Characterization of heme oxygenase 1 based therapy options in chronic persistent HCV infection

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submitted by:

Janine Kah

from Schierke am Brocken (Harz)

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Erstgutachter:

Prof. Dr. Gisa Tiegs

Institut für experimentelle Immunologie und Hepatologie

Zweitgutachter:

Prof. Dr. Thomas Dobner

HPI Abteilung Molekulare Virologie

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HPI Heinrich-Pette-Institut
Leibniz-Institut für Experimentelle Virologie

Carol Stocking, Ph.D.
Retrovirus Pathogenesis, Head
Phone: +49-40-48051 273
Fax: +49-40-48051 187
Email: stocking@hpi.uni-hamburg.de

Studienbüro Biologie
z.H. Frau Sült-Wüpping
MIN Fakultät
Universität Hamburg
Biozentrum Klein Flottbek
Ohnhorststr. 18
22609 Hamburg

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Sehr geehrte Damen und Herrn,

hiermit bestätige ich, dass die von Frau Janine Kah mit dem Titel " Characterization of heme oxygenase 1 based therapy options in chronic persistent HCV infection" vorgelegte Doktorarbeit in korrektem Englisch geschrieben ist.

Mit freundlichen Grüßen,

Carol Stocking

Dr. Carol Stocking

Leiterin der FG Retrovirus Pathogenesis
Heinrich-Pette-Institut
(Amerikanerin)

Meinen Eltern Wolfgang und Ines-Judith Kah

Contents

1. Introduction

1.1 Background of the study

Approximately 80% of patients infected with the Hepatitis C Virus (HCV) develop a chronic infection that subsequently increases the risk of developing hepatocellular carcinoma (HCC). The heme degrading, stress response enzyme heme oxygenase 1 (HO-1 or heat shock protein (Hsp32)) exhibits protection against occurrence of apoptosis and inflammation both, *in vitro* and *in vivo*. The induction of HO-1 can be achieved by several means e.g. by Cobalt-protoporphyrin-IX (CoPP) or aspirin application, as well as after the application of statins in endothelial cells [1], smooth muscle cells [2], and macrophages [3]. We and others recently described that CoPP-mediated induction or over-expression of HO-1 significantly interferes with HCV replication *in vitro* [4;5;6]. This antiviral effects could be attributed to the heme degradation product biliverdin (BV) [6], which has been shown to increase endogenous antiviral interferon signaling [6], and to directly inhibit the HCV protease NS3/4A [7]. While a combinational therapy of interferon and ribavirin has been considered for years the best option for treatment of chronic HCV infection, treatment regimens leading to sustained virologic responses have now markedly improved through the application of new direct antiviral agents (DAAs), such as HCV protease (telaprevir, borceprevir, simeprevir) and polymerase inhibitors (sofosbuvir) in combination with pegylated interferon (PEG-IFN) alpha [8]. Nevertheless, escape mutants may occur [9]. Therefore additional strategies targeting host components and being also able to affect HCV-driven inflammatory responses, e.g. through HO-1 induction in infected cells, might be useful to support therapy.

1.2 The Hepatitis C Virus

1.2.1 Classification and genome organization

The Hepatitis C virus (HCV) is a member of the genus hepacivirus within the Flaviviridae family. Altogether, this viral family contains four genera, flavivirus, pestivirus, and a newly defined genus pegivirus [10]. Novel hepaciviruses have been described for and isolated from bats, rodents, primates, horses and dogs [11;12]. The Hepatitis C virus has a narrow host range, which is due to the high species-specificity and restricted tissue tropism. A very recent study revealed the presence of at least 7 genotypes and 67 subtypes [13]. Moreover it has been reported that inter-subtypes and inter-genotypes HCV recombinants occur [14], whether this events appear to be rare.

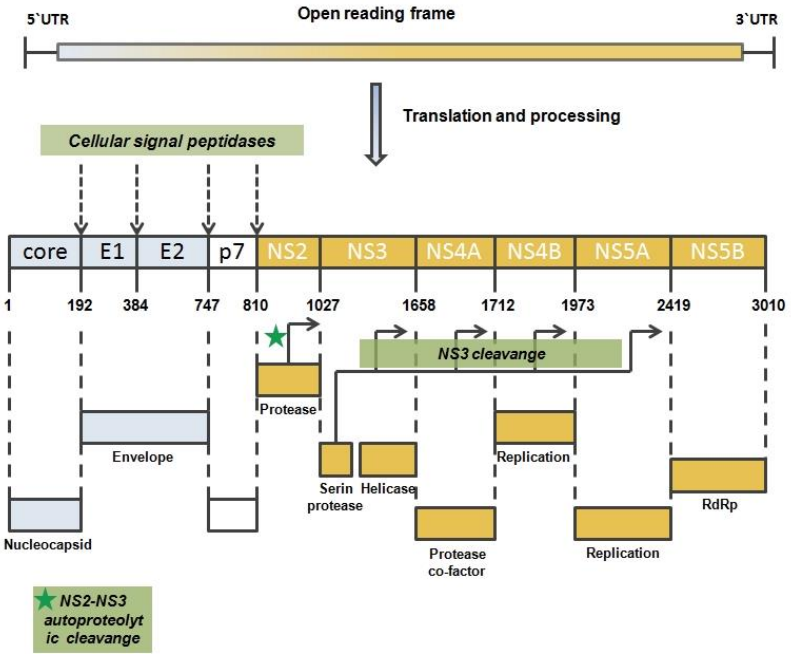

Figure 1: Illustration of the organization of the HCV genome

The 9.6-kb positive-strand RNA genome is schematically depicted at the top. Internal ribosome entry site (IRES)-mediated translation yields a polyprotein precursor that is processed into the mature structural and nonstructural (NS) proteins. Functions of the structural and nonstructural proteins are mentioned below. Processing steps / peptides are illustrated by arrows and described in green boxes.

The genome as illustrated in Figure 1 is a positive-strand (+ ss) RNA molecule of approximately 9.6 kb with an open reading frame (ORF) encoding a polyprotein of 3000 amino acids. As illustrated in Figure 1, the structural proteins were encoded by the N-terminal part of the ORF, whereas the remaining portion of the ORF codes for the nonstructural proteins. The ORF is flanked in 5' and 3' by untranslated regions (UTR) which play an important role in polyprotein translation and RNA replication [15]. Translation of the HCV genome is induced by the 5´UTR which functions as an internal ribosome entry site (IRES) [16] in contrast to the RNA organization of the flavi- and pestivirus where the 5´UTR represents the so-called Cap-structure. During viral replication, the polyprotein cleaved by viral, as well as host enzymes (Figure 1; green boxes) into the three structural proteins (core, E1, E2; Figure 1; light blue; Table 1) and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B; Figure 1; Table 1). Recently, the presence of an additional protein (termed F [frameshift] or ARF [alternate reading frame]) has been reported [17]. This protein is predicted as a result of ribosomal frameshifting during translation within the core region of the genomic RNA. Moreover, anti-F-protein antibodies were detectable in HCV-infected subjects, leading to the point, that the protein is indeed expressed during infection *in vivo* [18].

Table 1: Size and function of HCV structural and nonstructural proteins

protein nomenclature	protein size in kDa	function
C	14	Capsid-protein; interaction with RNA genome
E1	31-35	Membrane protein; glycosylated
E2	68-72	Membrane protein; glycosylated
p7	7	Assembly; release of virions
NS2	21	Zn^{2+} - Metalloproeinase; associated with NS3-protease
NS3	70	Serinprotease; helicase
NS4	8-10	Associated with NS3-protease; membrane-associated
NS4B	27	membrane- associated
NS5A	56-58	phosphorylated
NS5B	68-70	RNA-depending RNA poloymerase

1.2.2 Life cycle of the hepatitis C virus

Due to the development of small animal models the opportunity has been offered to investigate the several steps of the viral replication. As illustrated in Figure 2 the HCV core protein (C-protein) and the envelope glycoproteins (E1- and E2-protein) are the principal protein components of the virion. E1- and E2-protein are presumably anchored to a host cell-derived double-layer lipid envelope that surrounds a nucleocapsid, composed by multiple copies of the C-protein and containing the genomic RNA. HCV particles circulate in various forms in the infected host. They can be associated with low-density lipoproteins (LDL) and very-low-density lipoproteins (VLDL) but also circulate as virions bound to immunoglobulins, as well as free virions [19].

The hepatocytes are the major target cells supporting HCV replication but infection of B cells, dendritic cells and other cell types has also been reported [20-;21;22]. The viral entry into its target cell is currently descript as a multi-step process depending on CD81, a tetra spanning protein, the LDL receptor (LDLR), the scavenger receptor class B type I (SR-BI), the tight junction proteins claudin-1 and occludin [23], epidermal growth factor receptor (EGFR) and ephrin receptor (EphA2) [24]. Interestingly, latest investigations identify the receptor tyrosine kinases (RTKs) and the Niemann-Pick C1-like 1 (NPC1L1) cholesterol uptake receptor as cofactors for the HCV entry [25]. Only the cellular molecules cluster of differentiation 81 (CD81) and SR-BI have been reported to interact with HCV E2-protein and can be therefore considered as necessary receptors for the HCV entry [26]. Together with glycosaminoglycans (GAGs), the LDLR and other cell surface proteins involved in serum lipoprotein binding and metabolism might serve as primary collectors of HCV particles for further targeting to CD81 and additional receptor components as illustrated in Figure 2. HCV E2-protein also binds to dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and liver/lymph node-specific intercellular adhesion molecule- 3-grabbing integrin (L-SIGN). L-SIGN is a calcium-dependent lectin expressed on liver sinusoidal endothelial cells that may facilitate the infection process by trapping the virus for subsequent interaction with hepatocytes. However, certain cell types found to be non-permissive despite expression of CD81, SR-BI and claudin-1, indicating that one or more additional HCV entry factor(s) remain to discover.

After binding to the different host membrane factors, HCV enters the cell in a pH-dependent manner indicating that the virus internalized via clathrin-mediated endocytosis.

The acidic environment within the endosomes is assumed to trigger HCV E1-E2 glycoprotein-mediated fusion of the viral envelope with the endosome membrane [27]. In result the genomic HCV RNA is released into the cytoplasm of the host cell. Direct interaction of the IRES structure (5`UTR region) with cellular ribosomal units initiates translation of the viral genome.

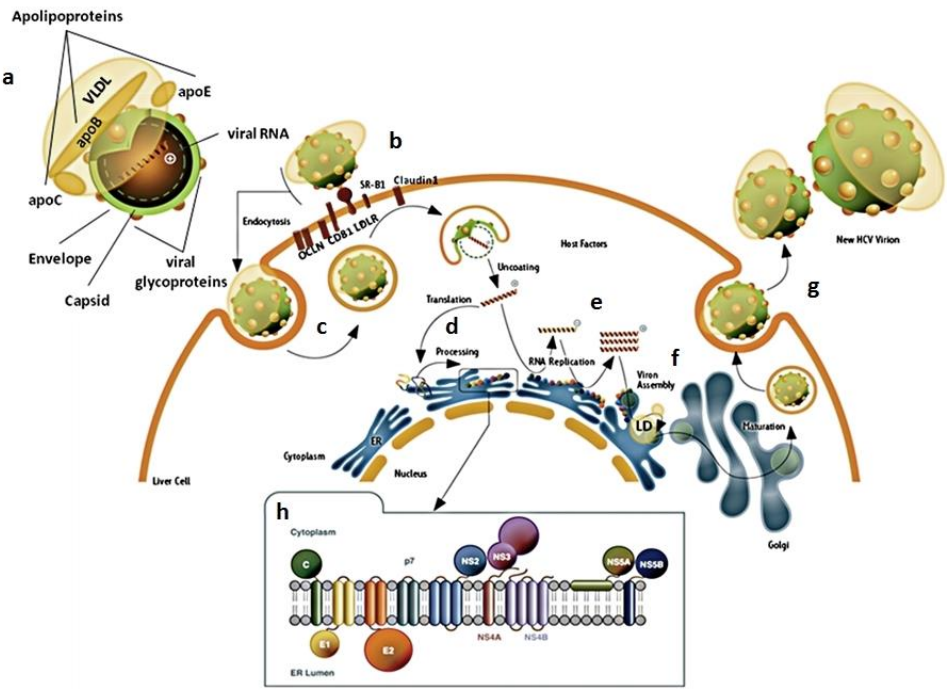

Figure 2: Illustration of the HCV lifecycle

The steps of the viral life cycle are depicted schematically. The circulating viral Lipoparticle (**a**) is illustrated including compounds of the viral as well as the Lipid-droplet (LD). Receptor binding; endocytosis (**b**); cytoplasmic release and uncoating (**c**); IRES-mediated translation and polyprotein processing (**d**); RNA replication (**e**) followed by packing and assembly and subsequently virion maturation and release (**f**). The topology of HCV structural and nonstructural proteins at the endoplasmic reticulum (ER) membrane is shown (**h**). [Illustration adapted from the **Journal of online Hepatology; Lecture: HCV viral kinetics; 2007** modified]

The translated polyprotein is further processed by viral and host proteases (Figure 1) to form the membrane-associated replication complex (Figure 2) which is composed of viral proteins, replicating RNA genomes cellular membranes, as well as additional host cell factors [28;29].

Sense (+) RNA molecules are transcribed into anti-sense (-) RNA molecules by the NS3-5B replicase and then used as templates for multi-copy transcription of the viral genomes. These may be used again as templates of viral replication or are enveloped to be released as HCV progeny. The HCV genome is stored within the viral capsid through interactions between the (+) RNA molecule and the structural C-proteins. Viral particles pass through budding, which takes place at the endoplasmatic reticulum (ER)-membrane before being released as lipoparticles.

1.2.3 Clinical view on HCV-infection and manifestation

The hepatitis C virus infection is a disease with a significant global impact. According to the World Health Organization there are 130-170 million people (2-2.5% of the world`s population) currently infected with HCV. Parental exposure to HCV is the most effective way of transmission, while the majority of patients infected with HCV in Europe and the United States acquire the disease through intravenous drug abuse or blood transfusions. Since the onset of routine HCV testing of the blood supply in the early 1990s, the latter became rare. Transplant recipients who receive organs from HCV-positive donors have a high risk to develop HCV-infection transmission vary form 30-80% [30,31].

Symptoms of HCV infection like fatigue, muscle ache, loss of appetite or nausea are unspecific and in the most cases mild or even absent. As a consequence the infection with HCV may remain unrevealed for many years so that only a minority of patients (30-50%) infected with HCV are aware of their disease and contribute to viral transmission [32]. Untreated hepatitis C advances to a chronic state in up to 80% of people, leading to liver cirrhosis in 20-40% with a correlated risk of hepatic decompensation, hepatocellular carcinoma and death [33].

For diagnostic of HCV both serological and nucleic acid-based molecular assays are available [34]. It is estimated that the hepatitis infection after persistence of six months became chronic. A constant escape from the immune recognition may result from the genetic diversity of the virus as well as its tendency to rapid mutation.

1.2.4 From HCV infection to HCC

Chronic inflammation of the liver, which is characterized by repetitive cycles of cell death and regeneration thus promoting the accumulation of genetic mutations, is responsible for the development of hepatocellular carcinoma (HCC). Chronic HCV infection represents a major risk factor for the development of HCC worldwide (25% of the cases [35]). It is estimated that HCC represents the fifth most common cancer of males and the eighth most common cancer in females, with around 560.000 new cases every year. Because of the poor prognosis, HCC represents the third leading cause of cancer death worldwide. Both viral hepatitis and non-virus-mediated HCC development is a multistep process involving different genetic alterations promoting the malignant transformation of the hepatocyte. Detailed analysis of HCC development in animal models has identified a variety of genomic and molecular alterations [36]. There are at least 4 pathways known to be involved in regulating either cell proliferation or cell death (phosphor-retinoblastoma (pRb), p53, transforming growth factor beta 1 (TGF-beta1) and beta-catenin pathways) which are affected in hepatocarcinogenesis [37;38;39]. Although resistance to audiogenic seizures (Ras) family oncogenes were found to be activated in spontaneous and chemically induced rodent hepatocardiogenesis models and the

cyclin D was found to be amplified in 10-20% of the cases [40], no consistent pattern of proto-oncogene activation has emerged in human HCCs so far.

1.2.5 Models in HCV studies

1.2.5.1 In vitro models

The establishment of the genotype 1b replicon system in 1999 was a milestone for understanding and targeting intracellular replication, allowing replication in Huh7-derived hepatoma cells under selection [41]. Consecutively, replicons of genotype 1-4, as well as intergenotypic replicons, were developed [42]. The genotype 2a isolate JFH1 is unique in the case that no adaptation was necessary for RNA replication in Huh7-derived cell culture. Beginning in 2005, this isolate and its chimeric and adapted variants resulted in the first robust cell culture virus production systems [43;44;45;46]. JFH1 further allowed establishment of intergenotypic recombinants of the core-NS2, NS3-NS4 protease and NS5A regions [47;48]. Recent developments also led to the establishment of efficient culture-adapted full-length systems for isolates of genotypes 1a, 2a and 2b [49;50], so that these *in vitro* systems can recapitulate the entire viral life cycle of different HCV strains and permit a first screening of the antiviral efficacy of all DAAs [51;52]. While these cell culture systems represented a breakthrough for HCV replication studies, because of the divergence between hepatoma cells and primary hepatocytes, there is still a limitation for studies aiming at understanding mechanism of HCV pathogenesis and viral interactions with the natural host cell.

1.2.5.2 In vivo models

To date, the only model for studies of adaptive immunity and vaccine response is the chimpanzee [53;54;55]. However, current guidelines strongly limited the use of chimpanzees for infection research. As alternative approaches, HCV has been adapted to infect mouse cells [56] or mice that have been genetically humanized to allow HCV entry [57]. This approach is limited since only low viral replication and virus production levels can be achieved.

Immunodeficient mice (SCID mice) engrafted and reconstituted with primary human hepatocytes (USB mice) offer the opportunity to study viral entry, replication and interactions with the natural target of infection, the human hepatocyte, although not adaptive immune responses can be investigated [58]. Briefly, the basic principle of the transgenic USB model is to induce damage of mouse hepatocytes upon expression of an Alb-promoter-driven urokinase-type plasminogen activator (uPA) transgene. This toxic effect supplies the opportunity for xenogenic hepatocytes to repopulate the diseased mouse liver. Crossbreeding the uPA mice with the immunodeficient mice (SCID) guarantees the permanent repopulation of the mouse liver with e.g. human hepatocytes. 3 weeks old uPA/SCID (USB) mice are genrally used for transplantation and hence to generate mouse livers with high levels of human chimerism [123]).

1.3 The Heme oxygenase 1 enzyme

1.3.1 Classification of heme oxygenase enzymes

The heme oxygenase (HO; encoded by *HMOX* genes) enzymes catabolize iron (Fe) protoporphyrin (IX) (heme) into equimolar amounts of inconstant Fe, carbon monoxide (CO) and biliverdin [59]. Heme is a prosthetic group and permits the incorporation of Fe^{2+} into the tertiary structure of hemoproteins. Free heme catalyzes the production of free radicals through Fenton chemistry [60]. This pro-oxidant effect is highly controlled by insertion of heme into the heme target pockets of the hemoproteins, which allows controlling the rate of electron exchange between Fe-heme and a variety of ligands under homeostasis conditions. In case of oxidative stress hemoproteins release their prosthetic heme groups resulting in disposability of free heme and further leading to production of free radicals. The inducible HO-1 isoenzyme enables the cells to avoid pro-oxidant effects and subsequently prevent the initiation of the programmed cell death by rapid increasing the catabolism-rate of free heme. The constitutively expressed and moreover not inducible HO-2 isoenzyme seems to be less important in the case of cytoprotection.

1.3.2 Regulation of HO-1 expression

With some exceptions, HO-1 expression is known to be regulated at the transcriptional level, since both hypoxia [61] and acidosis [62] were shown to promote mRNA stabilization. Moreover, HO-1 enzymatic activity can be also regulated, as demonstrated for hyperoxia [63]. The transcription of HO-1 can be induced by a variety of signal transduction pathways mediating the activation of different transcription factors [64] which recognize the specific DNA-binding

elements in the proximal (−0.3 kb) and distal (−4 kb- E1 and −10 kb-E2) regions of the promoter [65] (Figure 3). This implicates that most forms of oxidative stress are related to the rapid increase of heme catabolism, which is mediated by the induction of HO-1 transcription. By linking oxidative stress to HO-1 induction, cells ensure that the rates of free heme are not acting in a cytotoxic manner [66]. In case of oxidative stress, Bach1, the transcriptional repressor that binds to several stress responsive elements (StREs) within the HO-1 promoter, inhibit HO-1 transcription [67;68]. Bach1 has also heme-binding sites [69], by which heme can induce Bach1 conformational modifications leading to the loss of Bach1-binding activity to StREs.

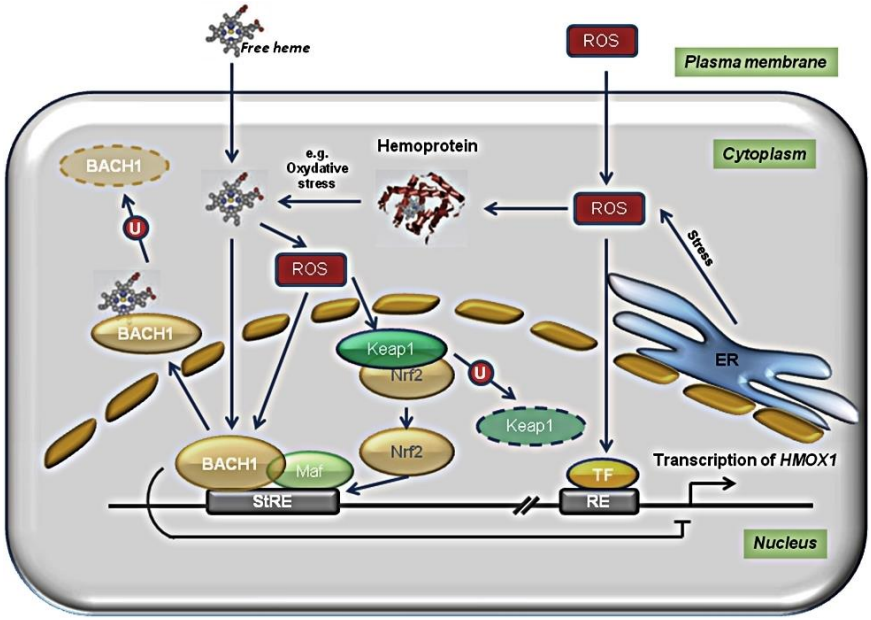

Figure 3: Transcriptional regulation of HO-1 (encode by the *HMOX1* gene)

Signal pathways, inducing HO-1 transcription with regard to free heme and reactive oxygen species (ROS). ROS (endogenous or/and cellular stress) causes the release of heme from its hemoproteins; leading to oxidative stress and triggers HO-1 induction. Multiple DNA-responsive elements (RE) in the HO-1 promoter bind specific transcription factors (TF), which is activated in response to oxidative stress. Under homeostasis Bach1/small Maf dimers bind constitutively to stress responsive elements (StREs) in the HO-1 promoter and inhibit transcription. In response to oxidative stress, Bach1 is exported from the nucleus, ubiquitinated (red circled u) and degraded (dotted lines), releasing transcriptional repression. Stress-induced Keap1 ubiquitination-degradation (u/dotted line), allowing the transcription factor NF-E2-related factor-2 (Nrf2) to translocate into the nucleus. Nrf2 /small Maf protein heterodimers bind to StRE and promote transcription of HO-1. Most probably the Bach1/Nrf2 transcriptional system interacts functionally with other transcription factors to regulate HO-1 transcription. [**partly adapted from: Gozzelino et al (2010).** Mechanisms of Cell Protection by HO-1. Annu. Rev. Pharmacol. Toxicol].

