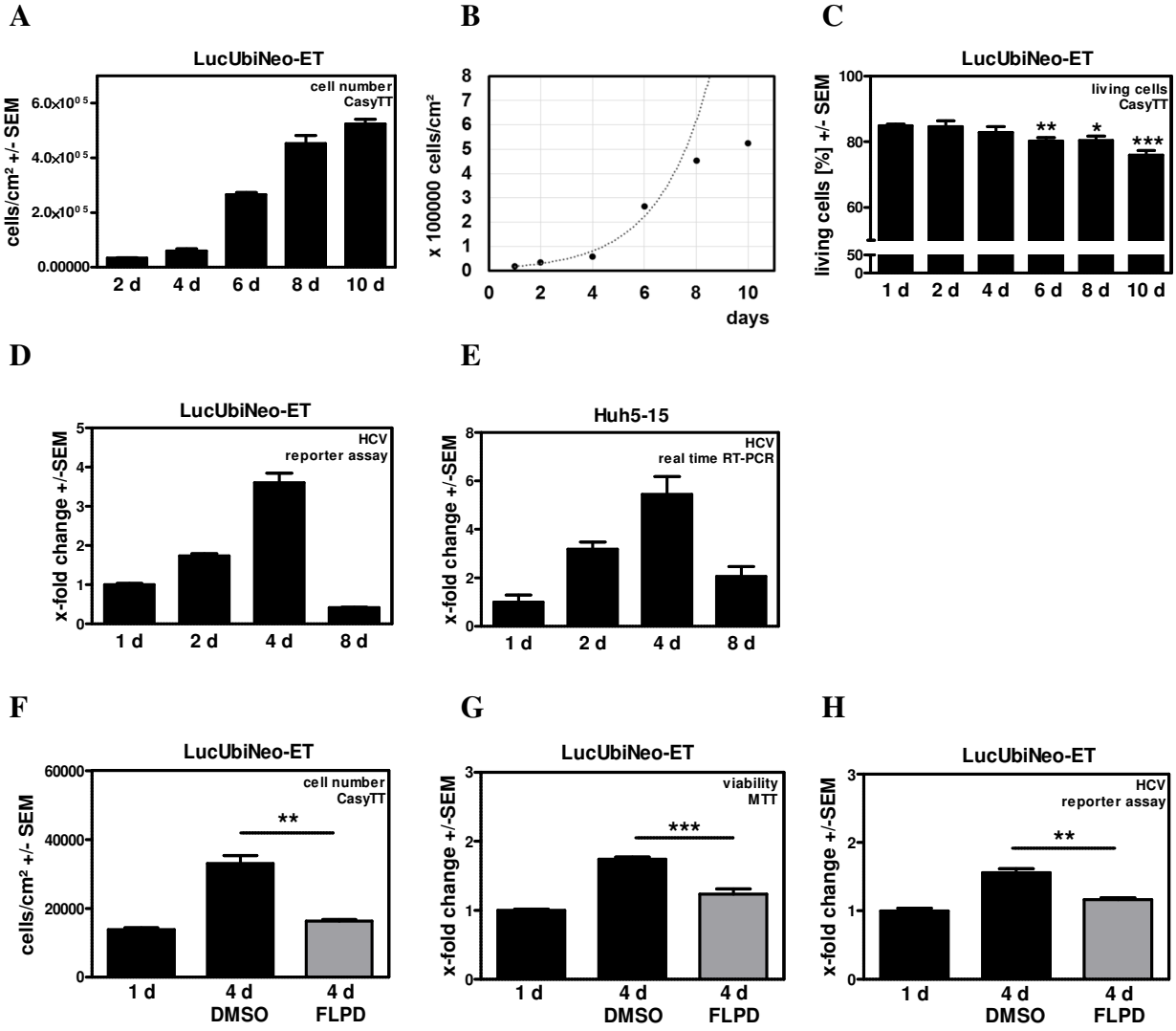

Figure 18: Expression of HCV proteins depends on cell proliferation
A, B, C: HCV replicon cell line LucUbiNeo-ET cells were cultivated for 10 days, medium was changed daily. Cells were counted (A, B) and vitality was determined (C) with CasyTT on day 2, 4, 6, 8, and 10. **B:** Dots are illustrating mean values and interrupted line is depicting exponential growing. **D, E:** HCV replicon cell lines LucUbiNeo-ET (D) or Huh5-15 (E) cells were cultivated for 8 days, medium was changed daily. Expression of HCV proteins was measured by luciferase reporter assay (D) or real time RT-PCR (normalized to GAPDH) (E). **F, G, H:** LucUbiNeo-ET cells were cultivated for 24 h and subsequently treated with DMSO, as a solvent control, or Flavopiridol (FLPD) [100 nM] to down-regulate cell proliferation. Medium was changed daily. On day 1 and day 4 cells were counted with CasyTT (F), viability was determined with MTT (G), and expression levels of HCV proteins were measured by luciferase reporter assay (H). *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

5.8 Expression of HCV proteins has no effect on proliferation or viability

It has been shown, that the Wnt pathway is activated in HCV replicon cell lines and that the expression of HCV proteins depends on Wnt signaling pathway activity and cell proliferation (5.5; *Loscher, Keller et al.