UNIVERSITÉ DE STRASBOURG

ÉCOLE DOCTORALE 414

UMR_S 1110 Institut de Recherche sur les maladies virales et hépatiques

Thèse

présentée par

Katharina HERZOG

soutenue le 29 Octobre 2019

Strasbourg, France

Pour obtenir le grade de : **Docteur de l'Université de Strasbourg**

Discipline/Spécialité : Sciences de la Vie et de la Santé

Aspects moléculaires et cellulaires de la Biologie

Impact of OGT on late steps of the hepatitis C virus replication cycle

THÈSE dirigée par :

Dr. Mirjam B. ZEISEL

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AUTRE MEMBRE DU JURY :

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Success

He has achieved success

Who has lived well, laughed often, and loved much;

Who has enjoyed the trust of pure women, the respect of intelligent men and the love of little children;

Who has filled his niche and accomplished his task;

Who has left the world better then he found it whether by an improved poppy, a perfect poem or a rescued soul;

Who has never lacked appreciation of Earth's beauty or failed to express it;

Who has always looked for the best in others and given them the best he had;

Whose life was an inspiration;

Whose memory a benediction.

[Bessie Anderson Stanley]

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Table of Contents

List of Figures

List of Tables

ABBREVIATIONS

aa	Amino acids
ApoB	Apolipoprotein B
ApoC	Apolipoprotein C
ApoE	Apolipoprotein E
CD81	Cluster of differentiation 81
CLDN1	Claudin-1
DAA	Direct-acting antiviral
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
gt	Genotype
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVcc	Cell culture-derived HCV
HCVpp	HCV pseudoparticles
HDL	High density lipoprotein
HIV	Human Immunodeficiency Virus
Huh7	Human hepatoma 7
INF-α	Interferon-alpha
INF-γ	Interferon-gamma
IRES	Internal ribosome entry site
JFH1	Japanese Fulminant Hepatitis I
kb	Kilobase
kDa	Kilodalton
LD	Lipid droplet
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LPL	Lipoprotein lipase
LVP	Lipoviral particle

miR	micro-RNA
MLV	Murine leukemia virus
mRNA	messenger RNA
MTP	Microsomal triglyceride transfer protein
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
ncRNA	non-coding RNA
OGA	β-N-acetylglucosaminidase
OGT	O-linked N-acetylglucosamine transferase
OCLN	Occludin
PHH	Primary human hepatocytes
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
RNAi	RNA interference
SCID	severe combined immunodeficiency
siRNA	small interfering RNA
SR-BI	Scavenger receptor class B type I
$TCID_{50}$	50% tissue culture infectious dose
TJ	Tight junction
TLR	Triglyceride-rich lipoprotein
uPA	urokinase plasminogen activator
UTR	Untranslated region
VLDL	Very low-density lipoprotein

I. HEPATITIS C – An Introduction

Hepatitis C is an infectious liver disease caused by the hepatitis C virus (HCV) that in most subjects chronically infects the human liver. HCV infection is one of the major etiologies, besides hepatitis B virus (HBV) infection, alcoholic liver disease (ALD) and non-alcoholic fatty liver disease, resulting in chronic hepatitis and progressive liver disease and thereby leading to development of lethal complications, i.e. cirrhosis and hepatocellular carcinoma (HCC), the second leading cause of cancer mortality worldwide (El-Serag and Davila 2011; Ryerson, Eheman et al. 2016).

HCV itself is mainly transmitted through direct blood-to-blood contact occurring through medical treatment with unsterilized needles, blood transfusions and more recently through needle-sharing drug abuse, also through sexual contact and mother-to-child transmission. Upon its discovery in 1989 (Choo, Kuo et al. 1989), enormous progress has been made in the field to develop diagnostic and therapeutic strategies for HCV treatment. These efforts resulted in the development of highly efficient direct-acting antivirals (DAAs) targeting specific HCV proteins and enabling cure in more than 95% of infected individuals by achieving sustained virologic response (SVR) since 2013. Indeed, the combination of different DAAs resulted in cure rates over 96% regardless of the HCV genotype of patients and with a relative short duration of treatment (Bourliere, Pietri et al. 2018). Nevertheless, major clinical and scientific challenges still remain: DAA therapy is cost-intensive and still only available to a fraction of HCV-infected patients (Chung and Baumert 2014), some infected individual developed resistance to DAAs, and an urgently needed HCV vaccine to prevent the global spread of infection is still lacking due to the high diversity of the virus, limited models for testing vaccines and a partial knowledge of protective immune response (Bailey, Barnes et al. 2019). Of note, HCC risk remains high for decades even after SVR (Morgan, Baack et al. 2013; Kanwal, Kramer et al. 2019).

An estimated 71 million individuals are persistently infected with HCV with big geographical differences worldwide (WHO Global hepatitis report, 2017). The prevalence of chronic HCV infection is high, including developed countries as shown in Figure 1.

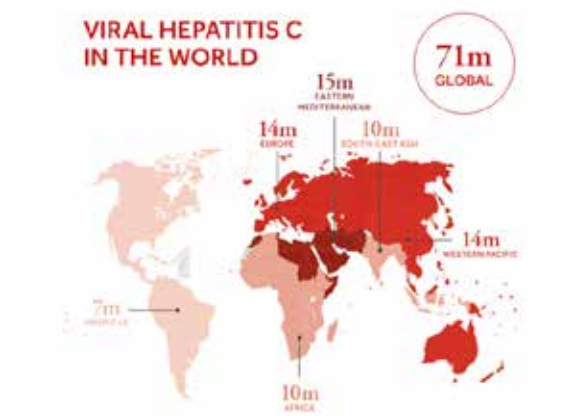

Figure 1. Estimated global HCV prevalence in 2015.
In general, there are variations in prevalence across and within countries. One could observe a higher affection of the European and Eastern Mediterranean regions by HCV infection (WHO Global Hepatitis Report, 2017).

1.1 Pathogenesis of HCV infection

Hepatitis C is caused by HCV infection of hepatocytes resulting in acute (20-30% of infected patients) or more often in chronic HCV infection (70-80% of infected patients) (reviewed in Zeisel, Cosset et al. 2008). The difference between both outcomes of infection is in principle pretty simple: Acute infections consequently lead to clearance of virus through an effective immune response mediated by HCV-specific neutralizing antibodies and T-cell responses, contrarily in chronic infections the immune system is not able to clear the virus resulting in high levels of viral replication and in dysfunctionality of the immune system (see Figure 2) (reviewed in Zeisel, Cosset et al. 2008; Park and Rehermann 2014).

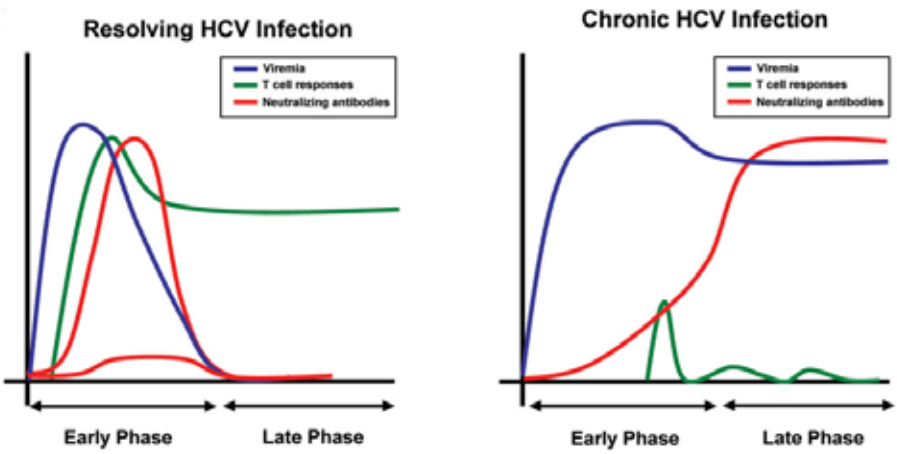

Figure 2. Acute (resolving) HCV infection versus chronic HCV infection.
While in resolving HCV infection the HCV RNA levels decrease due to effective immune responses through rapid induction of neutralizing antibodies and HCV-specific T-cell responses (left), while the contrary result can be observed during chronic HCV infection (right). (Zeisel, Cosset et al. 2008).

Obviously, chronic HCV predisposes to progressive fibrosis, cirrhosis and HCC either directly through changes of the cellular metabolism or indirectly as a result of persistent chronic inflammation.

Approximately 1-4% of patients with HCV-related cirrhosis develop HCC (NIH 2002) which is, per basic definition, a randomly occurring genomic and epigenomic change leading to an alteration of cellular gene expression and an abnormal proteome. Epigenetic and gene expression alterations observed in hepatitis C patients can be associated with high risk to HCC, even after the virus could be eradicated by treatment with DAAs (Hamdane, Juhling et al. 2019; Perez, Kaspi et al. 2019). Additionally to epigenetic changes , four pathways and biological processes can be involved in the progression of hepatocarcinogenesis: (i) oxidative stress pathways, (ii) p53 cell cycle pathways, (iii) PI3K/Akt/mTOR and MAPK pathways as well as (iv) WNT/β-catenin pathways (Zucman-Rossi, Villanueva et al. 2015).

1.2 HCV – Basic virology

1.2.1 Genomic organization of HCV RNA

HCV, a member of the *Flaviviridae* family, is an enveloped, single-stranded positive-sense RNA virus that is mainly restricted to hepatocytes. Its genome encompasses approximately 9.6 kb encoding a single polyprotein precursor of around 3000 amino acids (aa) and is flanked by 5'-and 3'-untranslated regions (UTRs). The internal ribosome entry site (IRES), located at the 5'UTR of the genome, mediates cap-independent translation of the polyprotein contributing to an efficient translation (Tsukiyama-Kohara, Iizuka et al. 1992; Wang, Sarnow et al. 1993). Moreover, miR-122, a crucial liver-enriched host factor, binds to two target sites close to the 5'UTR promoting HCV translation (Henke, Goergen et al. 2008) and replication (Jopling, Yi et al. 2005) by stabilizing HCV RNA (Shimakami, Yamane et al. 2012) (see Figure 3).

The synthesized polyprotein is co-and post-translationally processed by host and viral proteases into at least ten viral proteins: The three structural proteins Core, and the glycoproteins E1 and E2 building up the virion as well as the seven nonstructural (NS) protein mainly involved in replication and assembly through distinct enzymatic activities, namely p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (see Figure 3).

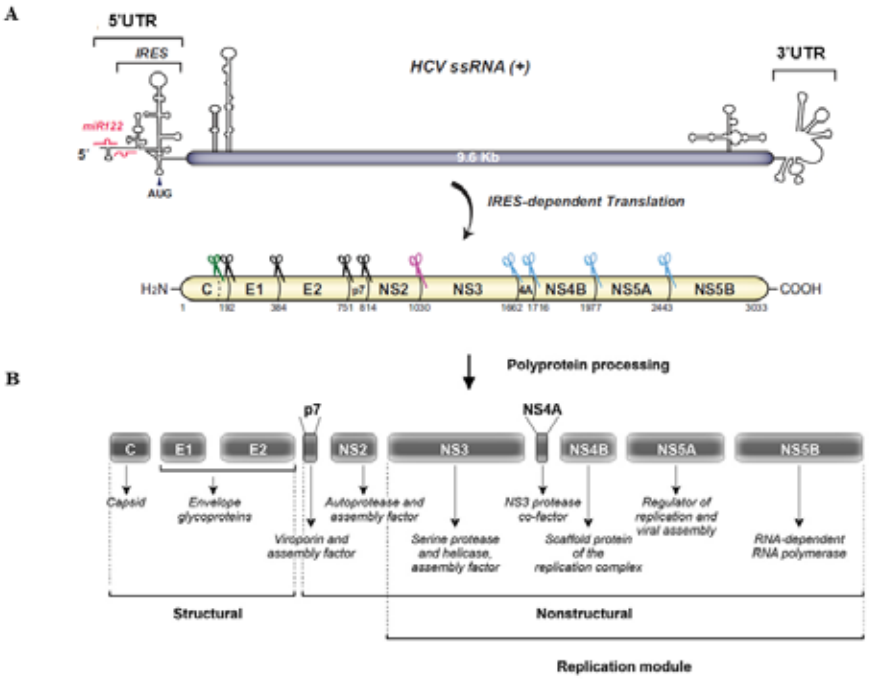

Figure 3. Overview of (A) HCV genome organization and IRES-dependent translation into polyprotein and (B) viral proteins and their function.
Scissors indicate proteolytic cleavage sites of the polyprotein. UTR: untranslated region; IRES: internal ribosome entry site; C: Core; NS: nonstructural (adapted from Dubuisson and Cosset 2014; Paul, Madan et al. 2014).

1.2.2 Genetic diversity of HCV – One challenge for vaccine design

HCV can be classified into seven genotypes and several subtypes demonstrating its high degree of genetic heterogeneity and thereby the challenges for research regarding efficient vaccine design: (i) HCV is able to rapidly mutate and (ii) to closely interact with the host lipid metabolism, both leading to escape from protective immune responses.

The genotypes (gt) are numbered from 1 to 7, whereas a variable number of sub-genotypes is designated with a lower case letter forming the basis of classifying HCV into genotypes such as 1a, 1b etc. (Simmonds, Bukh et al. 2005; Smith, Bukh et al. 2014). There is a designated distribution of all HCV strains worldwide: While genotype 1, the (from global perspective) most common genotype (almost half of all infections), dominates in Europe and the Americas, genotype 3, the second most common genotype (around 20-30% of infections), is mostly found in Asia and Northern Europe. Genotype 2 and 4 are less widespread but often found in North Africa and Middle East (around 10% of infections) (Gower, Estes et al. 2014).

1.2.3 HCV virion structure: The lipoviral particle

HCV is an enveloped virus that contains core proteins forming a nucleocapsid around the viral RNA genome which is surrounded by an endoplasmic reticulum (ER)-derived envelope with incorporated viral glycoproteins E1 and E2 which are involved in binding and entry into host cells (reviewed in Lindenbach 2013).

One outstanding characteristic of HCV particles is their tight link with the host cell lipid metabolism; even more their close association with host lipoproteins (Felmlee, Hafirassou et al. 2013; Lavie and Dubuisson 2017). To understand why one speaks of an important hallmark of the virus, it is absolutely essential to understand the process underlying the lipid homeostasis taking place in the liver: Lipids (e.g. triacylglycerides) are synthesized within the liver and their transport through aqueous medium (blood) is enabled through binding with proteins. A process in which triacylglycerides are pooled together with cholesterol and a variable number of proteins into hydrophilic lipoprotein complexes which can be subdivided upon their density into five groups: Chylomicrons (mostly generated in the intestine), very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). The association of lipids with these so-called apolipoproteins (Apo) is not only important for the transport of lipids through the organism to their organs of need but also for the uptake process into cells through specific membrane receptors.

In the process of VLDL assembly, ApoB and microsomal triglyceride transfer protein (MTP) are required: After synthesis and translocation into the rough ER lumen (rER), ApoB is charged with phospholipids and cholesterol via MTP leading to formation of a neutral lipid core that is transformed into a sphere-shaped particle. This so called VLDL2 contains exchangeable ApoE and ApoC and can be released after movement to a distal compartment in the secretory pathway. Alternatively, luminal lipid droplets (luLDs) are formed as second precursors at the smooth ER (sER) following triglyceride enrichment via MTP and close association with ApoE but not with ApoB. Fusion of VLDL2 with this second precursors is leading to a triglyceride-rich lipoprotein (TLR), namely VLDL1 (reviewed in Olofsson, Bostrom et al. 2009; Shelness and Sellers 2001. Due to the differences in density, lipoproteins can be separated via ultracentrifugation.

To come back to HCV, the virus itself hijacks the host cell lipid metabolism, more particularly, parts of the VLDL and LDL secretion pathway for production of infectious virions. In fact, infectious viral particles could be found in patient-derived serum associated with VLDLs or LDLs forming a so-called lipoviral particle (LVP) (Thomssen, Bonk et al. 1992; Andre, Komurian-Pradel et al. 2002; Nielsen, Bassendine et al. 2006). As a consequence, LVPs show distinct biophysical properties than VLDLs or LDLs: (i) obviously, LVPs are rich in cholesterol and triacylglycerides displaying (very) low density, and (ii) are containing apolipoproteins (e.g. ApoB, ApoA, ApoE and ApoC) and more interestingly, (iii) patient-derived HCV particles differ in density between 1.25g/ml to below 1.06g/ml with infectivity inversely correlated to density meaning that low-density viruses are more infectious. In fact, infectious LVPs have a density between 1.03 to 1.10g/ml and thus can be separated as well as lipoproteins via density gradient ultracentrifugation (Gastaminza, Cheng et al. 2008; Piver, Boyer et al. 2017; Catanese, Uryu et al. 2013; Meunier, Russell et al. 2008) (see Figure 4). Findings that could be confirmed using *in vivo* (Nielsen, Bassendine et al. 2006; Thomssen, Bonk et al. 1992; Andre, Komurian-Pradel et al. 2002) as well as *in vitro* (Gastaminza, Kapadia et al. 2006; Lindenbach, Meuleman et al. 2006; Merz, Long et al. 2011) model systems. Interestingly, recent evidence suggests that HCV LVPs contain several exchangeable ApoE and one non-exchangeable ApoB molecule (Piver, Boyer et al. 2017).

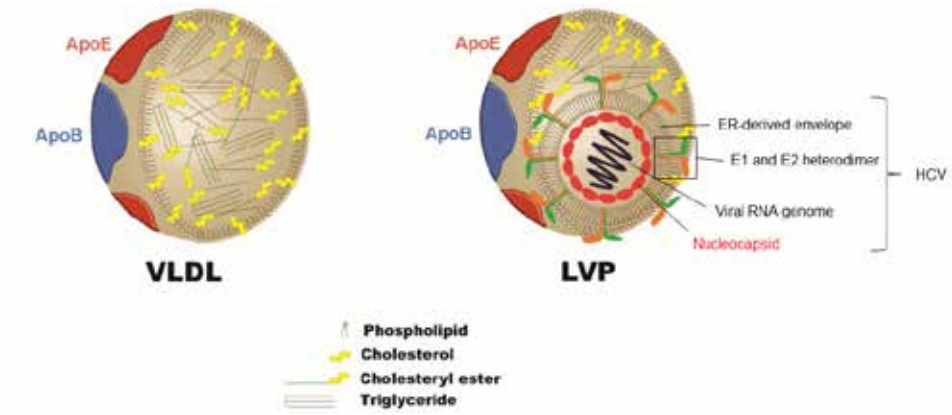

Figure 4. Infectious LVP versus VLDL.
Infectious HCV particles (LVP, right) are composed of ApoE and ApoB, which are also part of very-low-density lipoproteins (VLDL, left), and viral components. Consequently LVPs share common features with VLDLs (Felmlee, Hafirassou et al. 2013).

Consequently, through the association with VLDLs or LDLs and different kinds and amounts of apolipoproteins, HCV particles are heterogeneous and thus vary in shape and size ranging from 40 to 80 nm in diameter.

In fact, the association with ApoB and ApoE does not only lead to the masking of viral epitopes preventing the virus to be neutralized by HCV-specific antibodies (Catanese, Uryu et al. 2013; Merz, Long et al. 2011; Fauvelle, Felmlee et al. 2016) but even more this association helps to facilitate HCV entry into hepatocytes (reviewed in Zeisel, Felmlee et al. 2013).

1.2.4 The HCV life cycle

a. Virion attachment and entry

The initial step of the HCV life cycle is its attachment and entry into hepatocytes, a complex, multistep process involving both viral envelope glycoproteins E1/E2 and lipoprotein components which makes it difficult to entirely clear the exact sum of events and details that occur during this first step of the life cycle. The process itself can be subdivided into three key steps: (i) viral attachment to the hepatocyte, (ii) receptor-mediated endocytosis, and (iii) endosomal fusion.

Since LVPs are transported via bloodstream, initial attachment occurs at the basolateral membrane of the hepatocytes through binding of virion-associated ApoE to cell surface heparan sulfate proteoglycan (HSPGs) syndecan-1 or syndecan-4 and low-density lipoproteins receptor (LDLR) (Jiang, Cun et al. 2012; Shi, Jiang et al. 2013; Lefevre, Felmlee et al. 2014; Xu, Martinez et al. 2015; Grigorov, Reungoat et al. 2017).