As consequence, Bach1 undergoes nuclear export, poly-ubiquitination by the heme-responsive E3 ubiquitin-protein ligase HOIL-1, and subsequent degradation by the 26S proteasome pathway [68] (Figure 3). The release of Bach1 from its target elements permits the binding of the oxidative-stress responsive transcription factor NF-E2-related factor-2 (Nrf2) to HO-1 promoter and induction of HO-1 transcription [70]. The pro-oxidant activity of the free heme might also mediate the release of Bach1 and hence favor HO-1 induction (Figure 3).

1.3.3 HO-1 Polymorphisms

A number of polymorphisms in the gene encoding for HO-1 (*HMOX1*), such as a (GT)n microsatellite polymorphism in the promoter region, appear to be associated with the incidence or progression of various diseases [71]. The HO-1 expression level correlates with the GT repeats length which ranges from 15 to 40. In individuals harboring a lower then average number of GT repeats the HO-1 mRNA levels appeared higher as in individuals with more GT repeats [71]. This microsatellite (GT)n polymorphism regulates HO-1 expression by modulating *HMOX1* transcription as well as translation. Several studies suggested that individuals with fewer (GT)n repeats and presumably stronger induction of HO-1 expression are less likely to develop pathologies than individuals with a higher number of (GT)n repeats [71].

1.4 3-Hydroxy-3-methylgutaryl CoA reductase inhibitors

1.4.1 Classification and functional properties

Previous studies indicated that the induction of HO-1 can be achieved by cholesterol lowing drugs, as reported in endothelial cells [72], smooth muscle cells [73], and macrophages [74]. There are several classes of lipometabolism-regulating drugs available, like bile acid-binding resins (e.g. cholestyramine, colestipol, colesevalam), nicotinic acid (niacin), fibrates (e.g. fenofibrate, clofibrate, gemfibrozil, bezafibrate), as well as cholesterol-absorption inhibitors (e.g. ezetimibe). The hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are among the most commonly prescribed lipid-modifying therapies. The effectiveness of these drugs depends on the competitive inhibition of HMG-CoA reductase, which represents the rate-limiting enzyme in the cholesterol synthesis. The HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate (Figure 4) and results in a modest decrease in cellular cholesterol concentrations. This decreased cholesterol concentration moreover leads to activation of the sterol regulatory element binding protein (SREBP), which in turn increases LDLR gene expression, resulting in higher LDLR cell surface presentation on the hepatocytes and increased uptake of plasma LDL. This, in turn, can decrease plasma LDL-cholesterol (LDL-C) concentrations [75]. Increased concentrations of high-density lipoprotein cholesterol (HDL-C) as well as decreased levels of circulating triglycerides are also beneficial effects induced by statins [76].

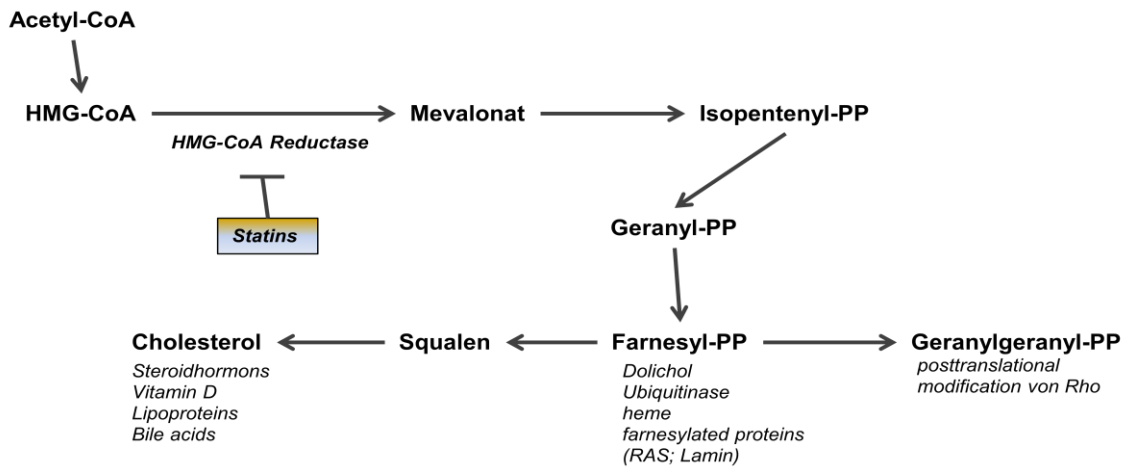

Figure 4: The mammalian mevalonate pathway including the point of statin-effectiveness

The main working point of statins is to bind the key enzyme of the mammalian mevalonate pathway; the HMG-CoA reductase. That results in the inhibition of the initial step of the pathways. PP: pyrophosphate.

Statin-mediated alteration of atherogenic lipoproteins levels including reduced levels of hepatic synthesis of apolipoprotein B100 and triglyceride-rich lipoproteins has been descript [77;78]. Moreover statins have been shown to exhibit beneficial cardiovascular effects [79]. An explanation for these pleiotropic effects mediated by statins is the interference of the synthesis of nonsteroidal isoprenoid compounds, which were produced from mevalonic acid (Figure 4) [80]. Moreover, it has been described in large-scale clinical trials that statins are able to reduce cardiovascular-related morbidity and mortality in patients with and without existing coronary heart disease (CHD) [81;82;83;84;85;86;87;88]. Thus, application of statins has been shown to slow the progression or even promote regression of coronary atherosclerosis which results in fewer lesions and total occlusions compared to untreated hypercholesterolaemic patients [80;89;90]. Taken together, seven remarkably safe and well-tolerated statins despite the withdrawal of cerivastatin [91] are currently approved for clinical use (Hypercholesterolemia; Coronary atherosclerosis). Apart from this, statins exhibit other biological activities

e.g. antiproliferative effects in cancer [92]. With respect to viral infections, statins have been shown to interfere with HIV replication and release *in vitro* [93,94], while a combination of statins and caffeine was shown to inhibit influenza virus infection in mice [95]. Despite the known benefits of statin medication, the antiviral effects of statins in HCV-infected patients remain controversial [96;97;98].

1.4.2 Chemical structure of statins

While lova-, prava- and simvastatin are fungal-derived statins, atorva-, ceriva-, fluva-, pitava- and rosuvastatin are fully synthetic compounds [99]. The chemical structure of these statins, as well as their classification can be broadly divided into three parts (Figure 5) [100]: an analogue of the target enzyme substrate, HMG-CoA; a complex hydrophobic ring structure that is covalently linked to the substrate analogue and is involved in binding of the statin to the reductase enzyme; side groups on the rings that define the solubility properties of the drugs and therefore many of their pharmacokinetic properties. All statins are competitive inhibitors of HMG-CoA reductase with respect to the binding of the substrate, HMG-CoA, but not the co-enzyme Nicotinamidadenindinukleotidphosphat (NADPH) [101]. The mechanism for statin inhibition was elucidated by solving crystal structures of the catalytic portion of the enzyme bound to several statins [102]. The structures indicate that statins act by binding to the active site of the enzyme, sterically preventing the substrate from binding. Moreover the substrate-binding pocket also undergoes a rearrangement that enables the inflexible, hydrophobic ring structures of the statins to be suitable.

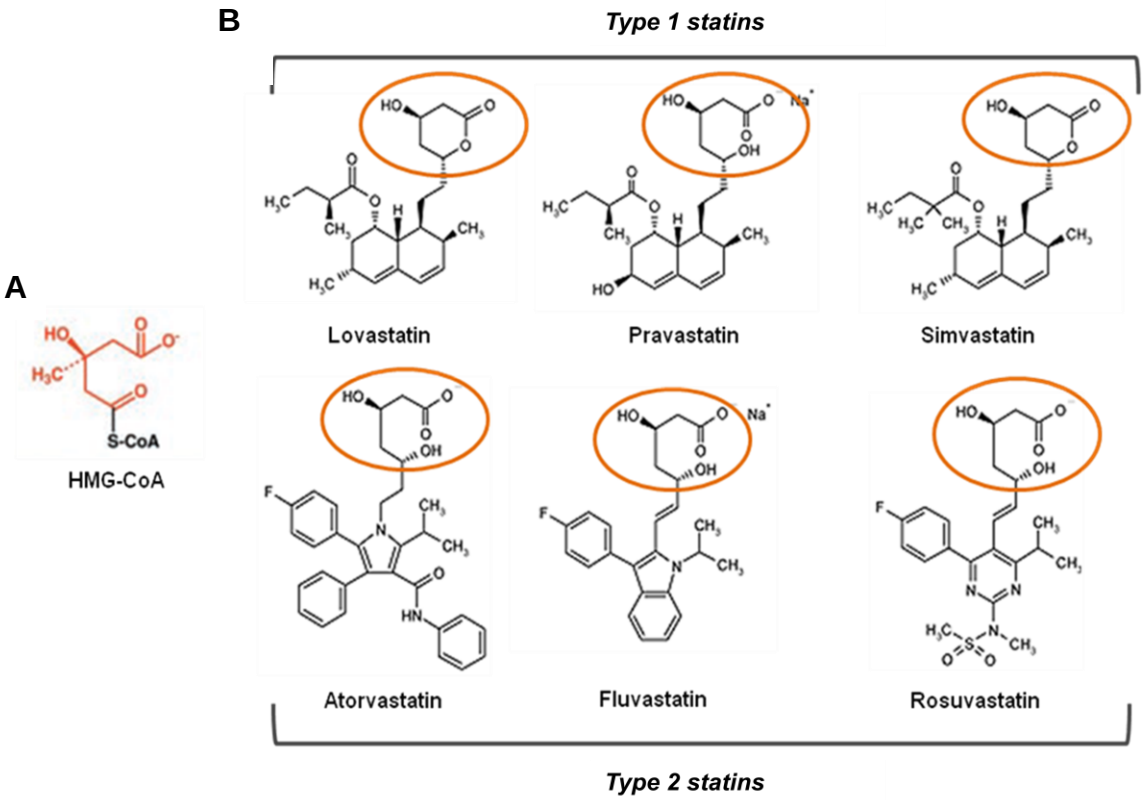

Figure 5: Structural formulas of HMG-CoA inhibitors

Structure of the substrate for the HMG-CoA reductase; HMG-CoA is illustrated on the left side **(A)** in comparison to the statins on the right side **(B)**, which were used in the study. The statins were classified in type 1 and type 2 statins coursed by their development. Type 1 statins (fungal-derivates) resemble the sustained decalin-ring structure of mevastatin while type 2 stains are fully synthetic inhibitors with lager side-groups linked to the HMG-CoA moiety.

By comparing the resulting statin-enzyme complexes subtle differences were detected in their way of binding. In atorvastatin and rosuvastatin enzyme complexes additional hydrogen bond has been found [103]. Moreover a polar interaction unique to rosuvastatin was detectable, leading to the most prominent binding interactions with HMG-CoA reductase of all the statins. Whether additional bonding properties may lead to an increased binding potency of statins to their target enzymes, the full meaning remains to be elucidated.

1.4 Aim of the study

The emphasis of this study was given to the characterization and evaluation of the antiviral effects of the inducible enzyme HO-1 and its product biliverdin i*n vivo*, as well as to the evaluation of antiviral and anticancer effects of statins *in vitro*. Both Cobalt-protoporphyrin-IX (CoPP) and statins have been shown to provoke the induction of the HO-1 enzyme. Since CoPP-mediated suppression of HCV replication was previously observed *in vitro* [6], in this study we aimed to evaluate the antiviral properties of human HO-1 *in vivo* using immunodeficient mice (USB mice) engrafted with human hepatocytes and infected with HCV genotype 1a. Moreover, some clinical studies report that fluvastatin monotherapy could reduce HCV replication [96] and enhanced the efficacy of IFN alpha therapy [104], while others could not confirm this observation [105,106] or described only a rapid decrease of viral loads without significant effects on sustained virologic response [107]. In order to evaluate the ability of statins to suppress HCV replication, we aimed to identify the factors responsible for the inconsistent antiviral effects described in clinical studies [96;97;98]. For this purpose genotype 1b replicon systems (Huh5-15 and LucUbiNeoET) were used for *in vitro* investigations. We compared HO-1-induction and antiviral activity of different statins, like fluva- (FLV), simva- (SMV), rosuva- (ROV), atorva- (ATV) and pravastatin (PRV), *in vitro*. In addition, statins have been shown to exert antiproliferative effects and interfere with cancer [93]. Therefore the named statins were also analyzed in respect to their antiproliferative efficiency and specificity on human hepatoma cell lines (HepG2; Huh7 and Hep3B) as well as for mechanisms of anticancer activity.

2. Material and Methods

2.1 Material

2.1.1 Devices and general Reagents

Table 2: List of Instruments

Device	Producer	Country
ChemiDoc XRS Imaging Station	BioRAD Laboratories	USA
Chemilumineszenz-Mikropartikel Immunoassay Archtitect	Abbott Laboratories	USA
CFX^{TM} Real-Time system	Biorad	Germany
CFX^{TM} Real-Time software	Biorad	Germany
ELISA Readers MRX TC 2.02	Dynex Technology	USA
Light Cycler Capillaries (20 µl)	Roche Diagnostics	Germany
Light Cycler Software 3.5	Roche Diagnostics	Switzerland
Lightcycler 1.5 Real-time PCR System	Roche Diagnostics	Switzerland
Mikroskop Biorevo BZ-9000	Keyence	Japan
QuantityOne Software	BioRAD Laboratories	USA
QubitFluorometer	Invitrogen™ (Life Technologies)	USA
Rotationsmikrotom Frigocut 2800	(Reichert Jung)	Germany
Thermomixer compact	Fa. Eppendorf	Germany
Thermocycler iCycler	Biorad	Germany
Tischzentrifuge – Centrifuge 5415R	Fa. Eppendorf	Germany
Tischzentrifuge – Centrifuge 5417C	Fa. Eppendorf	Germany
Vortexer MS2 Minishaker	IKA	Germany
ViiA™ 7 System	Life Technologies GmbH	Germany
ViiA™ 7 Software	Life Technologies GmbH	Germany

Table 3: List of general Reagents

Reagents	Producer	Country
Ammoniumacetat	Sigma-Aldrich	USA
Anchored-oligo(dT) Primer (cDNA)	F.Hoffmann-La Roche	Switzerland
Aqua ad inectabilia	B. Braun Melsungen AG	Germany
ATP 100mM	Biozym Scientific GmbH	Germany
AW1 Puffer	Quiagen	Netherlands
AW2 Puffer	Quiagen	Netherlands
BSA (Bovines Serumalbumin)	PAA Laboratories GmbH	Österreich
Dako Mounting Medium	Dako	Dänemark
Dako-Polymer	Dako	Dänemark

Desoxynucleotide Mix (cDNA)	F.Hoffmann-La Roche	Switzerland
DNA Master (HybProbe)	Roche	Switzerland
DNase I Stock-Solution (DNAse Kit)	Quiagen	Netherlands
Ethanol 100%	Th. Geyer GmbH & Co	Germany
Ethanol 75%	Th. Geyer GmbH & Co	Germany
Extraktion Solution	Sigma-Aldrich	USA
FlurophoreTyramide	Perkin Elmer	USA
GelRed	GeneON GmbH	Germany
Glykogen	F.Hoffmann-La Roche	Switzerland
H_2O_2	Merck	Germany
Hepatocyte Wash Medium	Invitrogen™ (Life Technologies)	USA
Hoechst	Hoechst AG	Germany
HRP Conjugated Goat anti-Human Albumin Detection Antibody A80-129P	Bethyl Laboratories	USA
Aceton	Th. Geyer GmbH & Co	Germany
Isofluran	Baxter International	USA
Isopropanol	Baxter International	USA
LightCycler FastStart DNA Master SYBR Green I	F.Hoffmann-La Roche	Switzerland
Methanol	Th. Geyer GmbH & Co	Germany
$MgCl_2$	F.Hoffmann-La Roche	Switzerland
NaCl	Carl Roth GmbH & Co	Germany
NaCl 0,9%	B. Braun Melsungen AG	Germany
Neutralisation Solution	Sigma-Aldrich	USA
PBS	Invitrogen™ (Life Technologies)	USA
ProtectorRNase Inhibitor	F.Hoffmann-La Roche	Switzerland
Protein PrecipitationReagent	Epicentre	USA
Proteinase K	Epicentre	USA
Puffer AL	Quiagen	Netherlands
Puffer Tango	Thermo Fisher Scientific	USA
QIAGEN Protease	Quiagen	Netherlands
Quant-iT Puffer	Invitrogen™ (Life Technologies)	USA
Quant-iTReagent	Invitrogen™ (Life Technologies)	USA
RDD-Puffer	Quiagen	Netherlands
Reaction Buffer	Epicentre	USA
REDExtract-N-Amp PCR Reaction mix	Sigma-Aldrich	USA
RLT-Puffer	Quiagen	Netherlands
RNA	F.Hoffmann-La Roche	Switzerland
RNase-freies Wasser	Quiagen	Netherlands
RPE-Puffer	Quiagen	Netherlands
RW1-Puffer	Quiagen	Netherlands
Schwefelsäure	Carl Roth GmbH & Co	Germany

Serum-freiem Medium	Invitrogen™ (Life Technologies)	USA
Standard Quant-it	Invitrogen™ (Life Technologies)	USA
TAE (Tris-Acetat-EDTA-Buffer)	Sigma	USA
Tissue Preparation Solution	Sigma	USA
Transcriptor Reverse Transcriptase	F.Hoffmann-La Roche	Switzerland
Transcriptor RT Reaction Buffer	F.Hoffmann-La Roche	Switzerland
Tris	Sigma-Aldrich	USA
Trypanblau	Invitrogen™ (Life Technologies)	USA
Tween	Sigma-Aldrich	USA
Uracil-DNA Glycosylase	F.Hoffmann-La Roche	Switzerland

2.1.2 Oligonucleotides and Probes

Table 4: List of Oligonucleotides obtained from Metabion International AG

Oligonucleotide	Sequence 5´-3´
5’GAPDHhum	5´-TGA TGA CAT CAA GAA GGT GG-3´
3’GAPDHhum	5´-CGA CCA CTT TGT CAA GCT C-3´
5’mATPsynthase	5´-GCC CAC TTC TTA CCA CAA GG-3´
3’mATPsynthase	5´-GCG ACA GCG ATT TCT AGG AT-3´
5'PCNA	5´-GGC GTG AAC CTC ACC AGT AT-3´
3'PCNA	5´-TCT CGG CAT ATA CGT GCA AA-3´
5’p53	5´-CTC CTG GCC CCT GTC ATC GT-3´
3’p53	5´-GCT CAG TGG GGG AAC AAG AAC-3´
5’BACH1	5’-GCAGATTGCCCACTTTCATT-3’
3’BACH1	5’-AGAGGTGGCTGTGGACATCT-3’
5’GAPDH	5’-TGATGACATCAAGAAGGTGG-3’
3’GAPDH	5’-CGACCACTTTGTCAAGCTC-3’
5’HO-1	5’-CCTGCTCAACATCCAGCTC-3’
3’HO-1	5’-CTACAGCAACTGTCGCCAC-3’
5’IFNalpha 2	5’-GCAAGTCAAGCTGCTCTGTG-3’
3’IFNalpha 2	5’-GATGGTTTCAGCCTTTTGGA-3’
5’IFNalpha 17	5’-AGGAGTTTGATGGCAACCAG-3’
3’IFNalpha 17	5’-CATCAGGGGAGTCTCTTCCA-3’
5’KLF2	5’-CACCAAGAGTTCGCATCTGA-3’
3’KLF2	5’-ACAGATGGCACTGGAATGG-3’
5'LDLR	5´-GTGCTCCTCGTCTTCCTTTG-3’
3'LDLR	5’-TAGCTGTAGCCGTCCTGGTT-3’
5’OAS 1	5’-CAAGCTCAAGAGCCTCATCC-3’
3’OAS 1	5’-TGGGCTGTGTTGAAATGTGT-3’
5’OAS 2	5’-ACAGCTGAAAGCCTTTTGGA-3’
3’OAS 2	5’-GCATTAAAGGCAGGAAGCAC-3’

Table 5: List of TaqMan probes obtained from Applied Biosystems

Gene	Probe
MX1	Hs00895608_m1
ISG15	Hs00192713_m1
OAS1	Hs00973637_m1
ISG20	Hs00158122_m1
CXCL10/IP10	Hs00171042_m1
STAT1	Hs01013989_m1
STAT3	Hs01051722_s1
HLA-E	Hs03045171_m1
Wnt5A	Hs00998537_m1
TGFbeta1	Hs00171257_m1
HO-1	Hs01110250_m1
LDLR	Hs00181192_m1
SRB1	Hs00969821_m1
CD81	Hs01002167_m1
Actb	Mm00607939_s1
RPL30	Hs00265497_m1
GAPDH	Hs99999905_m1
5´UTR HCV	Pa03453408_s1
IFN alpha	Hs00855471_g1
IFN beta	Hs00277188_s1
IFN gamma	Hs04193049_gH

2.1.3 Reagents for specific experimental settings

HMG-CoA-reductase inhibitors fluva- and simvastatin (Cayman Chemical, Ann Arbour, MI, USA), atorvastatin (Sortis, Pfizer Pharma GmbH, Darmstadt, Germany), rosuvastatin (Crestor, Astra Zeneca, Wedel, Germany) and lovastatin (Tocris Bioscience; Bristol, UK) as well as NS3/4A protease Inhibitor telaprevir (Janssen-Cilag Pharma GmbH, Wien, Austria) were dissolved in DMSO. As a vehicle control, DMSO was dissolved to the concentrations used on statin incubated cells and measured in parallel. Mevalonate (MVLT), geranylgeranyl-pyrophosphate (GGPP), and cholesterol were purchased from Sigma-Aldrich

Chemie GmbH; Steinheim, Germany. Final concentrations (as indicated in the Figures and Figure legends) were obtained by dilution in medium. The Rho-kinase inhibitor Hydroxyfasudil (HA1100) (Tocris Bioscience, Bristol, UK) was dissolved in sterile water. Recombinant interferon alpha-2b (Intron A) was purchased from Essex Pharma, München, Germany. CoPP was purchased from Frontier Scientific Europe Ltd., Carnforth, Lancashire, UK). Biliverdin was purchased from MP Biomedicals (Heidelberg, Germany).