*, unpublished). Next it was investigated, whether the expression of HCV proteins plays a role in promoting cell proliferation. HCV replicon cell lines Huh5-15 and LucUbiNeo-ET and their background cell line Huh7 were cultivated and counted with CasyTT over a period of 10 days (Figure 19 A). A higher proliferation was observed for the parental cell line Huh7 in comparison to the HCV replicon cell lines (Figure 19 A), even though the proliferation relevant Wnt signaling pathway was shown to be more active in HCV replicon cell lines than in the parental cell line (Figure 15). The difference of growth rate already indicates that expression of viral proteins does not lead to a higher proliferation rate. The lower proliferation of HCV replicon cell lines could in part be explained by the selection pressure of Geneticin, which eliminates cells that have lost viral genes. To investigate this more specifically, HCV protein expression was down-regulated in HCV replicon cell line LucUbiNeo-ET 24 h after seeding with Telaprevir (TLPV) for 3 days. The successful down-regulation of HCV protein expression was shown by real time RT-PCR (normalized to GAPDH) (Figure 19 B), while cell numbers (Figure 19 C), total cell protein (Figure 19 D) or viability (Figure 19 E) were unchanged. In a nutshell, HCV down-regulation has no influence on cell viability, even though HCV replicon cells have a higher Wnt signaling pathway activity.