Four main host-derived entry factors have been described, namely tetraspanin CD81 (Pileri, Uematsu et al. 1998), scavenger receptor BI (SR-BI) (Scarselli, Ansuini et al. 2002), and the tight junction proteins Claudin 1 (CLDN1) (Evans, von Hahn et al. 2007) and Occludin (OCLN) (Ploss, Evans et al. 2009), as well as numerous cofactors, especially two receptor tyrosine kinases, epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2) (Lupberger, Zeisel et al. 2011). Interactions of virion-associated ApoB-100 with SR-BI is proposed to induce lipoprotein-HCV dissociation (Scarselli, Ansuini et al. 2002; Dreux, Dao Thi et al. 2009; Maillard, Huby et al. 2006) leading to direct interaction of HCV glycoprotein E2 with SR-BI and CD81 (Dao Thi, Granier et al. 2012; Scarselli, Ansuini et al. 2002; Pileri, Uematsu et al. 1998; Bartosch, Vitelli et al. 2003). At this stage of HCV entry, additional entry factors are required: CLDN1 and OCLN (Evans, von Hahn et al. 2007; Ploss, Evans et al. 2009; Liu, Yang, Shen et al. 2009). Two models have been proposed. Recently, imaging-based studies in a three-dimensional polarized hepatoma system reported that an initial co-localization of HCV with SR-BI, CD81 and EGFR at the basolateral membrane occurs, leading to trafficking and accumulation of HCV virions at the tight junctions, where the interaction with CLDN1 and OCLN takes place (Baktash, Madhav et al. 2018). Furthermore, HCV may also interact with CLDN1 on the basolateral membrane of hepatocytes. Indeed, although CLDN1 and OCLN are classified as tight junction proteins, a minority of these proteins can be found on the basolateral membrane (reviewed in Zeisel, Dhawan et al. 2018). Using a CLDN1-targeting monoclonal antibody (mAb) and confocal microscopy, Mailly et al could observe a minority pool of CLDN1 found on basolateral membrane of hepatocytes in chimeric mouse liver as well as in normal liver tissue (Mailly, Xiao et al. 2015). Interestingly, fluorescence resonance energy transfer-based studies could show that using CLDN1-sprecific mAb leads to the perturbation of CD81-CLDN1 co-receptor formation at hepatocyte basolateral membrane and inhibition of HCV entry (Mailly, Xiao et al. 2015; Fofana, Krieger et al. 2010).

Host cell kinases have been shown to contribute to the regulation of viral entry by promoting co-receptor association between CD81 and CLDN1 which is essential for HCV entry (Harris, Farquhar et al. 2008; Harris, Davis et al. 2010; Farquhar, Harris et al. 2008; Lupberger, Zeisel et al. 2011). Indeed, inhibition of EGFR and EphA2 via the protein kinase inhibitors erlotinib and dasatinib, respectively, led to disruption of CD81-CLDN1 co-receptor formation resulting in inhibition of HCV entry (Lupberger, Zeisel et al. 2011). These results propose the direct contribution of EGFR and EphA2 in CD81-CLDN1 co-receptor complex (Lupberger, Zeisel et al. 2011) and moreover, the identification of HRas, a GTPase acting downstream of EGFR signaling, and its association with CD81 and CLDN1 supports the model that kinase signaling pathways play a role in this formation process (Zona, Lupberger et al. 2013). In fact, CD81-CLDN1 complex formation could also been disrupted using a protein kinase A inhibitor leading to an intracellular localization of CLDN1 and

consequently to an impaired viral entry (Farquhar, Harris et al. 2008). It could also be shown that binding to CD81 triggers the autophosphorylation of EGFR (Diao, Pantua et al. 2012), resulting in basolateral diffusion of CD81; which in turn leads to an association with CLDN1 and the formation of the CD81-CLDN1 co-receptor complex (Harris, Farquhar et al. 2008; Harris, Davis et al. 2010). In contrast to CD81, SR-BI and CLDN1, the role of OCLN in the viral entry process has been less well studied. Nonetheless, one group was able to create mutants of OCLN proteins blocking HCV cell entry via specific antibodies. It could be demonstrated that OCLN is required in late steps of HCV entry and may be directly interacting with HCV virions *in vitro* (Sourisseau, Michta et al. 2013). Indeed, Shimizu et al. were able to develop functional mAbs directed against extracellular domains of OCLN confirming that OCLN is required in late steps of HCV entry, and inhibition of OCLN via mAbs resulted in inhibition of HCV infection *in vitro* and *in vivo*. Moreover, by using these mAbs HCV cell-free and cell-to-cell transmission was efficiently blocked (Shimizu, Shirasago et al. 2018). CD81-CLDN1 bound HCV virions are internalized via clathrin- and dynamin-dependent endocytosis (Blanchard, Belouzard et al. 2006; Farquhar, Hu et al. 2012), a process in which EGFR-signaling appears to be required (Baktash, Madhav et al. 2018).

These processes describe the complex mechanism of cell-free HCV entry, while an alternative route of HCV entry is cell-cell transmission (Timpe, Stamataki et al. 2008; Brimacombe, Grove et al. 2011). In contrast to cell-free HCV entry, HCV cell-cell transmission is insensitive to most neutralizing antibodies and thus represents the main mode of viral spread. There is an overlap of host factors required for both entry routes; however, while cell-free HCV entry is strictly dependent on CD81 interactions, cell-cell transmission can also occur in a CD81-independent mode of action (Witteveldt, Evans et al. 2009; Jones, Catanese et al. 2010). Consequently, HCV-infected hepatocytes are found in discrete clusters inside the liver in an abundance of one to fifty-four percentage of all hepatocytes (Wieland, Makowska et al. 2014).

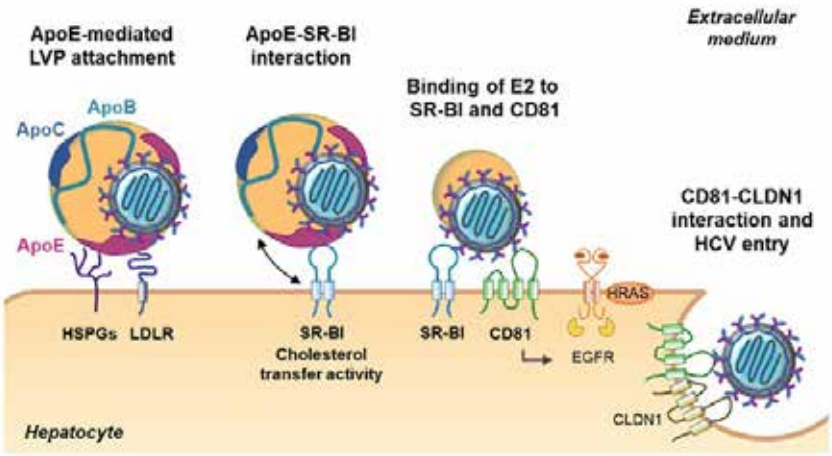

Figure 5. HCV attachment and entry into hepatocytes (Wrensch, Crouchet et al. 2018).

Membrane fusion is mediated by viral glycoproteins E1 and E2, after acidification of HCV-bearing endosomes leading to uncoating and cytoplasmic release of the genome (Douam, Dao Thi et al. 2014; Lavillette, Bartosch et al. 2006; Sharma, Mateu et al. 2011).

b. Genome translation and co-translational processing

Upon its release into the cytoplasm, the positive-strand HCV RNA genome directly serves as a template for viral polyprotein synthesis at the rough ER. Basically, a polyprotein precursor of 3000 aa in length is synthesized and co-and post-translationally cleaved by cellular (e.g. signal peptidases) and viral proteases (NS2, NS3-4A heterodimer) into 10 mature viral proteins.

In contrast to eukaryotic messenger RNA (mRNA), HCV RNA genome lacks a 5'-terminal cap and a 3'-terminal poly(A) tail. To overcome this genetic differences, the 5'UTR shows some special structural finesse: (i) the presence of a functional IRES within the 5'UTR of the viral genome allows to anchor in the ribosome and ensure cap-independent translation initiation (Lukavsky 2009), and (ii) the direct targeting of liver-enriched miR-122, on two target sites within the 5'UTR as well as three additional target sites in the coding region and the 3'UTR, stabilizes HCV RNA and contributes to HCV translation and replication (Niepmann, Shalamova et al. 2018; Jopling, Yi et al. 2005); even more, this interaction prevents degradation of the viral genome by host degradation machinery through exo -and endonucleases (reviewed in Li, Yamane et al. 2015). miRNAs are a class of small non-coding RNA molecules that normally target specific mRNA by base-pairing with a complementary site typically located at the 3'UTR, thus post-transcriptionally regulating gene expression (Saliminejad, Khorram Khorshid et al. 2019). In fact, miR-122 acts completely contrary to normal destabilizing actions of miRNAs on host mRNAs by the binding and stabilization of the 5'UTR of HCV RNA genome and thus promoting viral replication and persistence (reviewed in Li, Yamane et al. 2015).

After initiation of translation, protein synthesis is blocked by a signal sequence between core and E1 targeting the ribosome to the translocon complex of the ER where translation proceeds. The nascent polypeptide is cleaved by the signal peptidases of the ER and the 191 aa core precursor is released into the cytosolic side of the ER (Santolini, Migliaccio et al. 1994) and further processed into the mature core protein of 21 kDa size by an ER-residing cellular protease (McLauchlan, Lemberg et al. 2002). E1 and E2 are the next viral proteins that are released from the polyprotein precursor after cleavage by host signal peptidases, both containing a large N-terminal ectodomain and a C-terminal hydrophobic anchor (Dubuisson 2000). E1 and E2 assemble to form a non-covalent heterodimer (Dubuisson 2000); additionally, the C-termini of both glycoproteins are co-translationally integrated

and anchored into the ER membrane resulting in protruding of their ectodomains into the ER lumen (Cocquerel, Op de Beeck et al. 2002). NS2 is able to release itself from the polyprotein by its autocatalytic activity (Grakoui, McCourt et al. 1993), while the remaining nonstructural proteins are cleaved by NS3-4A protease (Tomei, Failla et al. 1993). All nonstructural viral proteins are anchored within the ER and oriented towards the cytosolic side.

Of note, one could see miR-122 as well as the presence and expression rates of the HCV entry factors (see p.7) and the close association with host-derived lipoproteins (see p.5) as possible reasons explaining the strict hepatotropism of HCV (Dubuisson and Cosset 2014).

c. Genome replication

Importantly, the HCV genome does not integrate into the host genome and therefore continuous replication of the viral genome is required for the maintenance of chronic infection. After processing of the viral proteins, the nonstructural proteins NS3 to NS5B induce distinct membrane alterations that contain the sites of viral RNA replication.

Through this enormous rearrangements of intracellular ER membranes, the HCV replication complex, also known as membranous web, is formed by activity of viral NS4B (Gouttenoire, Penin et al. 2010) and of the cellular lipid kinase phosphatidylinositol-4-kinase (PI4Kα), whose lipid kinase activity is initiated by interaction with NS5A and NS5B (Paul, Hoppe et al. 2013; Ferraris, Beaumont et al. 2013; Reiss, Rebhan et al. 2011). Additionally, the activated replication machinery also requires NS4A acting as a co-factor forming a heterodimer with NS3 and triggering NS3 protease function (Bartenschlager, Lohmann et al. 1995), and host factor cyclophilin A (CyPA) (Liu, Yang, Robotham et al. 2009; Kaul, Stauffer et al. 2009). Of note, the viral RNA-dependent RNA polymerase, NS5B, plays the key role in HCV RNA synthesis (Lohmann, Korner et al. 1997; Behrens, Tomei et al. 1996). The membranous web is a typical morphological feature of positive-strand RNA viruses (Miller and Krijnse-Locker 2008), and can be visualized via electron microscopy of HCV-infected hepatocytes, often in close proximity with lipid droplets (LDs) indicating the tight link of HCV with host cell lipid metabolism (see Figure 6); indeed HCV core (McLauchlan, Lemberg et al. 2002) and NS5A (Shi, Polyak et al. 2002) could be found in association with LDs implicating a possibly key role in coordination of viral replication and virion morphogenesis (Bartenschlager, Penin et al. 2011) (see p.13).

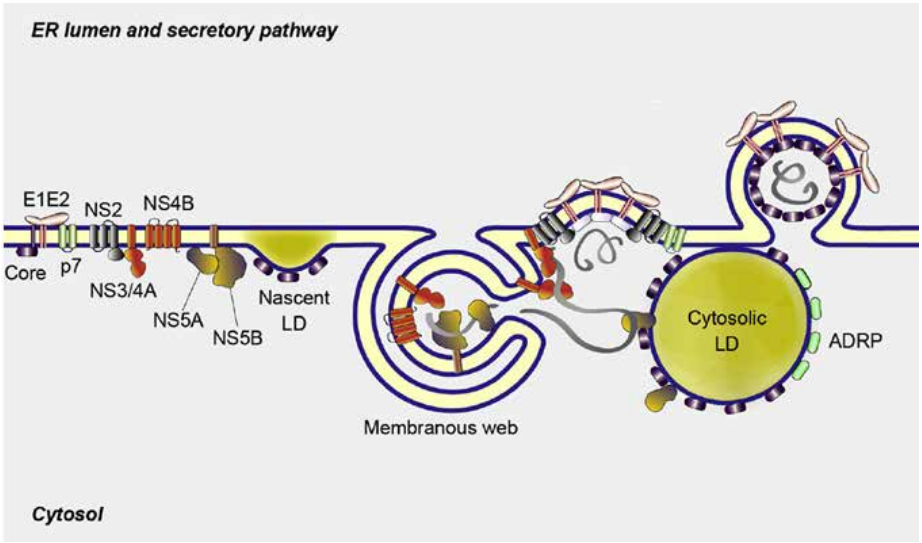

Figure 6. HCV replication and formation of the membranous web (adapted from Dubuisson and Cosset 2014).

In the primer-independent initiation step of RNA replication, a negative-strand of the genome is generated serving as template for progeny positive-strand viral genomes. After formation of a dinucleotide that acts as a primer, elongation of the nascent negative-strand RNA chain occurs mediated by NS5B proceeding in a 3'-to-5' direction resulting in a positive-strand RNA molecule without the help of other factors (Lohmann 2013). About termination only little is known, but one can imagine that the polymerase dissociates from the template after reaching its end. Of note, the polymerase NS5B does not work properly, but rather error-prone resulting in high genetic variability of HCV isolates. The newly generated positive-strand RNA copies either serve as template for continuing viral protein synthesis or move to LDs resulting in assembly of progeny virions or they remain within the membranous web undergoing negative-strand RNA synthesis for replication (Lohmann 2013). HCV is able to create an environment conducive to its replication and assembly: the formation of the membranous web is one example. Moreover, the ratio of neutral to membrane lipids is reduced upon HCV infection and membrane lipids as cholesterol and phospholipids were gathered in microsomal fractions of HCV-infected cells (Hofmann, Krajewski et al. 2018). Additionally, HCV seems to recruit various cytoplasmic nuclear pore complexes (Nups) to site of replication where they could be found in increased numbers and accumulated at the membranous web and even more co-localized with core or NS5A (Levin, Neufeldt et al. 2014; Neufeldt, Joyce et al. 2013). It is proposed that cytoplasmic Nups form channels across the double membrane structures of the membranous web to serve as gatekeepers by facilitating movement of several HCV proteins and host proteins without being recognized by the pattern recognition receptors (PRRs) of the innate immune system (see Figure 7) (Levin, Neufeldt et al. 2014; Neufeldt, Joyce et al. 2013). Upon Dengue and hepatitis A virus (HAV) infection, a similar redistribution of cytoplasmic Nups could be observed

implicating a possible conserved role of cytoplasmic Nups upon positive-strand RNA virus infections (Neufeldt, Joyce et al. 2013).

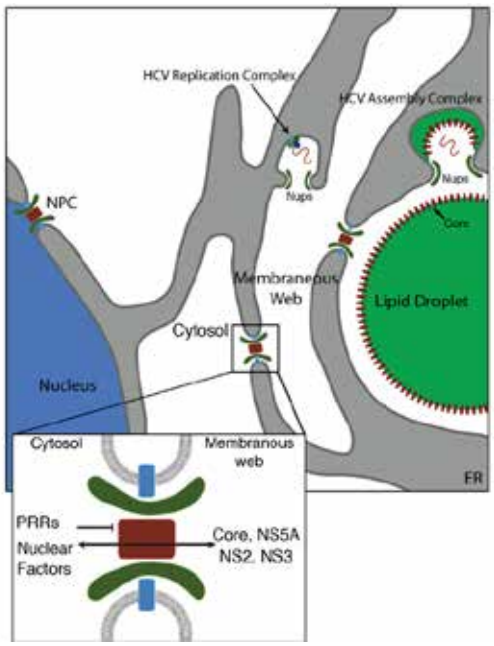

Figure 7. Potential function of the cytosolic nuclear pore complexes in HCV replication and assembly (Neufeldt, Joyce et al. 2013).

d. Viral assembly and egress

After replication of the viral genome, RNA progeny can be used for encapsulation into new virions which can be released using a noncytolytic pathway related to the VLDL secretory pathway (Chang, Jiang et al. 2007; Jiang and Luo 2009; Gastaminza, Cheng et al. 2008; Huang, Sun et al. 2007). Several viral and host proteins are involved in the viral assembly process that can be divided into an early and late phase of assembly, whereupon detailed information on different steps is still lacking. During the early phase, the nucleocapsid is formed by the involvement of different recruitment processes with the aim to localize in close proximity to LDs and to the assembly site in the ER lumen: (i) Shuttle between HCV core protein and LDs , (ii) movement of the replication complex through action of NS5A, (iii) recruitment of glycoproteins E1 and E2 by interaction with NS2 (reviewed in Popescu, Riva et al. 2014).

HCV core protein attaches to LDs through its hydrophobic domain, replacing adipose differentiation-related protein (ADRP, Figure 6) leading to an accumulation of LDs in perinuclear regions (Boulant, Douglas et al. 2008; Boulant, Montserret et al. 2006). In terms of assembly efficiency, core-LD association is a crucial step resulting in downstream recruitment of viral proteins (Miyanari, Atsuzawa et al. 2007). However, an inverse correlation of core-LD motility and assembly efficacy of

HCV was determined implicating that core is see-sawing from LD-ER interface to the ER or *vice-versa* (Shavinskaya, Boulant et al. 2007). Diacylglycerol acyltransferase 1 (DGAT1) is the key host factor involved in this step of assembly by facilitating recruitment of core onto the LD by direct core-DGAT1 interaction (Herker, Harris et al. 2010).
Since HCV RNA replication takes plays within the membranous web, two possible models are proposed where assembly starts at ER membrane or at the surface of cytosolic LDs (cLDs): (1) Transfer of HCV core protein onto the surface of cLDs and re-recruitment to ER membrane at assembly sites where interaction of core with NS5A occurs concluding that cLDs might serve as trafficking vehicles to transport core from sites of translation and replication to sites of viral assembly; or (2) Initiation of nucleocapsid formation occurs on the surface of cLDs, while distributions of HCV RNA to core protein is mediated by NS5A that is colocalized onto surface of cLDs. Currently, discrimination between both hypotheses is not easy by considering the low assembly efficiency of HCV per cell (reviewed in Bartenschlager, Penin et al. 2011).

NS5A as well as DGAT1 seem to play a central role in the early stage of viral assembly. Indeed, an interaction between the hyperphosphorylated form of NS5A and core could be observed leading to the relocation of NS5A to LDs (Masaki, Matsunaga et al. 2014), while the hypophosphorylated form of NS5A correlates with genomic replication (Evans, Rice et al. 2004). Given the direct interaction of core-DGAT1, also NS5A directly interacts with DGAT1 resulting in its recruitment to LDs (Camus, Herker et al. 2013). Additionally, an interaction between NS5A and ApoE is also suggested to contribute to the recruitment of this apolipoprotein to assembly sites (Benga, Krieger et al. 2010). Besides, NS5A was also reported to interact with Rab18, a small G protein, to facilitate its recruitment to LD and HCV assembly site (Salloum, Wang et al. 2013). The transition from replication to assembly is represented by the recruitment of NS5A together with the replication complex to LDs (reviewed in Lindenbach and Rice 2013).

The last step of the early phase, and in fact the first step of late phase, of viral assembly is the recruitment of envelope proteins at assembly sites. Several groups reported that the viral envelope glycoproteins E1 and E2 are bound in complexes composed of NS2, p7 and NS3 (Jirasko, Montserret et al. 2010; Popescu, Callens et al. 2011; Stapleford and Lindenbach 2011; Ma, Anantpadma et al. 2011) resulting in an accumulation of these complexes in close proximity of NS5A, core and LDs (Jirasko, Montserret et al. 2010; Popescu, Callens et al. 2011). However, the concrete nature and composition of this complex is not yet fully understood, however, it could be determined that NS2 and HCV E2 are associated with detergent-resistant membranes (DRMs) which are required for efficient HCV assembly (Shanmugam, Saravanabalaji et al. 2015); moreover, the entire replication complex as well as the HCV structural proteins could be shown to be associated with those DMRs

(Aizaki, Morikawa et al. 2008; Aizaki, Lee et al. 2004; Paul, Hoppe et al. 2013; Shi, Polyak et al. 2002). Recently, Boyer et al had a deeper look on the nature of this NS2 complex and found a direct protein-protein interaction of NS2 and E1E2 using immunoprecipitation assays revealing an association of NS2 with NS3 via DRMs. Contrarily, NS5A and E1E2 do not associate but rather interact separately with NS2-E1-E2-NS3 complex via unstable DMRs (Boyer, Dreneau et al. 2019). Interestingly, core was found to interact with NS2 and E1E2 through unstable DMRs suggesting a crucial role of DMRs transporting the NS2-E1-E2-NS3-NS5A-core complex for HCV assembly initiation (Boyer, Dreneau et al. 2019).