2.3 Methods

2.3.1 Cell culture

The human hepatoma cell lines Huh7 [108] and HepG2 [109] as well as the replicon cell lines Huh-5-15 [41] and LucUbiNeo-ET [110] were cultured in DMEM containing 10% fetal calf serum (FCS) (both: Invitrogen GmbH, Karlsruhe) and 1% penicillin/streptomycin (Biochrom AG Seromed, Berlin, Germany). The mouse hepatoma cell line Hepa1-6 [111] was maintained in RPMI 1640 medium (10% FCS; 1% penicillin/streptomycin). For experimental procedures cells were seeded into 24- or 96-well plates and allowed to adhere overnight.

2.3.2 Transfection and Transduction

Transfections were performed using Lipofectamine™ 2000 (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's instructions. SiRNA target sequences: siKLF2: CTG CGG CAA GAC CTA CAC CAA (Qiagen GmbH, Hilden, Germany); siControl (GFP: AAT CTC AGG GTT CCT GGT TAA; Eurogentec Deutschland GmbH, Köln, Germany). ShRNA expressing vectors were based on the lentiviral pLKO.1 construct (RNAi Consortium vector collection [112]; and

purchased from Sigma Aldrich GmbH (Steinheim, Germany). Target sequences for shRNA: HO-1: TGG GTC CTT ACA CTC AGC TTT CT; GFP: CAA CAA GAT GAA GAG CAC CAA [113]; p53: TGG GTC CTT ACA CTC AGC TTT CT and TTA TCG CGC ATA TCA CGC G for E. coli DNA polymerase as a control gene (shneg). Transfected cells were selected with puromycin (2 µg/mL). For virus production the plasmid pFK$_{I389}$RLuc2ACore-3'-Jc1 harbouring the genome of a monocistronic reporter virus, referred to as JcR-2A, derived from the JC1 chimera [114] was used. Electroporation of Huh-7 cells and *in vitro* transcription of HCV RNA was performed as described previously [115].

2.3.3 Infection with virus particles

Huh-7.5 cells were seeded into 12-well plates at 2×10^5 cells/well 24 h prior to infection. Cells were infected with the HCV genotype 2a strain JC1 at an MOI of 0.5. After 2 h cells were washed 3 times with PBS, infection medium was changed and cells were incubated as indicated.

2.3.4 Preparation of Polyacrylamide Gel Supports

Polyacrylamide (PAA) gel supports of various elasticity were prepared on glass cover slips as described previously [116,117], using modifications to the method initially described by Pelham and Wang [118].

2.3.5 Luciferase assay

Luciferase activity of LucUbiNeo-ET replicon cells was measured using the Luciferase Assay System (Promega, Mannheim, Germany), and normalized to the protein content of the individual sample.

2.3.6 Generation of human chimeric mice

Alb-uPA transgenic mice (Jackson Laboratories) crossed with SCID/beige mice (Taconic Farms) were housed and maintained under specific pathogen–free conditions in accordance with institutional guidelines under approved protocols. The presence of the uPA transgene and the maintenance of the SCID phenotype were determined as reported [119]. Generation of humanized mice was conducted as previously described [120]. In short, three- to four-week-old USB mice were anesthetized with isoflurane and injected intrasplenically with 1 million cryopreserved human hepatocytes [121] derived from different donors. Animals were housed and maintained under specific pathogen-free conditions according to authorized protocols. All procedures were approved by the Ethical Committee of the city and state of Hamburg and accorded with the principles of the Declaration of Helsinki. Human hepatocyte repopulation levels were determined by measuring human serum albumin in mouse serum with the human albumin enzyme-linked immunosorbent assay quantitation kit (Bethyl Laboratories, Biomol GmbH, Hamburg, Germany). Human chimeric animals displaying human serum albumin concentrations of ≥ 3 mg/ml were used for HCV inoculation.

2.3.7 Infection and treatment of chimeric mice

To establish HCV infection, animals received a single intraperitoneal injection of purified human or mouse-derived HCV-positive serum (5×10^7 HCV-RNA copies/ml, genotype 1a). Mice developing a stable HCV infection (>3 weeks post injection) received Cobaltprotoporphyrin (CoPPIX) (5 mg/kg; twice/week) or Biliverdin (BV) (25 mg/kg; twice a week) intraperitoneally, while human peg-

interferon-alpha (PEG-IFNα) (2.5 ng/g.; twice/week) was given subcutaneously. Experimental settings are illustrated in Figure 6.

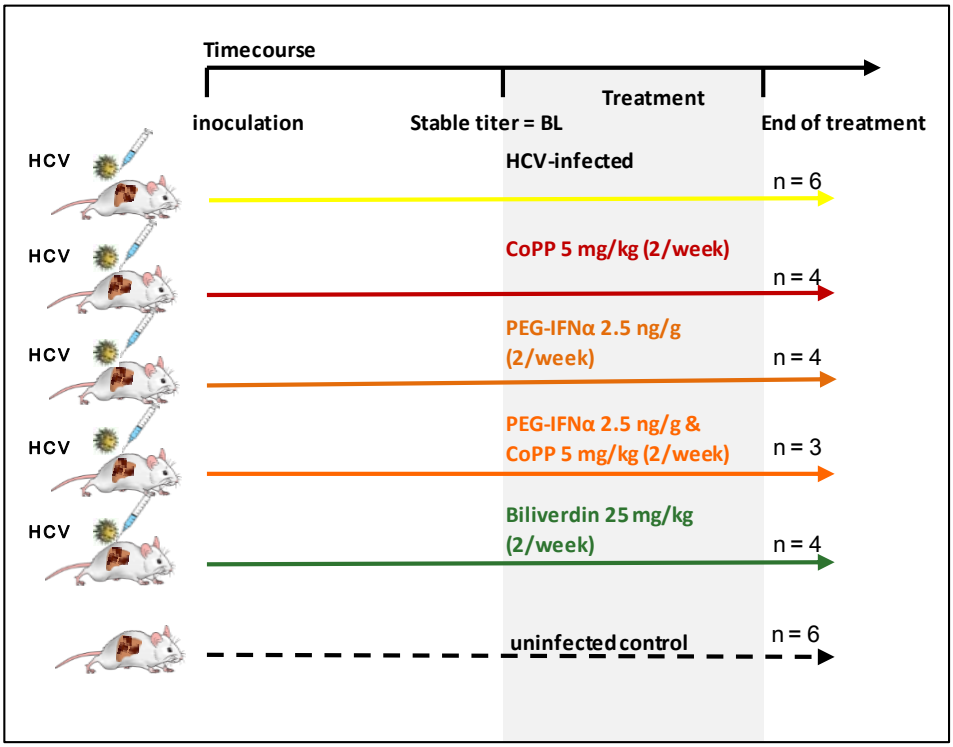

Figure 6: Treatment setting for reported *in vivo* investigations

After 3 weeks mice which displaying a stable HCV titer and were further used for treatment period. **BV (25 mg/ml)**, **CoPP(5 mg/ml)** and/or **PEG-IFNalpha (2,5 ng/µl)** were applicated twice a week for a total treatment period of 2 weeks. Infected untreated as well as uninfected untreated mice were used as control groups.

2.3.8 Isolation and treatment of primary hepatocytes

Primary hepatocytes were isolated by a modification of the two-step collagenase perfusion method of Seglen [122] and cultured in William's E+GlutaMAX^{TM}-I medium, supplemented with 10% FCS, 1% L-glutamine, 2% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% sodium pyruvate (all: Invitrogen GmbH; USA) and 1% penicillin/streptomycin (Biochrom AG Seromed; Berlin, Germany). Primary human hepatocytes were isolated as described previously

[123]. Hepatocytes isolated from humanized mice and from human livers were cultured on collagen coated 24-well plates for 24h before treatment.

2.3.9 Analysis of cell viability, proliferation and apoptosis

Cell viability was measured by using (3-4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma Aldrich GmbH; Steinheim, Germany) according to the manufacturer's instructions. Cellular proliferation and viability was measured by the xCELLigence real-time cell analyzing system (Roche Molecular Diagnostics, Mannheim, Germany). Briefly, 10,000 cells were seeded per well of a 96-well E-Plate and viability was measured continuously as impedance and expressed as an arbitrary unit named cell index. Data were normalized to the time point of seeding and represent means of triplicates as described previously [124]. Dead cells were visualized by trypan blue staining and cell counting using a Neubauer chamber (Carl Roth GmbH + Co. KG; Karlsruhe; Germany). Total cell numbers as well as the percentages of dead cells were determined at 72 hours of incubation. To quantify apoptosis, activation of caspase 3 was measured using the colorimetric assay (Sigma Aldrich GmbH; Steinheim, Germany) according to the manufacturer's instructions.

2.3.10 Analysis of GT-repeats polymorphism in HO-1 gene Promoter

DNA samples were extracted from donor liver tissue using the Quiagen Dneasy kit (Qiagen, Hilden, Germany) and the DNA amount was determined using a Qubit fluorometer (Invitrogen, Darmstadt, Germany). Pair of primers was obtained as described previously [125] to amplify a 137 to 200 bp fragment, depending on the number of repeats, containing GT-n repeats region from promoter of HO-1 gene by

PCR. The 5′ end of forward primer was labeled with FAM 5′-FAM-AGAGCCTGCAGCTTCTCAGA-3′ for using in analysis of DNA fragmentation by ABI capillary genotyping and an unlabeled antisense primer 5′-ACAAAGTCTGGCCATAGGAC-3′. The PCR products were subsequently run on a 8% denaturing polyacrylamide gel, followed by silver staining. PCR products were then mixed together with a Geno Type TAMRA DNA ladder (Size range 50–500 bp; GibcoBRL) and analyzed with an automated DNA sequencer (ABI Prism 377) Each size of the GT-repeats was calculated using GeneScan Analysis software (PE Applied Biosystem). Polymorphism lengths were classified into short (s; n<25), medium (m; n<30) and long (l; n>30) as described previously [126].

2.3.11 Detection of viremia and intrahepatic mRNA expression levels

Viral RNA was extracted from serum samples (5µl) using the QiAmp MinElute Virus Spin kit (Qiagen, Hilden, Germany). For absolute quantification of serum viral load, purified genotype 1a patient-derived serum was used. Intrahepatic RNA was isolated with the RNeasy Kit (Qiagen, Hilden, Germany), amounts determined using a Qubit fluorometer (Invitrogen, Darmstadt, Germany) and reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) to assess host transcriptional changes. One step RT-PCR was performed for viral load quantification while human and mouse specific mRNA expression levels were measured by RT-qPCR on the ViiA™ 7 System (Life Technologies GmbH, Darmstadt, Germany), using TaqMan probes (Table 5) specifically recognizing human or mouse transcripts, as summarized in 2.2.2. For normalization human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 60S ribosomal protein L30 (RPL30) expression were determined. Mouse gene

expression levels were normalized using primers specifically recognizing the murine beta-actin transcripts. To validate the species specificity of the primers, reverse transcribed complementary DNAs (cDNA) from murine, human and human chimeric samples were employed [120]. Real time analysis was also party performed using the CFX^{TM} Real-Time system (BioRad Laboratories GmbH) and reagents from ABgene® (Epsom, UK). Oligonucleotides for these measurements were obtained from Metabion International AG (Martinsried, Germany) and are summarized in 2.2.1. To confirm amplification specificity, PCR products were subjected to melting curve analysis.

2.3.12 Immunohistochemistry

Cryosections (12µm) of repopulated mouse livers were stained with anti-Calnexin monoclonal antibody (1:200 dilution, Cell signaling, USA) or polyclonal rabbit anti-SP100 antibody (1:1000 dilution, Sigma-Aldrich, Steinheim, Germany) recognizing human Calnexin or human SP100 respectively and not cross-reacting with mouse proteins. Human anti-HO-1 monoclonal antibody (1:200 dilution, BD Transduction Laboratories, USA) or anti-HCVcore monoclonal antibody (1:500 dilution, Abcam, Cambridge, UK) were used for detection of human HO-1 as well as HCV core particle. Specific signals were visualized with Alexa 488 or 546 labeled secondary antibodies or TSA Fluorescein System (Perkin Elmer, Waltham, MA) and nuclear staining was achieved by DRAQ5 (1:2000 dilution; Axxora, Lörrach, Germany). Stained sections were mounted with fluorescein mounting media (Dako) and analyzed by confocal laser scanning microscopy (Leika, Germany) using the same settings for the different experimental groups.

2.2.13 Immunofluorescence

To visualize HCV infection, E2-proteins were stained. Antibodies: human monoclonal A3R3 against E2 (a kind gift of Mansun Law, The Scripps Research Institute, La Jolla, CA, USA), chicken anti-human Alexa-488 (Molecular Probes, Life Technologies GmbH). The procedure included fixation (4% PFA; 20 min. at RT), permeabilization (0.1% Triton X100; 4°C for 10 min) and blocking (5% BSA; 20 min. at RT). Pictures were taken using an inverted microscope (CKX41; Olympus, Hamburg, Germany) with an LCachN/20X/0.40 Phc/1/FN22 UIS objective.

2.2.14 Western Blot

25-50µg of protein were fractionated by 12% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. Western Blots were developed using an enhanced chemiluminescence system (Amersham, GE Healthcare Europe GmbH, Munich, Germany) according to the manufacturer's instructions. Semi-quantitative evaluation was performed using the VersaDoc Imaging System (BioRad Laboratories GmbH, Munich, Germany). Antibodies: rabbit anti-p53 (1:1000; Santa Cruz Biotechnology, Inc., USA**)**, mouse anti-GAPDH (1:5000; HyTest Ltd., Turku, Finland), rabbit anti-HO-1 (1:1000; Stressgen Biomol, Hamburg, Germany) and mouse anti-NS4B (1:1000; Abcam, Cambridge, UK), and mouse anti-GAPDH (1:5000; HyTest Ltd., Turku, Finland).

3. Results

3.1 Suppression of HCV replication by HO-1 or BV enhances the antiviral effects of PEG-IFNα in humanized mice

3.1.1 Establishment of genotype 1a HCV infection in uPA/SCID mice repopulated with human hepatocytes

For *in vivo* investigations, the establishment of a HCV *in vivo* model was requited. Preliminary studies indicated that high levels of human hepatocyte chimerisms were necessary for a successful infection of the USB mice with HCV, since at least human serum albumin (HSA) levels of 2 mg/ml appeared to be required to establish HCV infection.

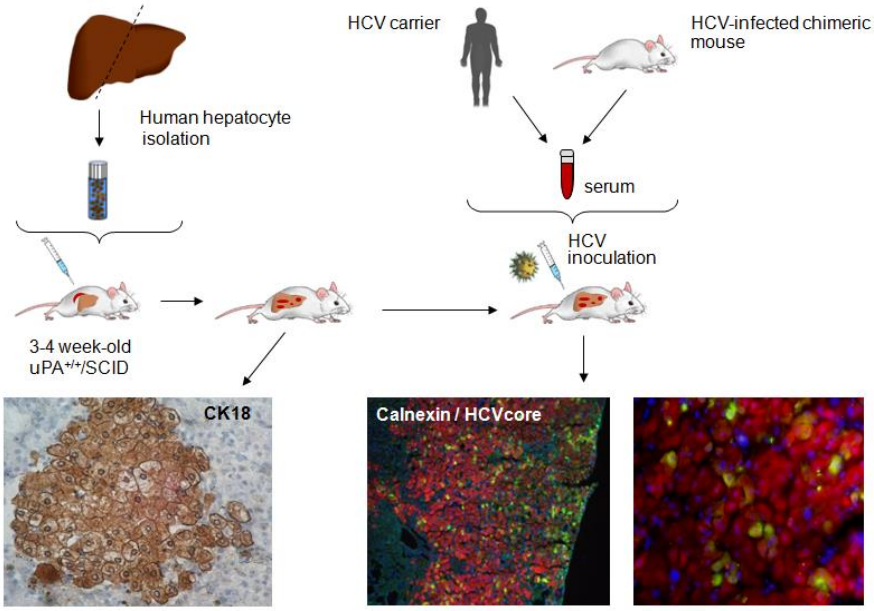

Figure 7: Generation of USB mice harboring "humanized livers" and establishment of the HCV infection

Workflow for generating chimeric uPA/SCID (USB) mice. First primary hepatocytes were obtained by collagen digestion and then cryoconserved allowing multiple rounds of experiments with the same cell source. Homozygous uPA/SCID mice were then transplanted at age of 3-4 weeks with the cryoconserved primary hepatocytes via injection into the spleen. 8-10 weeks after transplantation the murine liver was stable repopulated with human hepatocytes. Mice displaying high repopulation rates were used to establish stable *in vivo* HCV infection. For infection purified patient- and mouse-derived HCV-positive serum (genotype 1a) was used and injected intraperitoneal.

We also observed that after 2 weeks mice with high HSA levels developed HCV titer over 10^6 particles per ml serum as shown in Figure 9. The infection of the human hepatocytes was visualized by immunohistochemical staining (Figure 7).

3.1.2 HCV infection in correlation to the HO-1 promoter polymorphism

The Heme oxygenase 1 enzyme is a stress responsive protein which can be induced by various oxidative stresses [127]. Previous studies indicated that the expression level of HO-1 is related to the number of $(GT)_n$ repeats in its highly polymorphic promoter region [128,129] since longer $(GT)_n$ repeats have been associated with lower expression levels and shorter $(GT)_n$ repeats with higher HO-1 transcriptional levels [130]. According to these finding we characterized the $(GT)_n$ repeat length of human hepatocytes that were used to generate the chimeric mice. GeneScan analysis (2.3.10) was used to perform the characterization (results are listed in Table 6).

Table 6: Classification of human hepatocytes respective to the GT repeats

Number	Result of analysis	GTn length
TM-8	S	22
TM-11	S	22
TM-14	M	29
TM-17	S	19
TM-21	S	22
TM-40	S	22
TM-41	M	29
TM-42	S	22
TM-44	M	29
TM-45	M	29
TM-46	L	29

The geneScan analysis pointed out that 6/11 human donors sustain a short GTn repeat length, 4/11 sustain a medium GTn repeat length and 1/11 to sustain a long version. To assess the impact of the $(GT)_n$ repeat length on the induction of HO-1, we isolated hepatocytes from humanized mice repopulated either with short, medium or long $(GT)_n$ repeats. These hybrid isolates were cultivated for 24h on collagen coated 24-well plates and incubated for 6h with CoPP before determining. The expression levels of human HO-1 by qRT-PCR.

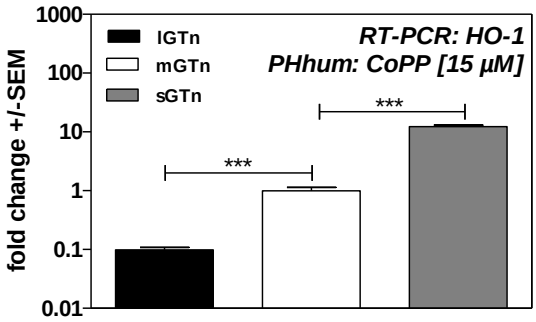

Figure 8: Transcriptional levels of HO-1 in correlation to different GTn lengths

Potency of HO-1 induction was identified in correlation to $(GT)_n$ repeat length in human hepatocytes after 6h of CoPP [15 µM] incubation. X-fold changes in RNA levels were referred to the medium $(GT)_n$ repeat length containing hepatocytes. Results were analyzed using Student's *t* test. Data are expressed as a mean ± SEM. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Our results show that hepatocytes containing a long or a medium GT-repeat cassette displayed none or only a modest induction of HO-1, while in hepatocytes contain a short GT-repeat cassette almost 12-fold induction was triggered (Figure 8) thus indicating that the potency of HO-1 induction is polymorphism-dependent (Figure 8). For this report, mice successfully infected with the HCV genotype 1a were repopulated with TM-17 and TM-44. Unfortunately, TM-46 transplanted mice did not display high repopulations rates, possibly because of damages occurred during cell isolation, or other unknown reasons. USB mice (listed in Table 7)

displaying high HSA levels and hence repopulations rates, exhibited stable viral loads (median 2.05 *10^6 particle/ml serum) 3 weeks after HCV inoculation, as summarized in Figure 9.

Table 7: Human serum albumin (HSA) level of mice before infection

Mice (number)	HSA level	GT-repeat
i882	4,5	S
5472	8,1	S
5464	10,1	S
5481	8,6	S
929	8,2	M
958	4,5	M
5415	7,2	S
5370	3,7	S
1035	3,7	M
1036	3,4	M
1051	5,6	M
5840	4,9	M
1123	3,2	S
1132	3,6	S
5502	6,4	S
5503	9,4	S
5565	5,8	S
5555	8,3	M
5841	3,0	M
5858	3,3	M
916	7,3	S

With regard to the polymorphism length, we observed even higher viral load in mice, which were repopulated with hepatocytes carrying the mGTn polymorphism of the HO-1 promoter. As listed in Table 7 human serum albumin level were comparable.

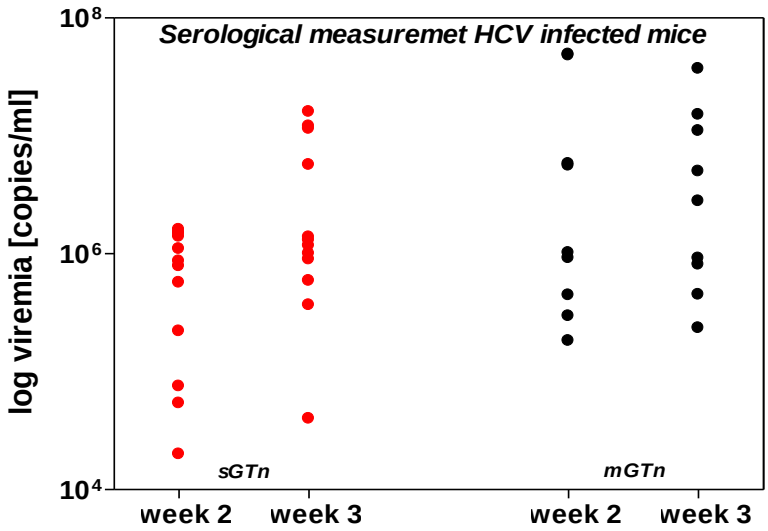

Figure 9: Measurement of viremia in mice infected with HCV genotype 1a

Viremic measurement form blood samples of mice 2 and 3 weeks post infection was performed by one step qRT-PCR and for absolute quantification of serum viral load, purified genotype 1a patient-derived serum was used. According to the HO-1 polymorphism results were subdivided into short (sGTn) and medium (mGTn) repeat length representatives. Resulting **median for sGTn: 8.63 *10^5 particle/ml after 2 weeks; 1.30 * 10^6 particle/ml after 3 weeks and for mGTn: 1.2 *10^6 particle/ml after 2 weeks; 2.8 * 10^6 particle/ml after 3 weeks**.

To get a more detailed view on this topic, intrahepatic expressions levels of the transcription repressor Bach1, as well as the transcription enhancer KLF2 (Figure 10) and the antioxidant enzyme HO-1 (Figure 11) were analyzed.