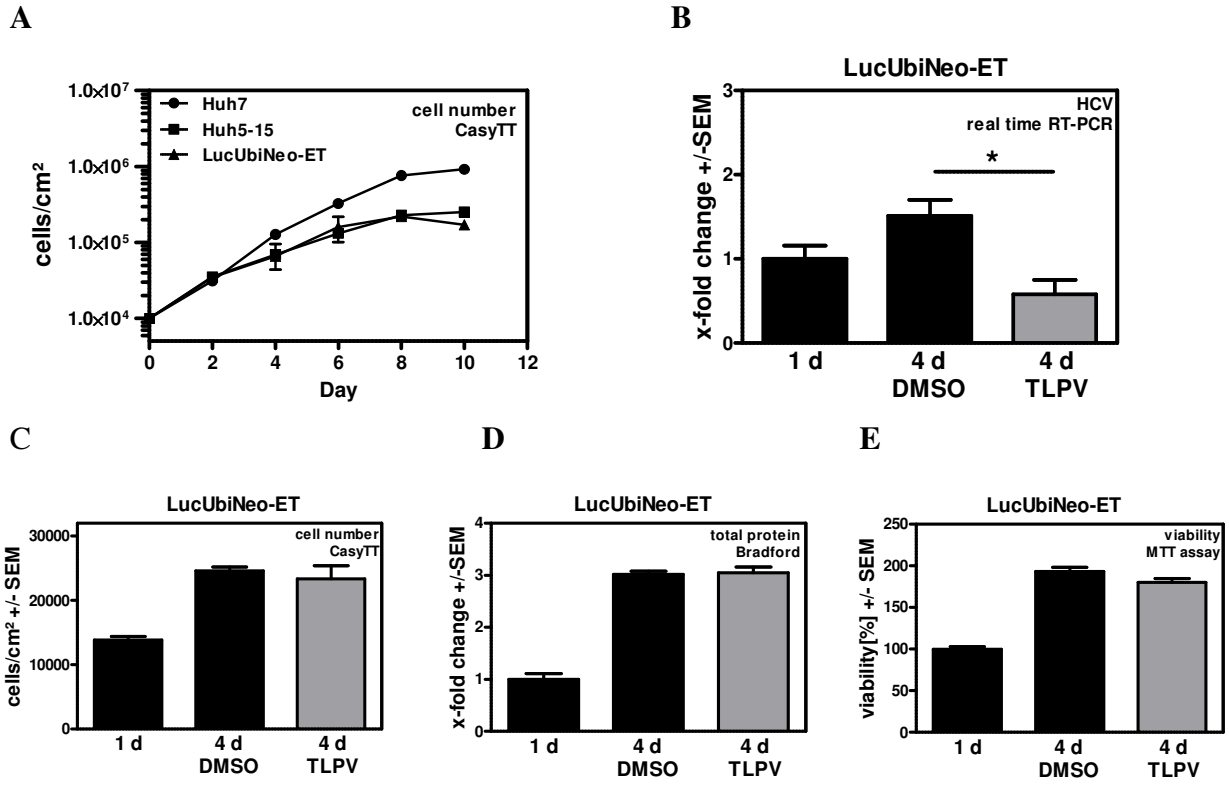

Figure 19: Expression of HCV proteins does not affect proliferation or viability
A: HCV replicon cell lines Huh5-15, LucUbiNeo-ET, and their parental cell line Huh7 were cultivated for 10 days, medium was changed daily and cells were counted with CasyTT on day 2, 4, 6, 8, and 10. **B, C, D, E:** LucUbiNeo-ET cells were cultivated for 24 h, treated with DMSO, as a solvent control, or the polymerase inhibitor Telaprevir (TLPV) [250 nM] to down-regulate expression of viral proteins, medium was changed daily. On day 1 and day 4 HCV mRNA expression level (normalized to GAPDH) was measured by real time RT-PCR (B), cells were counted with CasyTT (C), total protein content was determined by Bradford assay (D) and viability of cells was measured with an MTT assay (E). $*P \leq 0.05$

5.9 OPN expression in the HCV replicon cell line changes throughout the growth period analogous to HCV

OPN and HCV were shown to be able to up-regulate the Wnt signaling pathway activity (Figure 14) (Figure 15), which is responsible for cell viability. Therefore, it was investigated whether the viability in turn regulates OPN expression. OPN mRNA expression was analyzed in dependence of cell viability according to the experimental set up of Figure 18 E. Expression level of OPN mRNA in Huh5-15 cells was measured on day 1, 2, 4, and 8 by real time RT-PCR (Figure 20). The highest OPN mRNA expression level was detected on day 4 by real time RT-PCR (normalized to GAPDH) (Figure 20), a time point at which the cells still grow exponentially (Figure 18 A, B, C). During continues growth, at the time point were cells switch from exponential growth to the stationary phase (day 6 to day 8), expression of OPN mRNA was reduced (Figure 20).

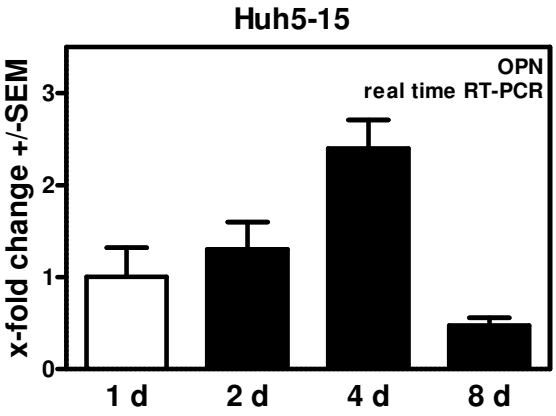

Figure 20: Alteration of OPN expression in Huh5-15 HCV replicon cell line depends on proliferation
HCV replicon cell line Huh5-15 was cultivated for 8 days, medium was changed daily. Cells were harvested at the indicated time points to analyze OPN mRNA expression by real time RT-PCR (normalized to GAPDH).