The late phase of viral assembly is the maturation and release of HCV particles in tight association with the host VLDL pathway within the ER lumen (Chang, Jiang et al. 2007; Jiang and Luo 2009; Gastaminza, Cheng et al. 2008; Huang, Sun et al. 2007). Indeed, it could be shown that HCV core accumulates in lipid fractions which contain high amounts of cholesterol and sphingolipids (Matto, Rice et al. 2004); these two lipids are observed to be enriched in viral particles and in turn involved in their infectivity (Aizaki, Morikawa et al. 2008). The nucleocapsid could be integrated into the core of luminal LDs (luLDs); however, how E1 and E2 are integrated into virions remains unclear. It is proposed that there is an interaction between NS3, E1 and E2 by either NS2 alone or together with p7 (Yi, Ma et al. 2009; Phan, Beran et al. 2009). Indeed, two studies observed that NS2 brings together E1, E2, p7, NS3 and NS5A in close proximity of LD by protein-protein interactions between all involved components (Jirasko, Montserret et al. 2010; Ma, Anantpadma et al. 2011). Since HCV E2 is the well-studied HCV glycoprotein that plays the major role for neutralization of HCV-specific antibodies and the binding capacity to CD81, the involvement of HCV E1 in HCV replication cycle still remains to be elucidated. By studying the role of E1 for viral morphogenesis, Haddad et al used mutants of E1 observing first a shift in the receptor usage from CLDN1 to CLDN6 for two mutants, and second virus carrying one mutant (D263A) was assembled and released but devoid of viral RNA revealing a crucial role for E1 in incorporating HCV RNA into the nucleocapsid (Haddad, Rouille et al. 2017). The step in which the apolipoproteins incorporate into mature infectious particles is also not fully clear. It is assumed that ApoB-positive precursors, formed in the rough ER (rER), fuse together with ApoB-negative precursor which incorporate the viral nucleocapsid resulting in LVPs associated with nonexchangeable ApoB (see p.5). ApoE and ApoC, both exchangeable apolipoproteins, could be associated to the LVPs within the ER lumen. However, there are differences between HCV particles depending in which cell type they are produced (detailed comparison is found on p.20).

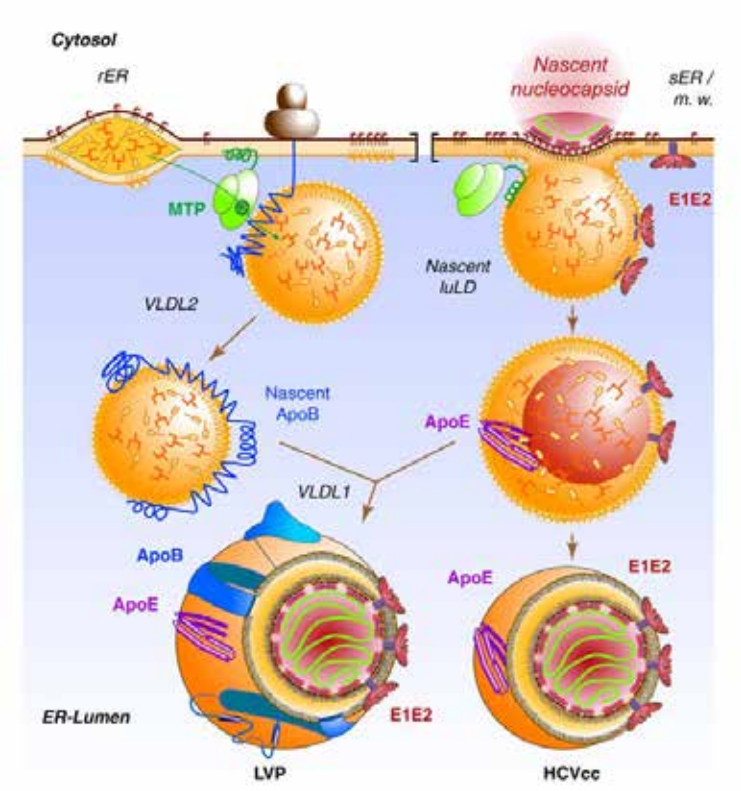

Figure 8. Model of the formation of LVPs and comparison to cell culture-derived HCV (HCVcc).
Precursors are formed: (1) VLDL2 resulted from the translocation of nascent ApoB into the ER lumen and the association with phospholipids, triglycerides and ApoE/C which are not shown for clarity (left), (2) luminal LD (luLD) is generated at the smooth ER (sER) or the membranous web (m.w.) and enriched with triglycerides via MTP (right). E1 and E2 associate with luLD after release from the ER membrane and the nucleocapsid is proposed to insert within the hydrophobic core of the luLD. In primary human hepatocytes the nucleocapsid-loaded luLD fuses with the VLDL2 resulting in a LVP, while in the cell culture model VLDL1 formation is lacking, cell culture-derived (HCVcc) is released without ApoB association (more details of in vitro models see on p.20). For clarity reasons, VLDL1 is generated as a fusion of VLDL with the triglyceride-riche luLD (Bartenschlager, Penin et al. 2011.

LVPs are transported along the VLDL secretory pathway (reviewed in Bartenschlager, Penin et al. 2011) to the Golgi, where the HCV glycoproteins E1 and E2 are post-translationally modified (reviewed in Vieyres, Dubuisson et al. 2014). The exact mechanism of HCV budding at the plasma membrane is still lacking. Immunoprecipitation and electron microscopy (EM) analysis revealed that HCV LVPs assemble in the ER and are transported to Golgi through vesicular transport mediated by COPII vesicles to enter the Golgi secretory pathway (Syed, Khan et al. 2017). In line with these findings, Takacs et al could observe that the secretion of ApoE and ApoB is differentially controlled by Rab1b which is a major regulator of transport from the ER to Golgi, and thus regulates secretion of HCV as well (Takacs, Andreo et al. 2017). Golgi protein 73 (GP73) has been demonstrated to be involved in HCV secretion: normally as a Golgi membrane resident protein and upregulated upon HCV infection, GP73 is colocalized with the HCV replication complex in Huh7.5.1 cells and is able to directly mediate an interaction of NS5A and ApoE and thus promoting the secretion of HCV (Zhang, Wang et al. 2016). These studies all supported the idea that HCV secretion and budding occurs through the Golgi secretion pathway. However, on the other hand could be demonstrated that

HCV is hijacking proteins of the endosomal sorting complex required for transport (ESCRT), e.g. hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), for budding at the plasma membrane. Indeed, HRS is able to directly interact with NS2 and NS5A in HCV-infected cells supporting efficient viral assembly as well as viral budding (Barouch-Bentov, Neveu et al. 2016). In line with this, another group found viral particles and structural proteins in endosomal compartments but not in compartments of the Golgi assuming that release of HCV occurs through noncanonical secretory route which is different from the canonical Golgi-mediated secretion (Bayer, Banning et al. 2016).

In sum, the above described processes followed the hypothesis that virions exist as hybrid lipoviral particles protecting from neutralization with HCV-specific antibodies (Andre, Komurian-Pradel et al. 2002). However, in a proposed two-particle model, the virus and serum lipoproteins stably interact as separate particles via protein-protein interaction. Indeed, when cholesterol is chemically removed from HCVcc particles their infectivity is lost, while adding back exogeneous cholesterol led to restorage (Aizaki, Morikawa et al. 2008). Additionally, another group could observe a rapid shift of buoyant density of viral particles in serum of fasting HCV patients concluding that virions and serum lipoproteins associate in a transient and exchangeable way (Felmlee, Sheridan et al. 2010). Both hypotheses could be imaginable, but they still need to be confirmed.

1.3 Model systems to study HCV

1.3.1 *In vitro* model systems

After its discovery in 1989 (Choo, Kuo et al. 1989), several efforts to culture HCV *in vitro* failed through lacking robustness, inefficient replication and thus failure for detailed molecular studies of the viral life cycle (reviewed in Bartenschlager and Lohmann 2000). After ten years of research, several breakthrough developments led to improvement to study the virus *in vitro*: the replicon system in 1999 followed by retroviral pseudoparticles in 2003 and finally the cell culture system recapitulating the entire viral lifecycle in 2005 (see Figure 9).

a. HCV replicon system

The establishment of sub-genomic replicons that autonomously amplify in cultured human hepatoma cells was a first major breakthrough to study viral replication: the genotype 1b HCV genome that was derived from a chronically infected patient was trimmed to those components essential for replication (NS3 to NS5B), while the viral structural genes (core, E1, E2) as well as p7 and NS2 were deleted

(see Figure 9). Practically, this shortened HCV genome was cloned into a plasmid resulting in a replicon encoding the 5'UTR of HCV, the first 12 codons of the core protein fused with a G418 selection cassette, the IRES from encephalomyocarditis virus (EMCV) allowing translation of the non-structural proteins, as well as viral NS3, NS4A, NS4B, NS5A, NS5B, and 3'UTR. After undergoing *in vitro* transfection, the construct is transfected into human hepatoma 7 (Huh7) cells (see p.20) resulting in direct translation of viral RNA and G418 selection of those Huh7 cells displaying high levels of HCV RNA replication (Lohmann, Korner et al. 1999). Subsequently, full length HCV replicons were developed, ultimately leading to the establishment of recombinant full-length infectious HCV (see HCVcc below).

b. HCV Pseudoparticles (HCVpp)

Four years after developing the replicon system, the next important achievement was the generation of retroviral pseudoparticles displaying functional HCV glycoproteins, E1 and E2, for dissection of HCV entry process. In basic principal, pseudoparticles are retroviral capsids with incorporated viral glycoproteins in their envelopes. Consequently, these pseudoparticles allow to study the relevance and need of those glycoproteins in viral attachment and entry processes; the latter can be easily quantified using a reporter gene located inside the pseudoparticles. HCVpp could be successfully developed by integrating HCV glycoproteins in retroviral particles: A system in which 293T cells are co-transfected with expression vectors encoding HCV E1 and E2, the gag-pol proteins of either murine leukemia virus (MLV) or human immunodeficiency virus (HIV), and a retroviral genome encoding a reporter gene such as green fluorescent protein (GFP) or luciferase (Bartosch, Dubuisson et al. 2003; Hsu, Zhang et al. 2003) (see Figure 9). Using HCVpp is an elegant way to study HCV entry independently of the entire viral life cycle which in turn highlights the limitation of HCVpp to the entry process: they are produced in a non-liver cell line and thus assemble as retroviruses on plasma membranes without getting associated with lipoproteins, contrarily liver-generated HCV particles assemble in the ER in close association with lipoproteins (see p.5 and 13).

c. Cell culture-derived HCV (HCVcc)

After successful development of sub-genomic replicons and the HCVpp system, the challenge to establish an HCV permissive cell culture system remained.

The initial production of cell culture-derived HCV particles is based on the genotype 2a HCV strain, namely JFH1 (Japanese Fulminant Hepatitis 1) that was isolated from a Japanese patient with acute viral hepatitis (Kato, Furusaka et al. 2001). Based on the wild-type JFH1 genome or chimeras consisting of the JFH1 replicase genes (NS3 to NS5B) and core to NS2 regions of alternative HCV

genomes, three groups proposed a robust *in vitro* system recapitulating the entire HCV life cycle in Huh7 cells (Lindenbach, Evans et al. 2005; Wakita, Pietschmann et al. 2005; Zhong, Gastaminza et al. 2005) which since then is the laboratory standard for *in vitro* HCV studies. These produced particles, well-known as cell culture-derived HCV (HCVcc), are able to infect new target cells, thereby completing the entire HCV life cycle (see Figure 9). This achievement led to the development of several chimeras that consist of JFH1 replicase genes NS3 to NS5B and core to NS2 of alternative HCV genomes allowing to study HCV entry, neutralization and assembly of all seven known HCV genotypes. Among all these chimeras, Jc1, consisting of the JFH1 nonstructural proteins (NS3 to NS5B) and core-E1-E2-p7 and partially NS2 of J6 (HCV gt 2a), produces infectious virus titers that are around 100 to 1000-fold higher than the original JFH1 strain (Pietschmann, Kaul et al. 2006). Over the years, chimeras of all seven known genotypes of HCV based on JFH1 have been developed giving the possibility to study the entire viral life cycle and additionally, neutralization via HCV-specific antibodies (Gottwein, Jensen et al. 2011; Gottwein, Scheel et al. 2009; Scheel, Gottwein et al. 2008; Scheel, Gottwein et al. 2011; Jensen, Gottwein et al. 2008; Gottwein, Scheel et al. 2007), however, with differences concerning infectivity. In order to rapidly and efficiently detect viral replication and infection, reporter genomes of different HCV chimeric genomes containing luciferase or GFP were generated (reviewed in Vieyres and Pietschmann 2013). Furthermore, non JFH1-based HCVcc enabling the production of recombinant HCV of different genotypes have been established but JFH1-based HCVcc remain the most widely used and efficient models.

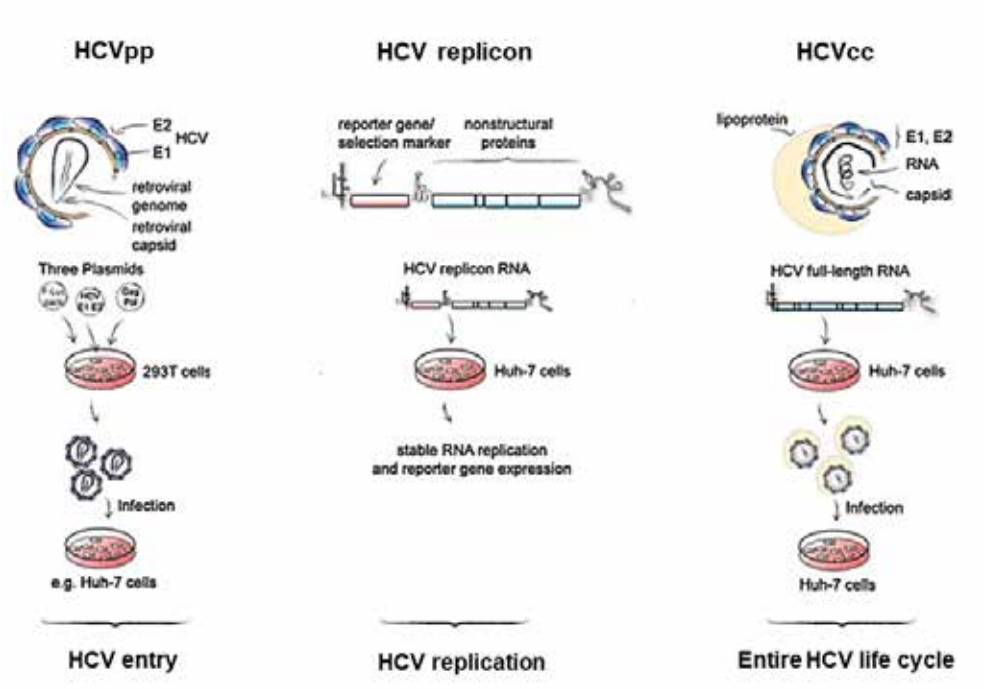

Figure 9 *In vitro* HCV cell culture models.
In vitro HCV cell culture models to investigate different steps of the viral life cycle: HCV pseudoparticle system (left) to study viral entry, the HCV replicon (middle) system to study viral replication, and full-length recombinant cell culture-derived HCV (right) system to investigate the entire viral replication cycle. (Adapted from Steinmann and Pietschmann 2013).

d. HCV-permissive host cells

Since HCV primarily replicates in human hepatocytes, cultured primary human hepatocytes (PHH) from liver resection of patients represent the most physiological *in vitro* model closest to the natural host (Ploss, Khetani et al. 2010). However, PHH display several disadvantages that make them not easy to handle in laboratories: difficult to obtain, high donor-dependent variability and limited time of culture (time-dependent dedifferentiation and approximately two weeks before apoptosis occurs); even worse, once infected with HCV PHH only show low-level replication (Fournier, Sureau et al. 1998; Molina, Castet et al. 2008; Rumin, Berthillon et al. 1999), hence making it difficult to conduct complex experiments.

Thus, the most HCV-permissive and widely used cell line are Huh7 human hepatoma cells that were originally isolated from HCC of a Japanese patient (Nakabayashi, Taketa et al. 1982). Several Huh7 subclones with increased HCV permissiveness could be obtained, by selection of replicon-containing Huh7 cells. The subclones Huh7.5 (Blight, McKeating et al. 2002), Huh7.5.1 (Zhong, Gastaminza et al. 2005), and Huh7-Lunet (Friebe, Boudet et al. 2005) cells were obtained after cure of the HCV-transfected Huh7 cells from the replicon via treatment with INF-α, INF-γ or selective inhibitors. It is not entirely clear why these Huh7 subclones yield higher levels of HCV RNA replication. However, in case of Huh7.5 cells mutations in the retinoic acid-inducible gene I (RIG-I) were shown to be involved in increased viral replication (Sumpter, Loo et al. 2005).

However, Huh7-derived cells are dedifferentiated and asynchronously dividing cancer cells, in contrast to primary hepatocytes which are differentiated and quiescent (Michalopoulos and DeFrances 1997). However, they show hepatocyte-specific gene expression and arrested cell growth by adding 1% of dimethyl sulfoxide (DMSO) to the culture medium, while permissiveness to HCV is maintained (Sainz and Chisari 2006); and thus are closer to hepatocytes.
Importantly, cultured Huh7-derived cells and *in vivo* hepatocytes show differences in their capability in producing lipoproteins, and thus HCV particles differ in their properties in dependency of the cells in which they are produced. In infected patients, the heterogenous density of circulating HCV particles and their infectivity is negatively correlated meaning low-density viruses are more infectious (Bradley, McCaustland et al. 1991; Hijikata, Shimizu et al. 1993). Those HCV particles can be immunoprecipitated via ApoB-specific antibodies confirming the association of HCV with triglyceride-rich lipoproteins (TRLs) within hepatocytes and their circulation via the bloodstream as LVPs (Andre, Perlemuter et al. 2005; Nielsen, Bassendine et al. 2004). In comparison to patient-derived LVPs, the negative correlation for density and infectivity could also been shown for HCVcc (Miyanari, Atsuzawa et al. 2007; Podevin, Carpentier et al. 2010; Haid, Pietschmann et al. 2009). Nevertheless, HCVcc were shown to be captured via ApoE (Chang, Jiang et al. 2007; Merz, Long et

al. 2011) - and not ApoB-specific antibodies (Merz, Long et al. 2011; Huang, Sun et al. 2007) highlighting the tight association of HCVcc with ApoE and ApoC and the inefficiency of Huh7-derived cells in producing authentic VLDL (reviewed in Bartenschlager, Penin et al. 2011; Vieyres and Pietschmann 2019). However, Piver et al described that at least a small fraction of HCVcc derived from Huh7.5 cells displayed ApoB and ApoE as well as E1E2 on the surface after immunogold staining following immunocapture (Piver, Boyer et al. 2017). Recently, it was confirmed that Huh7.5 cells growing in serum-free medium produced immature HCV particles, however, when incubating those cells/particles with physiological serum conditions and concentrations of lipoproteins resulted in the maturation of HCV particles to fully lipidated and notably, ApoB-containing infectious virions displaying low density very similar to patient-derived LVPs (Denolly, Granier et al. 2019). In addition to HCV maturation through the cell secretory pathway, also extracellular lipidation of particles may occur through serum itself properly after egress (Denolly, Granier et al. 2019). In contrast, HCV nucleocapsids derived from patients and cell culture appeared to be similar in size and structure and interestingly those nucleocapsids are found to be surrounded by an irregular, detergent-sensitive crescent which may be consistent with lipids (Piver, Boyer et al. 2017).

1.3.2 *In vivo* model systems

Cell culture model systems to study HCV are of high importance, however, the complex immunological host responses and liver disease progression cannot be fully answered *in vitro*. Since lacking control parameters such as time and dose of infection strongly limited clinical research in patients, attention of research was directed to animal models such as chimpanzees, tree shrews and rodents by keeping in mind that ideally, similarly to HCV-infected patients, HCV-infected animals should develop chronicity followed by liver disease progression towards cirrhosis and HCC.

Chimpanzees (*Pan troglodytes*) are with more than 98% genetic identity the closest living relatives to human and susceptible to HCV infection (Abe, Kurata et al. 1993), and therefore represented an ideal model to study HCV *in vivo* for over 20 years. After HCV infection, viremia as well as antiviral innate and adaptive immune responses are detectable following a mild acute hepatitis. In contrast to humans, chimpanzees rarely develop chronicity (Bassett, Brasky et al. 1998; Major, Dahari et al. 2004), and thus do not develop progressive liver disease (Walker 1997). High costs and increased ethical concerns limited the use of this animal model (National Research Council Committee on the Use of Chimpanzees in and Behavioral 2011), although it is the only well-studied model to study

protective immunity against HCV, probably leading to vaccine development (Cooper, Erickson et al. 1999).