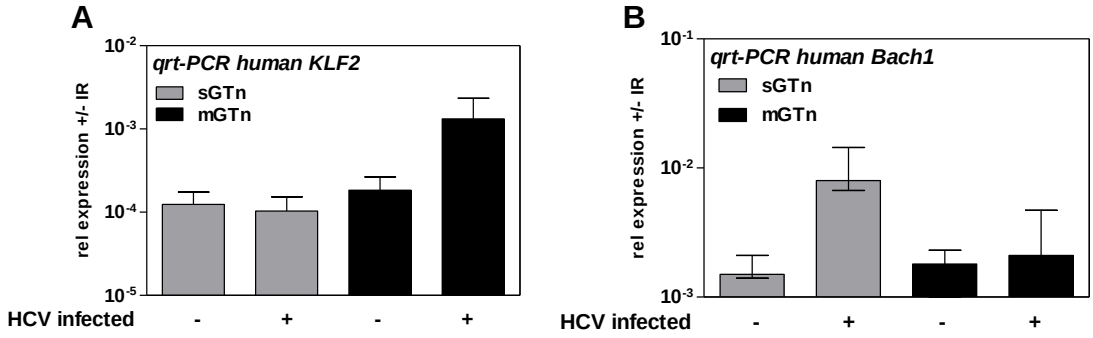

Figure 10: Expression profile of the transcription repressor Bach1 and the transcriptional factor KLF2 in relation to the promoter polymorphism

Basic expression (genes were normalized against the HK median) levels of KLF2 **(A)** and Bach1 **(B)** were measured by qRT-PCR in correlation to the HO-1 polymorphism uninfected control mice (n=6 (3/6 = sGTn; 3/6 = mGTn)) versus HCV stably infected (n=6 (4/6 = sGTn; 2/6 = mGTn)) mice. Statistical analysis was performed with the GraphPad Prism 5 software. Results were analyzed using Student's *t* test. All data are expressed as a mean ± interquartile range (IR).

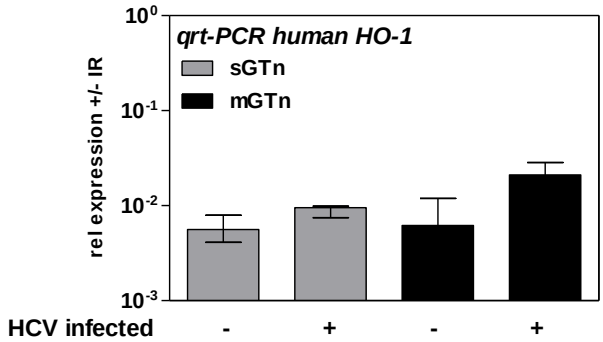

Figure 11: Expression profile of HO-1 in relation to its promoter polymorphism

Basic expression (genes were normalized against the HK median) levels of HO-1 were measured by qRT-PCR in correlation to the HO-1 polymorphism uninfected control mice (n=6 (3/6 = sGTn; 3/6 = mGTn)) versus HCV stably infected (n=6 (4/6 = sGTn; 2/6 = mGTn)) mice. Statistical analysis was performed with the GraphPad Prism 5 software. Results were analyzed using Student's *t* test. All data are expressed as a mean ± interquartile range (IR).

As shown in Figure 11, HO-1 induction seemed to be more prominent in mice repopulated with medium GT repeat length hepatocytes. In line with these results, we found the transcription factor Krueppel-like factor (KLF) 2 was strongly induced after 2 weeks of HCV infection (Figure 10B) in mice containing $m(GT)_n$ hepatocytes. Interestingly Bach1 was found to be induced in $s(GT)_n$ representatives (Figure 10A).

3.1.3 Gene expression profile after HCV infection of humanized mice

Human liver chimeric mice infected with HCV offer the opportunity to study the expression profile of HCV-infected primary human hepatocytes *in vivo*. Figure 12 show that HCV genotype 1a infection significantly induced human genes of the innate immune response. Transcription of the Major Histocompatibility Complex I protein HLA-E, as well as of wingless-related MMTV integration site (Wnt) 5A which has been described to induce interferon stimulated genes, were significantly increased after HCV infection (Figure 12A). Moreover, we determined a strong

induction of human signal transducer and activator of transcription (STAT) 1 while STAT3 expression levels remained unchanged (Figure 12 A).

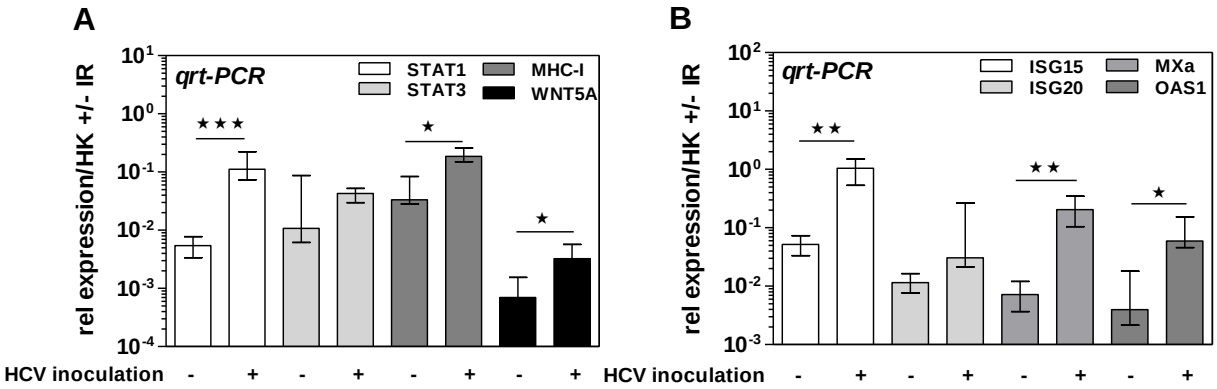

Figure 12: HCV-driven changes in the expression profile of human genes

Basic expression (genes were normalized against the HK median) levels of interferon-stimulated gens were measured by qRT-PCR in uninfected control mice (n=6) versus HCV stably infected (n=6) mice. Statistical analysis was performed with the GraphPad Prism 5 software. Results were analyzed using Student's *t* test. All data are expressed as a mean ± interquartile range (IR). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Representative analysis of classical interferon stimulated genes (ISGs) indicated that HCV infection sensed by the innate immune system of the human hepatocytes in these mice (Figure 12B). In addition, expression levels of known entry HCV receptors (i.e. LDLR, CD81 and SRB1) were found to be enhanced in HCV-infected mice (Figure 13 A).

Transcription levels of human cytokines as TGFbeta1 and IP10 were increased post infection (Figure 13B) although levels of human and murine interferon's (alpha and beta) remained under detection (data not shown).

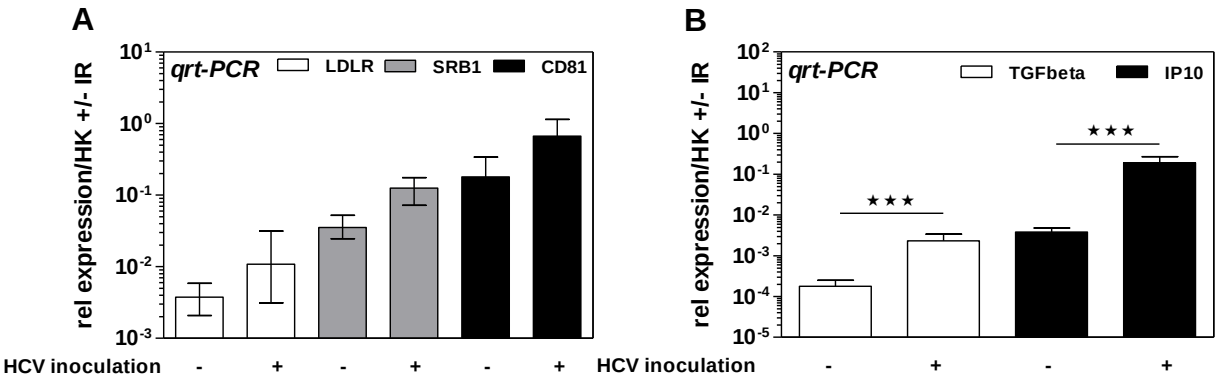

Figure 13: HCV driven changes of human HCV entry molecules and small Cytokines

Basic expression levels of some entry receptors (A) as well as small cytokines (B) were measured by qRT-PCR in uninfected control mice (n=6) versus HCV stably infected (n=6) mice. Statistical analysis was performed with the GraphPad Prism 5 software. Results were analyzed using Student's *t* test. All data are expressed as a mean ± IR. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

3.1.4 HO-1 induction or application of BV, alone or in combination with peg-IFNα, strongly suppress HCV replication in humanized uPA/SCID mice

CobaltprotoporphyrinIX (CoPP) has been shown to be a strong HO-1 inducer *in vitro*. HO-1 and its downstream product BV reported to interfere with HCV replication *in vitro* [6]. In this study, humanized mice highly repopulated with human hepatocytes (summarized in Table 7) and inoculated with HCV developed stable HCV viremia after 2-3 weeks post infection as shown in Figure 9 and subjected to treatment as illustrated in Figure 6.

Our results show that CoPP (5 mg/kg; twice per week) administration alone or in combination with PEG-IFNα significantly increased HO-1 expression levels in HCV-infected humanized mice (Figure 14A), as well as Bach-1 expressions levels (Figure 12B). HO-1 induction mediated by CoPP, as well as BV or PEG-IFNα administration resulted in a viremia decrease of about 1 log (Figure 15).

However, combination treatment of CoPP and PEG-IFNα resulted in a stronger reduction of HCV viremia (3 log; Figure 15).

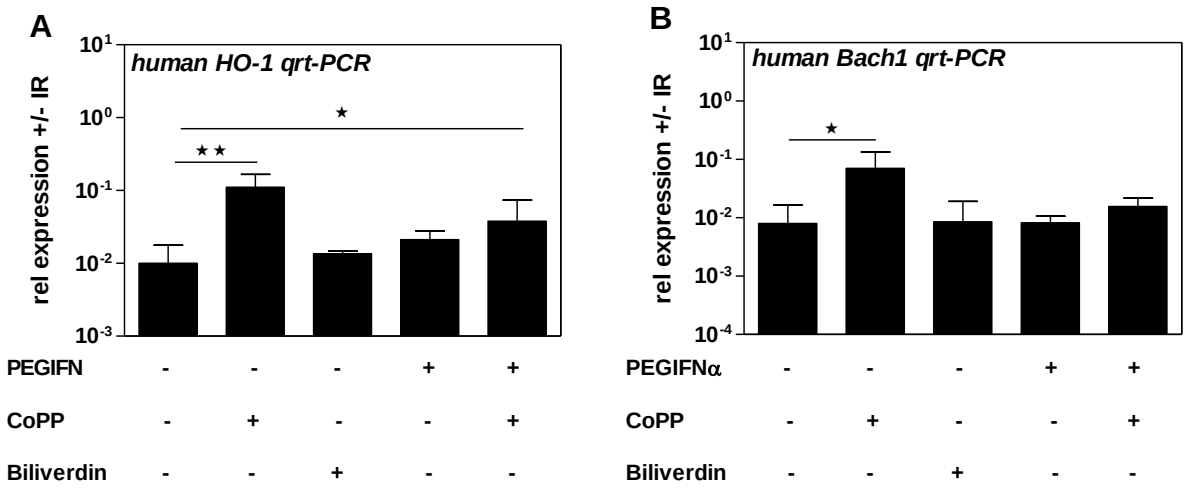

Figure 14: HO-1 and Bach1 expression levels after 2 weeks of treatment

Expression levels of HO-1 were detected in HCV-infected untreated mice (n=6) as well as treated mice (CoPP: n= 4; BV: n=4; PEG-IFNα: n= 4; PEG-IFNα + CoPP: n= 3) in comparison to uninfected control mice (n=6). Statistical analysis was performed with the GraphPad Prism 5 software. Results were analyzed using Student's *t* test. All data are expressed as a mean ± IR. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

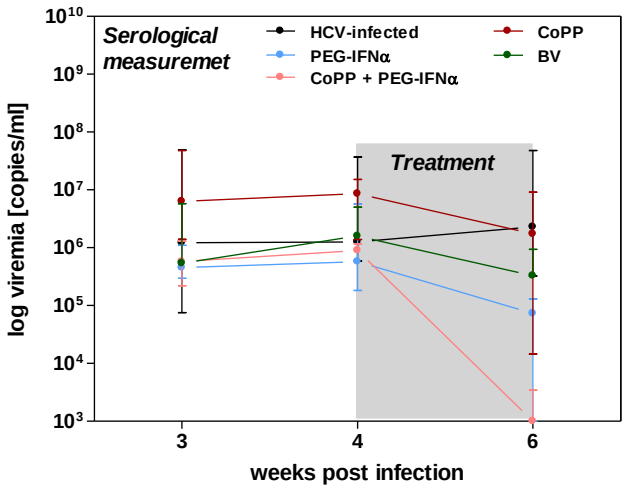

Figure 15: Serological measurement addressed on the HCV titer 2 weeks after treatment

Viremic measurement form blood samples of infected mice before and after treatment period was performed by one step qRT-PCR and for absolute quantification of serum viral load, purified genotype 1a patient-derived serum was used.

To exclude the occurrence of possible treatment-mediated cell toxicity and hence loss of human hepatocytes, HSA levels were detected in Figure 16. Taken together, there was no decreased HSA level detectable in relation to the treatment.

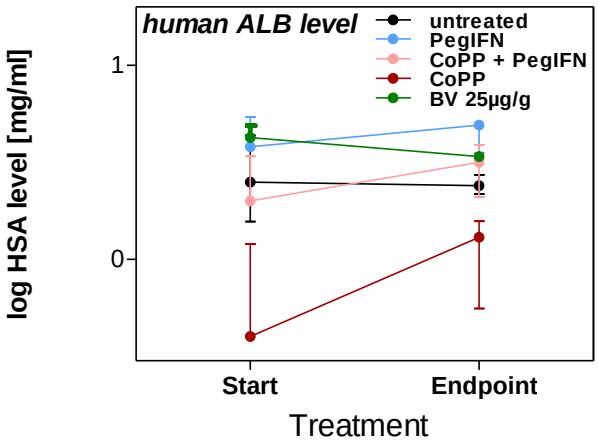

Figure 16: Human Serum Albumin level before and after treatment

Human hepatocyte repopulation levels were determined by measuring human serum albumin in mouse serum with the human albumin enzyme-linked immunosorbent assay quantitation kit before and after treatment.

To confirm our serological findings, posttranslational levels were visualized (Figure 17).

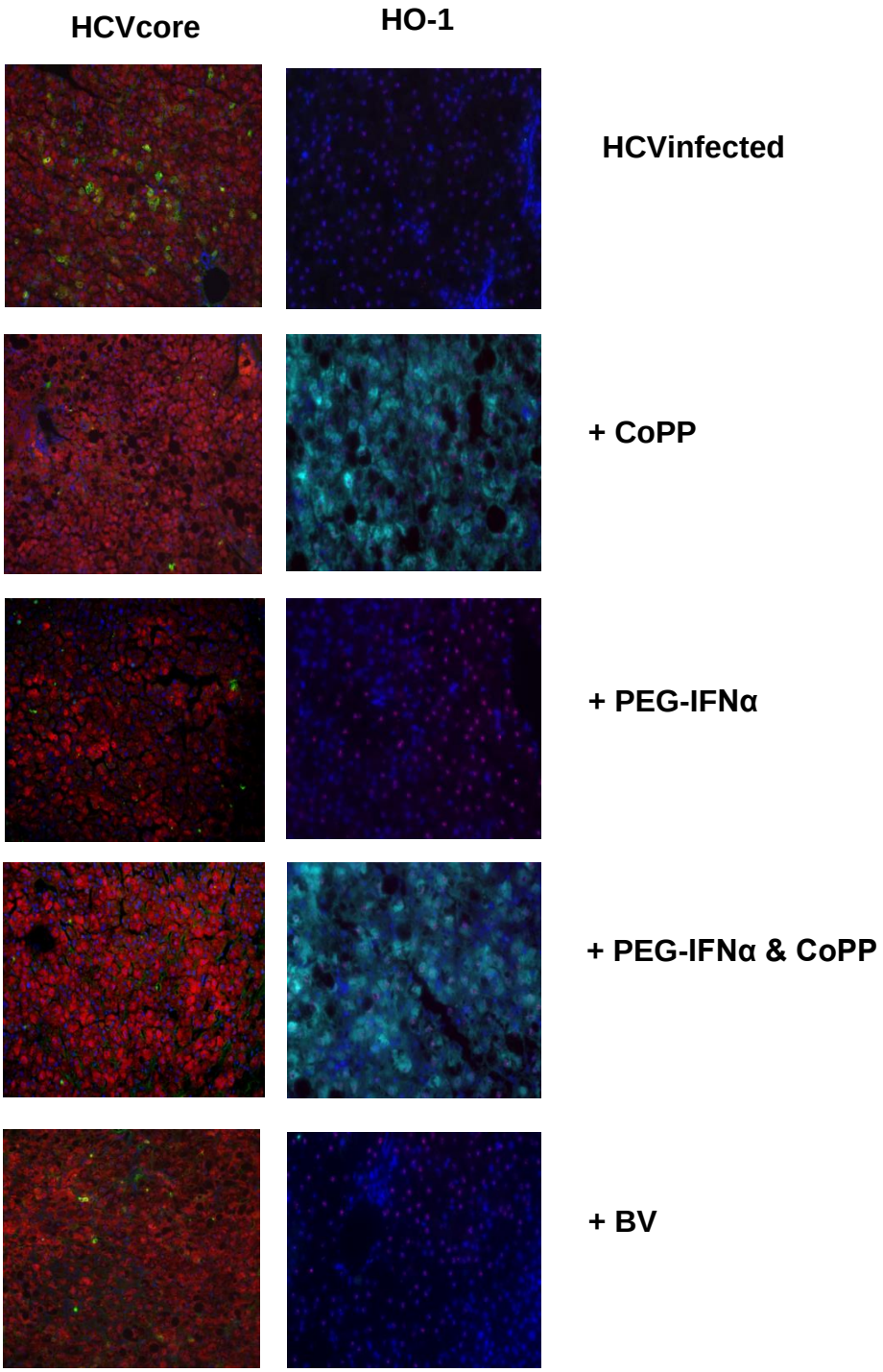

Figure 17: Immunohistochemical stainings of liver tissue slides

Liver tissue slides (12µm) of humanized HCV-infected mice with or without treatment were used for histological staining. Human hepatocytes were identified by using human sp100-Ab (red; right panels) and human Calnexin-Ab (red; left panels). HCV core protein frequency was visualized in green (upper panel). CoPP-induced antioxidant enzyme HO-1 was detected posttranslational (light blue; right panel).

The reduction of HCV C-protein (Figure 17; left panel; green staining) correlated to the induction of HO-1 (Figure 17; right panel; light blue) as visualized by immunohistochemical staining of liver tissue slices derived from these mice.

Notably, HO-1 enzyme was not detectable by immunofluorescence in liver tissue sections from untreated HCV-infected mice, as well as from mice treated with BV or PEG-IFNα, while groups treated with CoPP showed a clear increase of HO-1 protein levels (Figure 17; right panel; light blue). Regarding the HCV core, a strong reduction was visible already in groups receiving monotherapy (CoPP, PEG-IFNα and BV), while almost no HCV C-protein was detectable in the combination group (CoPP + PEG-IFNα). Thus, the synergistic effect of PEG-IFNα treatment in combination with HO-1 induction demonstrated both in blood samples (Figure 15), and in liver tissues (Figure 17). The reduction of HCV C-protein (Figure 17; left panel; green staining) correlated to the induction of HO-1 (Figure 17; right panel; light blue) as visualized by immunohistochemical staining of liver tissue slices derived from these mice.

3.1.5 Induction of HO-1 attenuate HCV-mediated gene expression in humanized mice

Taking into account that HCV inoculation increases levels of innate immune response-related genes and that high pretreatment levels of ISG were shown to inversely correlate with treatment responses, we analyzed the gene expression profile of some human ISGs during different treatment regimens.

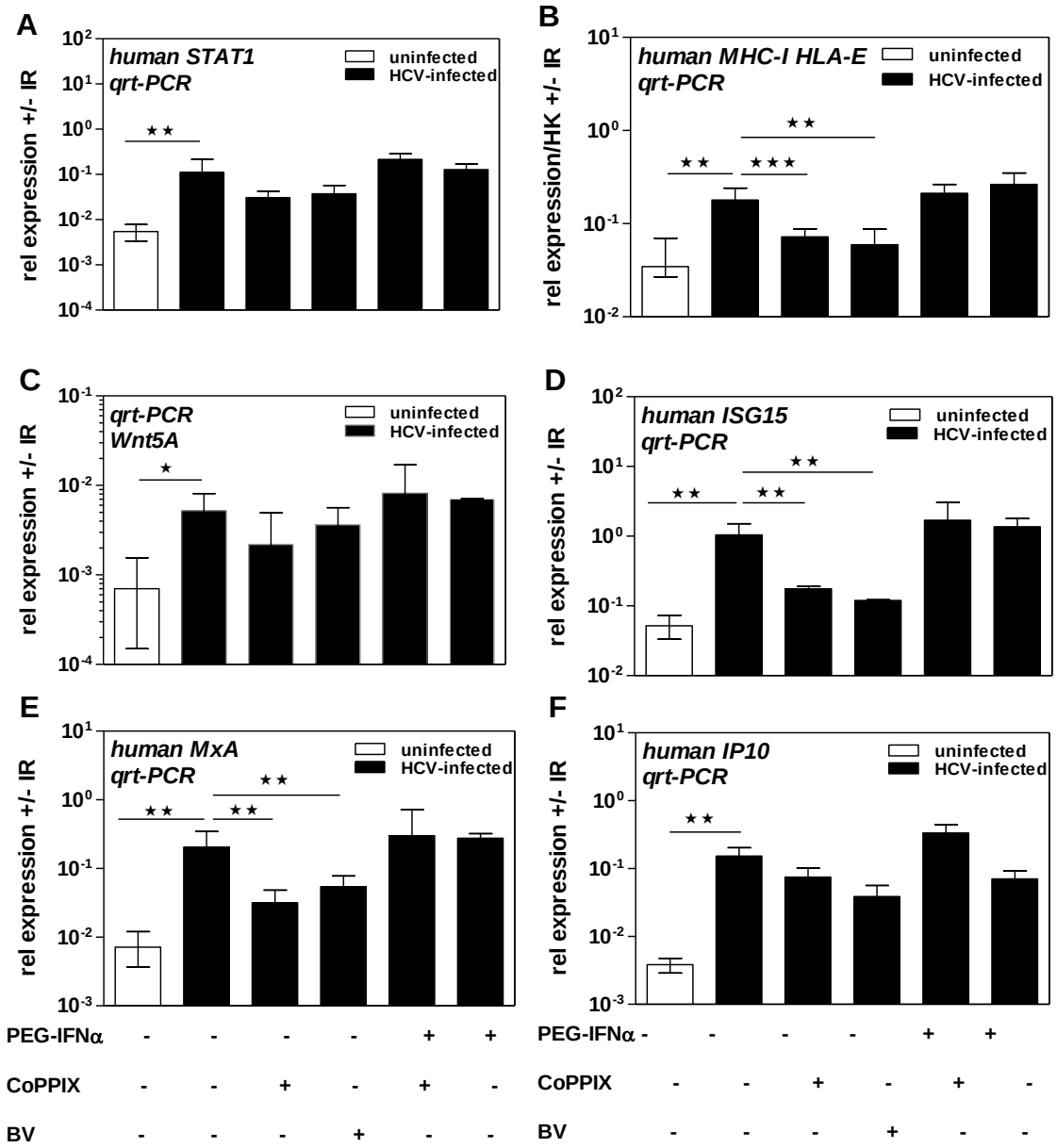

Figure 18: Transcriptional changes of human interferon stimulated genes and cytokines after treatment in humanized uPA/SCID mice

Basic expression levels of human STAT1 **(A)**; MHC-I gene loci HLA-E **(B)**; Wnt5A **(C)**; ISG15 **(D)**; MxA **(E)** and IP10 **(F)** were quantified by qRT-PCR of uninfected control mice vs. infected mice with or without treatment. Statistical analysis was performed with the GraphPad Prism 5 software. Results were analyzed using Student's *t* test. All data are expressed as a mean ± IR. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Notably, also the transcription levels of small cytokine interferon gamma-induced protein 10 (IP10) were slightly reduced after CoPP or BV treatment (Figure 18F),

indicating that suppression of HCV replication leads to a clear decline of inflammatory innate signaling.