Between days 1 through 8 the course of OPN mRNA expression looks similar to the expression levels of HCV proteins (Figure 18 D, E). In consideration of the results concerning the dependency of OPN on HCV protein expression (Figure 8, Figure 11, Figure 12) it is possible that OPN is regulated directly by HCV.

5.10 OPN does not regulate viability although it increases Wnt signaling pathway activity

As the Wnt signaling pathway is activated by OPN (Figure 14), it was investigated whether this influences cell viability. OPN was down-regulated with siRNA against OPN (siOPN) and cell viability was measured in Huh7, Huh5-15, and HepG2 cells after 24
h and 48 h. The MTT assay revealed, that OPN knockdown had no effect on cell viability (Figure 21).

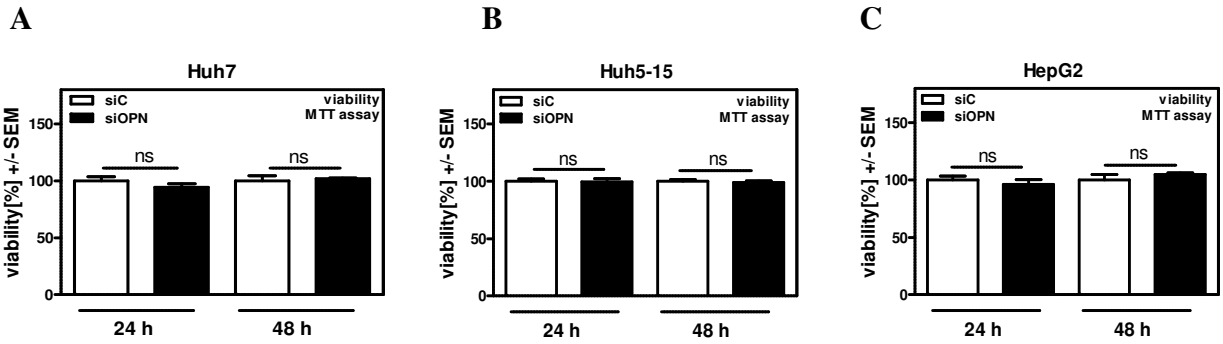

Figure 21: OPN does not influence cell viability
Huh7 (A), Huh5-15 (B) or HepG2 (C) cells were cultivated for 24 h and subsequently transfected with a control siRNA (siC) or with siRNA directed against OPN (siOPN) for 24 h or 48 h, to down-regulate OPN expression. Cell viability was measured by MTT assay. ns = not significant

6 *Discussion*

Hepatitis can be induced by multiple factors, for example by viral infections, which lead to acute or chronic inflammation that can progress to fibrosis and cirrhosis. A chronic inflammation may result in accumulation of mutations, which might support the development of an hepatocellular carcinoma (HCC).[7] However, the multifactorial mechanisms of HCC development are poorly understood. For instance, OPN expression is known to be increased in cancer[47], involved in different steps of hepatic inflammation[32], and induced via Hepatitis C Virus (HCV)[33], but it is not clearly understood, how OPN regulates the different mechanisms of a hepatitis. For a better understanding of how OPN influences chronic hepatic inflammation and cancer, the mechanisms of OPN-mediated effects in relation to HCV protein expression and tumor cell survival was investigated in this thesis (Figure 22). Various pathways are involved in hepatitis, thereof the Wnt signaling pathway is known to be up-regulated in patients with chronic HCV infection[46] and in HCC tumor tissue[51], as is OPN. Given that there is a connection between OPN and Wnt signaling pathway in hepatitis it was aimed to investigate the influences of OPN on the Wnt pathway and subsequent regulation of HCV protein expression or tumor cell survival.