The tree shrew (*Tupaia belangeri*), a squirrel-like mammal originally found in South-East Asia, has also been shown to be susceptible to HCV infection (Xu, Chen et al. 2007; Xie, Riezu-Boj et al. 1998) followed by chronicity and development of liver disease in some tree shrews (Amako, Tsukiyama-Kohara et al. 2010). Moreover, ethical concern and lack of tool to analyze HCV-host interactions in these animals preclude a wide usage of this model.

Rodent models represent a cheap and easy to breed model to study HCV. In general, mice and rats are not susceptible for HCV infection and need to undergo experimental modifications. The most commonly used rodent models can be divided into (i) the human liver-chimeric mouse models and (ii) the transgenic mouse models:

(i) In human liver-chimeric models, mouse livers are humanized by xenotransplantation of primary human hepatocytes or hepatoma cell lines resulting in a subsequent susceptibility to HCV infection. To successfully xenograft human hepatocytes into mouse livers, the xenograft recipients require specific genetic defects leading to death of the original mouse hepatocyte and to dysfunctionality of the mouse immune system. Currently, two major types of mice are used: first, mice overexpressing the urokinase plasminogen activator (uPA) gene under albumin promotor control were crossed with mice that suffer from a severe combined immunodeficiency syndrome (SCID) resulting in uPA-SCID mice; once xenografted with human hepatocytes, these mice are efficiently infected with HCV followed by chronic infection (Mercer, Schiller et al. 2001) and led to achievements concerning preclinical studies of antiviral compounds (Mailly, Xiao et al. 2015) and understanding of steps in the viral lifecycle (e.g. composition of LVPs, role of HCV-specific antibodies or viral entry) *in vivo* (Lindenbach, Meuleman et al. 2006; Vanwolleghem, Bukh et al. 2008; Lacek, Vercauteren et al. 2012; Meuleman, Catanese et al. 2012).
Second, mice that have fumarylacetoacetate hydrolase ($Fah^{-/-}$) deficiency accumulate hepatotoxic metabolites consequently leading to liver failure (Grompe, al-Dhalimy et al. 1993). These $Fah^{-/-}$ mice were crossed with mice suffering from severe immune system dysfunctionality ($Rag2^{-/-}$ $IL2R\gamma^{null}$) resulting in FRG mice. The liver degeneration resulting from depletion of Fah can be prevented by a drug, namely NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)1,3-cyclohexanedione)). Hence, this model provides an advantage over uPA-SCID mice: the timing of human hepatocyte transplantation can be anticipated by removing NTBC (Azuma, Paulk et al. 2007; Bissig, Le et al. 2007).

(ii) In contrast to human liver-chimeric mouse model, where mice show severe immune defects, the genetically humanized mouse models serve basically to study adaptive immune responses and

immunoevasion mechanisms. The mouse livers in these model systems are provided with all human host factors required to enable (parts of) the HCV replication cycle in mouse hepatocytes. Expression of SR-BI, OCLN, CLDN1 and CD81 under an albumin promotor enables to detect HCV entry in mouse livers (see p.7); of note, in line with results from *in vitro* experiments (see p.7) expression of human OCLN and CD81 is the minimum requirement enabling the study of viral entry in mouse livers (Dorner, Horwitz et al. 2011). Additionally, knockout of STAT1, IRF1, IRF7 and IFNAR1, all important players in innate immunity, results in low-level HCV RNA replication and production of infectious virus in mice (Dorner, Horwitz et al. 2013).

1.4 HCV treatment

As we now deep dived into the HCV molecular biology to which knowledge has markedly advanced, molecular mechanisms of disease progression and vaccine development still needs to be figured out. By thinking about viral infection, the aim of every viral therapy is eradication of virus, known in hepatitis C clinical context as sustained virologic response (SVR) which is defined as undetectable HCV RNA levels over 12 to 24 weeks after end of therapy.

Throwback to the 80's and 90's, the only available therapy for HCV infection was based on interferon (INF). INF-α exerts its antiviral effect by inducing IFN-stimulated genes that in turn inhibit HCV replication. Until 1998, three INF-α injections a week for up to 48 weeks were the therapy of choice, unfortunately resulting in cure of only one-fifth of infected patients (Carithers and Emerson 1997; Hoofnagle and di Bisceglie 1997). This application was improved by injection of INF-α combined with orally taken ribavirin up to 24 to 48 weeks, an antiviral medication which could increase the antiviral effect of INF-α resulting in around 35-45% of cure in infected patients (McHutchison, Gordon et al. 1998). The addition of polyethylene glycol to INF-α (PEG-INF-α) gave rise to increased half-life of the drug and in combination with ribavirin an increased effectivity (Manns, McHutchison et al. 2001) was achieved replacing the standard INF-α for more than ten years. Of note, HCV-infected patients responded differently to the PEG-INF-α/ribavirin therapy depending on their HCV genotype: while a SVR of around 50% was achieved for patients infected with HCV genotype 1, up to 80% of SVR was shown for patients infected with genotypes 2, 3, 5 and 6 (Antaki, Craxi et al. 2010). Nevertheless, the INF-based therapy had many side-effects including flu-like symptoms, gastritis or even worse depression (Manns, Wedemeyer et al. 2006).

The achievement of uncovering key viral proteins involved in the HCV replication cycle led to the development of direct-acting antivirals (DAAs) which are specifically targeting nonstructural proteins of HCV. DAAs can be classified into four main groups: NS3/NS4A protease inhibitors,

NS5B nucleoside and non-nucleoside polymerase inhibitors and NS5A inhibitors. In 2011, a NS3/4A protease inhibitor was combined with the PEG-INF-α/ribavirin increasing SVRs for genotype 1. Nevertheless, this triple therapy was only available for genotype 1-infected patients combined with severe side-effects (Bacon, Gordon et al. 2011; Jacobson, McHutchison et al. 2011; Zeuzem, Andreone et al. 2011). In 2014-2015, new interferon-free DAA regimen, including sofosbuvir (NS5B inhibitor) and daclatasvir or ledipasvir (NS5A inhibitor), became much more efficient (SVR around 90%) with the big plus of only little side-effects (Afdhal, Zeuzem et al. 2014; Afdhal, Reddy et al. 2014; Sulkowski, Gardiner et al. 2014). The combination of different DAAs - that are now the current standard of care - lead to highly improved SVR rates and shortened therapy duration; moreover, defined DAA combinations are efficient for all HCV genotypes resulting in SVR rates of 95% (reviewed in (Asselah, Boyer et al. 2016; Li and De Clercq 2017). Currently recommended DAAs are: dasabuvir (DSV), elbasvir (EBR), glecaprevir (GLE), grazoprevir (GZR), ledipasvir (LDV), ombitasvir (OBV), pibrentasvir (PIB), paritaprevir (PTV), ritonavir (r), sofosbuvir (SOF), velpatasvir (VEL) and voxilaprevir (VOX). Among all these recommended DAAs, the indication is depended on the HCV genotype, the severity of liver disease as well as prior therapy. Combinations of DAAs are very efficient, even if combinations of 2 regimens is preferred to triple combination in order to avoid drug-drug interactions and severe side-effects (see Table 1) (EASL 2018).

Genotype	Pangenotypic regimens			Genotype-specific regimens		
	SOF/ VEL	GLE/PIB	SOF/ VEL/ VOX	SOF/ LDV	GZR/ EBR	OBV/ PTV/r + DSV
Genotype 1a	Yes	Yes	No*	Yes[a]	Yes[b]	No
Genotype 1b	Yes	Yes	No*	Yes	Yes	Yes
Genotype 2	Yes	Yes	No*	No	No	No
Genotype 3	Yes[c]	Yes	Yes[d]	No	No	No
Genotype 4	Yes	Yes	No*	Yes[a]	Yes[e]	No
Genotype 5	Yes	Yes	No*	Yes[a]	No	No
Genotype 6	Yes	Yes	No*	Yes[a]	No	No

Table 1. Combinational DAA treatment.
Combination of DAA treatment. Recommendation for treated and non-treated patients without or with compensated cirrhosis (a-e). *Triple combination. DSV: dasabuvir, EBR: elbasvir, GLE: glecprevir, GZR: grazoprevir, LDV: ledipasvir, OBV: ombitasvir, PIB: pibrentasvir, PTV: paritaprevir, r: ritonavir, SOF: sofosbuvir, VEL: velpatasvir, VOX: voxilaprevir (EASL 2018).

Even if may appear that with the achievement of DAAs HCV research could be disregarded, there are still some important challenges that remain (reviewed in Bartenschlager, Baumert et al. 2018). First, DAAs are only available to around 10% of infected patients (Edlin 2016) and the high costs of DAA-based therapies limit the access to a minority (Iyengar, Tay-Teo et al. 2016). Second, there are some difficult-to-treat patient populations existing with decreased SVR in response to DAAs, even

more in the presence of advanced liver disease progression. These patients are less able to clear the virus suggesting that there is still need to investigate the fine tuning of optimal DAA combination, treatment duration and dosages for difficult patient populations (Hezode, Fontaine et al. 2013; Ferenci 2015). Third and in line with the second limitation, the risk to develop HCC can be reduced after SVR, but HCC can occur after years of viral clearance suggesting that there is a "point-of-no-return" when virus-induced liver disease came to the point where HCC will develop even in the absence of HCV (Baumert, Juhling et al. 2017). Fourth, a minority of patients can develop resistance to DAA due to the error-prone HCV RNA polymerase which creates a pool of genetic variants in each patient leading to viral polymorphism in DAA-targeted regions resulting in resistance to DAAs (Esposito, Trinks et al. 2016; Pawlotsky 2016).

In addition to DAAs, the standard of care for chronic hepatitis C, alternative strategies have been developed. When reminiscing about the HCV replication cycle, several host factors play a crucial role in different steps. When reminiscing about the HCV replication cycle, several host factors play a crucial role in different steps.

Indeed, host-targeting agents (HTAs) appeared to be of therapeutic interest. Monoclonal antibodies specifically targeting CD81 (Fofana, Xiao et al. 2013), CLDN1 (Mailly, Xiao et al. 2015; Fofana, Krieger et al. 2010) or SR-BI (Meuleman, Catanese et al. 2012; Zahid, Turek et al. 2013) were developed and could be shown to efficiently inhibit viral entry *in vitro* and *in vivo* in liver chimeric mice. Additionally, small molecule inhibitors of SR-BI, ITX 5061 and ITX 7650, could efficiently block HCVcc and HCVpp infection of PHH or human hepatoma cell lines (Syder, Lee et al. 2011). ITX 5061 has been evaluated in phase 1 clinical trials (ClinicalTrials.gov identifier: NCT01165359 and NCT01292824). Moreover, a monoclonal CLDN1-specific antibody was shown to cure chronic HCV infection in the uPA-SCID chimeric mouse model (Mailly, Xiao et al. 2015; Colpitts, Tawar et al. 2018). Of note, synergy between host-targeting entry inhibitors combined with DAA-based treatment could be demonstrated (Xiao, Fofana et al. 2015; Paciello, Urbanowicz et al. 2016).

Another important host factor for the HCV life cycle is liver-enriched miR-122 that has been shown to play a crucial role in the stabilization of HCV RNA and consequently in HCV replication and translation (Jopling, Yi et al. 2005). miR-122 antagonists significantly decrease HCV replication (Jopling, Yi et al. 2005; Jopling, Schutz et al. 2008) and miravirsen and RG-101 demonstrated efficacy against HCV in clinical trials (van der Ree, de Vree et al. 2017; Stelma, van der Ree et al. 2017). In fact, these inhibitors can be regarded as pan-genotypic antivirals due to the conserved miR-122 binding sites across HCV genotypes (Li, Gottwein et al. 2011; van der Ree, de Vree et al. 2017). Cyclophilin A (CypA) inhibitors, namely alisporivir or DEB025, could efficiently block CypA interaction with NS5A leading to inhibition of HCV replication and additionally, these inhibitors are able to rehabilitate the innate immune response against HCV (Daito, Watashi et al. 2014; Hopkins,

Bobardt et al. 2012; Naoumov 2014). Phase 3 clinical trials that examined a triple therapy with alisporivir (DEB025), PEG-INF and RBV have been completed (ClinicalTrials.gov identifier: NCT01446250, NCT01318694).

In fact, the given examples represent only a small overview of HTAs that have been studied for their ability to improve antiviral treatment, several other host factors involved in the viral life cycle are at different stages of preclinical or clinical development (reviewed in Crouchet, Wrensch et al. 2018; Zeisel, Crouchet et al. 2015; Zeisel, Lupberger et al. 2013 (see Figure 10).

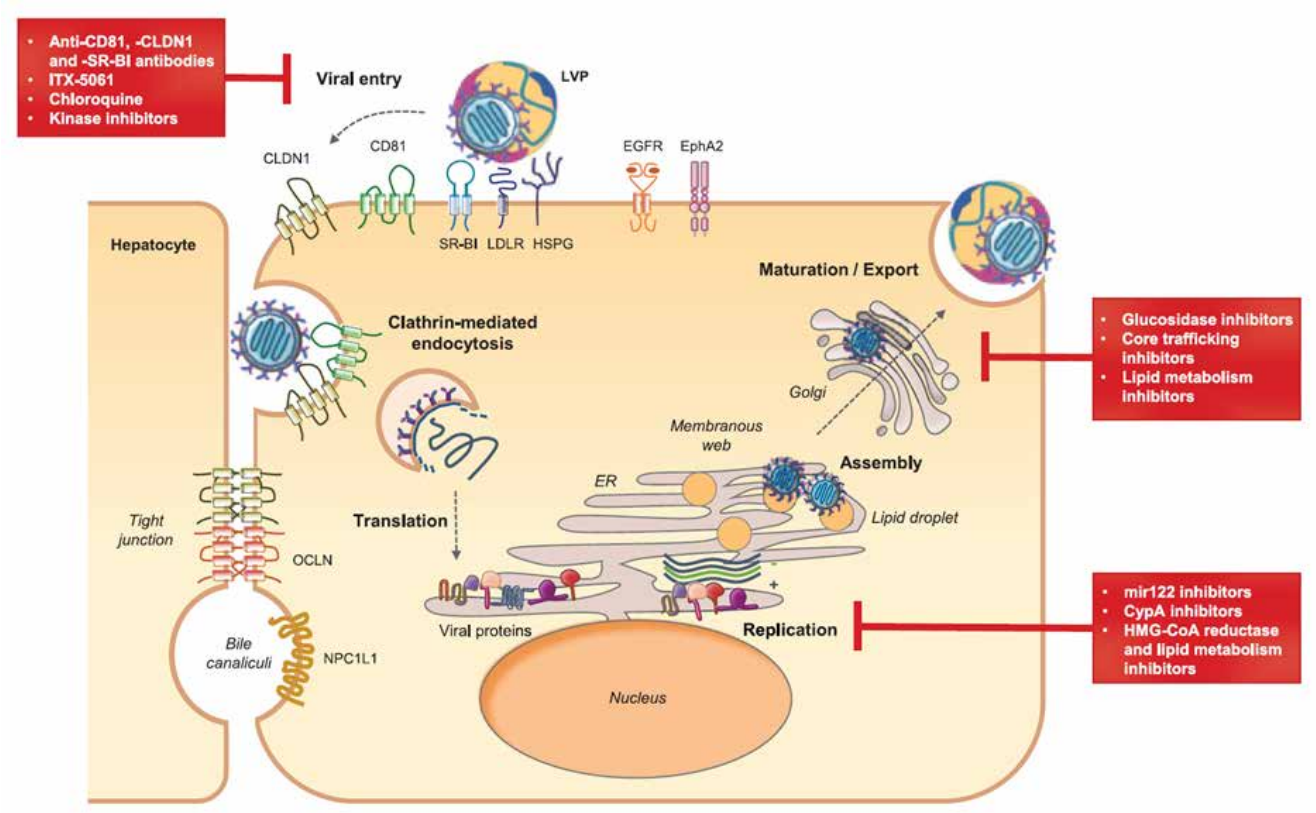

Figure 10. Host-targeting agents (HTAs) at different steps of the HCV replication cycle (Crouchet, Wrensch et al. 2018).

Taken together, HCV infection can now be regarded as curable disease, although some limitations and challenges still exist. Since HCC can develop even after successful eradication of HCV, a current question is what kind of molecular imprinting is left by HCV in the host genome that drives carcinogenesis even after SVR in patients (see HEPATITIS C – An Introduction).

II. PROTEIN GLYCOSYLATION

2.1 Post-translation modifications of HCV proteins

Post-translational modifications (PTMs) are as the name implies covalent and enzymatic modifications of proteins after completed translation and needed for protein maturation and activation. Different PTMs are known, some occur more often than others: e. g. phosphorylation, glycosylation, ubiquitination, sumoylation, nitrosylation, methylation, acetylation and lipidation; proteolysis can also be regarded as a PTM. In general, proteins are either directly modified after translation for stabilization, mediation of correct folding or recruitment of the nascent protein to their cell compartment of need; or PTMs occur after folding and localization to activate or inactivate proteins (cell signaling) contributing to the biological activity of the protein. Of note, both possibilities how and when PTMs takes place do not exclude each other but are often combined in a stepwise mechanism. While protein PTMs can be reversible, proteolysis processes are permanent.

Since HCV replication cycle is depended on the interaction with several host cell factors, it is not surprising that HCV proteins undergo PTMs for protein function and regulation as well. One can summarize a few key modifications that occur during the viral replication cycle: the proteolytic cleavage of the polyprotein, glycosylation of the HCV envelope glycoproteins E1 and E2, as well as some PTMs of the nonstructural proteins. Among the latter, phosphorylation of NS5A has been well-described: NS5A is found in a hypo-or hyper-phosphorylated form which is important for regulation of its function (Chong, Hsu et al. 2016; Appel, Pietschmann et al. 2005). Indeed, it could be shown that knockout of kinases important for this phosphorylation consequently led to reduced NS5A phosphorylation resulting in lower HCV RNA levels (Lee, Chen et al. 2016), suggesting that the phosphorylation status of NS5A plays a crucial role for HCV replication (Appel, Pietschmann et al. 2005). It is suggested that PTMs are important for the location of HCV proteins, however, questions about the function and biological relevance of these PTMs still need to be answered (reviewed in Hundt, Li et al. 2013).

The focus here are glycosylation and to a small extent phosphorylation, the most common PTMs, that could compete for the same target protein/residue.

Phosphorylation is defined in simple words as the dynamic and reversible addition or removal of phosphate residues to tyrosine, serine and threonine residues by kinases and phosphatases, respectively, resulting in activation or inactivation of proteins. Glycosylation, on the other hand, is the addition of sugar-moieties to proteins, ranging from simple monosaccharide modifications to highly complexed branched polysaccharide changes. The sugar-moieties are added either to amino

group of asparagine (N-linked) in the ER or to the hydroxyl group of serine/threonine (O-linked) of nuclear and cytoplasmic proteins (reviewed in Levine and Walker 2016) (see Figure 11).

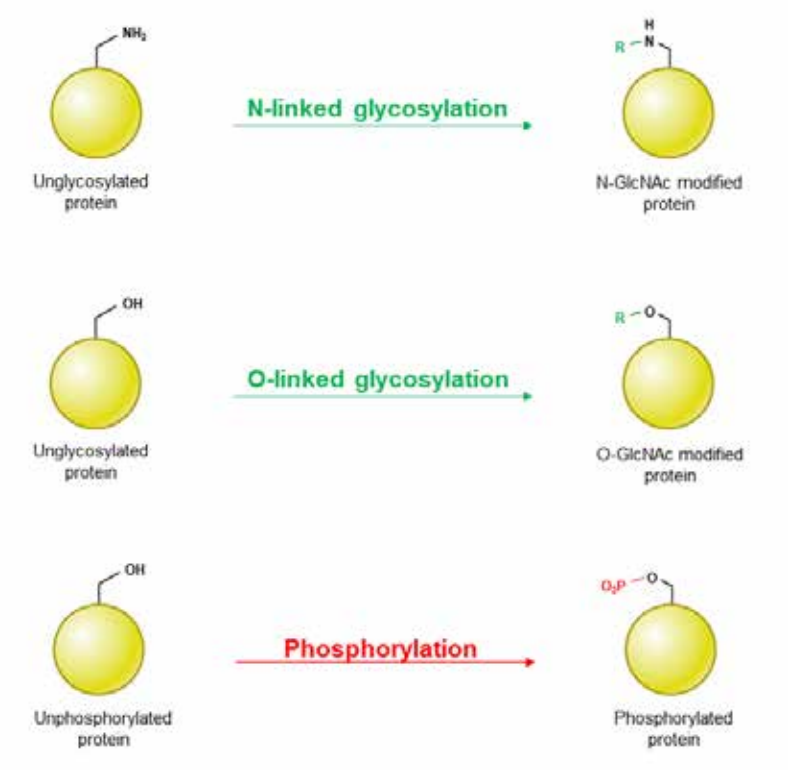

Figure 11. Basic principle of N-and O-linked glycosylation, and phosphorylation.
-R: sugar residues, -PO_3: phosphate residues (adapted from Levine and Walker 2016).