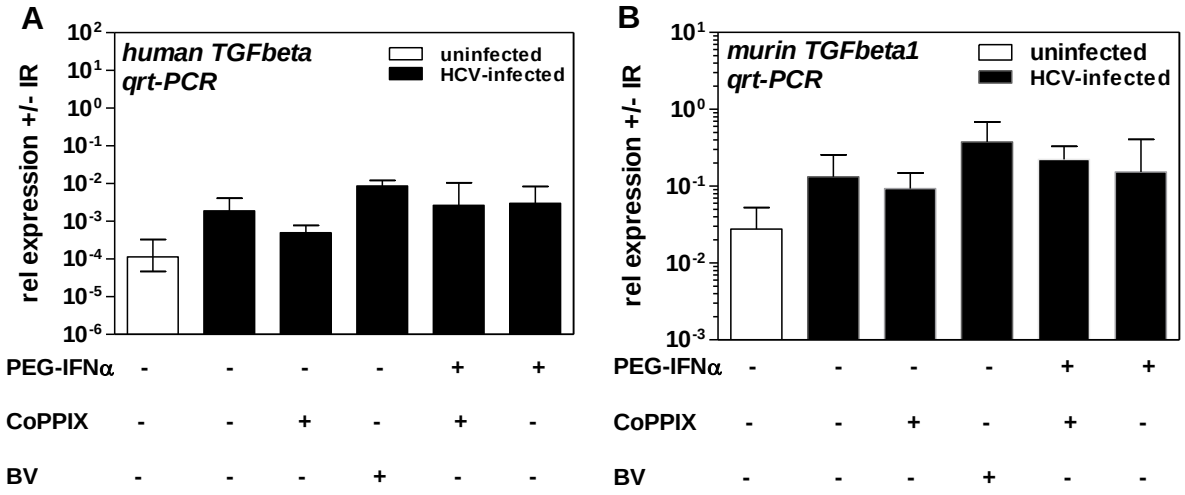

Figure 19: Transcriptional changes of TGFbeta1 after treatment in humanized uPA/SCID mice

Basic expression levels of human TGFbeta1 **(A)**, as well as murin TGFbeta1 **(B)** were quantified by qRT-PCR of uninfected control mice vs. infected mice with or without treatment. Statistical analysis was performed with the GraphPad Prism 5 software. Results were analyzed using Student's *t* test. All data are expressed as a mean ± IR.

Significant decreases in RNA expression were obtained by detecting HLA-E, interferon stimulated genes like ISG15 (Figure 18B, D) after 2 weeks of CoPP or BV administration, as well as myxovirus resistence 1 (MX1) (Figure 18E) have been shown to be strongly decreased. Transcript levels of the ISG-inducing Wnt5A and of the mediator STAT1 were also slightly reduced after HO-1 induction or BV application (Figure 18C, A). In addition, we detect a reduced expression level of TGFbeta1 after treatment (Figure 19).

3.1.6 HCV suppression occur in a polymorphism-dependent manner while the ability to decrease the pro-inflammatory cytokine milieu remain comparable

In agreement with the findings mentioned in 3.1.2, the HO-1-mediated HCV suppression determined *in vivo*, occurred in a polymorphism-dependent manner, while such differences were not observed upon BV administration. Our results show that the reduction of HCV viremia caused by CoPP application was more prominent in mice repopulated with human hepatocytes containing short $(GT)_n$ repeats (Figure 20A) and in contrast to mice that received BV for 2 weeks (Figure 20B).

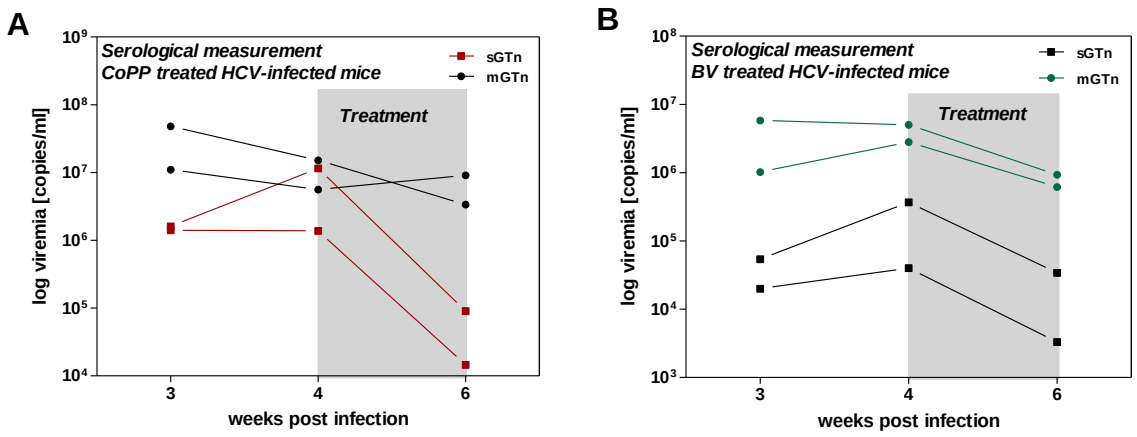

Figure 20: Serological measurement of CoPP and BV treated mice during Treatment periode

Viremic measurement form blood samples of infected mice before and after treatment period was performed by one step qRT-PCR and for absolute quantification of serum viral load, purified genotype 1a patient-derived serum was used. Virologiocal changes in infected humanized mice after 2 weeks of CoPP **(A)** and BV **(B)** treatment.

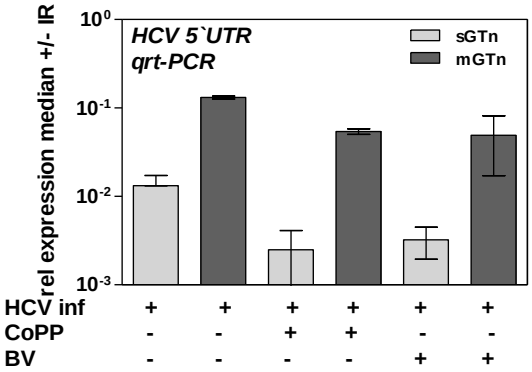

Figure 21: Intrahepatic measurement of the 5`UTR structure in correlated to the HO-1 promoter polymorphism

Viral RNA levels were detected by one step qRT-PCR after treatment period for mice repopulated with short $(GT)_n$ repeat length donors (sGTn) and medium $(GT)_n$ repeat length donors (mGTn) in comparison to infected untreated controls. Statistical analysis was performed with the GraphPad Prism 5 software. Results were analyzed using Student's *t* test. All data are expressed as a mean ± IR.

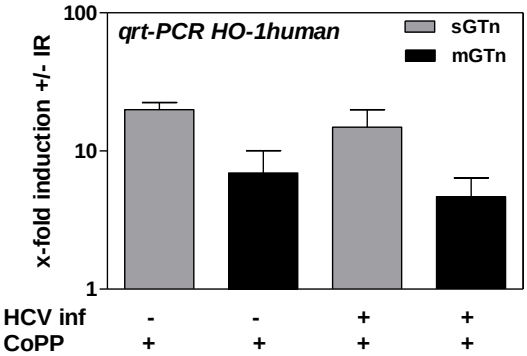

Figure 22: CoPP-mediated HO-1 induction correlated to the HO-1 promoter polymorphism

To determine the polymorphism depending intensity of HO-1 induction, uninfected as well as HCV-infected humanized uPA/SCID mice were treated with CoPP [5mg/kg] for 2 weeks and analyzed by qRT-PCR. Measurements were analyzed against the untreated control mice of every group. Statistical analysis was performed with the GraphPad Prism 5 software. Results were analyzed using Student's *t* test. All data are expressed as a mean ± IR.

These observations were confirmed at intrahepatic level by detecting HCV RNA levels of the 5´UTR region (Figure 21). The extent of HO-1 induction was measured in HCV-infected as well as in uninfected mice containing a short or a medium $(GT)_n$ repeat length after 2 weeks of CoPP application (Figure 22). These results indicated that also the basal levels of HO-1 were stronger in hepatocytes containing a short (GT)-polymorphism, although HO-1 induction was

achieved in both cases and regardless of the HCV infection status (Figure 22). Even if the potency of HO-1 mediated HCV suppression appeared polymorphim-dependent, we detected no differences in the HO-1 mediated capacity to lower the inflammation related genes (Figure 23).

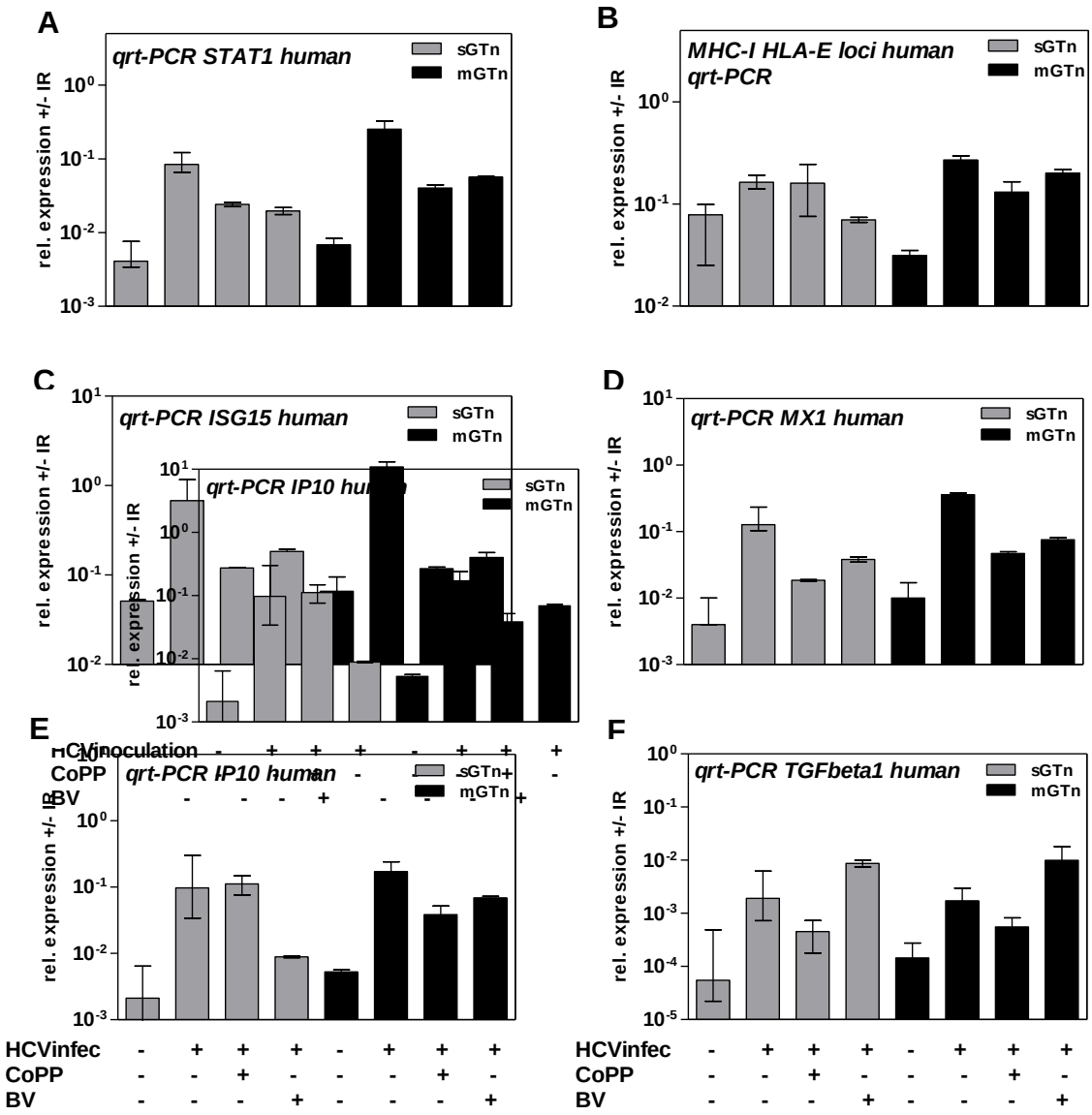

Figure 23: Transcriptional changes of some human ISGs and cytokines in correlation to the HO-1 promoter polymorphism $(GT)_n$ repeat length.

Basic expression levels of human STAT1 **(A)**, HLA-E **(B)**, ISG15 **(C)**, Mx1 **(D)**, IP10 **(E)** and TGFbeta1 **(F)** were detected by qRT-PCR and normalized against human GAPDH and RPL30. Expression was measured in mice repopulated with short as well as middle $(GT)_n$ donors treated with CoPP 5mg/kg and BV 25 mg/kg for 2 weeks. Statistical analysis was performed with the GraphPad Prism 5 software. Results were analyzed using Student's *t* test. All data are expressed as a mean ± IR.

3.2 Matrix conditions and KLF2-dependent induction of heme oxygenase-1 modulate inhibition of HCV replication by Fluvastatin *(date of publication: 06 May 2014; PlosOne)*

3.2.1 FLV, SMV, ROV and ATV, but not PRV interfere with HCV replication and induce HO-1

Statins are widely used drugs to control biosynthesis of cholesterol and to reduce amounts of LDL-cholesterol by inducing LDL-receptor (LDLR) expression. Therefore, biological activity of statins used in our experiments was verified by measuring their ability to increase LDLR expression by RT-qPCR (Figure 24).

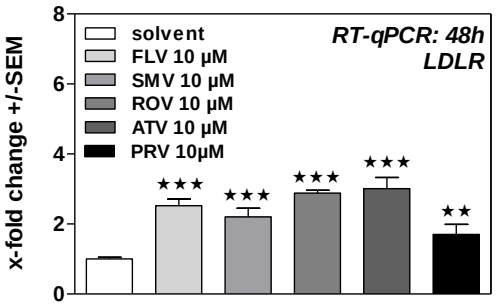

Figure 24: LDLR expression in correlation to statin application *in vitro*

LucUbiNeo-ET replicon cells were incubated in the presence of fluvastatin (FLV), pravastatin (PRV), simvastatin (SMV), rosuvastatin (ROV) and atorvastatin (ATV) at 10 µM for 48 hours. Expression of the LDL-receptor (LDLR) was measured by real time RT-qPCR. Statistical analysis was performed with the GraphPad Prism 5 software. Results were analyzed using Student's *t* test. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Investigating statin effects on HCV replication we found that FLV (Figure 24A), SMV (Figure 25B), ROV (Figure 25C), and ATV (Figure 25D) reduced HCV replication in the subgenomic replicon system in a dose- and time-dependent manner. On the other hand, PRV, while perfectly dissolved, was not able to reduce HCV replication (Figure 25E). According to these findings, antiproliferative properties of statins which interfered with HCV replication at [10 µM] were excluded by cell counting after 72 h of incubation (Figure 25F).

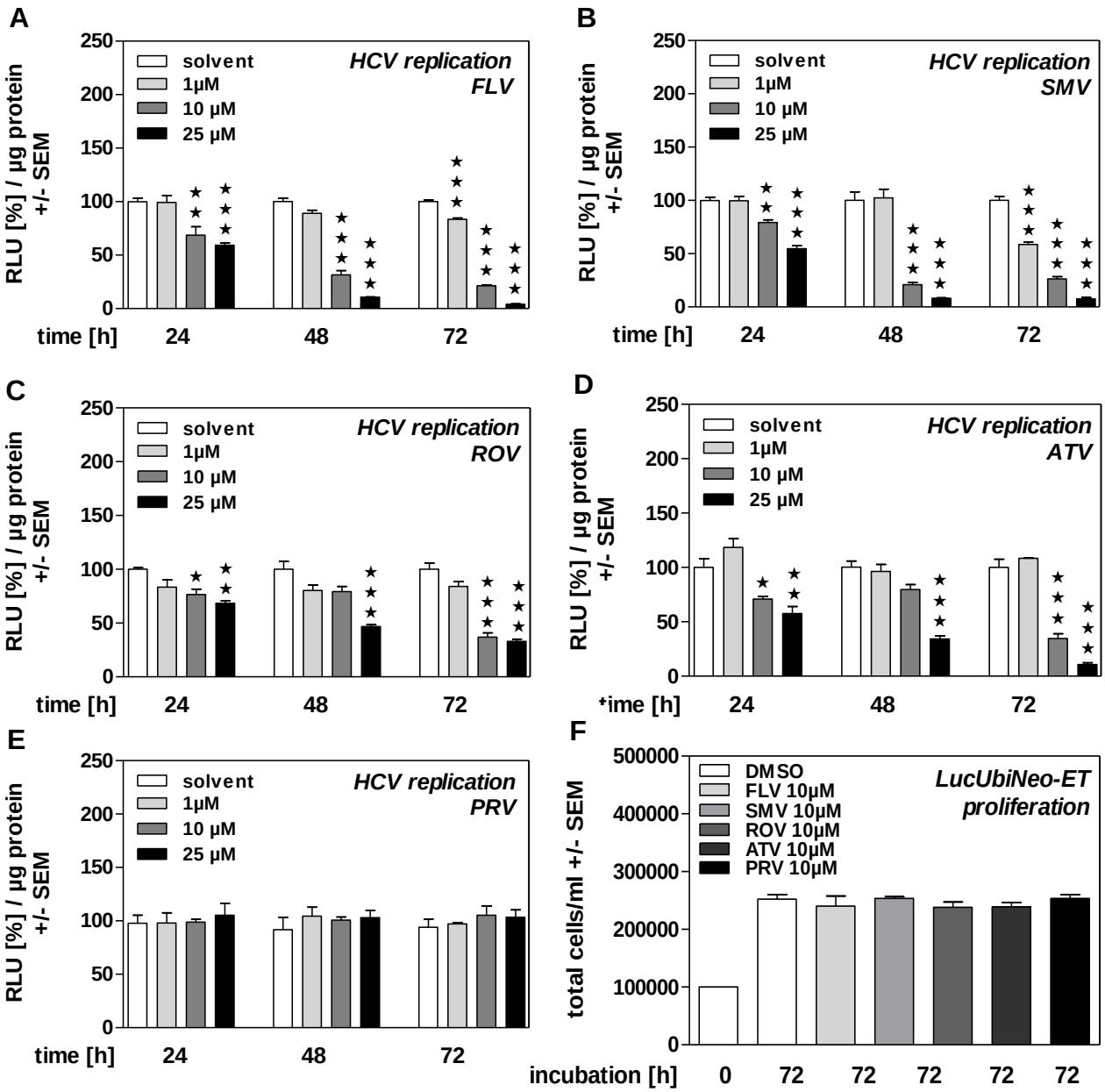

Figure 25: Statins interfere with HCV replication depended on their concentration

LucUbiNeo-ET replicon cells were incubated in the presence of fluvastatin (FLV) **(A)**, simvastatin (SMV) **(B)**, rosuvastatin (ROV) **(C)**, atorvastatin (ATV) **(D)** or pravastatin (PRV) **(E)** at the indicated concentrations for 24, 48 or 72 hours. HCV replication was measured by luciferase reporter assay. Cell proliferation was measured after 72 hours of incubation **(F)** Statistical analysis was performed with the GraphPad Prism 5 software. Results were analyzed using 1way ANOVA test. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Results obtained by luciferase reporter assays were verified by Western Blot. As an example, Figure 26 shows reduced HCV-nonstructural protein NS4B expression after 72 hours of incubation with FLV.

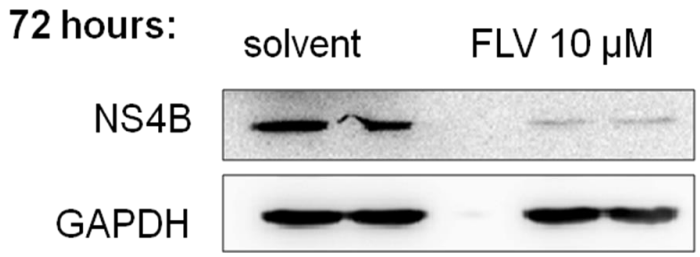

Figure 26: FLV interferes with HCV replication visualized by NS4B protein level detection

LucUbiNeo-ET replicon cells were incubated in the presence of fluvastatin (FLV) at 10 µM for 72h. HCV replication (NS4B) was measured on the protein level by Western Blot.

Using FLV we compared results obtained in the subgenomic replicon system (Figure 25A; Figure 26) to the infectious HCV cell culture system. Here FLV reduced replication of the full-length HCV clone JcR2A, as shown by luciferase reporter assay (Figure 27) and staining of JC1-infected cells for the HCV structural protein E2 (Figure 28; upper center panel).

Figure 27: Inhibition of HCV replication by statins

Huh-7.5 cells were transfected with the full length HCV clone JcR2a. HCV replication was measured by luciferase reporter assay after 72 hours of FLV incubation. Statistical analysis was performed with the GraphPad Prism 5 software. Results were analyzed using Student's *t* test. * $p \leq 0.05$.

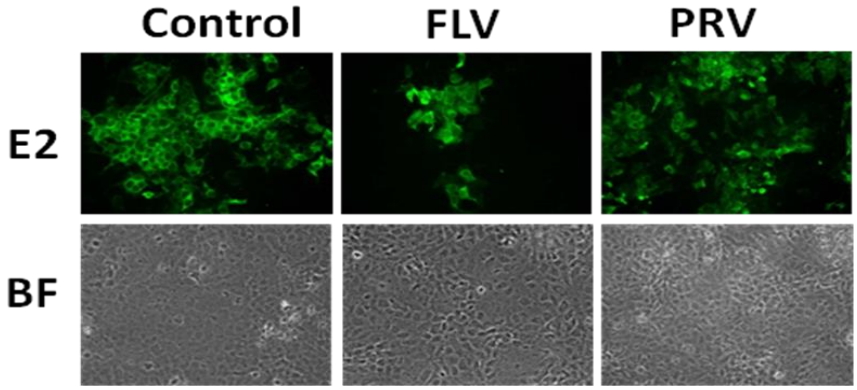

Figure 28: Inhibition of HCV replication by FLV not PRV

Huh-7.5 cells were infected with the HCV genotype 2a strain JC1 and incubated with FLV or PRV for 24 hours. Viral replication was visualized by immunofluorescent staining of the HCV protein E2 **(upper panel)**. Cell viability was verified by bright field (BF) microscopy **(lower panel)**. Representative images pairs are shown.