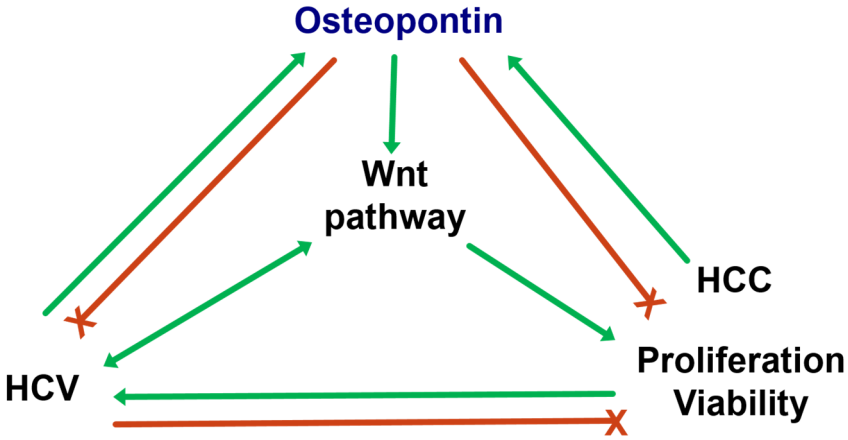

Figure 22: Schematic summary of the results
HCV = Hepatitis C Virus; HCC = Hepatocellular carcinoma

It was shown that OPN can be induced in HCC[47] and that an increased OPN level is related to higher metastatic potential and poor prognosis[50]. This thesis shows increased, but inhomogeneous OPN levels in murine liver tumors (Figure 9) and the human hepatoma cell lines HepG2 and Huh7 (Figure 10). Different expression levels of OPN are described consistently[47, 50, 52] and can be explained with the various regulators of OPN expression, which occur in dependence of tissue- and microenvironment. As one example, the higher OPN

expression level in HepG2 cells compared to Huh7 cells, can be explained by a higher availability of p53 in HepG2 cells.[25] It was shown that OPN is directly induced by p53 due to a binding sequence for p53, upstream of the first exon of OPN.[53] However, in contrast to OPN, p53 is not directly linked to poor prognosis, thus other OPN regulators are involved in OPN-mediated detrimental effects in patients.

It has previously been shown, that the Wnt signaling pathway is up-regulated in murine HCC.[46] The work presented in this thesis not only shows that OPN is up-regulated in murine HCC (Figure 9) or in human hepatoma cell lines as well, but that it is also directly correlated to the Wnt signaling activity. This has been shown by the fact, that down-regulation of OPN via siRNA and up-regulation via an OPN-plasmid, respectively, affected the Wnt signaling pathway activity (Figure 14). Although the regulation of Wnt signaling pathway by OPN is not well understood, it has been shown that OPNko mice, treated with synthetic OPN analogs, showed increased Wnt signaling pathway activity. This correlation between OPN and Wnt signaling pathway was explained with the OPN mediated up-regulation of MMPs, which cleaves N-cadherin, which in turn binds β-catenin and supports cell attachment. Cleavage of N-cadherin leads to a release of β-catenin, which consequently can act as a coactivator of transcription factors for Wnt signaling pathway target genes.[48] This could be the mechanism, how OPN increases transcription of Wnt signaling pathway target genes in our study as well.
We analyzed the connection between HCV and Wnt signaling pathway activity, because OPN regulates the Wnt signaling pathway (Figure 14) and this pathway is associated with higher HCV replication[46]. For instance, livers of patients with a chronic HCV infection show higher expression levels of beta-catenin, a key component of the Wnt signaling pathway.[49] This can be explained by findings, that the HCV NS5A-protein[49] as well as the HCV core-protein[54] activate the Wnt signaling pathway. This meets our previous observations, that Wnt signaling pathway activity is significantly enhanced in the HCV replicon system in comparison to its parental cell line Huh7 (Loscher, Keller et al., unpublished). In this thesis, up-regulation of Wnt signaling pathway by the HCV NS3-protein was revealed (Figure 15) and up-regulation by the HCV NS5-protein was confirmed for in Huh7 cells. Previously it was shown that down-regulation of Wnt signaling pathway activity decreases HCV protein expression (*Loscher, Keller et al.*, unpublished) and in this study up-regulated expression of HCV proteins was detected after activation of the Wnt pathway (Figure 16). This implicates that regulation of the Wnt signaling pathway correlates with HCV protein expression.