When looking retrospectively at section 1.2.2, it was mentioned that the big challenges of HCV vaccine development are the genetic heterogeneity of the virus, its ability to rapidly mutate and its close interaction with the host lipid metabolism leading to escape from protective immune responses. However, another important key challenge regarding vaccine design is high glycosylation of the ectodomains E1 and E2 with N-glycans resulting in a glycan shield and representing one-third of the heterodimer mass (Goffard, Callens et al. 2005) (reviewed in Lavie, Hanoulle et al. 2018). This shield enables the virus to avoid recognition by the humoral immune response and prevents neutralization by HCV-specific antibodies, since glycans are host-derived and thus recognized as self-patterns.

There are N-glycosylation sites that are conserved among the seven genotypes but there are some N-glycosylation sites present only in distinct genotypes, however all of the N-glycosylation sites have been confirmed to be engaged by glycans (Goffard, Callens et al. 2005). Four N-glycosylation sites can be found for E1 at positions 196 (E1N1), 209 (E1N2), 234 (E1N3), and 305 (E1N4) on the reference strain H77 (gt 1a), additionally N-glycosylation sites at position 250 (gt 1b and 6) and position 299 (gt 2b) could be found (Helle, Goffard et al. 2007). Whereas E2 contains nine conserved N-glycosylation sites at position 417 (E2N1), 423 (E2N2), 430 (E2N3), 448 (E2N4), 532 (E2N6),

556 (E2N8), 576 (E2N9), 623 (E2N10), and 645 (E2N11); two additional glycosylation sites can be detected at position 476 (E2N5) (except for gt 1b) and position 540 (E2N7) (gt 3 and 6) (see Figure 12).

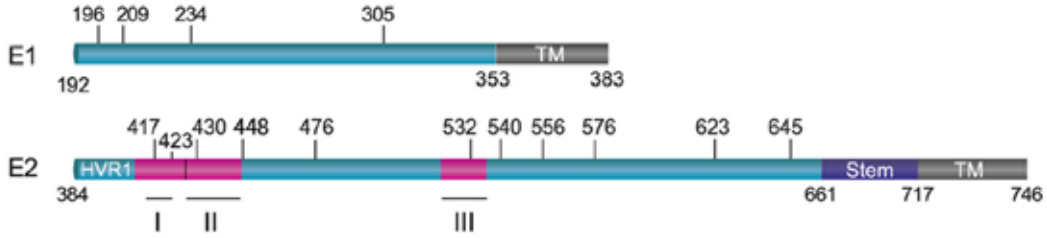

Figure 12. Schematic representation of N-glycosylation sites of E1 and E2.
Numbers and verticals bars correspond to position of glycosylation sites with the reference strain H77 (gt 1a). TM: Transmembrane domain, HVR1: Hypervariable region 1. I (412-423), II (427-446) and III (523-535) represent the three major neutralizing epitopes on E2 (Lavie, Hanoulle et al. 2018).

There are three major N-glycans on HCV E1E2 based on N-acetylglucosamines and associated with: (i) several mannose residues (high mannose glycans), (ii) galactose, and sometimes sialic acid and fucose (complex oligosaccharides), and (iii) galactose, mannose and probably sialic acid (hybrid glycans). In fact, the latter two are produced during the transfer of proteins through the Golgi by enzymatic addition or removal of sugar residues mediated by glycosidases and glycosyl transferases. Indeed, after assembly in the ER and during transport along the VLDL secretory pathway, HCV glycoproteins are modified by the two latter enzymes. Differences in their glycosylation patterns could be observed between HCVpp and HCVcc resulting from the different assembly processes of HCVpp and HCVcc in the post-Golgi compartment or in an ER-derived compartment, respectively: while E1E2 derived from HCVpp contain a majority of complex-type glycans, HCVcc-derived E1E2 display high-mannose and complex N-linked glycans (Vieyres, Thomas et al. 2010) suggesting a diverse glycan processing and a different organization of proteins on the surface of HCVpp or HCVcc (reviewed in Lavie, Hanoulle et al. 2018). As a consequence, HCVpp and HCVcc act differently during their entry processes (Johansson, Voisset et al. 2007; Kapadia, Barth et al. 2007; Russell, Kawaguchi et al. 2009; Albecka, Montserret et al. 2011; Wasilewski, Ray et al. 2016).

Two benefits of N-glycans on HCV glycoproteins E1 and E2 are obvious: First, N-glycans, normally involved in protein folding (Helenius and Aebi 2001), can affect the binding affinity of E1E2 for receptors. Second, N-glycans on E1E2 are effective in shielding E1 and E2 from the humoral immune response and thus prevent the virus from being neutralized by HCV-specific antibodies, consequently to a non-effective immune response (de Jong, Dorner et al. 2014). In fact, HCV E2 is the main target of neutralizing antibodies (reviewed in Ball, Tarr et al. 2014). When looking again at Figure 12, the three major neutralizing epitopes of E2 (mentioned as I-III) are N-glycosylated resulting in an

effective shield of those epitopes and an evasion of the host immune response. Additionally, one group could observe escape mutants from an E2-specific antibody (AP33) displaying a glycan shift (Pantua, Diao et al. 2013) suggesting that the N-glycosylation of HCV glycoproteins and the evasion of the host immune response is not a fixed but rather dynamic process.

Apart from N-glycosylation, O-glycosylation sites have been predicted on the E2 envelope protein as well (Bandiera and Zeisel, unpublished data and (reviewed in Lavie, Hanoulle et al. 2018), however, they have not been well-described.

2.2 *O*-linked *N*-acetylglucosaminylation (*O*-GlcNAcylation)

O-linked *N*-acetylglucosaminylation (*O*-GlcNAcylation) is a dynamic and reversible PTM that mainly takes place in the cytoplasmic, mitochondrial, and nuclear compartments of the cell (Issad, Masson et al. 2010), and appears to show similar features to phosphorylation, since the modification sites for both are serine and threonine residues. Indeed, an extensive crosstalk between *O*-GlcNAcylation and phosphorylation could be observed, either by competing for the same serine or threonine residue or by *O*-GlcNAcylation influencing phosphorylation of adjacent residues (Leney, El Atmioui et al. 2017) (see Figure 13).

The substrate of *O*-GlcNAcylation, Uridine-diphosphate *N*-acetylglucosamine (UDP-GlcNAc) is synthesized as an end product of the hexosamine biosynthetic pathway (HBP); an alternative pathway within the glucose metabolic pathway that converts up to 2%-5% of cellular glucose into UDP-GlcNAc involving several enzymes (reviewed in Buse 2006). UDP-GlcNAc serves as the donor for the addition of GlcNAc to serine and threonine residues mediated by *O*-linked *N*-acetylglucosamine transferase (OGT), whereas the removal of *O*-GlcNAc is mediated by *O*-GlcNAc hydrolase (OGA) (reviewed in Levine and Walker 2016). Of note, OGT and its counterpart OGA are the unique enzymes regulating *O*-GlcNAc levels within cells (see Figure 13).

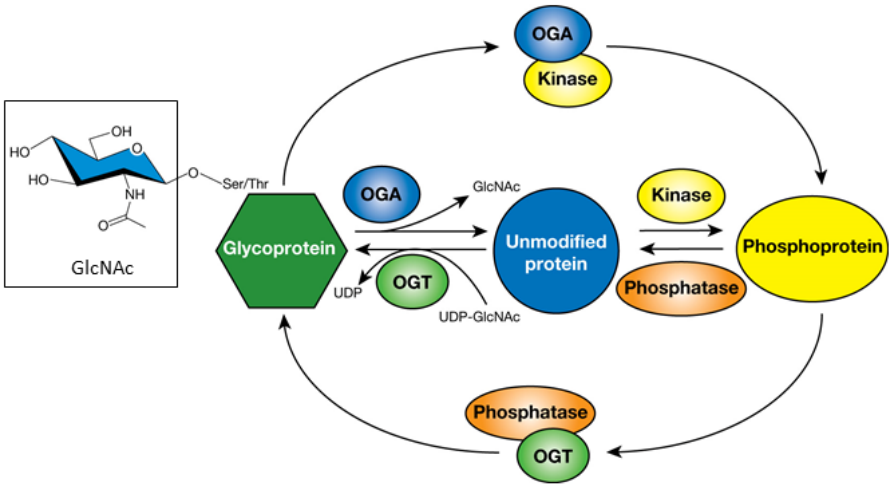

Figure 13. *O*-GlcNAcylation versus phosphorylation.
Dynamic addition and removal of *O*-GlcNAc mediated solely by OGT and its counterpart OGA. For some target proteins, *O*-GlcNAc and phosphorylation compete for serine and threonine residues (adapted from Zachara, Akimoto et al. 2015).

OGT is found in three isoforms in mammals (i) nucleocytoplasmic (ncOGT, 116 kDa), (ii) mitochondrial (mOGT, 103 kDa), and (iii) short (sOGT, 78 kDa), all containing a N-terminal domain consistent of tetraticopeptide repeat (TPR) motifs which are important to recognize the different protein substrates, and a C-terminal domain implying the glycosyltransferase activity (reviewed in Joiner, Li et al. 2019; Liu, Dai et al. 2015) (see Figure 14). In addition to its role in *O*-GlcNAcylation of nuclear, cytoplasmic and mitochondrial proteins, OGT has scaffold function and promotes the stable binding of proteins in multiprotein complexes (Hart, Housley et al. 2007).

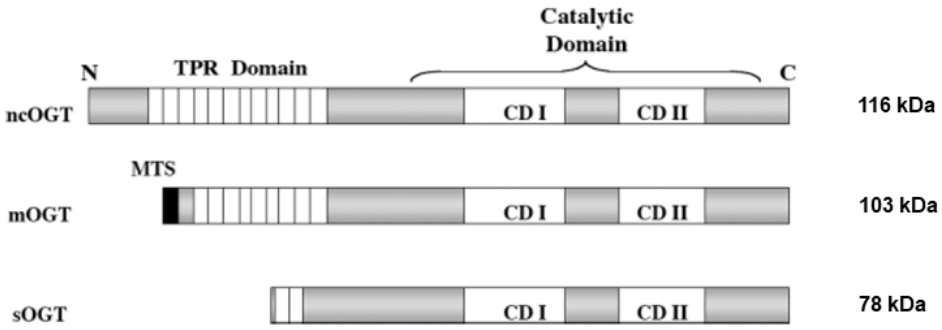

Figure 14. The three isoforms of OGT.
N-terminal domain contains tetraticopeptide repeat (TPR) motifs which are important for the recognition of protein substrates, while the C-terminal domain consists of the glycosyltransferase activity. Nucleocytoplasmic OGT (ncOGT, 116 kDa), mitochondrial OGT (mOGT, 103 kDa) and short OGT (sOGT, 78 kDa) (adapted from Lazarus, Love et al. 2006.

OGA, on the other hand, is expressed as two isoforms (i) long, nucleocytoplamic (OGA-L, 102 kDa) and (ii) short, nuclear (OGA-S, 76 kDa) (reviewed in Liu, Dai et al. 2015).

Since knockout of OGT in mammals (Shafi, Iyer et al. 2000) and OGA in mice (Yang, Song et al. 2012) is lethal on a developmental or single cell level, respectively, the design of effective inhibitors raised the ability to study *O*-GlcNAcylation *in vivo*. Gloster *et al.* generated metabolic inhibitors of glycosyltransferases within cells using sugar analogues. After its uptake, Ac-5SGlcNAc -one of those inhibitors- is converted to UDP-5SGlcNAc which inhibits activity of OGT by direct binding leading to decreased levels of *O*-GlcNAc in cells (Gloster, Zandberg et al. 2011). Other small molecules to inhibit OGT have been reported with controversial observations: (i) alloxan that completely blocks activity of OGT *in vitro* but is not specific and inhibits other critical enzymes (Lee, Alborn et al. 2006), (ii) benzoxazolinone also inhibits OGT by forming a carbonyl crosslink in the OGT active site which is an irreversible process (Jiang, Lazarus et al. 2011), (iii) OSMI-1, which is a cell-permeable molecule that is able to inhibit OGT in several mammalian cell lines (Ortiz-Meoz, Jiang et al. 2015), however, high cytotoxicity was observed in human hepatoma cells (Herzog and Zeisel, unpublished data) or (iv) ST045849, which leads to decrease of total *O*-GlcNAcylation in prostate cancer cells (Itkonen, Gorad et al. 2016), while in human hepatoma cells increased levels of *O*-GlcNAcylation have been observed (Herzog and Zeisel, unpublished data). On the other hand, O-GlcNAcylation in cells is increased by efficient inhibition of OGA via Thiamet G (Yuzwa, Macauley et al. 2008) and GlcNAcstatin C (Dorfmueller, Borodkin et al. 2006) *in vitro* (Mariappa, Sauert et al. 2011) as well as *in vivo* (Yuzwa, Shan et al. 2012). Recently, a metabolic precursor inhibitor that acts as an analogue of N-acetylglucosamine, termed 5SGlcNHex, was shown to decrease O-GlcNAc levels in time-and dose-dependent manner in various tissues. Moreover, a correlation between decreased O-GlcNAc levels and lower levels of transcription factor Sp1 and satiety-induced hormone leptin could be demonstrated *in vitro* in adipocytes as well as *in vivo* in mice proposing an association between decreased O-GlcNAc levels and nutrient sensing in peripheral tissues of mammals (Liu, Zandberg et al. 2018).

O-GlcNAcylated proteins of different functional classes have been detected, including transcription factors, metabolic enzymes and signaling proteins (see Figure 15) highlighting a crucial role of *O*-GlcNAcylation in many biological processes as transcription, translation, metabolism, signal transduction and autophagy (Butkinaree, Park et al. 2010; Ferron, Denis et al. 2018). Additionally, proteins with intrinsic disorders can be non-specifically modified by OGT as well (e.g. tau and nuclear pore proteins) (reviewed in Yang and Qian 2017).

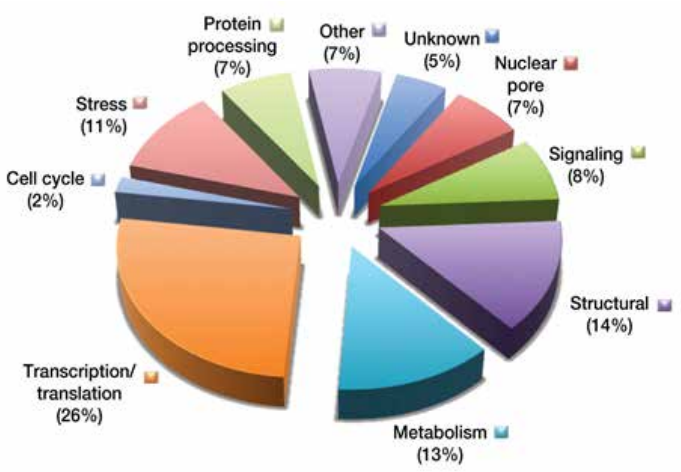

Figure 15. Functional distribution of identified *O*-GlcNAcylated proteins (adapted from Zachara, Akimoto et al. 2015).

2.3 OGT and O-GlcNAcylation in disease and cancer- Current knowledge for liver disease and HCC

O-GlcNAcylation plays a crucial role in many different biological processes and is solely performed by OGT and OGA; due to this fact, it is obvious that deregulated *O*-GlcNAcylation has been associated with a variety of human diseases ranging from diabetes and neurodegenerative and cardiovascular diseases to cancer (reviewed in Banerjee, Lagerlof et al. 2016; Pinho, Verde et al. 2018; de Queiroz, Carvalho et al. 2014; Fardini, Dehennaut et al. 2013). However, detailed knowledge how and which *O*-GlcNAcylation contributes to these diseases remains incomplete.

In fact, the present thesis is based on HCV and virus-host interactions. Since it is known that HCV infection, even after SVR, can lead to progressive liver disease and HCC, I will focus here, for clarity reasons, on the current knowledge about *O*-GlcNAcylation and its contribution to cancer development, especially for HCC development.

Basically, cancer cells are characterized by uncontrolled proliferation and show a specific metabolic phenotype in which ATP is primarily produced through anaerobic glycolysis rather than through oxidative phosphorylation. The metabolic shift is widely known as “Warburg effect”, after the discovery made by Otto Warburg resulting in an up-regulation of glucose uptake and consequently in increased flux of metabolites through the HBP (Warburg 1956). This phenomenon of metabolic shift is characterized through an increased *O*-GlcNAcylation, observed in a wide range of cancers, resulting in an altered glycosylation profile leading to deregulated signaling and transcriptional pathways (reviewed in (Ferrer, Sodi et al. 2016; Liberti and Locasale 2016) (see Table 2).
Tumorigenesis, invasion and metastasis of breast, lung and colon cancer is enhanced through aberrant O-GlcNAcylation (Caldwell, Jackson et al. 2010; Gu, Mi et al. 2010; Mi, Gu et al. 2011; Ferrer, Lu et al. 2017). It has been demonstrated that aberrant flux through HBP (reviewed in Bond and Hanover

2015) resulted in alteration of cell surface *O*-Glycans (Alisson-Silva, Freire-de-Lima et al. 2013) as well as *N*-Glycans (Dennis, Lau et al. 2009), however, the focus here is on *O*-GlcNAcylation. One outcome of the deregulated signaling pathways is the gain of function of oncogenes and a loss of function of tumor suppressors. Indeed, it could be demonstrated that *O*-GlcNAcylation is able to modify several tumor-associated proteins as for example Nuclear factor κB (NF-κB), c-Myc, β-catenin, p53 and Ras (reviewed in de Queiroz, Carvalho et al. 2014; Nie and Yi 2019). Additionally, it has been demonstrated that several HBP genes were overexpressed in human prostate cancer as well (Itkonen, Minner et al. 2013).

HCC is the most frequently occurring primary liver malignancy worldwide, in fact liver transplantation (LT) is the only treatment of choice available for patients with early stage of HCC (reviewed in El-Serag and Davila 2011; El-Serag and Rudolph 2007). Nevertheless, tumor recurrence following LT remains so far un unsolved problem preventing patients from long-term survival. The addressed question is which are the molecular mechanism of tumor recurrence after LT? Zhu et al. tried to elucidate this question by studying *O*-GlcNAcylation levels in HCC tissues of patients with different etiologies (Zhu, Zhou et al. 2012). Indeed, it has been shown that *O*-GlcNAcylation levels of HCC tissues was significantly increased as compared to healthy liver tissue; moreover, HCC tissue of those patients that suffered from tumor recurrence after LT had significantly enhanced *O*-GlcNAcylation levels (Zhu, Zhou et al. 2012). While OGT expression could not be correlated with a prognosis for HCC recurrence after LT, low OGA expression in tumors has been elevated with an increased risk of tumor recurrence after LT (Zhu, Zhou et al. 2012).
NF-κB, a transcription factor known to play a crucial role in cancer-related processes as cell proliferation, apoptosis ad metastasis (reviewed in Patel, Horgan et al. 2018) displays two O-GlcNAcylation sites at its subunit p65, at threonine 322 and 352. The latter glycosylation site is responsible for an increased transcriptional activity (Yang, Park et al. 2008). Interestingly, another group observed a tumor-promoting role of OGT in fatty liver-associated HCC by regulating lipid metabolism through increasing palmitic acid leading to ER stress and ER-related NF-κB signaling pathways proposing an oncogenic role for OGT in fatty-liver associated HCC (Xu, Zhang et al. 2017). Most recently, another group could reveal an increase of total *O*-GlcNAcylation and an increase of OGT protein expression in HCC (Cao, Duan et al. 2019). Moreover, it could be shown that eukaryotic initiation factor 4E (eIF4E), a key translation factor of protein synthesis, is *O*-GlcNAcylated by OGT leading to stability of protein and protection against proteasomal degradation; as a result, it is proposed that *O*-GlcNAcylation contributes to a stem-like cell potential of hepatoma cells (Cao, Duan et al. 2019).

Taken together, O-GlcNAcylation has a crucial role for reprogramming metabolic networks of cancer cells and in turn in promoting tumor growth and carcinogenesis. Table 2 gives an overview of deregulated O-GlcNAcylation and aberrant OGT/OGA protein expression in different kind of cancers.