As observed in the subgenomic replicon system (Figure 25E) PRV, in contrast to FLV, did also not reduce HCV replication and infection (Figure 28, upper right panel). Cell viability was verified by bright field microscopy (Figure 28, lower panel).

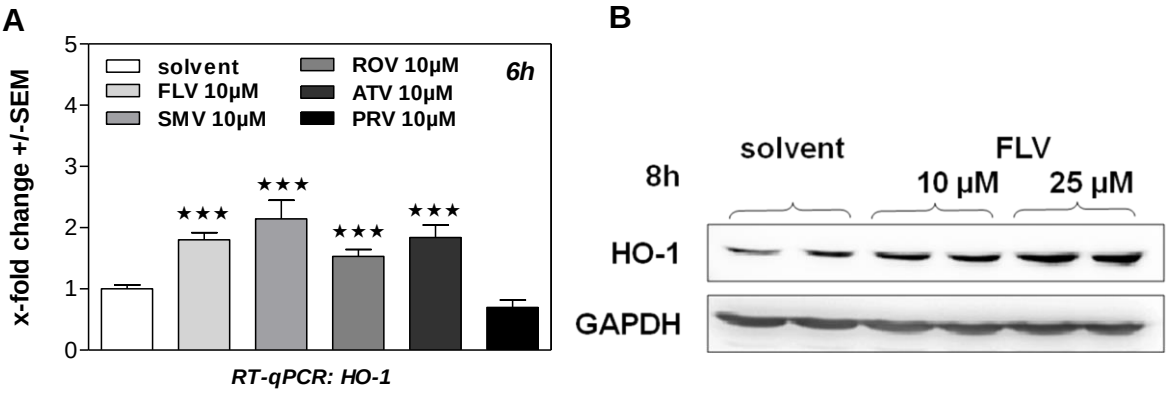

Figure 20: Statin-induced HO-1 expression interferes with HCV replication

Corresponding to previous findings HO-1-induction was measured by RT-qPCR after 6 hours **(A)** and Western Blot **(B)** after 8 hours of statin incubation in Huh-5-15 replicon cells. Statistical analysis was performed with the GraphPad Prism 5 software. Results were analyzed using Student's *t* test. All data are expressed as a mean ± SEM. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Further investigations revealed that all statins, except PRV, significantly induced expression of the antiviral enzyme HO-1 within 6-8 hours of incubation, shown by RT-qPCR (Figure 29A) and Western Blot (Figure 29B).

3.2.2 Statin-induced HO-1 expression contributes to inhibition of HCV replication

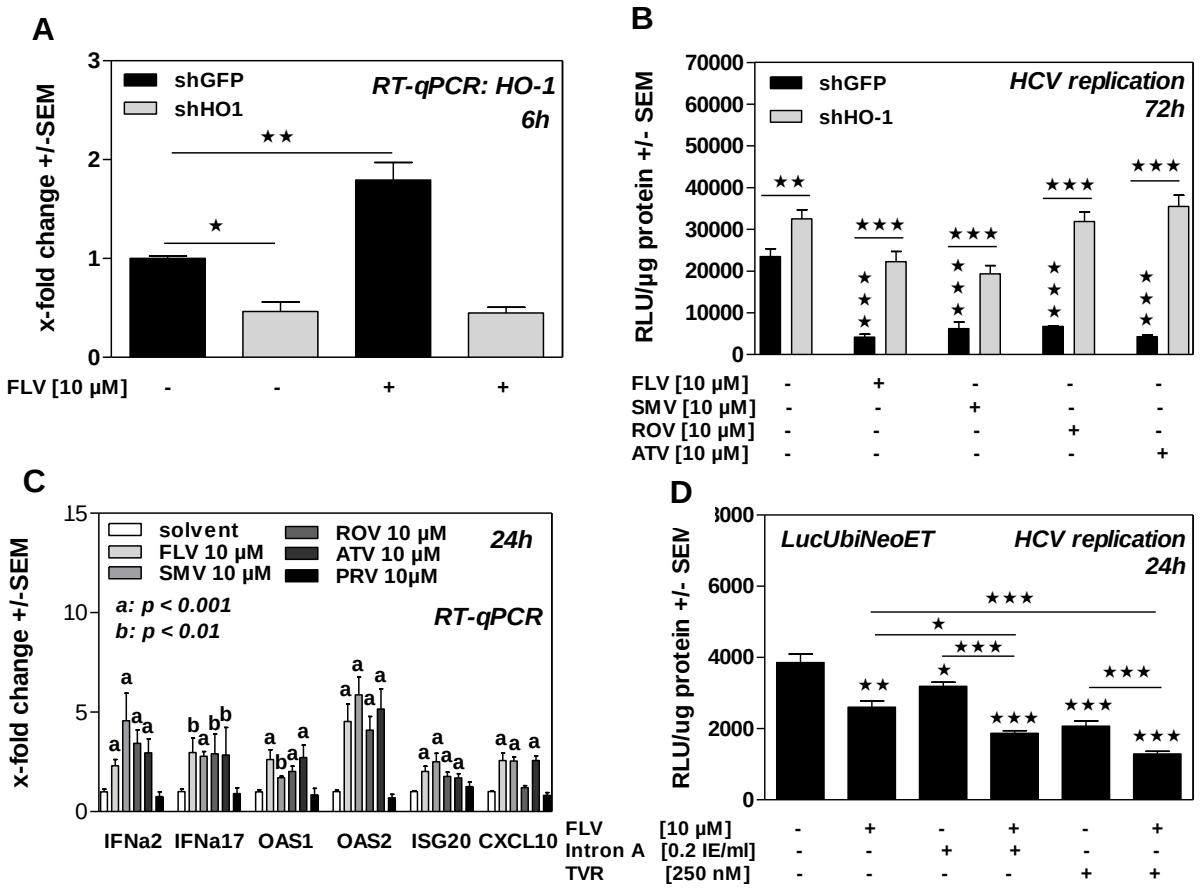

Figure 30: Statin-induced HO-1 expression contributes to inhibition of HCV replication and endogenous interferon response

FLV-mediated induction of HO-1 in LucUbiNeo-ET replicon cells stably expressing shRNA against HO-1 (shHO1) or a control gene (shGFP) was detected after 6 hours by RT-qPCR **(A)**. HCV replication of stable knock down was measured by luciferase reporter assay after 72 hours with or without statin incubation **(B)**. Replicon cells were incubated in the presence of statins for 24 hours. Expression levels of interferon alpha 2 and 17 as well as interferon response genes were analyze by RT-qPCR **(C)**. LucUbiNeo-ET cells were incubated for 24 hours with FLV, IFN alpha (Intron A) and TVR alone or in combination. HCV replication was measured by luciferase assay **(D)**. Results were analyzed using Student's *t* test, if two groups were compared and 1way ANOVA in combination with Bonferroni's Multiple Comparison Test if more than 2 groups were compared. All data are expressed as a mean ± SEM. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

To investigate the contribution of HO-1-induction to the antiviral effects of statins, we established replicon cell lines with a stable knockdown of HO-1 (shHO1) or a control gene (shGFP). In the shHO-1 cell line we observed an about 50% decrease of the HO-1 background expression, compared to the control cell line

containing shGFP, while FLV was not able to increase HO-1 expression in those cells (Figure 30A). Further investigations showed that a knockdown of endogenous HO-1 resulted in a significantly higher HCV replication in LucUbiNeoET cells (Figure 30B) and interfered with statin-mediated inhibition of HCV replication (Figure 30B). In a previous study, we could show that antiviral effects of HO-1 are mediated by its degradation product biliverdin and that biliverdin is able to induce endogenous interferon response [6]. We now combined statin incubation with interferon- or telaprevir -incubation and found that all statins, except PRV, were able to induce endogenous interferon response (Figure 30C) and furthermore support antiviral effects induced by interferon or telaprevir (Figure 30D).

3.2.3 Statins induce HO-1 expression by a Bach1- and KLF2-dependent mechanism

Investigating the mechanism of HO-1-induction by statins, we detected significantly reduced expression of the HO-1 transcriptional repressor Bach1 in statin-incubated cells (Figure 31A). Here PRV incubation did not significantly reduce Bach1 expression, but showed a slight tendency (Figure 31A). Expression levels of KLF2, a cofactor of NRF2, which is involved in HO-1-induction [131], were found to be increased by all statins except PRV (Figure 31B). Expression levels of NRF2 itself, or other factors involved in HO-1- induction, like Kelch-like ECH-associated protein 1 (KEAP1) or Hypoxia-inducible factor 1 (HIF1) alpha, were not increased by statin incubation (data not shown).

Again using FLV as a model statin, we found that a knockdown of KLF2 by transfection of siRNA (siKLF2) significantly reduced endogenous (Figure 28A) as well as statin-induced (Figure 32C) KLF2- and HO-1 expression.

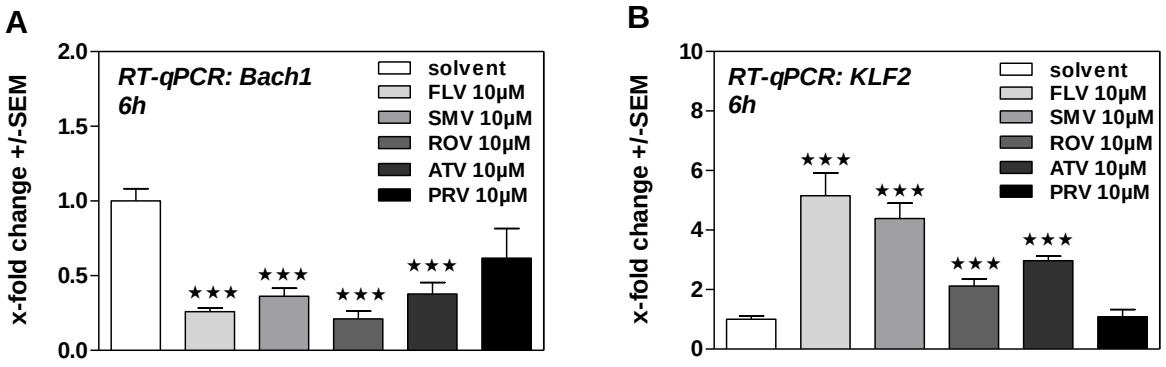

Figure 31: Expression of HO-1 Transcriptions factors after statin incubation

Expression of Bach1 **(A)** and KLF2 **(B)** was measured in Huh-5-15 cells by real time RT-qPCR after 6 hours of statin-incubation. Results were analyzed using Student's *t* test. All data are expressed as a mean ± SEM. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

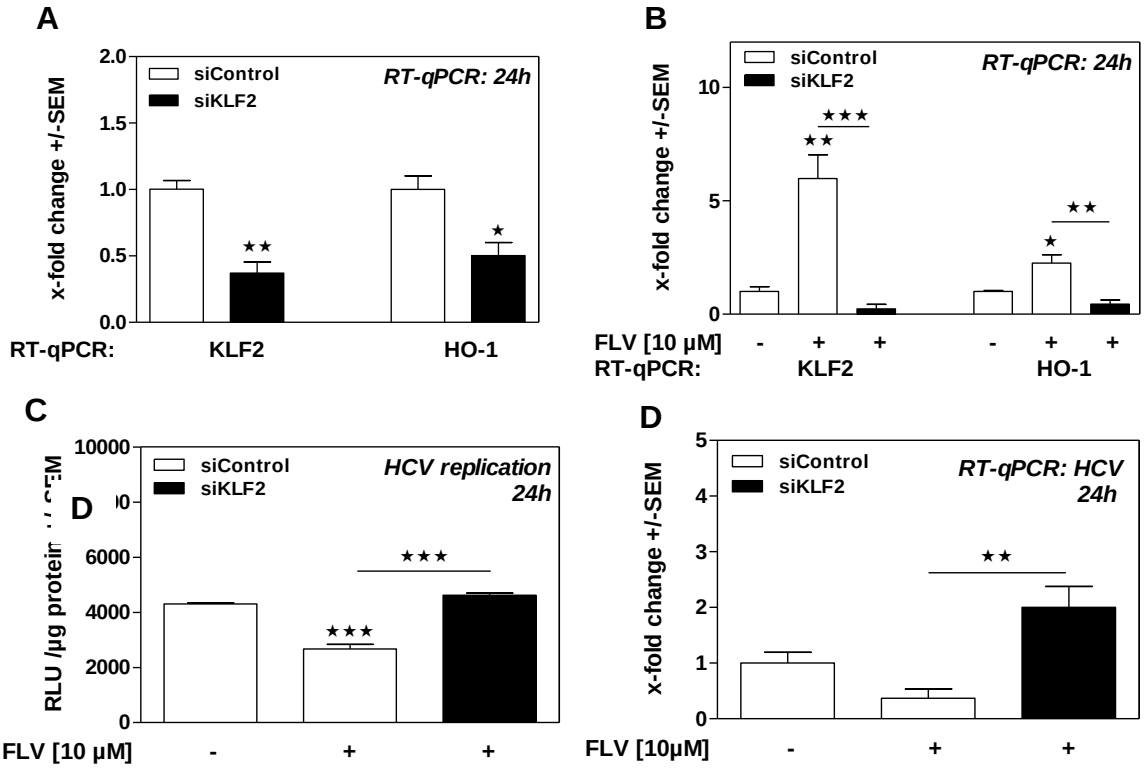

Figure 32: Statins promote HO-1 expression by reduction of Bach1- and induction of KLF2- expression

LucUbiNeo-ET replicon cells were transfected with siRNA directed against KLF2 (siKLF2) or against a control gene (GFP; siControl) **(A, B)** at [10 nM]. Expression of KLF2 and HO-1 was measured by RT-qPCR with **(B)** or without **(A)** 24 hours of FLV incubation. HCV replication was detected by luciferase reporter assay **(C)** or RT-qPCR **(D)**. Results were analyzed using Student's *t* test, if two groups were compared and 1way ANOVA in combination with Bonferroni's Multiple Comparison Test if more than 2 groups were compared. All data are expressed as a mean ± SEM. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

In consequence, a knockdown of KLF2 showed abrogated antiviral effects of FLV as measured by luciferase reporter assay (Figure 32C) or RT-qPCR (Figure 32D).

3.2.4 Matrix stiffness as a requirement for antiviral activity and HO-1-induction by statins

Viral infections of the liver frequently result in chronic inflammation and formation of fibrosis. In order to investigate how these conditions might influence antiviral effects of statins we prepared PAA gels of different stiffness as supports for the replicon cell culture system, mimicking physiological (soft) or fibrotic (stiff) liver tissue. In fact, HCC cell morphology as well as proliferation has been shown to be stiffness-dependent [117].

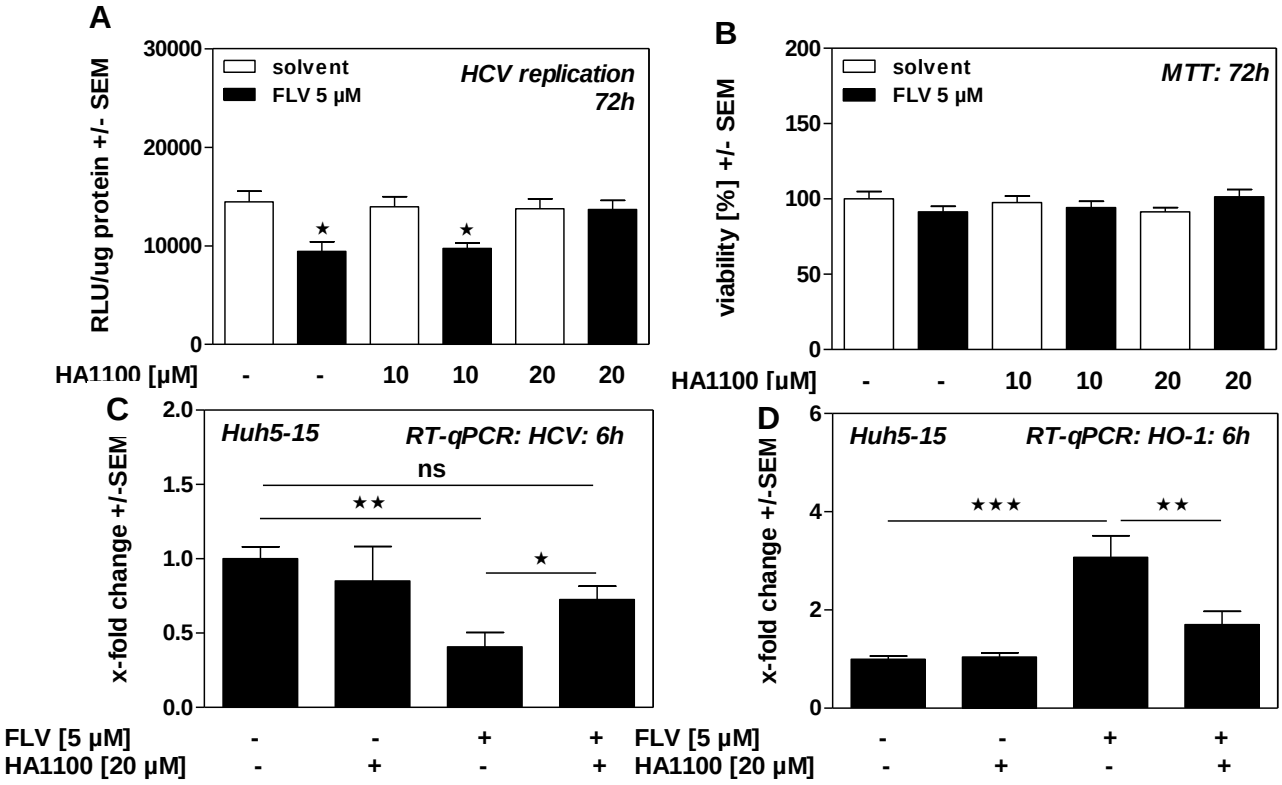

Figure 33: Matrix conditions predict antiviral properties of statins

LucUbiNeo-ET replicon cells were incubated with increasing concentrations of the Rho kinase inhibitor HA1100 for 72 hours. HCV replication was measured by luciferase assay **(A)**. Cell viability was measured by MTT assay **(B)**. Huh-5-15 replicon cells were incubated with FLV with or without HA1100 for 6 hours. HCV replication **(C)** and HO-1 expression **(D)** were measured by RT-qPCR. Results were analyzed using 1way ANOVA. All data are expressed as a mean ± SEM. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Our results show that the antiviral activity of FLV was decreased when replicon cells were growing on a soft matrix (Figure 33A), while there were no adverse

effects on cell viability (Figure 33B). In a second approach we used the Rho kinase inhibitor HA1100 to mimic decreased environmental stiffness by interfering with the matrix stiffness sensing of these cells In this environment antiviral effects of statins were also decreased, as measured by luciferase reporter assay (Figure 33A) and RT-qPCR for viral replication (Figure 33C), while cell viability was not affected (Figure 33B). Furthermore, incubation of cells with the Rho kinase inhibitor reduced the ability of FLV to induce HO-1 (Figure 33D).

3.3 Selective induction of apoptosis by statins in hepatoma cells and dependence on p53 expression

(published 2012; Oncology reports)

3.3.1 FLV, SMV and LOV dose-dependently and selectively reduce viability of mouse hepatoma cells

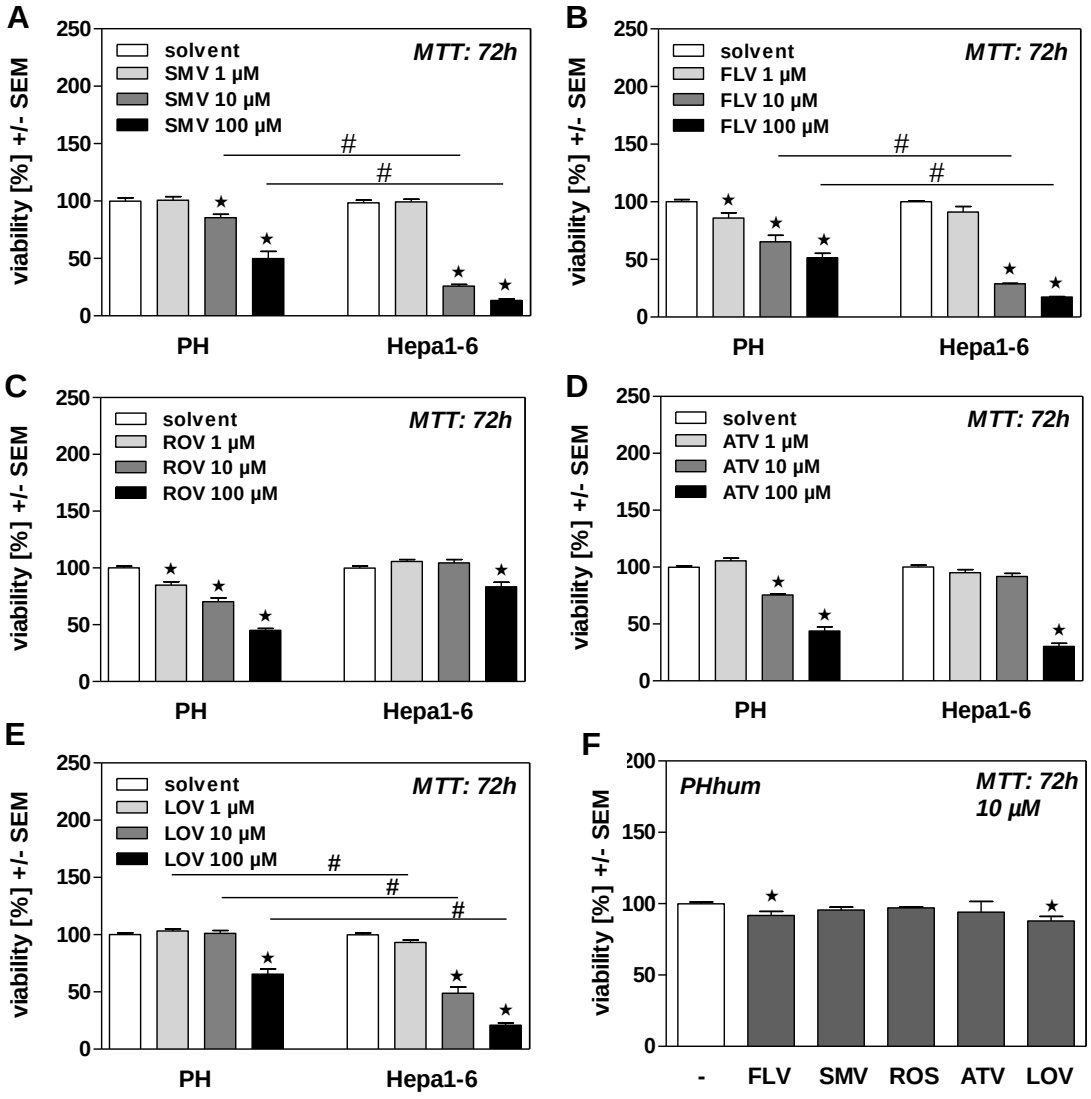

Figure 34: FLV, SMV and LOV dose-dependently and selectively reduce viability of mouse hepatoma cells

Primary mouse hepatocytes (PH) or mouse hepatoma cells (Hepa1-6) were incubated in the presence of fluvastatin (FLV; **A**), simvastatin (SMV; **B**), rosuvastatin (ROV; **C**), atorvastatin (ATV; **D**), or lovastatin (LOV; **E**) at 1, 10 or 100 µM for 72 h. Cell viability was measured by MTT assay. * $P \leq 0.05$ for statin vs. solvent incubated cells; # $P \leq 0.05$ for statin incubated PH vs. Hepa1-6 cells. (**F**): Primary human hepatocytes (PHhum) were incubated in the presence of FLV, SMV, ROV, ATV, or LOV at 10 µM for 72 h. Cell viability was measured by MTT assay. * $P \leq 0.05$ for statin vs. solvent incubated cells

The use of statins has been shown to affect hepatic tumor growth in patients [132], but also bears the risk of severe side effects when over-dosed or applied to patients with e.g. impaired liver function [108;109]. To investigate which statin might have the highest efficacy in HCC therapy and to predict the probability of side effects we first compared viability of freshly isolated primary mouse hepatocytes (PH) in the presence of increasing concentrations of statins to mouse hepatoma cells (Hepa1-6) *in vitro*. Using fluva- (FLV), simva- (SMV), rosuva- (ROV), atorva- (ATV), as well as lovastatin (LOV) we found that within 72 hours of incubation, FLV (Figure 34A), SMV (Figure 34B) and LOV (Figure 34E) significantly reduced viability of hepatoma cells in comparison to PH, while ROV (Figure 34C), and ATV (Figure 34D) did not. At a concentration of 10 µM FLV, SMV and LOV effects on mouse hepatoma cells were significantly more pronounced than effects on primary mouse hepatocytes. We also chose this concentration to compare the impact of all 5 statins on viability of primary human hepatocytes (PHhum). Our results show that PHhum in general were much more resistant towards statin incubation than mouse hepatocytes, with only mild toxic effects being observed for FLV and LOV (Figure 34F).