As shown by several groups[18, 20] and this work, induction of the Wnt signalling pathway enhances cellular proliferation, hence this proliferation might support viral replication. Furthermore this study shows a connection between active Wnt signaling pathway, cell proliferation (Figure 16) and increased HCV protein expression (Figure 18). The correlation between high HCV protein expression in exponentially growing cells and less HCV protein expression in resting cells was previously shown.[55] This observation was explained with different availability of kinases, during cell cycle phases, which regulate phosphorylation of viral proteins. Likewise, this study reveals a decreased expression of HCV proteins (Figure 18 H), when proliferation was impaired by Flavopiridol (FLPD) or during stationary cell growth of cultured cells. Several groups have shown that different viruses enhance Wnt signaling pathway activity, for example, mediated by the latent membrane protein 2A of Epstein-Barr virus[56], the Tax-protein of human T-cell leukemia virus[57], X-protein of Hepatitis B virus[58], NS5A-protein of HCV[49], or the core-protein of HCV[54]. These are viral proteins with different functions, but all of them are up-regulating the Wnt signaling pathway via inactivation of glycogen synthase kinase 3β (GSK3β). We conclude, that HCV needs cell proliferation for efficient replication and that activation of the Wnt signaling pathway might be a viral survival strategy. Besides proliferation there are Wnt signaling target genes that are involved in inflammation or cell migration and thereby influence the course of HCC development. As some examples, Wnt signaling pathway regulates cell fate specification or differentiation[59], is involved in epithelial-mesenchymal transition (EMT) via the interaction between cadherin and β-catenin[60], or may increase production of reactive oxygen species (ROS) that are known to cause DNA damage[61].

Although OPN regulates Wnt signaling pathway activity and Wnt signaling pathway regulates HCV protein expression, this study does not reveal a direct regulation of HCV protein expression mediated by OPN. This may be explained by the fact that, in this in vitro system, OPN mediated effects on the Wnt signaling pathway are still below the threshold needed to induce cell proliferation, which in turn is necessary for the increased expression of viral proteins. According to literature, it is not known whether OPN directly influences HCV replication. In various experiments, in which we reduced the OPN expression level (data not shown), we achieved different grades of decreased Wnt signaling pathway activity. If we achieved not only changes in the expression of the β-catenin reporter, as shown before, but down-regulation of Wnt signaling pathway target genes like conductin, expression of HCV proteins appeared to be decreased as well. Hence, for future investigations of the role of OPN in the regulation of the Wnt signaling pathway, a more efficient knock-down or knock-out of

OPN should be achieved. Nevertheless, the Wnt target genes may be counter-regulated by other pathways.

Similar to the effect of OPN, different expression levels of HCV proteins influence the Wnt signaling pathway activity, while they show no effect on cell proliferation in this study. This is partly in contrast to published data, in which HCV core-protein is described to enhance cell proliferation mediated by an activated Wnt signaling pathway.[54] However, in this in vitro system manipulation of the HCV protein expression does not influence the Wnt signaling pathway activity sufficiently to achieve further effects on cell proliferation.