Tumor type	O-GlcNAc/OGT/OGA	References
Liver cancer	↑/↑/↓	Cao, Duan et al. 2019; Xu, Zhang et al. 2017; Zhu, Zhou et al. 2012
Breast cancer	↑/↑/↓	Liu, Huang et al. 2017; Ferrer, Lu et al. 2017; Caldwell, Jackson et al. 2010; Gu, Mi et al. 2010; Barkovskaya, Seip et al. 2019
Prostate cancer	↑/↑/NI	Kamigaito, Okaneya et al. 2014; Itkonen, Minner et al. 2013; Itkonen, Gorad et al. 2016; Lynch, Ferrer et al. 2012; Itkonen, Urbanucci et al. 2019
Colon cancer	↑/↑/↓	Yehezkel, Cohen et al. 2012; Mi, Gu et al. 2011; Phueaouan, Chaiyawat et al. 2013; Biwi, Clarisse et al. 2019; Fuentes-Garcia, Castaneda-Patlan et al. 2019
Bladder cancer	NI/↑/↓	Wang, Chen et al. 2018; Rozanski, Krzeslak et al. 2012
Leukemia	↑/NI/NI	Shi, Tomic et al. 2010
Endometrial cancer	NI/↑/↑	Krzeslak, Wojcik-Krowiranda et al. 2012
Pancreatic cancer	↑/↑/↓	Ma, Vocadlo et al. 2013; Sharma, Gupta et al. 2019
Lung cancer	↑/↑/ND	Mi, Gu et al. 2011; Szymura, Zaemes et al. 2019; Lin, Lin et al. 2018
Thyroid cancer	↓/NI/↑	Krzeslak, Jozwiak et al. 2011; Zhang, Wang et al. 2015; Cheng, Li et al. 2016

Table 2. O-GlcNAc dynamics in different types of cancer.
↑: increase; ↓: decrease; ND: No difference; NI: Not identified.

Even if an increased global *O*-GlcNAcylation and increased OGT protein expression could be shown to contribute to cancer development, downregulation of *O*-GlcNAcylation could be demonstrated to drive tumorigenesis as well (e.g. thyroid cancer). Overall, one can assume that uncovering key *O*-GlcNAcylated proteins and deregulated OGT/OGA expression could reveal novel biomarkers for

early detection of cancer, and especially for HCC these potential markers could help to predict patient risk of recurrence and could be potential targets for efficient therapy.

III. OBJECTIVES

Infection of human hepatocytes is a complex process involving both viral and host factors involved in the HCV replication cycle such as microRNAs (miRNAs). Within the past years, it has been shown that to establish chronic infection, HCV hijacks and modulates host cell-derived miRNAs that are required for the viral life cycle. Uncovering host factors hijacked by HCV contributes to a better understanding of virus-host interactions underlying the HCV life cycle and involvement in the establishment of chronic HCV infection as well as to the identification of potential targets for treatment of liver disease and prevention of HCC (Zeisel and Baumert 2017; Zeisel, Crouchet et al. 2015).

To systematically uncover human miRNAs affecting the HCV replication cycle, the laboratory had performed a genome-wide screen in human hepatoma Huh7.5.1 cells using a genomic miRNA mimic library and a two-step infection assay with a *Renilla* luciferase reporter HCV virus (JcR2a) (Herzog, Bandiera et al. 2019, figure 1A). Through this miRNA mimic screen 427 miRNAs could be identified that significantly modulated HCV infection (Herzog, Bandiera et al. 2019, figure 1C). One hundred eighty-six miRNAs out of the 427 miRNAs affected HCV entry and replication, while the remaining 309 miRNAs modified viral assembly and release (including 68 hits affecting both HCV entry/replication and assembly/release) (Figure 16).

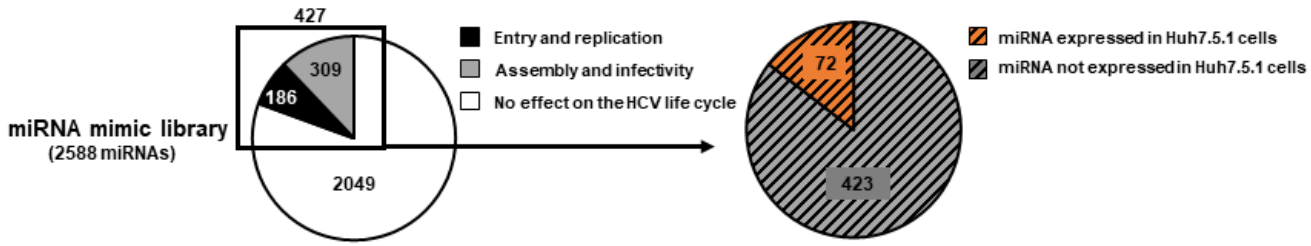

Figure 16. miRNAs involved in the HCV life cycle and expression in Huh7.5.1 cells.

Thus, it was decided to focus on miRNA hits that modulated late steps of the HCV replication cycle since these processes steps still remain incompletely understood. Among the 309 miRNAs, miR-501-3p and miR-619-3p appeared to markedly enhance HCV infection suggesting that these miRNAs may target host genes which contribute to control virus assembly and release (Herzog, Bandiera et al. 2019, figure 1D). By using a combination of computational and functional approaches to study miRNA targeting, OGT was uncovered as a common target for miR-501-3p and miR-619-3p and

demonstrated to be markedly involved in assembly and infectivity (Herzog, Bandiera et al. 2019, table 1 and figure 2).

Hence, the aim of my PhD project was to characterize the role of OGT in HCV-host interactions. The specific aims of my PhD project were i) to analyze the interplay of miR-501-3p and miR-619-3p with their predicted target OGT, ii) to unravel how OGT may contribute to HCV morphogenesis and, iii) to investigate the role of miR-501-3p and OGT in HCV-induced liver disease and HCC development.

IV. RESULTS

To systematically uncover miRNAs that could play a role in HCV infection, the team of Mirjam Zeisel and Thomas Baumert had conducted a genome-wide screen in human hepatoma Huh7.5.1 cells using a genomic miRNA mimics library and a two-step infection assay with a luciferase reporter virus (JcR2a), which allowed us to functionally assess the role of miRNAs during the early steps (viral entry/translation/replication) and the late steps (viral assembly/release/infectivity) of the HCV life cycle. This screen identified a set of miRNAs whose overexpression overall impairs HCV infection by affecting early and/or late steps of the HCV replication cycle. The analysis was focused on miRNAs that modulate late steps of the HCV life cycle, as the molecular mechanisms underlying HCV assembly/release still remain only partially understood. The screen had identified 11 miRNAs that increased and 230 miRNAs that decreased late steps of HCV infection without affecting early steps of infection. Among the miRNAs that increased HCV infection, miR-140-3p, miR-501-3p, miR-619-3p and miR-4778-5p had not been associated with HCV before. The effect of two out of these 4 miRNAs, namely miR-501-3p and miR-619-3p, on late steps of the HCV life cycle were confirmed in independent experiments. The targets of these two miRNAs were predicted using several bioinformatics tools and the list of potential targets were refined by considering only those genes that are i) expressed in Huh7.5.1 cells as assessed using a microarray analysis and ii) belonging to pathways potentially involved in infectious virus production, such as lipid metabolism and cholesterol biosynthesis, protein maturation and processing at the ER and components of the endosomal sorting complex. This led to a list of 28 candidate targets whose functional role in HCV infection was subsequently assessed using a two-step infection assay with a luciferase reporter virus (JcR2a) and siRNA directed against these 28 targets. Interestingly, only the silencing of OGT phenocopied the effect of miR-501-3p and miR-619-3p on HCV infection, suggesting that OGT modulates HCV assembly, release and/or infectivity.

During my PhD, to assess whether OGT could be indeed targeted by miR-501-3p and miR-619-3p, OGT expression was analyzed at both the mRNA and the protein levels in Huh7.5.1 cells following overexpression of miR-501-3p and miR-619-3p. We demonstrated that neither miRNA had an impact on the OGT mRNA levels (Herzog, Bandiera et al. 2019, figure 3A) but that miR-501-3p significantly decreased OGT protein expression (Herzog, Bandiera et al. 2019, figure 3B). Using a dual luciferase reporter construct, we showed that co-transfection of miR-501-3p mimic with the wild-type 3'UTR reporter (RLuc wt *OGT* 3'UTR) significantly decreased luciferase activity as compared to the empty vector while the repression was lost when the reporter with the mutated miR-501-3p binding site (RLuc mt *OGT* 3'UTR) was used (Herzog, Bandiera et al. 2019, figure 3C). These data indicate that

miR-501-3p mediates post-transcriptional regulation of OGT by decreasing its expression at the protein level.

To investigate the effect(s) of OGT on HCV assembly, release and/infectivity, we determined infectious virus titer (TCID50), HCV RNA levels and the specific infectivity of HCVcc particles generated in Huh7.5.1 cells with silenced OGT. We observed a significant increase in the TCID50 as well as in the specific infectivity of HCVcc generated in OGT-silenced Huh7.5.1 cells as compared to control HCVcc (Herzog, Bandiera et al. 2019, figure 4A). Furthermore, using pharmacological inhibitors to inhibit either OGT ($Ac_4$5s-GlcNAc) or its counterpart OGA (Thiamet G), we demonstrated that O-GlcNAcylation modulates HCVcc infectivity.

By analyzing the structural and biophysical properties of HCVcc produced in OGT-silenced Huh7.5.1 cells, we demonstrated that silencing of OGT led to the production of more infectious HCVcc with higher density (Herzog, Bandiera et al. 2019, figure 5A-B) as well as higher ApoE concentrations (Herzog, Bandiera et al. 2019, figure 5C) suggesting that OGT/O-GlcNAcylation affects the biophysical properties of HCVcc. Electron microscopy (EM) analysis and counting of HCVcc particles revealed a shift towards bigger size of sucrose-cushion purified HCVcc generated in OGT-silenced Huh7.5.1 cells as compared to control HCVcc (Herzog, Bandiera et al. 2019, figure 6A-B) suggesting that OGT-silencing affects the lipidation of HCVcc.

Since the silencing of OGT promotes HCVcc infectivity, we also assessed whether HCV infection in turn had an effect on miR-501-3p and OGT expression. In Huh7.5.1 cells, HCV infection led to significant decrease of OGT mRNA as well as proteins levels, while a small but significant increase of miR-501-3p expression could be observed (Herzog, Bandiera et al. 2019, figure 7A-B and supplementary figure 1B) which may promote viral infection given the proviral and antiviral roles of miR-501-3p and O-GlcNAcylation, respectively (Herzog, Bandiera et al. 2019, figure 1C-D and 4D). In liver tissue from HCV-infected patients, HCV RNA levels were not correlated with OGT expression suggesting that in patients there is likely no direct effect of HCV on OGT expression (Herzog, Bandiera et al. 2019, figure 7C).

Given the fact that O-GlcNAcylation has been associated with a variety of cancers, we also studied OGT expression in chronic liver disease and HCC. While a trend of increased OGT expression in liver tissue from HCV-infected patients with fibrosis and inflammation could be observed (Herzog, Bandiera et al. 2019, figure 7D-E), OGT levels were markedly and significantly elevated in the tumor tissue of patients chronically infected with HCV or hepatitis B virus, and patients with alcoholic liver disease or non-alcoholic fatty liver disease as compared to non-tumor tissue (Herzog, Bandiera et al. 2019, figure 7F). These data suggest that OGT expression increases in HCC in an etiology-

independent manner. Taken together, these results suggest that OGT expression likely increased in HCV-induced liver disease and cancer trough inflammation and fibrosis rather than by HCV itself.

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A functional microRNA screen uncovers O-linked N-acetylglucosamine transferase as a host factor modulating hepatitis C virus morphogenesis and infectivity

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Abstract

Objective: Infection of human hepatocytes by the hepatitis C virus (HCV) is a multistep process involving both viral and host factors. microRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate gene expression. Given that miRNAs were indicated to regulate between 30% and 75% of all human genes, we aimed to investigate the functional and regulatory role of miRNAs for the HCV life cycle.

Design: To systematically reveal human miRNAs affecting the HCV life cycle, we performed a two-step functional high-throughput miRNA mimic screen in Huh7.5.1 cells infected with recombinant cell culture-derived HCV. miRNA targeting was then assessed using a combination of computational and functional approaches.

Results: We uncovered miR-501-3p and miR-619-3p as novel modulators of HCV assembly/release. We discovered that these miRNAs regulate O-linked N-acetylglucosamine (O-GlcNAc) transferase (OGT) protein expression and identified OGT and O-GlcNAcylation as regulators of HCV morphogenesis and infectivity. Furthermore, increased OGT expression in patient-derived liver tissue was associated with HCV-induced liver disease and cancer.

Conclusion: miR-501-3p and miR-619-3p and their target OGT are previously undiscovered regulatory host factors for HCV assembly and infectivity. In addition to its effect on HCV morphogenesis, OGT may play a role in HCV-induced liver disease and hepatocarcinogenesis.

Significance of this study

What is already known about this subject?

- To establish chronic infection, the hepatitis C virus (HCV) hijacks cellular factors including microRNAs (miRNAs), known to post-transcriptionally regulate gene expression.
- miRNAs may positively or negatively modulate HCV infection either by directly targeting the viral genome or indirectly by regulating virus-associated cellular pathways.

What are the new findings?

- A functional miRNA mimic screen uncovered miR-501-3p and miR-619-3p to enhance late steps of HCV infection.
- miR-501-3p regulates the expression of O-linked N-acetylglucosamine transferase (OGT) at the protein level.
- Silencing of OGT expression or inhibition of O-linked N-acetylglucosaminylation (O-GlcNAcylation) leads to an increase in the infectivity and size of HCV particles.
- OGT expression increases in patient-derived liver tissue during liver disease progression and cancer.

How might it impact on clinical practice in the foreseeable future?

- As upregulation of OGT and increased O-GlcNAcylation of proteins have been associated with various forms of cancer, OGT may play a dual role in HCV morphogenesis as well as pathogenesis of HCV-induced liver disease and carcinogenesis.

Introduction

Chronic hepatitis C is a major cause of chronic liver disease and hepatocellular carcinoma (HCC). Since the approval of pan-genotypic direct-acting antivirals (DAAs), it is considered a curable disease in more than 90% of treated patients. Nonetheless, an estimated 71 million individuals are still infected by the hepatitis C virus (HCV) and several challenges remain; viral cure reduces but does not eliminate the HCC risk in patients with advanced fibrosis[1], the majority of infected patients has limited access to therapy and DAA failure/viral resistance has been reported in a subset of patients[2, 3]. To overcome these limitations, approaches to target host factors involved in HCV infection and pathogenesis are developed[4, 5]. Interestingly, defined host factors that contribute to the establishment of chronic HCV infection and represent potential antiviral targets, e.g. epidermal growth factor receptor[6], also play a role in liver disease pathogenesis and represent candidate targets for treatment of advanced liver disease and HCC prevention[7]. Thus, uncovering host factors usurped by HCV not only contributes to a better understanding of virus-host interactions underlying the HCV life cycle but also to the identification of potential targets for treatment of liver disease and prevention of HCC.

The establishment of various models to study HCV infection has shed light on the molecular mechanisms that govern the HCV life cycle, which can be subdivided into early steps, including viral entry, translation and replication as well as late steps, including assembly and release of new virions. Each step of the HCV replication cycle relies on specific virus-host interactions that involve host proteins and microRNAs (miRNAs)[5], small non-coding RNAs that regulate gene expression at the post-transcriptional level. One miRNA can target numerous messenger RNAs (mRNAs) by base-pairing with a complementary site that is typically located within the 3′ untranslated region (3′UTR) of the mRNA. Accumulating evidence indicates that miRNAs participate to HCV replication by exerting pro- or antiviral effects. The breakthrough discovery of the direct targeting of HCV by miR-122, the most abundant miRNA in the liver, revealed the crucial role of this miRNA for HCV translation/replication that contributes to progression to chronic HCV infection[8, 9]. miR-122

antisense oligonucleotides were subsequently developed as host-targeting antivirals[10, 11]. Other miRNAs can indirectly target HCV by regulating host factors that participate in antiviral responses and immune surveillance[12, 13, 14]. Since up to 60% of all human protein-coding genes were reported to be under miRNA-mediated regulation and miRNAs are involved in basically every biological process, we hypothesized that miRNAs provide a tool for loss-of-function approaches to uncover novel HCV host factors. We performed genome-wide high-throughput modulation of the human miRNome and analyzed their impact on HCV infection by combining computational and functional approaches.

Material and methods

Cells, cell culture conditions, viruses, virus purification, infectivity assays, miRNAs, antagomiRs, siRNAs, antibodies, immunoblot, immunocapture, electron microscopy analysis of viral particles and gene expression analysis in liver tissue are described in the Supplementary information.

Functional miRNA/siRNA screens. Huh7.5.1 cells were transfected with the miRIDIAN human miRNA mimic library (mIRBase 19) comprising more than 2000 mature miRNAs or 28 ON-TARGETplus smart pool siRNAs (20 nM, Dharmacon) using Interferin HTS (Polyplus) in a 96-well format[6]. After 48h, a viability test (Presto Blue, Thermo Scientific) was performed prior to a two-step infection assay[15, 16, 17]. During part 1 of the protocol, 50 µL of HCV cell culture-derived particles (HCVcc, JcR2a) were incubated with cells during 4h. The inoculum was removed and cells were incubated with 150 µl of medium for 48h. In part 2, supernatants from part 1 cells were transferred onto naïve Huh7.5.1 cells and part 1 cells were lysed to determine luciferase activity[17, 18]. After 72h, part 2 cells were lysed to determine luciferase activity[17]. siCD81 (20 nM), antagomiR-122 (100 nM) and siApoE (20 nM) were used as positive controls[17]. A non-targeting siRNA with no sequence complementarity to any human gene or homology to any human miRNA was used as negative control.

Inhibitor treatment. Four hours following HCV RNA electroporation[6], Huh7.5.1 cells were incubated with vehicle or inhibitors of OGT (peracetylated 5-thio-N-acetylglucosamine ($Ac_4$5S-GlcNAc)[19]) or OGA (Thiamet G (Sigma))[20]. After 96h, supernatants were transferred onto naïve Huh7.5.1 cells for 72h prior to determination of luciferase activity while electroporated cells were lysed to determine luciferase activity.

Gene expression analyses. Total RNA was purified[17] and transcribed into cDNA using Maxima reverse transcriptase (Thermo Scientific). *GAPDH* and *OGT* mRNA was detected by real time qPCR using iTaq™ Universal Probes Supermix (Bio-Rad) and TaqMan Gene Expression Assay (Thermo Scientific). Relative *OGT/GAPDH* gene expression was calculated by the ΔΔCt method[21].

Dual luciferase reporter gene assay. The human *OGT* 3'UTR sequence was retrieved from NCBI (NM_181672.2) and Ensembl genome browser (ENST00000373719.3). A fragment of the *OGT* 3′UTR (positions 3380-3837, NM_181672.2) (Thermo Fisher Scientific GENEART) was cloned between the *Not*I and *Xho*I sites downstream of a *Renilla* luciferase cassette in a psiCHECK2 plasmid (Promega). A mutated version of this construct (9-bp substitution in the predicted miR-501-3p target site) was generated as described[22]. The functionality of the *OGT* 3′UTR was assessed as described[23]. The miRIDIAN mimic negative control 1 was used as control. *Renilla* and *firefly* luciferase activity was assessed 48h after transfection into HeLa cells using Dual-Luciferase Reporter assay (Promega).

Bioinformatic and statistical analysis. Data analysis and statistical treatment for the miRNA mimic screen were performed in R (www.r-project.org). Cell measurement data used in further analysis were cell viability and luciferase activity. In total 26 sets of plates (performed in triplicate) were tested. The presence of multiple wells with negative and positive controls on each plate allowed stepwise normalization intra- and inter-plate. First, intra-plate zonal bias was examined and a model of median effects across the entire screen

determined using the median-polish algorithm[24] and all plates corrected accordingly. Then the dataset was examined for outlier plates, i.e. plates where all individual measurements correlate very poorly with the other remaining replicates. Three and 9 plates were excluded for part 1 and part 2 of the screen, respectively, based on poor median correlation ($r < 0.7$) so that the remaining plates correlation improved substantially (> 40%). Next, the plates were normalized inter replicates using the particularly robust quantile-quantile approach[25]. Finally, the data were tested using a moderated t-test (empirical Bayes shrinkage, R-package limma[26]) for the null-hypothesis of no change of a given miRNA compared to the negative control. The resulting *p*-values for independent testing of each miRNA where corrected for the multiple testing situation and expressed as local false discovery rate (lfdr, R-package fdrtool[27]). The testing was performed independently for part 1 and 2 of the screen and candidate miRNAs selected for each part. For data from part 1, a lfdr threshold of 0.00027 was used. Data from part 2 were subject to increase inherent stochastic noise and for this reason the minimum acceptable relative risk of false positives was increased to 0.1226 (i.e. maximum 15% risk for each of the retained hits).

Other datasets were analyzed using the two-tailed Mann-Whitney test, Wilcoxon test, Spearman correlation or the two-tailed unpaired t-test for data with normal distribution as assessed by D'Agostino and Pearson omnibus and Shapiro-Wilk normality tests (GraphPad Prism v.6 package).