3.3.2 FLV, SMV and LOV most efficiently reduce viability of human hepatoma cell lines HepG2 and Hep3B, but not Huh7

Incubating the human hepatoma cell lines Huh7 and HepG2 in the presence of 10 µM of statins we found that FLV and LOV showed mild toxic effect on Huh7 cells, while none of the other statins reduced viability of this cell line (Figure 35A). In contrast, all statins significantly interfered with viability of HepG2 cells (Figure 35A). Again, most pronounced effects were observed for FLV, SMV and LOV,

while effects of ATV were intermediate and ROV had only moderate effects on viability of HepG2 cells (Figure 35A).

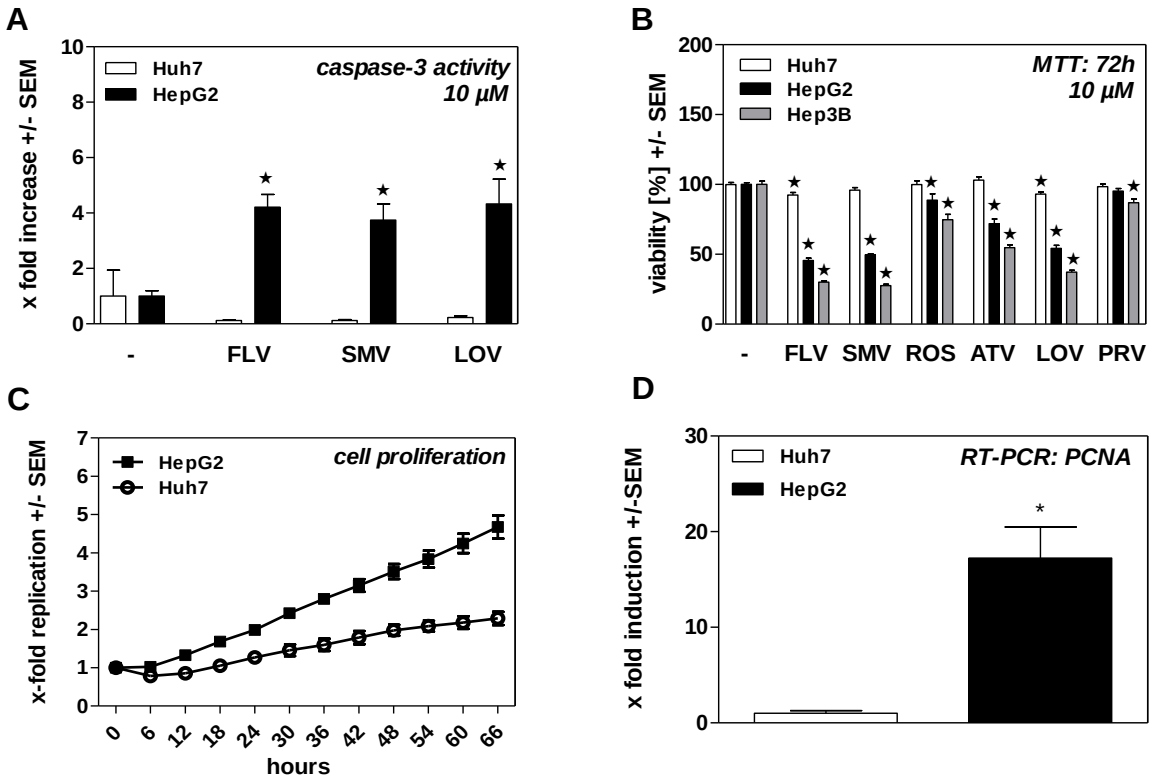

Figure 35: FLV, SMV and LOV efficiently and selectively reduce viability of human hepatoma cell lines

Human hepatoma cells (Huh7; HepG2; Hep3B) were incubated in the presence of fluvastatin (FLV), simvastatin (SMV), rosuvastatin (ROV), atorvastatin (ATV), or lovastatin (LOV) at 10 µM for 72 h. Cell viability was measured by MTT assay. * $P \leq 0.05$ for statin vs. solvent incubated cells **(A)**. Apoptosis induction was measured in Huh7 and HepG2 cells after 48 hours of statin incubation by caspase-3 activity assay. * $P \leq 0.05$ for statin vs. solvent incubated cells **(B)**. Proliferation of Huh7 and HepG2 cells was measured continuously over a period of 66 h using the impedance-based xCELLigence real-time cell analyzing system. Results are expressed as mean cell index normalized to the time-point of seeding to 96 well E-Plates **(C)**. Expression of proliferating cell nuclear antigen (PCNA) was measured in Huh7 and HepG2 human hepatoma cell lines by real time RT-PCR. * $P \leq 0.05$ **(D)**.

We also measured apoptosis induction by FLV, SMV and LOV in Huh7 and HepG2 cells and found that these statins induced apoptosis in HepG2, but not in Huh7 human hepatoma cells (Figure 35B). These results indicate that statins in principle are able to induce apoptosis in tumor cells. Measuring real time proliferation of HepG2 and Huh7 cells we found that HepG2 cells were proliferating much faster

than Huh7 cells (Figure 35C). This finding was in line with results from real time qRT-PCR, where expression of proliferating cell nuclear antigen (PCNA) was found to be significantly higher in HepG2 cells (Figure 35D).

3.3.3 Protection of Huh7 cells against statin induced cytotoxicity seems to depend on over-expression of p53

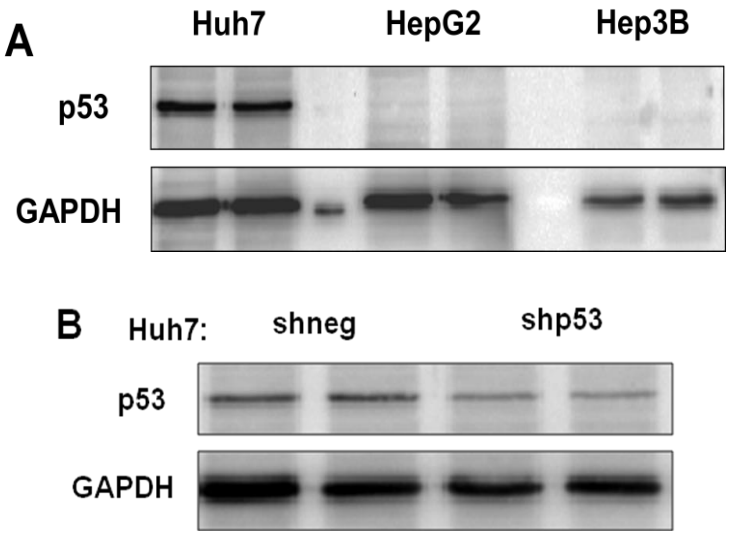

Figure 36: Detection of p53 Protein level in HCC celllines

p53 protein expression was measured in human hepatoma cells (Huh7; HepG2; Hep3B) by Western Blot **(A)**. p53 protein expression was measured by Western Blot in Huh7 cells with stable knockdown of p53 (shp53) or Huh7 cells expressing control shRNA directed against E.coli polymerase (shneg) **(B)**.

As shown in Figure 36A and B Huh7 human hepatoma cells did not exert reduced viability or increased apoptosis induction following statin incubation. One fundamental difference between these cells and HepG2 cells is their content of p53 tumor suppressor protein. While HepG2 express normal amounts of p53, Huh7 cells contain significantly higher amounts, due to a block in p53 degradation [133]. We could confirm this observation in our cell lines by Western Blot (Figure 36A). Based on the hypothesis that high amounts of p53 would interfere with cell proliferation, which might be the reason for decreased sensitivity of Huh7 cells

towards statin-induced cytotoxicity, we generated Huh7 cells with a stable knockdown of p53 (shp53). Western Blot analysis (Figure 36B) revealed a p53 knockdown of about 50% on protein level in comparison to Huh7 cells expressing shRNA directed against E. coli polymerase (shneg) as a control for unspecific knockdown.

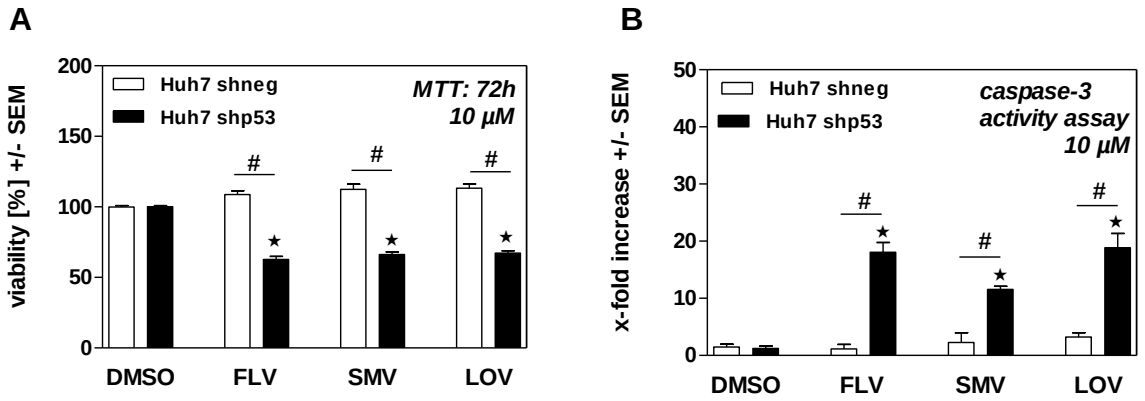

Figure 37: Statin mediated reduction of cell viability and induction of caspase-3 in relation to p53 knock down

Huh7shneg or Huh7shp53 cells were incubated in the presence of 10 µM fluvastatin (FLV), simvastatin (SMV) or lovastatin (LOV) for 72 hours. Cell viability was measured by MTT assay **(A)**, apoptosis induction was measured by caspase-3 activity assay **(B)**. * $P \leq 0.05$ for Huh7shneg vs. Huh7shp53 cells.

Incubation of both cell lines in the presence of FLV, SMV or LOV revealed significantly reduced cellular viability (Figure 37A) and increased apoptosis induction (Figure 37B) by statins in the presence of the p53 knockdown, providing evidence that p53 might contribute to resistance against anti-tumor effects of statins. Our hypothesis was further supported by the finding that p53 knockdown increased expression of proliferating cell nuclear antigen (PCNA) (Figure 38).

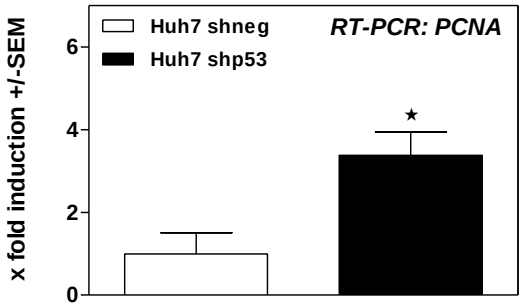

Figure 38: Expression profile of PCNA in shp53 Huh7 cells

Expression of proliferating cell nuclear antigen (PCNA) was measured by real time RT-PCR. * P ≤ 0.05 for Huh7shneg vs. Huh7shp53 cells.

3.3. 4 Statins induce tumor cell apoptosis by interfering with geranyl-geranylation

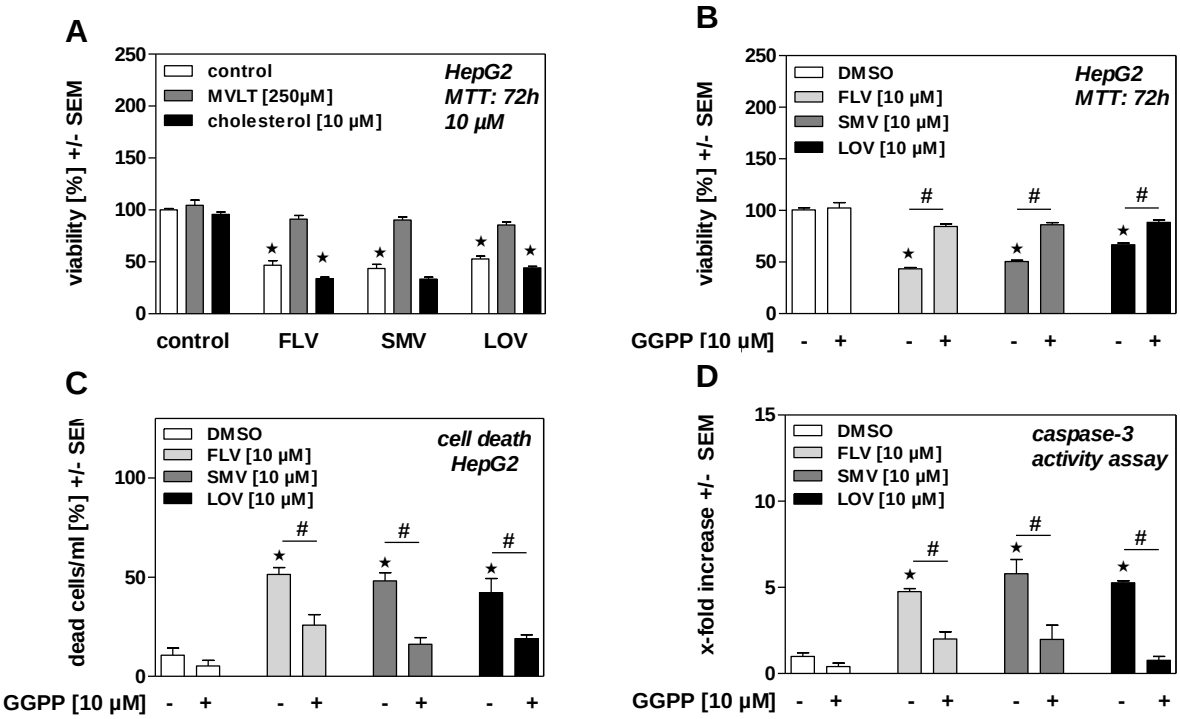

Figure 39: Statins reduce viability and induce apoptosis of tumor cells by interfering with geranyl-geranylation

HepG2 human hepatoma cells were incubated in the presence of 10 µM fluvastatin (FLV), simvastatin (SMV) or lovastatin (LOV) with or without co-incubation with cholesterol [10 µM] or mevalonate (MVLT) [250 µM] for 72 hours **(A)**. Cell viability was measured by MTT assay. * P ≤ 0.05 for statin vs. solvent incubated cells. HepG2 cells were incubated in the presence of statins with or without co-incubation with geranyl-geranyl-pyrophosphate (GGPP) [10 µM]. Cell viability was measured by MTT assay **(B)**. The percentage of dead cells was measured by trypan blue staining and cell counting **(C)**. Apoptosis induction was measured by caspase-3 activity assay **(D)**.* P ≤ 0.05 for statin incubated vs. solvent incubated cells. # P ≤ 0.05 for statin incubated cells with or without co-incubation with GGPP.

To further characterize apoptosis induction by statins in hepatoma cells we investigated if reduced biosynthesis of cholesterol itself or an intermediate step in the cholesterol biosynthesis pathway might be responsible. Therefore, HepG2 cells were incubated in the presence of FLV, SMV or LOV with or without cholesterol or mevalonate (MVLT) supplementation. Our results show that cholesterol was not able to restore cell viability (Figure 39A), while mevalonate (MVLT) did, indicating that inhibition of the cholesterol synthesis pathway rather than a lack of cholesterol seems to be responsible for induction of hepatoma cell death by statins. By interfering with the cholesterol biosynthesis pathway statins deprive cells of signaling molecules, e.g. geranyl-geranyl-pyrophosphate (GGPP), necessary for prenylation of small G-proteins and subsequent maintenance of cellular integrity. This might be especially important for fast proliferating tumor cells. To investigate this hypothesis we incubated HepG2 cells in parallel with statins and GGPP and found a partially restoration of cell viability (Figure 39B) and inhibition of cell death, as measured by counting dead cells as a percentage of whole cell numbers (Figure 39C) or apoptosis induction (Figure 39D). The same effects were observed when incubating mouse hepatoma cells (Hepa1-6) in the presence of statins and GGPP (data not shown), indicating a generalized mechanism. In summary, our results implicate that FLV, SMV and most prominently LOV induce apoptosis in hepatoma cell lines by interfering with cellular integrity.

4. Discussion

4.1 Part I: Suppression of HCV replication by HO-1 or BV enhances the antiviral effects of PEG-IFNα in humanized mice

Since the HO-1 enzyme also known as heat shock protein Hsp32 has became the topic of several investigations more and more pleiotrophic effects of the enzyme have been described. In previous findings, the antioxidant enzyme HO-1 has been shown to interfere with the HCV life cycle in replicon cell lines. We aimed to analyze the HO-1 mediated antiviral effects *in vivo* in the use of a small animal model, the uPA/SCID (USB) mouse, which was stable infected with the HCV genotype 1. HCV infection was detected 2-3 weeks after viral inoculation by performing serological measurements and immunohistochemical staining of tissue slides, as mentioned in 3.1.1 and 3.1.2. With regard to the HO-1 promoter polymorphism we characterized transplanted human hepatocytes by determining the GTn length (3.1.2). It has been shown that in individuals harboring a short GTn length the HO-1 mRNA levels appeared higher as in individuals with more GTn repeats [71]. By assessing the variation of HO-1-inducing potency related to its promoter polymorphism, we also found that from mouse isolated and then cultured primary human hepatocytes containing a short $(GT)_n$ repeat length exhibited the most prominent induction rates (Figure 8). Besides characterization of the $(GT)_n$ repeat length as ($s(GT)_n$, $m(GT)_n$ and $l(GT)_n$), we obtained USB mice repopulated with $s(GT)_n$ as well as $m(GT)_n$ which displayed high levels of human liver chimerisms and could be infected with HCV (3.1.2). Our results gave also a hint that mice harboring the $m(GT)_n$ repeat length appeared more prone to achieve higher infection titers, pointing out that the potency of HO-1 induction might have influenced the course of HCV infection even in human liver chimeric mice.

Determining the HCV-driven expression profile of HO-1 and its regulating genes Bach1 and KLF2, polymorphism related alternations have been detected as illustrated in 3.1.2. It has been shown, that the expression of HCV C-protein in hepatocyte cell lines expressing HCV C-protein but not envelope or nonstructural proteins leads to increased oxidative stress, resulting in increased HO-1 expression levels [134]. In liver samples of HCV-infected patients HO-1 mRNA likewise protein levels were decreased [135]. Moreover, the down regulation of HO-1 expression by HCV was reproduced in cell lines [134]. Furthermore, HO-1 induction in response to cytotoxins is impaired in HCV C-protein expressing hepatocytes [136]. In line with these findings, we found HCV-driven induction of HO-1 mRNA as shown in 3.1.2 (Figure 11). KLF2 transcription factor, which is involved in HO-1-induction [131], was also induced (Figure 10A). This effect was visible 3 weeks post infection in mice harboring $m(GT)_n$ hepatocytes, while in $s(GT)_n$ hepatocytes there was already a light suppression detectable, possibly due to Bach1 induction (Figure 10B). One explanation for these results could be that the higher potency of $s(GT)_n$ hepatocytes to induce HO-1 resulted in a faster HCV-driven recognition. While in $m(GT)_n$ hepatocytes the lower potency of induction resulting to a later recognition of the antiviral acting enzyme. In order to this explanation the highest peak of HO-1 in $s(GT)_n$ hepatocytes supposed to be at a earlier time point. To evaluate this hypothesis it would be necessary to investigate the intrahepatic expression of liver containing short repeats 2 weeks post infection. This explanation would be also true for the lower viral load in mice with short repeats in comparison to medium repeat length.

Various experimental systems such as cell culture models [137], transgenic mice with humanized livers [138], experimental inoculated chimpanzees [139] likewise

human liver samples of HCV-infected patients [140] have been used to characterized IFN response induced by HCV. Taken together, these studies already indicated that HCV can induce the upregulation of Interferon stimulated genes (ISGs). By determining the expression level of human ISGs and related genes of the innate immune response in infected human hepatocytes in chimeric livers we could confirm that HCV triggers upregulation of most ISGs in the infected cells as shown in 3.1.3 (Figure 12B). Thus several groups reported an upregulation of IFN III in liver samples of patients and in infected chimpanzees [141;142].

Also a strong induction of STAT1 has been reported. Amongst others, overexpression of IFN-λ4 fusion protein has been shown to induce phosphorylation of STAT1 and leads to suppression of HCV replication [143]. Recently, WNT5A was proposed to be an inflammatory mediator inducing ISGs in livers of treatment-resistant interleukin 28 (IL28B) minor genotype (MI) patients [144]. The same report pointed out that WNT5A as well as FZD5 were significant upregulated in IL28B MI patients. Thus, they point out that WNT5A-FZD5 signaling participates in the induction of ISG expression, but preserves HCV replication likewise infection by increasing stress granule (SG) protein levels. Interestingly in a present study, WNT5A was also shown to be significantly increased post infection as illustrated in 3.1.3 (Figure 12A). In this context, the observed effects of WNT5A could be a promising explanation for the reported IFN response pattern while IFN I, II and even III remain under detection line.