Given that the OPN expression level is increased in HCV infection (Figure 8) or HCV replicon cell lines (Loscher, Keller et al., unpublished), (Figure 11) and that OPN, as well as HCV, is able to up-regulate the Wnt signaling pathway activity, mRNA expression level of OPN was analyzed in parallel to that of HCV in dependence of the cell growing phase. Due to the observation that OPN mRNA expression levels were changed throughout the growth period analogous to HCV mRNA expression levels (Figure 20, Figure 18), HCV might be involved in the regulation of OPN. Inhibition of HCV protein expression, indicated that OPN expression is suppressed just as well. It was shown by other groups and us, that HCV proteins regulate the Wnt signaling pathway[49, 54] and that OPN is a Wnt target gene[62]. The OPN promotor exhibits, among others, a binding sequence for the Wnt signaling pathway relevant transcriptionfactor-4 (Tcf-4) which can be activated by β-catenin[62]. We also detected increased OPN expression by trend after up-regulating Wnt signaling pathway activity with Kenpaullone in vitro (data not shown), indicating that Wnt signaling pathway could be one mechanism of how OPN is regulated by HCV proteins. To validate this correlation for the human disease, humanized uPA/SCID mice or human primary material should be used for further investigations.

Taken together, the OPN level is up-regulated in livers of HCV infected uPA/SCID mice or HCC of MDR2 knock out mice, however, at very diverse levels. OPN is thus more than just a tumor marker. This thesis revealed that it regulates the tumor relevant Wnt signaling pathway. Furthermore, it could be shown that changing the activity of this pathway can influence HCV protein expression, mediated by cell proliferation. The work presented here could not detect a significant decrease of HCV protein expression after down-regulation of OPN, which may be due to the insufficient effects of OPN down-regulation on the Wnt signaling pathway activity. Therefore, sufficient down-regulation of OPN or one of its effector molecules in the Wnt

signaling pathway might not only represent a target to interfere with tumor growth, but might also become a novel approach for HCV therapy. OPN and the Wnt signaling pathway may influence the progression of hepatitis also independently of proliferation, as they also promote inflammation or cell migration.

7 Outlook

This thesis showed that OPN manipulation affected the tumor relevant Wnt signaling pathway activity, which was shown to influence HCV protein expression. However, manipulation of OPN did not regulate HCV protein expression. This may be explained by the insufficient effects of OPN regulation on the Wnt signaling pathway activity, since it is expected that the Wnt signaling pathway supports HCV production via the enhancement of proliferation. Hence, for further investigations or to become an approach for therapy, a more efficient knock-down or knock-out of OPN should be achieved. Additionally, worthwhile results could be achieved by identification of OPN's effector molecules within the Wnt signaling pathway, which could be regulated in a therapeutic approach. Nevertheless the risk remains that other pathways could counter-regulate these target genes of interest. Progression of hepatitis can be influenced by inflammation or cell migration, mediated by OPN or Wnt signaling pathway, independently of proliferation. For a translational approach, analysis of inflammatory responses could be performed in HCV infected, humanized uPA/SCID mice or human primary material, to validate the influences of OPN and Wnt signaling pathway for the human disease.

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9 Abbreviations

°C	degree Celsius
ad	fill-up to
AP-1	activatorprotein-1
ATM	ataxia telangiectasia mutated
caspase	cyclic-aspartic protease
CD	cluster of differentiation
CDK	cyclin dependent kinase
cDNA	complementary DNA
CK	Casein Kinase
CRT	β-catenin related transcription
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
E1	envelope glycoprotein 1
E2	envelope glycoproteins 2
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
FCS	fetal calf serum
FLDP	Flavopiridol
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GSK3β	glycogen synthase kinase 3β
h	hours
HCC	hepatocellular carcinoma
HCV	Hepatitis C Virus
HSC	hepatic stellate cells
IL	interleukin
IRES	internal ribosome entry site
kDa	kilo Dalton
kg	kilo gram
ko	knock-out
KPLN	Kenpaullone
LDL	low density lipoproteins
LEF	Lymphocyte-Enhancer-Factor
LPS	Lipopolysaccharide
LSEC	liver sinusoidal endothelial cells
M	molar
mA	milliampere
mATPsy	mitochondrial ATP synthase β subunit
Mdr2ko	multi drug resistant transporter 2 knock-out
mg	milligram
min	minute