Results

Genome-wide identification of human miRNAs affecting the HCV life cycle. We performed a genome-wide screen in human hepatoma Huh7.5.1 cells using a genomic miRNA mimics library and a two-step infection assay[17] with a luciferase reporter virus (JcR2a), which allowed us to functionally assess the role of miRNAs during the early steps (part 1 - viral entry/translation/replication) and the late steps (part 2 - viral assembly/release/infectivity) of the HCV life cycle (Fig. 1A). Silencing of *CD81* and *ApoE*,

two essential host factors required for HCV entry or assembly, respectively, was performed in parallel using small interfering RNA (siRNA) as controls. Silencing of *CD81* resulted in a reduction of HCV infection in part 1 and consequently in part 2 of the screen since reduced viral entry in the first part of the assay leads to a reduced production of viral particles (Fig. 1B)[17]. Silencing of *ApoE* resulted in a marked inhibition of HCV infection only in part 2 of the assay, consistent with the role of ApoE in HCV assembly (Fig. 1B)[17]. The screen identified 427 miRNAs (corresponding to about 16% of the library) that significantly modulated HCV infection (lfdr < threshold, Supplementary Table 1 and Fig. 1C): 186 miRNAs affected HCV infection in part 1, 309 miRNAs affected HCV infection in part 2, including 68 hits in part 1 and part 2. The limited number of part 1 and 2 hits may be due to the fact that a single miRNA may modulate the expression of several proteins, which may have different roles in the viral life cycle. Most hits were observed to dampen HCV infection independently of any significant alteration of cell viability (data not shown). The 186 miRNAs modulating the early steps of HCV infection all decreased viral infection. Among the 309 miRNAs that had an impact in part 2, 11 miRNAs increased HCV infection by at least 3-fold while 298 miRNAs inhibited HCV infection by at least 2.7-fold. Hits from the screen included the let-7 family[12, 28], miR-27a[29] and miR-29 family[30] that were already shown to inhibit HCV infection, as well as miR-21[31] and miR-146a-5p[17] that were shown to stimulate HCV infection thus supporting the relevance of our findings. Collectively, our screen identified a set of miRNAs whose overexpression overall impairs HCV infection by affecting viral entry/translation/replication and/or virion assembly/egress/infectivity.

miR-619-3p, miR-501-3p and OGT play a role in late steps of the HCV life cycle. We focused our analysis on miRNAs that modulate late steps of the HCV life cycle, as the molecular mechanisms of HCV assembly/release remain only partially understood. Our screen identified 241 miRNAs that modulated late steps without affecting early steps of infection: 11 miRNAs increased HCV infection while 230 miRNAs decreased HCV infection. Among the miRNAs that increased HCV infection, miR-140-3p, miR-501-3p, miR-619-3p and

miR-4778-5p have not yet been associated with HCV. Since they enhanced HCV infection in part 2 without affecting part 1, these miRNAs may target host genes that control virus assembly/egress/infectivity. We first confirmed the effect of these miRNAs in independent experiments using the same protocol as for the screen. Overexpression of miR-619-3p or miR-501-3p consistently led to an increase in the infection of progeny virions (Fig. 1D) while infection was decreased with progeny virions from antagomiR-transfected cells (Supplementary Figure S1A). miR-619-3p or miR-501-3p were thus selected for further investigation. To study the molecular mechanisms by which these miRNAs affect HCV infection, we generated a list of predicted miRNA targets using DIANA, TargetScan Human v6.2 and miRDB databases, and selected candidate targets based on their expression in our Huh7.5.1 cells as assessed by microarray (data not shown). Ingenuity Pathway Analysis enabled us to refine the gene list by selecting 28 genes involved in the following functional networks or pathways that contribute to the HCV life cycle[32, 33, 34]: lipid metabolism and cholesterol biosynthesis, protein maturation and processing at the endoplasmic reticulum (ER), components of the endosomal sorting complex, adipocyte biogenesis, cellular morphology and cell inflammation (Table 1).

To assess whether knock-down of these 28 candidate targets affects virus production, we performed a siRNA-based screen using siRNA pools exhibiting strong silencing without cytotoxicity (Fig. 2). Silencing of *CD81* and antagomiR-122 served as controls for part 1; knock-down of *ApoE* served as control for part 2 (Fig. 2). Hits were defined as genes whose knock-down modulated HCV infection in at least one part of the screen with high significance (Fig. 2, *p*-value < 0.0001, Mann-Whitney U-test). HCV entry/translation/replication was significantly modulated by silencing of *PPP3CA, CEBPA, MID1, WDFY3, DCX* and *SLC35D1*. HCV assembly/egress/infectivity was significantly modulated by knock-down of *PPP3CA, CSDE1, GAN, USP37, CEBPA, MID1, WDFY3, DCX, MAPK9, SLC35D1, DCC, RNF144A, PPP2R2C* and *OGT*. Strikingly, only the silencing of *OGT* was associated with an enhancement of HCV assembly/release/infectivity (*p*-value = 0.0002), while that of the other hits was associated with reduced HCV infection (Fig. 2).

These results indicate that the down-regulation of *OGT* phenocopies the effect of miR-501-3p and miR-619-3p on HCV infection (Fig. 2) and suggest OGT as a novel player in the HCV life cycle.

miR-501-3p post-transcriptionally regulates OGT expression. To study whether miR-501-3p and miR-619-3p target OGT, we analyzed OGT RNA and protein levels in Huh7.5.1 cells following overexpression of miR-501-3p or miR-619-3p. While neither miRNA had an impact on OGT RNA levels (Fig. 3A), up-regulation of miR-501-3p significantly decreased OGT protein expression by ~65% (Fig. 3B, *p*-value < 0.05, t-test). miR-619-3p also decreased OGT expression but less robustly than miR-501-3p (Fig. 3B), prompting us to focus our investigation on miR-501-3p. To assess whether OGT is a functional target of miR-501-3p, we subcloned a fragment of the *OGT* mRNA 3′UTR that harbors the predicted miR-501-3p target site in the *Renilla* luciferase expression cassette (RLuc) of a dual luciferase reporter construct. Co-transfection of miR-501-3p mimic with the wild-type 3'UTR reporter (RLuc wt *OGT* 3'UTR) significantly decreased luciferase activity as compared to the empty vector (Fig. 3C, p-value < 0.05, t-test). In contrast, the repression of luciferase expression was lost when the reporter with mutated miR-501-3p binding site (RLuc mt *OGT* 3'UTR) was used (Fig. 3C). These data are consistent in indicating that miR-501-3p mediates post-transcriptional regulation of OGT.

O-GlcNAcylation modulates HCVcc infectivity. To investigate whether OGT modulates HCV assembly and/or infectivity, we determined infectious virus titer (TCID50) and HCV RNA levels to calculate the specific infectivity of HCVcc particles generated in OGT-silenced Huh7.5.1 cells. Interestingly, OGT-silencing led to a significant increase in the TCID50 and the specific infectivity of HCVcc (Fig. 4A, *p*-value < 0.05, Mann-Whitney test). Noteworthy, the effect of OGT on HCVcc infectivity was genotype-independent as demonstrated by increased infectivity of HCVcc bearing the envelope glycoproteins of genotypes 1a, 1b and 2a upon OGT-silencing (Fig. 4B). We next sought to investigate how OGT could modulate

HCVcc infectivity. OGT is the only enzyme that catalyzes the addition of N-acetylglucosamine (O-GlcNAc) to serine and threonine residues of proteins. Moreover, OGT has a scaffold function and promotes binding of proteins in multiprotein complexes[35]. To assess whether the enzymatic activity of OGT modulates HCVcc infectivity, we used pharmacological inhibitors of OGT ($Ac_4$5S-GlcNAc) or O-GlcNAcase (OGA) (Thiamet G), the OGT counterpart that removes O-GlcNAc (Fig. 4C). $Ac_4$5S-GlcNAc led to a significant enhancement of HCVcc infectivity in a dose-dependent manner, while the opposite effect was observed with Thiamet G (Fig. 4D, *p*-value < 0.05, Mann-Whitney test). Collectively, these results demonstrate that O-GlcNAcylation modulates HCVcc infectivity.

OGT-silencing affects HCVcc biophysical properties and size distribution. To further assess how OGT may impact HCVcc morphogenesis, we analyzed the structural and biophysical properties of HCVcc produced in siCtrl- and siOGT-transfected Huh7.5.1 cells following iodixanol gradient ultracentrifugation. Silencing of OGT led to the production of more infectious HCVcc with higher density (Fig. 5A-B) as well as higher ApoE concentrations (Fig. 5C) suggesting that OGT/O-GlcNAcylation affects the biophysical properties of HCVcc. No change in apoB concentrations were observed between HCVcc produced from siCtrl- or siOGT-transfected cells (Fig. 5D), in line with the model that HCV lipoviroparticles contain several exchangeable ApoE molecules and one non-exchangeable apoB[36]. We also visualized HCVcc by electron microscopy (EM) following anti-E2 antibody immunocapture[36] to assess whether OGT-silencing had an impact on HCVcc size. Particle size distribution was assessed from a series of randomly acquired electron micrographs. A shift towards bigger sizes was observed for sucrose-cushion purified HCVcc generated in OGT-silenced Huh7.5.1 cells as compared to control HCVcc (Fig. 6A-B). This shift was also observed in different fractions of iodixanol gradient-separated HCVcc (Fig. 6C-F) in line with the higher infectivity and ApoE concentrations of HCVcc generated in OGT-silenced Huh7.5.1 cells (Fig. 5A-C). These data suggest that OGT-silencing affects the lipidation of HCVcc.

OGT expression increases in liver disease. Since silencing of OGT promotes HCV infectivity, we assessed whether HCV infection in turn had an effect on miR-501-3p and OGT expression. In Huh7.5.1 cells, HCV infection lead to small but significant increase of miR-501-3p and decrease of OGT levels (Fig. 7A-B and Supplementary Fig. 1B; *p*-value < 0.05, Mann-Whitney test), which may promote viral infection given the pro- and antiviral roles of miR-501-3p and O-GlcNAcylation, respectively (Fig. 1C-D and 4D). In contrast, no significant difference of OGT expression was observed between the livers of HCV transgenic and wild-type mice[37] (data not shown) suggesting that HCV proteins do not directly modulate OGT expression. In liver tissue from HCV-infected patients, HCV RNA levels were not correlated with OGT expression (Fig. 7C, Spearman correlation: 0.06004019, p-value = 0.7661) suggesting that in patients there is likely no direct effect of HCV on OGT expression.

O-GlcNAcylation has been associated with a variety of cancers, including HCC recurrence linked to increased O-GlcNAcylation after liver transplantation[38]. We therefore investigated OGT expression in chronic liver disease and HCC. While there was a trend for increased OGT expression in liver tissue from HCV-infected patients with fibrosis and inflammation (Fig. 7D-E), OGT levels were markedly and significantly elevated in the tumor liver tissue of patients chronically infected with HCV or hepatitis B virus and patients with alcoholic liver disease or non-alcoholic fatty liver disease as compared to non-tumor tissue (Fig. 7F, *p*-value < 0.05, Wilcoxon test). These data suggest that OGT expression increases in HCC in an etiology-independent manner. Collectively, these results suggest that OGT expression is likely increased in HCV-induced liver disease and cancer through inflammation and fibrosis rather than by HCV itself.

Discussion

By focusing on miRNAs affecting late steps of the viral life cycle, we uncovered that i) miR-501-3p regulates the expression of OGT; ii) silencing of OGT expression or inhibition of its enzymatic activity increases the infectivity of HCV particles; and iii) OGT knock-down leads

to the release of bigger HCV particles. Our data suggest that O-GlcNAcylation affects HCV morphogenesis and infectivity.

While we were characterizing the role of OGT/O-GlcNAcylation for HCV morphogenesis, Li and colleagues published their functional genomics study of HCV-miRNA interactions[12]. By conducting genome wide miRNA mimic and hairpin inhibitor screens, they identified a set of miRNAs exhibiting a pro- or antiviral effect on HCV. Characterization of the underlying molecular processes showed that miR-25, let-7 and miR-130 families restrict viral infection by decreasing the expression of cellular HCV co-factors[12]. Despite similarities in the cell type and HCV infection models used here and by Li and colleagues, our screen only displays a small overlap with their study (9% common miRNA hits). This is not surprising given the small overlap between previous siRNA screens to uncover HCV host factors[6, 15] and is likely due i) to the different sizes of miRNA mimic libraries as the library used here was more than 2-times larger than the one used by Li and co-workers, and ii) to the markedly distinct pipelines for hit selection that were used in the two studies. Nonetheless, both screens were consistent in confirming the proviral role of miR-146a-5p in promoting HCV assembly/egress that we previously reported[17] and the global multistep inhibitory effects of the let-7 family on HCV infection[28], further corroborating the involvement of these miRNAs in fine-tuning the HCV life cycle. Both studies also consistently indicated that miR-518a-5p, miR-517-3p, miR-185 and members of the miR-302 family inhibit early steps of HCV infection, while miR-586, miR-620 and members of the miR-200 family inhibit late steps of viral infection. Since none of these miRNAs except miR-185 has been previously associated with HCV infection[39], it might be interesting to further characterize the involvement of these miRNAs in HCV-host interactions. Interestingly, an overall proviral effect of miR-501-3p was also observed by Li and colleagues[12], however the mechanism of action was not studied. By characterizing the role of miR-501-3p in the HCV life cycle, we uncovered OGT as a miR-501-3p target in liver-derived cells and showed for the first time a link between O-GlcNAcylation and HCV infection. These results indicate that genome-wide

miRNA functional screens represent a powerful strategy to dissect the role of miRNAs in pathogen-host interactions.

While N-glycosylation of HCV envelope glycoproteins plays an important role for escape from virus-neutralizing antibodies[40], so far no functional association between HCV and O-glycosylation has been reported. In contrast to N-linked glycosylation that consists of the attachment of a glycan to a nitrogen of an asparagine residue of proteins in the ER/Golgi prior to their trafficking to the plasma membrane and/or their secretion, the glycosylation of serine and threonine residues with O-GlcNAc is a post-translational modification (PTM) of intracellular proteins that are localized in the nucleus, cytoplasm or mitochondria. The O-glycosylation/deglycosylation of proteins is catalyzed by a single pair of nucleo-cytoplasmic enzymes, OGT/OGA. O-GlcNAcylation is complementary to protein phosphorylation/dephosphorylation, another more broadly known abundant protein PTM that involves numerous kinases/phosphatases. OGT/OGA are often found in protein complexes that also include kinases/phosphatases and a protein can be either O-GlcNAcylated or phosphorylated on a same residue to fine-tune cellular signaling[41]. O-GlcNAcylation and phosphorylation on the same or neighboring serine or threonine residue is known as yin yang site[42].

O-GlcNAcylation plays a major role in the regulation of metabolic pathways in the liver, including insulin signaling, bile acid metabolism and lipogenesis[35]. The large number of OGT/OGA substrates and cellular pathways regulated by O-GlcNAcylation hampers a detailed characterization of the role of these proteins in HCV infection. Since i) HCV assembly takes place at ER-derived membranes, ii) OGT/OGA are not known to localize in the ER lumen, and iii) O-GlcNAcylation of extracellular proteins containing EGF-like domains is catalyzed by EGF domain-specific OGT (EOGT) in the ER lumen in an OGT-independent manner[43]), OGT/OGA most likely modulate HCV infection by post-translationally modifying one or several cellular factors required for HCV morphogenesis rather than by affecting viral proteins, although HCV glycoproteins contain putative O-GlcNAcylation sites as determined using OGlcNAcScan, OGTsite and YingOYang1.2 bioinformatics tools (data not shown).

Regarding HCV host factors that may be regulated by OGT/OGA, O-GlcNAcylation sites have been predicted in human CLDN1[44] and OCLN at serine sites that can also be phosphorylated and this has been suggested to potentially play a role for HCV entry[45]. However, in our experimental setting we did not observe a significant effect of OGT-silencing on the early steps of HCV infection, suggesting that O-GlcNAcylation of CLDN1 and/or OCLN likely does not play a major role in HCV infection. Other host factors important for the HCV life cycle are well-known O-GlcNAcylated proteins, as for example various nuclear pore complex proteins (Nups) including Nup98, Nup153 and Nup155 that are involved in HCV replication and assembly and/or may be associated with viral particles[46, 47, 48]. However, since depletion of Nups was reported to alter HCV replication and/or assembly but to have no impact on the specific infectivity of HCV particles[46] in contrast to the depletion of OGT as shown here, it is unlikely that a modulation of Nup O-GlcNAcylation accounts for the effects of OGT-silencing and/or OGT/OGA inhibitors on HCVcc infectivity observed in our study. This is in line with our observation that OGT knock-down had no effect on Dengue virus (DENV) replication and infectivity (data not shown), although Nup98 had been suggested to potentially play a role for DENV infection[46]. These data suggest that OGT does not broadly modulate the infectivity of viruses of the *Flaviviridae* family.

However, OGT and/or O-GlcNAcylation have been reported to play a role in the infection with other viruses[49, 50, 51]. Interestingly, while OGT expression modulates the levels of human papillomavirus 16 (HPV16) oncoproteins E6 and E7[52], E6 in turn can up-regulate OGT to increase O-GlcNAcylation and the oncogene activities of HPV[53], suggesting that OGT/O-GlcNAcylation could play a role in virus-induced cancer. In cell culture, HCV infection appeared to be associated with a minor decrease in OGT expression in line with an antiviral role of O-GlcNAcylation. In contrast, an increased OGT expression was observed in HCC tissues of HCV-infected patients. Since OGT has been suggested to activate oncogenic signaling pathways in non-alcoholic steatohepatitis-related HCC[54] and O-GlcNAcylation has been associated with HCC recurrence linked to increased O-GlcNAcylation after liver transplantation[38], these data suggest that in addition to their effect

on the HCV life cycle, OGT/O-GlcNAcylation may also play a role in HCV-induced hepatocarcinogenesis.

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Figure legends

Figure 1. High-throughput screen identifies human miRNAs that regulate the HCV life cycle. (A) Schematic outline of the miRNA mimic screen strategy. Huh7.5.1 cells were transfected with miRNA mimics or controls prior to infection with *Renilla* luciferase HCVcc (JcR2a) two days later (part 1). Cell supernatants of part 1 were used to inoculate naïve Huh7.5.1 cells (part 2). Cells from part 1 and part 2 were lysed at the end of each infection step (2 and 3 days post infection, respectively) to determine luciferase activity. (B) Modulation of HCV entry and replication (part 1) and/or assembly and infectivity (part 2) upon transfection of control non-targeting siRNA (siCtrl, negative control), siCD81 (inhibiting viral entry) or siApoE (inhibiting viral assembly). By inhibiting HCV entry, siCD81 impacts part 1 as well as part 2. In contrast, by specifically impairing late steps of HCV replication cycle, siApoE inhibits HCV infection only in part 2. The box plots show the sample lower quartile (25th percentile; bottom of the box), the median (50th percentile; horizontal line in box) and the upper quartile (75th percentile; top of the box) of relative light units (RLU) in each lysate. The whiskers indicate s.d. Data are from three independent experiments. (C) Effects of miRNA overexpression on each part of the HCV life cycle. Data were tested using a moderated t-test (empirical Bayes shrinkage, R-package limma[26]) for the null-hypothesis of no change of a given miRNA compared to the negative control. The resulting p-values for independent testing of each miRNA where corrected for the multiple testing situation and expressed as local false discovery rate (lfdr, R-package fdrtool[27]). miRNAs having a significant effect on either part 1 or 2 of the screen are below the thresholds indicated by dashed lines (lfdr < 0.00027 or 0.1226, respectively). miRNAs that were previously reported to impact on HCV infection as well as miR-140-3p, miR-501-3p, miR-619-3p and miR-4778-5p are highlighted in blue (Log2(FC) < 0) or red (Log2(FC) > 0). Data are from three independent experiments. (D) Effect of miR-140-3p, miR-501-3p, miR-619-3p and miR-4778-5p on the HCV life cycle. Huh7.5.1 cells were transfected with siCtrl (Ctrl), miR-140-3p, miR-501-3p, miR-619-3p or miR-4778-5p and infection experiments were carried out as described in A. HCV infection was determined as luciferase activity. Results represent mean

percentage ± s.d. from three independent experiments in triplicate. The dashed line indicates values from control-transfected cells set at 100%. Statistics**: ***, *p*-value < 0.05, Mann-Whitney test.

Figure 2. OGT is a novel host cell factor involved in the late steps of the HCV life cycle. Huh7.5.1 cells were transfected with a set of siRNAs against 28 predicted targets of miR-501-3p and/or miR-619-3p, and infected with HCVcc JcR2A according to the two-step protocol depicted in Fig. 1A. siCD81, antagomiR-122 and siApoE were used as loss-of-function controls to perturb HCV entry, translation/replication and assembly, respectively. miR-501-3p and miR-619-3p, which were ineffective in part 1 of the screen but enhanced HCV infection in part 2, were transfected in parallel. HCV infection was quantified as fold change of luciferase activity with respect to negative control (siCtrl). Results for different replicates are shown as individual points. For each gene, median fold change of luciferase activity ± s.d. is shown as black horizontal lines. The dashed line indicates a fold change of 1. Data are from three independent experiments in triplicate. Results for miR-501-3p, miR-619-3p and siOGT that increase HCV infection in part 2 are depicted in red. Results for siRNA targeting *PPP3CA, CEBPA, MID1, WDFY3, DCX, SLC35D1, CSDE1, GAN, USP37, MAPK9, DCC, RNF144A,* or *PPP2R2C* that significantly modulated HCV infection in part 1 and/or part 2 but did not phenocopy the effect of miR-501-3p and miR-619-3p are depicted in blue.