In this study, expression levels of HCV entry receptors have been detected to be increased post infection as shown in 3.1.3 Figure 13A. However, there has been no correlation to the mentioned HO-1 promoter polymorphism when elucidating the expression on this issue. As mentioned in 3.1.3 Figure 13B, we also observed

increased expression of small cytokines like IP10 and TGFbeta1in course of the HCV infection. These results indicate that the infected human hepatocyte itself is a source for the production of pro-inflammatory cytokines. For a more detailed view expression analysis of TLR 3/7 likewise IRF 3/7 will be helpful.

As mentioned before, the antioxidant enzyme HO-1 has been shown to interfere with the HCV life cycle in replicon cell lines. The main focus of my thesis was to evaluate these effects in HCV-infected humanized mice. As illustrated in 2.3.7 Figure 6, we identified post transcriptional and post translational induction of HO-1 in the mono- (CoPP) as in the combination (CoPP + Peg-IFNα) treatment (3.1.4 Figure 14 and Figure 17). Whether 1 log reduction of the viral load was obtained after HO-1 induction or PEG-IFNα application in mono-therapy, an additional effect was achieved when performing the combination treatment. The HO-1 downstream product BV has been reported to interfere with the HCV replication [4-6] and hence to act direct antiviral [7]. Here we could confirm the *in vitro* findings addressed on the suppression of HCV replication *in vivo*. Notably, expression of ISGs and interferon related genes were decreased after HO-1 induction or BV application (see 3.1.5 Figure 18). Interestingly, the HO-1 downstream product exhibited the same anti-inflammatory effects as the induction of the enzyme itself, except for the expression of human and murine TGFbeta1, leading to the hypothesis that HO-1 induction may influence the cytokine signaling in both murine and human hepatocytes. These investigations are in line with previous *in vitro* findings addressed on the hepatoprotective effects of HO-1 induction [6].

Moreover, while the suppression of HCV replication appeared to be correlated to the potency of HO-1 induction, the hepatoprotective action of HO-1 remained the same regardless of the type of HO-1 promoter polymorphism, as shown in 3.1.6

(Figure 23). Taken together these results indicate that the induction of the antioxidant enzyme HO-1 may contribute to the efficacy of combination treatment particularly for patients carrying a short GTn repeat length. While for a long time a combinational therapy of interferon and ribavirin has been the best option for treatment of chronic HCV infection, sustained virologic response has now been markedly improved by treatment with HCV protease inhibitors in combination with pegylated interferon (IFN) alpha 2 [8]. In studies evaluating the effects of e.g. Telaprevir, increased evidence for escape mutants has been reported [145]. It has to be taken into account, that the antiviral effect triggered by HO-1 induction is strongly depending on the condition of the liver cells. We demonstrated a strong relation between the potency of HO-1 effectively and its polymorphism.

Exerting anti-inflammatory activity compared to applied medications, the stress response enzyme may be useful as additional compound in HCV therapy, especially in combination with PEG-IFNα. In context with our findings discussed in chapter 4.2, this report hinted to suggest synergistic effects when performing further DAA combination experiments. However, CoPP is a useful tool to elucidate HO-1 related issues in the USB model the potent HO-1 inducer is not useable in therapy. Therefore substances exerting comparable effects regarding the induction of the stress response enzyme are necessary to identify.

4.2 Part II: Matrix conditions and KLF2-dependent induction of heme oxygenase-1 modulate inhibition of HCV replication by Fluvastatin

While the emphases of the first part given to the evaluation of previously *in vitro* observed antiviral properties of HO-1 in the humanized USB mice, the second part of this report gives a more detailed view on conditions supporting these effects *in vitro* with regard to the statin-mediated induction of HO-1. The primary indication for statin prescription is treatment of hypercholesteremia to prevent adjacent secondary events, e.g. coronary problems. Evidences were reported that statins might also display beneficial effects on other kinds of diseases [92,93,95,96]. Recently, it has been shown that statins, except PRV, enhance effects of HCV-targeted therapy by NS3- or NS5-inhibitors *in vitro*. This is in line with our results showing that a combination of FLV and the protease inhibitor telaprevir was significantly more effective in reducing HCV replication, compared to effects of each compound alone as demonstrated in 3.2.2 Figure 30D. In addition we observed that FLV significantly increased antiviral effects of interferon, giving rise to the speculation that statin supplementation might help reducing therapeutic levels of interferon and its adverse side effects in patients.

These results are promising but do not generally reflect antiviral statin effects in patients [146,107,147]. Knowledge of underlying mechanisms leading to reported properties of statins might help to understand under which circumstances statins might support antiviral therapy. To date, inhibition of prenylation has been suggested as the mechanisms behind antiviral activity of statins [148]. In the process of cholesterol biosynthesis inhibition, statins reduce generation of the isoprenoids, which are necessary for post-translational modifications and activation of cellular proteins [149], thereby regulating cell survival and cell growth [150].

It has to be taken into account that subgenomic replicon system are based on the fast proliferating human hepatoma cell line Huh7 and that cellular integrity is a prerequisite for cell proliferation and efficient HCV replication. In fact we observed a loss of cell viability in human and mouse hepatoma cell lines which was reverted by restoration of prenylation caused by higher concentrations or longer statin incubation time periods, as reported in chapter 3.3 [151]. However, the inhibition of prenylation is a general mechanism of statin action and does not provide an explanation why e.g. PRV does not interfere with HCV replication as shown in 3.2.1.

Statins have been shown to regulate gene expression. Deficiency in cholesterol leads to the activation of sterol regulatory element-binding proteins (SREBPs), transcription factors, which increase expression of LDLR, subsequently leading to reduction of LDL-cholesterol [152]. The in 3.2.2 reported results show that statins were able to induce expression of the anti-viral enzyme HO-1 in replicon-containing cells by reducing expression of Bach1, a transcriptional repressor for HO-1 [153], as well as by activation of NRF2 due to increased availability of its cofactor KLF2 as mentioned in 3.2.3 Figure 31. KLF2 in fact seems to be the key factor since its knockdown reduced HO-1 expression and restored HCV replication in the presence of statins as shown in 3.2.2 Figure 32A-B. It has recently been shown that HO-1 interferes with HCV replication [456] via its product biliverdin [6, 7].

Moreover, we could show that statins were not able to reduce HCV replication in cell lines carrying a stable knockdown of HO-1 or KLF2 (3.2.3 Figure 32C-D). The results also show that PRV was not able to induce HO-1- or KLF2-expression in replicon cells, providing a possible explanation for missing effects of PRV on HCV

replication (3.2.3 Figure 31). Taken together our results indicate that HO-1-induction might be a key event in antiviral activity of statins.

Aiming to provide a possible explanation on inconsistent antiviral effects of e.g. FLV in patients [96;97] we investigated the role of matrix stiffness, corresponding to degrees of cirrhosis. Matrix stiffness has so far been mostly neglected in the study of therapeutic responses in cell culture. Since hepatocytes under physiological conditions reside in a soft environment *in vivo*, the regular culture on plastic surfaces has to be considered a non-physiological stiff environment, resembling fibrotic or cirrhotic conditions. It has been reported that environmental stiffness affects interferon treatment and thereby therapeutic responses in HCV infection [154]. This study provided evidence that matrix stiffness modulates the response of HCV replicon cells towards therapeutically used statins summarized in 3.2.4. In contrast to observations regarding interferon, we found increased effects of fluvastatin on HCV replication in replicon cells cultured on stiff matrices as well as cell culture plastic material, while effects on HCV replication as well as HO-1 expression were largely diminished under soft matrix conditions. Although these results might suggest a benefit of statin use in adjunct to antiviral therapy in patients with advanced liver disease, further studies in animal models and observational studies in humans are needed to prove this speculation. While statins have so far been used cautiously in patients with advanced liver diseases, new data suggest, that they might be safely used even in patients with cirrhosis [155].

As an additional benefit we observed that statins were able to induce endogenous interferon response under cell culture conditions which are comparable to cirrhotic tissue conditions (3.2.2 Figure 30C). Since exogenous interferon treatment has

been shown to display severe side effects, stimulation of endogenous interferon response might be an alternative attempt or at least help to reduce therapeutic doses of exogenous interferon. Hence, FLV might further support therapy especially under stiff matrix conditions by inducing endogenous interferon response. The direct antiviral acting (DAA) compound telaprevir is metabolized in the liver by cytochrome P-450 (CYP) isoenzyme 3A4 to the inactive (α-ketoamide bond) metabolites and the active *R*-diastereomer. Telaprevir is both a substrate and an inhibitor of the isoenzyme [156]. Atorva-, lova- and simvastatin are metabolized by the CYP3A4 isoenzyme [157]. Therefore, a combination of telaprevir with these statins would decrease their metabolism leading to increased blood concentrations, with a potential risk of detrimental side effects. On the other hand FLV metabolized by the CYP2C9 isoenzyme [157], which is not known to be affected by telaprevir. Consequently DAAs metabolized by CYP3A4 in combination with FLV might be beneficial. To verify this hypothesis further studies are necessary.

4.3 Part III: Selective induction of apoptosis by HMG-CoA-reductase inhibitors in hepatoma cells and dependence on p53 expression

By definition hepatitis C is regarded to be chronic after persistence of more than six months. A very low rate of spontaneous cleavage is observed if chronic infection is once established. This manifestation of chronic hepatitis C can discharge to cirrhosis, as reported for 20-30% of chronically infected patients and moreover leading to hepatocellular carcinoma (HCC). Interestingly, besides already mentioned properties of statins, they also have been shown to exert effects on cancer [158,159]. Statin therapy additionally supports in a lower risk to develop HCC in diabetes patients [160]. In fact, it has been shown that e.g. FLV and SMV induce apoptosis and cell cycle arrest in hepatoma cell lines [161,162], ATV blocks both MYC phosphorylation and activation, suppressing tumor initiation and growth *in vivo* [163] and that SMV modifies the expression of cell adhesion molecules leading to reduced tumor cell growth and invasion [161]. Moreover, statins showed synergistic anti-tumor effects with e.g. the COX-2 inhibitor celecoxib [164] or the protein kinase C (PKC) beta inhibitor enzastaurin [165]. In this part of the study, we compared anti-tumor effects of FLV, SMV, ATV, ROV and LOV on human and mouse HCC cell lines to effects on primary human or mouse hepatocytes. The general observation is that anti-tumor effects of FLV, SMV and LOV on hepatoma cell lines were significantly more pronounced compared to effects on primary hepatocytes, which goes in line with chapter 4.2 regarding the usefulness of statins, especially in therapy of HCC patients. The results indicate that there are profound differences in the susceptibility of human hepatoma cell lines towards statin treatment, pointing to the fact that not every HCC patient might benefit from statin therapy as reported in 3.3.1. Anti-tumor effects of statins are

tightly linked to the proliferative capacity of cells. The slow proliferating Huh7 cells, like the *in vitro* almost quiescent primary human hepatocytes, were significantly less affected by statins than fast proliferating human hepatoma cell line HepG2 (3.3.2). Along with inhibition of cholesterol biosynthesis statins also deprive hepatocytes of intermediate products, e.g. mevalonate or the isoprenoids geranyl-geranyl-pyrophosphate (GGPP) and farnesyl-pyrophosphate (FPP), which are necessary for post-translational modifications and activation of cellular proteins, e.g. small G-proteins of the Ras superfamily [149]. These proteins are regulators of important biological processes, including cell survival and cell growth, organization of the cytoskeleton and cell motility, intracellular vesicle formation and trafficking as well as nucleo-cytoplasmic transport [150]. Fast growing tumor cells have a higher consumption of those intermediate products for reproduction of the cytoskeleton due to increased cell cycle activity and permanent cell division. In line with our observations shown in 3.3.4, statins have also been shown to affect prostate cancer cells by inhibition of prenylation [166]. On the other hand, these findings implicate that necessary regenerative processes within the liver might also be subject to statin toxicity. Therefore, only statins with high selectivity for tumor cells, i.e. FLV, SMV or LOV might be useful in supplementation of HCC treatment.

A reason for reduced proliferation and susceptibility of Huh7 cells towards statins might be their enhanced content of the tumor suppressor protein p53 as reported in 3.3.3 Figure 36A. Compared to HepG2 or Hep3B, Huh7 hepatoma cells accumulate p53 due to a block in its degradation [133] and show reduced cellular proliferation, also mentioned in 3.3.2. As shown in 3.3.3, the ability of statins to induce apoptosis in hepatoma cell lines directly correlated with the levels of p53. The p53 knockout line Hep3B was found to be most sensitive, while Huh7 cells did

not respond to statin incubation at concentrations achievable in patients (the maximum daily dose of FLV for a patient would correspond to about 30 µM *in vitro*). In Huh7 cells a knockdown of p53 by about 50% increased statin effects significantly (3.3.3 Figure 37), indicating that there seems to be a certain threshold level of p53 for sensitivity of dividing hepatoma cells, which is higher than levels observed in wild type hepatoma cells. This implicates that only HCC with a particularly high expression or availability of p53 might be protected against statin toxicity, which is a rare event and supports the potential use of statins in HCC therapy. Nevertheless, the p53 pathway seems to play a role in the mechanism of apoptosis induction by statins.

G1/S cell cycle arrest in human HCC cell lines has been proposed as a mechanism for statins-induced apoptosis [167]. Regarding the balance between tolerable statin concentrations for primary cells and a certain necessity for cell proliferation to achieve statin toxicity it has to be evaluated if repeated cycles rather than high doses of statin treatment might have a more beneficial impact on tumor regression rates. It also has to be evaluated if the combination of statins with antiproliferative tumor therapy is recommendable.

4.4 Conclusion

The multifunctional enzyme HO-1 has became a very interesting subject of consideration during the last years caused by exerting effects against occurrence of apoptosis and inflammation both *in vitro* and *in vivo*. We aimed to characterize and evaluate some of these properties in chronic HCV infection regarding HO-1 induction as possible support for current antiviral therapy or even as usable tool to elucidate the mechanism of pathogenesis.

In consequence we reported that the enhancement of the antioxidant enzyme HO-1 in human hepatocytes not only provoked significant suppression of viral replication, but moreover mitigated the pro-inflammatory cytokine milieu in HCV-infected livers. The observed synergistic antiviral effects hinted at a potential role for HO-1 in antiviral therapy, while providing therapeutic protection of the hepatocytes from HCV-mediated hepatocellular injury. It has to be taken into account that HO-1 induction in human hepatocytes and suppression of HCV occurred in a polymorphism-dependent manner. However, the ability to decrease the pro-inflammatory cytokine milieu in infected livers remained comparable.

Interestingly, the *in vitro* studies related the statin-mediated effects on HCV replication pointed out a requirement of HO-1-induction, which is more pronounced in a microenvironment resembling fibrotic liver tissue. This implicates that certain statins might be especially useful to support HCV therapy of patients at advanced stages of liver disease.

With regard to clinical manifestation of HCV infection culminating in hepatocellular carcinoma, a related issue concerning the properties of statin to exert anticancer effects was also elucidated in this report. While this investigation we indentify profound differences in the susceptibility of human hepatoma cell lines towards

statin incubation, pointing to the fact that not every HCC patient might benefit from statin therapy. Besides inhibiting cholesterol biosynthesis, statins deprive hepatocytes of intermediate products necessary for modifications and activation of important celluar regulatory proteins. Tumor cells with high division rates have higher consumption of these intermediate products. Therefore, only statins that showed a high selectivity to tumor cells might be useful in HCC therapy. Here we found that the ability of statins to induce apoptosis in hepatoma cell lines directly correlated with their expression of p53. The report implicates that HCCs with high p53 expression level might be protected against statin toxicity.

5. Abstract

Enhancement of host antioxidant enzymes, such as heme oxigenase-1 (HO-1), may attenuate hepatocyte injury. Besides displaying hepatoprotective effects, the induction of heme oxygenase-1 (HO-1) by cobalt-protoporphyrin-IX (CoPP) administration or the application of its heme degradation product biliverdin (BV) were shown to interfere with HCV replication *in vitro*. Moreover, $(GT)_n$-repeats length in the polymorphic region of the HO-1 promoter may affect its responsiveness to oxidative stresses. We found CoPP-mediated HO-1-induction is accompanied with significant viremia reduction, whereby these changes were shown to depend from the length of the HO-1 promoter (medium = Δ 0.5 log HCV reduction; short = Δ 2 log), whereas BV-mediated antiviral effects were not (Δ 1 log with m- or s-polymorphisms). Moreover, even stronger viremia suppression (3 log) was observed when CoPP and PEG-IFNα were given in combination versus PEG-IFNα as monotherapy (1 log). On the other hand, mechanisms and contribution of the statins-mediated HO-1-induction, the following interferences with HCV replication, as well as statin-mediated effects on cell viability of primary mouse and human hepatocytes, as of mouse (Hepa1-6) and human (Huh7, HepG2) hepatoma cell lines, were elucidated *in vitro*. On the other hand, mechanisms and contribution of the statins-mediated HO-1-induction, the following interferences with HCV replication, as well as statin-mediated effects on cell viability of primary mouse and human hepatocytes, as of mouse (Hepa1-6) and human (Huh7, HepG2) hepatoma cell lines, were elucidated *in vitro.* Statin-mediated effects on HCV replication seem to require HO-1-induction, which is more pronounced in a microenvironment resembling fibrotic liver tissue.

This implicates that certain statins might be especially useful to support HCV therapy of patients at advanced stages of liver disease. Furthermore the results present here indicate that fluva-, simva- and lovastatin show selective cytotoxic effects on hepatoma cells in comparison to primary hepatocytes. The anti-tumor effect of statins did not depend on a lack of cholesterol production, but was restored by supplementation of mevalonate or geranyl-geranyl pyrophosphate, prerequisites for prenylation of small G proteins. Additionally, our results implicate that anti-tumor activity of statins requires cell proliferation and is reduced by p53 overexpression.

6. Publications

Presentations on international and national liver congress		
GASL Kongress	2012 / 2013 / 2014	(poster Presentation)
ESAL Kongress	2013/ 2014	(poster Presentation)
AASLD Kongress	2013	(poster Presentation)
DGVS Kongress	2013	(oral presentation)

Articles in press

Selective induction of apoptosis by HMG-CoA reductase inhibitors in hepatoma cells and dependence on p53 expression.
Kah J, Wüstenberg A, Keller AD, Sirma H, Montalbano R et al. **(2012). Oncol Rep** 28: 1077-1083.

Matrix conditions and 1 KLF2-dependent induction of heme oxygenase-1 modulate inhibition of HCV replication by Fluvastatin.
Wuestenberg A*, **Kah J***, Singethan K, Sirma H, Keller AD, Schrader J, Loscher C ,Volz T, Bartenschlager R, Lohmann V, Protzer U, Dandri M, Lohse AW, Tiegs G, and Sass G. **(2014) PlosOne: [online 6 may]**
* both authors contributed equally to this work

Binding of Hepatitis B Virus to its cellular receptor alters the expression profile of genes of the bile acid metabolism in mice
Oehler N,* Volz T,* Bhadra OD, **Kah J**, Allweiss L, Giersch K, Bierwolf J, Riecken K, Pollok JM, Lohse AW, Fehse B, Petersen J, Urban S, Lütgehetmann M, Heeren J and Dandri M.**(2014) Hepatology [currently under author proof]**

7. List of figures

8. list of Tables

9. list of abbreviation

BV	bilverdin
CD	cluster of differentiation
CHD	coronary heart disease
CoPP	Cobalt-protoporphyrin-IX
COX	cytochrome c oxidase subunit
C-protein	HCV core protein
CRV	cervastatin
CYP	Cytochrome P-450
DAA	direct antiviral acting
DC-SIGN	DCcellspecific-intercellular-adhesion-molecule3grabbing-nonintegrin
EGFR	epidermal growth factor receptor
E-protein	HCV envelope protein
ER	endoplasmatic reticulum
FCS	fetal calf serum
FLV	fluvastatin
FZD	frizzled family factor
GAGs	glycosaminoglycans
GGPP	geranylgeranyl-pyrophosphate
HA1100	Hydroxyfasudil
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDL-C	high-density lipoprotein cholesterol
HIV	human immunodeficiency virus HLA
HMG-CoA	hydroxymethylglutaryl-coenzyme A

HO-1	heme oyxgenase 1
HSA	human serum Albumin
Hsp32	heat shock protein 32
IL	interleukin
IP10	interferon gamma-induced protein 10
IRES	internal ribosome entry site
IRF	interferon response factor
ISG	interferon stimulated gene
Kb	kilo base
KLF2	Krueppel-like factor 2
LD	lipid droplet
LDL	low-density lipoproteins
LDLR	low-density lipoprotein receptor
LOV	lovastatin
L-SIGN	liver/lymph node-specific intercellular adhesion molecule- 3-grabbing integrin
MTT	3-4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MVLT	Mevalonate
Mx1	myxovirus resistence 1
MYC	myelocytomatosis oncogene
NADPH	Nicotinamidadenindinukleotidphosphat
NPC1L1	Niemann-Pick C1-like 1
Nrf2	NF-E2-related factor-2
NS	non-structural
ORF	open reading frame

PAA	polyacryamid
PAV	pitavastain
PBS	phosphate buffered serum
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEG-IFNα	pegylated interferon alpha
PH	primary hepatocytes
PKC	protein kinase C
PRV	pravastatin
Ras	resistance to audiogenic seizures
ROS	rosuvastatin
RT	reverse transcription
RTKs	receptor tyrosine kinases
SMV	simvastatin
SRB1	scavenger receptor class B type I
SREBP	sterol regulatory element-binding proteins
STAT	signal transducer and activator of transcription
StRE	stress response element
SVR	sustained viral response
TGFbeta1	transforming growth factor beta 1
TLR	toll-like receptor
uPA	urokinase-type plasminogen activator
UTR	untranslated region
VLDL	very low-density lipoproteins
Wnt	wingless-related MMTV integration site

10. Curriculum Vitae

Persönliche Angaben

Name	Janine Kah
Geburtsdatum und – Ort	01.07.1983 in Wernigerode
Staatsangehörigkeit	deutsch
Familienstand	ledig

Schulische Ausbildung und Studium

07/1990 – 06/1994	Grundschule Schierke am Brocken
08/1994 – 07/2003	Hochharz Gymnasium Elbingerode **Abschluss:** Abitur
10/2003 – 06/2010	Universität Greifswald Diplomstudiengang Biologie **Vertiefung:** Genetik / Virologie / Biochemie **Abschluss:** Diplom
08/2009 - 06/2010	Anfertigung der Abschlussarbeit am HZI Braunschweig **Thema:** Zellspezifische Genexpression nach Infektion mit dem Influenza-A-Virus
01/2011 - 06/2014	Wissenschaftliche Angestellte am UKE Anfertigung der Promotion im Bereich der Hepatologie **Thema:** Charakterisierung HO-1 basierender Therapieoptionen in chronisch persistierender HCV Infektion

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13. Statement

Hiermit versichere ich an Eides Statt, dass ich die vorliegende Arbeit eigenständig und ohne fremde Hilfe verfasst, keine Quelle als die angegebenen benutzt und die den benutzen Werken wörtlich oder inhaltlich entnommen Stellen als solche kenntlich gemacht habe.

Die vorliegende Arbeit habe ich noch keiner anderen Universität vorgelegt, um ein Promotionsverfahren zu eröffnen.

Hamburg, den 24. April 2014

Janine Kah