mL	milliliter
MMP	matrix metalloproteinases
mRNA	messenger ribonucleic acid
MTT	3-4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
n	number
NS	nonstructural protein
OPN	Osteopontin
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PRR	pattern-recognition receptors
rcf	relative centrifugal force
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse-transcription-PCR
SCID	severe combined immunodeficiency
SDS	Sodiumdodecylsulfat
SEM	standard error of the mean
siC	siControl
siRNA	small interfering RNA
TCF	T-Cell Factor
Tcf	transcription factor
TLPV	Telaprevier
TNF	tumor necrosis factor
U	units
uPA/SCID	Urokinase-type plasminogen activator/ severe combined immunodeficiency
UTR	untranslated region
v	volume
w	week
w	weight

10 Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

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12 Publications and Posters

12.1 Publications

Wuestenberg A, Kah J, Singethan K, Sirma H, Keller AD, Rosal SR, Schrader J, Loscher C, Volz T, Bartenschlager R, Lohmann V, Protzer U, Dandri M, Lohse AW, Tiegs G, Sass G.; Matrix conditions and KLF2-dependent induction of heme oxygenase-1 modulate inhibition of HCV replication by fluvastatin; PLoS One. 2014 May 6;9(5):e96533. doi: 10.1371/journal.pone.0096533. eCollection 2014.

Brázdová M, Quante T, Tögel L, Walter K, Loscher C, Tichý V, Cincárová L, Deppert W, Tolstonog GV.; Modulation of gene expression in U251 glioblastoma cells by binding of mutant p53 R273H to intronic and intergenic sequences; Nucleic Acids Res. 2009 Apr;37(5):1486-500. doi: 10.1093/nar/gkn1085. Epub 2009 Jan 12.

Maenz C, Loscher C, Iwanski A, Bruns M.: Inhibition of duck hepatitis B virus infection of liver cells by combined treatment with viral e antigen and carbohydrates; J Gen Virol. 2008 Dec;89(Pt 12):3016-26. doi: 10.1099/vir.0.2008/003541-0.

12.2 Posters

Christine Loscher, Amelie Dorothea Keller, Andrea Wüstenberg, Volker Lohmann, Ralf Bartenschlager, Gabriele Sass, Gisa Tiegs, and Roja Barikbin; Mechanisms of Osteopontin-mediated effects on chronic hepatic inflammation and cancer; GASL 2015, München, Deutschland. Z Gastroenterol 2015; 53 – A5_39

Christine Loscher, Henning Wege, Ralf Bartenschlager, Volker Lohmann, Gabriele Sass, Gisa Tiegs, and Roja Barikbin.; Osteopontin mediated effects on tumor cell viability; Gastsprecher-Symposium 2014, Hamburg, Deutschland.

Christine Loscher, Roja Barikbin, Julia Schildgen, Jann-Hauke Wulf, Gisa Tiegs, and Gabriele Sass; Identification of HO-1/CO-induced tumor protective genes in HCC; GASL 2014, Tübingen, Deutschland. Z Gastroenterol 2014; 52 - P_4_23

Christine Loscher, Amelie Dorothea Keller, Sergio Réne Perez Rosal, Volker Lohmann, Ralf Bartenschlager, Gisa Tiegs, and Gabriele Sass; Inhibition of wnt signalling interferes with HCV replication; GASL 2013, Hannover, Deutschland. Z Gastroenterol 2013; 51 - P_5_38

Christine Loscher, Ralf Bartenschlager, Volker Lohmann, Gisa Tiegs, and Gabriele Sass; Down-regulation of Osteopontin interferes with HCV replication by inhibition of wnt signalling *in vitro*; GASL 2013, Hannover, Deutschland. Z Gastroenterol 2013; 51 - P_5_37

Christine Loscher, Amelie Dorothea Keller, Henning Wege, Ralf Bartenschlager, Volker Lohmann, Gisa Tiegs, and Gabriele Sass; Mechanisms of Osteopontin-mediated effects on chronic hepatic inflammation and cancer; Gastsprecher-Symposium 2012, Hamburg, Deutschland.