Figure 3. miR-501-3p mediates post-transcriptional regulation of OGT by decreasing its expression at the protein level. Huh7.5.1 cells were transfected with siCtrl (Ctrl), a pool of siRNA against *OGT*, miR-501-3p or miR-619-3p. After 96h, RNA and proteins were purified, and OGT expression analyzed by RT-qPCR and Western blot. (A) Percentage of *OGT* mRNA expression in miRNA-transfected cells as compared to negative control. Results are presented as mean ± s.d. and are from three independent experiments in triplicate. The dashed line indicates values from control-transfected cells set at 100%. Statistics: *, *p*-value

< 0.05, t-test (B) OGT protein expression. Left: percentage of OGT protein expression in siRNA- or miRNA-transfected cells as assessed by quantification of Western blots. OGT levels were normalized to actin levels using ImageLab™ 5.2.1 software (BioRad). Results are presented as mean ± s.d. and are from three independent experiments. The dashed line indicates values from control-transfected cells set at 100%. Statistics: *, *p*-value < 0.05, t-test. Right: representative Western blot analysis. (C) Analysis of miRNA targeting of *OGT* expression by dual luciferase reporter assay. Left: HeLa cells were co-transfected with a miR-501-3p mimic and a dual luciferase reporter plasmid containing either wild type miR-501-3p (RLuc wt *OGT* 3'UTR) or mutated miR-501-3p binding site (RLuc mt *OGT* 3'UTR) to modulate RLuc expression. Co-transfection of the miR-501-3p mimic and empty RLuc vector was used as control. Data are expressed as mean percentage of *Renilla* luciferase activity ± s.d. normalized to *firefly* luciferase, and relative to co-transfection of the vectors with non-targeting miRNA (miR-Ctrl). Results are from three independent experiments in triplicate. The dashed line indicates values from control-transfected cells set at 100%. Statistics: *, *p*-value < 0.05, t-test. Right: Schematic representation of the used constructs.

Figure 4. Silencing of *OGT* affects HCV morphogenesis and infectivity. (A) Analysis of HCV infectivity. Huh7.5.1 cells were transfected with siCtrl, a pool of siRNA against *OGT* or ApoE as a loss-of-function control to perturb HCV assembly, prior to infection with HCVcc (Jc1) two days later (entry and replication). Mock-transfected cells were used as control (Ctrl). After another 48h, intra- and extracellular HCVcc particles were used to infect naïve Huh7.5.1 cells (assembly and infectivity). Virus supernatants of Huh7.5.1 cells were assayed by (left) endpoint dilution assay (TCID50). Intra- and extracellular HCV RNA was purified and analyzed by RT-qPCR to calculate (right) the specific infectivity (TCID50/RNA). Data are expressed as mean percentage as compared to control ± s.d. Results are from four independent experiments in triplicate. The dashed line indicates values from control-transfected cells set at 100%. Statistics**: ***, *p*-value < 0.05, Mann-Whitney test. (B) Genotype-independent effect of OGT on HCV infection. Huh7.5.1 cells were transfected with siCtrl or

siOGT prior to infection with HCVcc JcR2a (genotype 2a), H77R2a (genotype 1a) or Con1R2a (genotype 1b). Experiments were carried out and analyzed as described in A. Data are expressed as mean percentage of Renilla luciferase activity as compared to control ± s.d. Results are from three independent experiments in quadruplicate. The dashed line indicates values from control-transfected cells set at 100%. Statistics**: ***, *p*-value < 0.05, Mann-Whitney test. (C) Activity of OGT/OGA inhibitors on O-GlcNAcylation. The activity of $Ac_4$5S-GlcNAc (OGT inhibitor) or Thiamet G (OGA inhibitor) on O-GlcNAcylation of proteins in Huh7.5.1 cells was demonstrated by Western blot as described in Supplementary Methods. (D) Effect of O-GlcNAcylation on HCV infectivity. Huh7.5.1 cells were electroporated with HCVcc (JcR2a), prior to treatment with increasing concentrations of $Ac_4$5S-GlcNAc (OGT inhibitor, left) or Thiamet G (OGA inhibitor, right) 4h later. After 96h, supernatants were transferred onto naïve Huh7.5.1 cells and electroporated cells were lysed to determine luciferase activity. Luciferase activity in infected Huh7.5.1 cells was assessed 72h later. Data are expressed as mean percentage as compared to control ± s.d. Results are from three independent experiments in quadruplicate. The dashed line indicates values from vehicle-treated cells set at 100%. Statistics**: ***, *p*-value < 0.05, Mann-Whitney test.

Figure 5. Silencing of *OGT* modulates HCVcc biophysical properties. (A) Separation of HCVcc by iodixanol density gradient ultracentrifugation. HCVcc were produced in non-targeting siRNA control- or siOGT-transfected Huh7.5.1 cells. After overlaying HCVcc (JcR2A) on a 4%-40% iodixanol step gradient and ultracentrifugation for 16h, fractions of HCV particles were used to infect naïve Huh7.5.1 cells in order to determine TCID50. HCV RNA of each fraction was purified and analyzed by RT-qPCR. Data are expressed as mean ± s.d. from three independent experiments. (B) Specific infectivity (TCID50/RNA) was calculated and the density was determined by weighting each fraction. Specific infectivity of each fraction is expressed as fold change as compared to the total infectivity of the control. Data are expressed as mean ± s.d. from three independent experiments. (C-D) ApoE and ApoB concentrations in the individual fractions were determined by ELISA. The dashed lines

indicate limits of quantification of the assays. Data are expressed as mean ± s.d. from three independent experiments.

Figure 6. Silencing of *OGT* increases the size of HCVcc. (A) Representative pictures of HCV particles generated in Huh7.5.1 cells transfected with non-targeting siRNA (siCtrl) or siOGT. (B-F) Comparative analysis of particle size distribution for immunocapture (IC) from HCV particles produced in Huh7.5.1 cells transfected with siCtrl or siOGT prior to infection with HCVcc (JcR2a) following sucrose-cushion purification (B) or iodixanol gradient fractionation (C-F) of HCVcc. HCVcc were transferred via anti-E2 antibody AR3A on electron microscopy (EM) grids through IC. Particle size distribution was assessed from a series of randomly acquired electron micrographs with Image-J software (NIH). Results from one of three (A-B) or two (C-F) independent experiments are shown. Black lines: size distribution of immunocaptured HCVcc produced in siCtrl-transfected cells. Grey lines: size distribution of immunocaptured HCVcc produced in siOGT-transfected cells.

Figure 7. *OGT* expression increases in HCC. (A-B) Huh7.5.1 cells were infected with HCV (JcR2a). After 72h, RNA and proteins were purified, and *OGT* expression analyzed by RT-qPCR and Western blot. (A) Percentage of *OGT* mRNA expression relative to uninfected Huh7.5.1 cells (Ctrl). Results are presented as mean ± s.d. from three independent experiments in duplicate. The dashed line indicates values from uninfected Huh7.5.1 cells set at 100%. Statistics: *, *p*-value < 0.05, Mann-Whitney test. (B) OGT protein expression. Left: percentage of OGT protein expression relative to uninfected Huh7.5.1 cells (Ctrl) following quantification of Western blots as described in Supplementary Methods. Results are presented as mean ± s.d. from three independent experiments. The dashed line indicates values from uninfected Huh7.5.1 cells set at 100%. Statistics: *, *p*-value < 0.05, Mann-Whitney test. Right: representative Western blot analysis of OGT and actin. (C) *OGT* expression and viral load in liver tissue from 22 HCV-infected patients and 6 patients not infected with HCV and normal histology (see Supplementary Material and Methods).

Spearman correlation: rho = 0.06004019, p-value = 0.77. (D-E) OGT expression in liver tissue from 22 HCV-infected patients and 6 patients not infected with HCV and normal histology according to fibrosis (D) or activity (E) scores (see Supplementary Material and Methods). Wilcoxon test: F1 vs F0 p-value = 0,38; F2 vs F0 p-value = 0,18; F3 vs F0 p-value = 0,43; F4 vs F0 p-value = 0,17; A1 vs A0 p-value = 0,28; A2 vs A0 p-value = 0,23; A3 vs A0 p-value = 0,09. (F) *OGT* expression in tumor (HCC) and non-tumor (Ctrl) liver tissue from HCV-infected patients (34 tumor samples including 5 paired tumor/non-tumor samples), hepatitis B virus (HBV)-infected patients (76 tumor samples including 7 paired tumor/non-tumor samples), patients with alcoholic liver disease (ALD) (72 tumor samples including 8 paired tumor/non-tumor samples) and patients with non-alcoholic fatty liver disease (NAFLD) (11 tumor samples including 2 paired tumor/non-tumor samples) as described in Supplementary Methods. *, *p*-value < 0.05, Wilcoxon test.

Table 1. Computational analysis of miR-501-3p and miR-619-3p targets and pathway enrichment.

miRNA ID	Target gene symbol	***Pathway* or network**
miR-501-3p	*MEF2A*; *PPP3CA*; *PPP3CC*	*Calcium signaling*
	HMGCS1	*Cholesterol biosynthesis*
	AFF4; *CHMP1B*; *CUX1*; *DCLK1*; *LMX1A*; *PTBP2*; *RBMS1*; *RC3H1*; *SCN2A*; *SEC63*; *ZFHX4*	Inflammatory response, dermatological diseases and conditions, inflammatory disease
	CDK6; *CSDE1*; *GLI2*; *HOXD10*; *LSM5*; *MEF2A*; *MYCN*; *OGT*; *PPP2R2C*; *PPP2R5E*; *SEMA3C*; *TFDP2*	Cellular development, nervous system development and function; organ morphology
	CIT; *COL10A1*; *FNBP1L*; *GAN*; *HERC1*; *KPNA4*; *NONO*; *SHPRH*; *STRN*; *TARDBP*; *UBE2H*; *USP37*	Cell death and survival; cellular compromise; free radical scavenging
	ATXN1; *CBLL1*; *CEBPA*; *DCC*; *PEX5L*; *RCC2*; *RNF144A*; *ZC3H12C*	Cell morphology, cellular assembly and organization; cellular function and maintenance
miR-619-3p	*RUNX1T1*; *SMAD3*	*Adipocyte biogenesis*
	FOXG1; *GPBP1*; *MID1*; *MKL2*; *MSI1*; *PCBP2*; *WDFY3*	Cell cycle; organismal injury and abnormalities; cancer
	ACVR2B; *DCX*; *ESRRG*; *MAPK9*; *OGT*; *PCBP1*; *PDE3B*; *SMAD3*; *SMARCC1*; *TGFB3*; *PAPOLA*	Carbohydrate metabolism, energy production; small molecule biochemistry
	RUNX1T1; *SHANK2*; *SLC35D1*	Gene expression, lipid metabolism, small molecule biochemistry

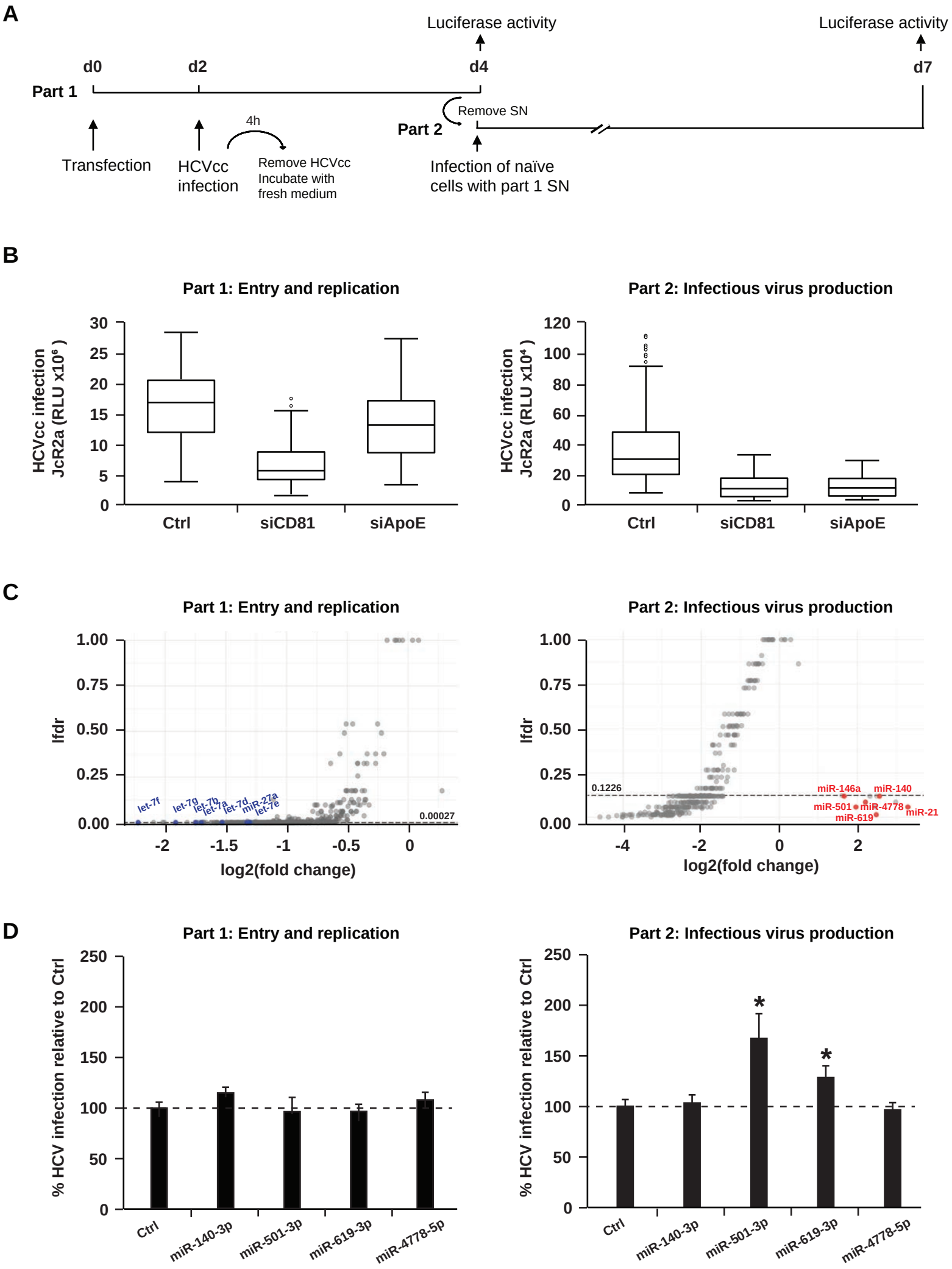
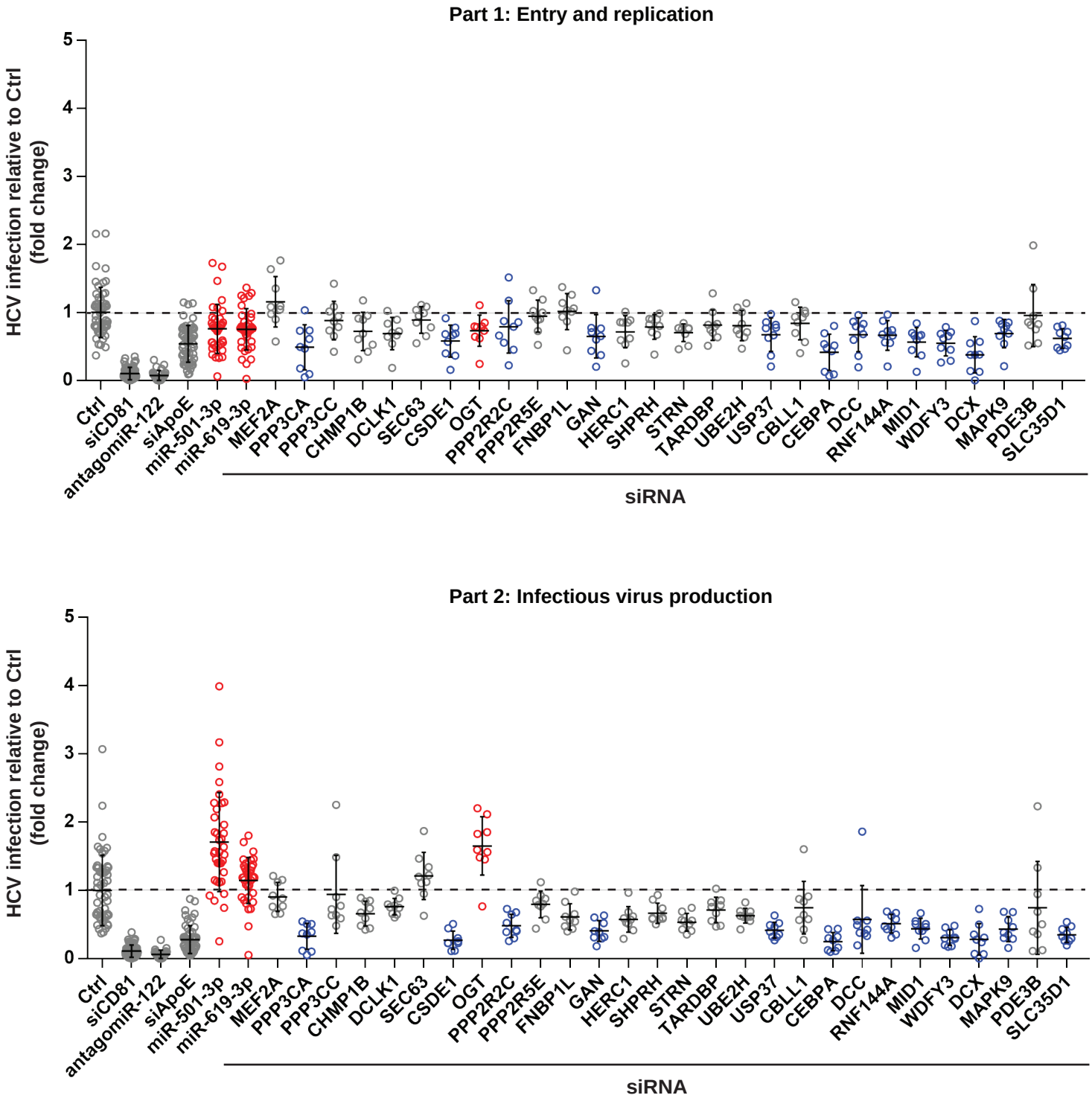

A
Luciferase activity
Luciferase activity
d0
d2
d4
d7
Part 1
Part 2
Remove SN
4h
Transfection
HCVcc infection
Remove HCVcc Incubate with fresh medium
Infection of naïve cells with part 1 SN
B
Part 1: Entry and replication
Part 2: Infectious virus production
HCVcc infection JcR2a (RLU x10⁶)
HCVcc infection JcR2a (RLU x10⁴)
Ctrl
siCD81
siApoE
C
Part 1: Entry and replication
Part 2: Infectious virus production
lfdr
log2(fold change)
let-7f
let-7g
let-7b
let-7a
let-7d
miR-27a
let-7e
0.00027
0.1226
miR-146a
miR-140
miR-501
miR-4778
miR-21
miR-619
D
Part 1: Entry and replication
Part 2: Infectious virus production
% HCV infection relative to Ctrl
Ctrl
miR-140-3p
miR-501-3p
miR-619-3p
miR-4778-5p

Figure 1


Part 1: Entry and replication
HCV infection relative to Ctrl (fold change)
0
1
2
3
4
5
Ctrl
siCD81
antagomiR-122
siApoE
miR-501-3p
miR-619-3p
MEF2A
PPP3CA
PPP3CC
CHMP1B
DCLK1
SEC63
CSDE1
OGT
PPP2R2C
PPP2R5E
FNBP1L
GAN
HERC1
SHPRH
STRN
TARDBP
UBE2H
USP37
CBLL1
CEBPA
DCC
RNF144A
MID1
WDFY3
DCX
MAPK9
PDE3B
SLC35D1
siRNA

Part 2: Infectious virus production
HCV infection relative to Ctrl (fold change)
0
1
2
3
4
5
Ctrl
siCD81
antagomiR-122
siApoE
miR-501-3p
miR-619-3p
MEF2A
PPP3CA
PPP3CC
CHMP1B
DCLK1
SEC63
CSDE1
OGT
PPP2R2C
PPP2R5E
FNBP1L
GAN
HERC1
SHPRH
STRN
TARDBP
UBE2H
USP37
CBLL1
CEBPA
DCC
RNF144A
MID1
WDFY3
DCX
MAPK9
PDE3B
SLC35D1
siRNA

Figure 2

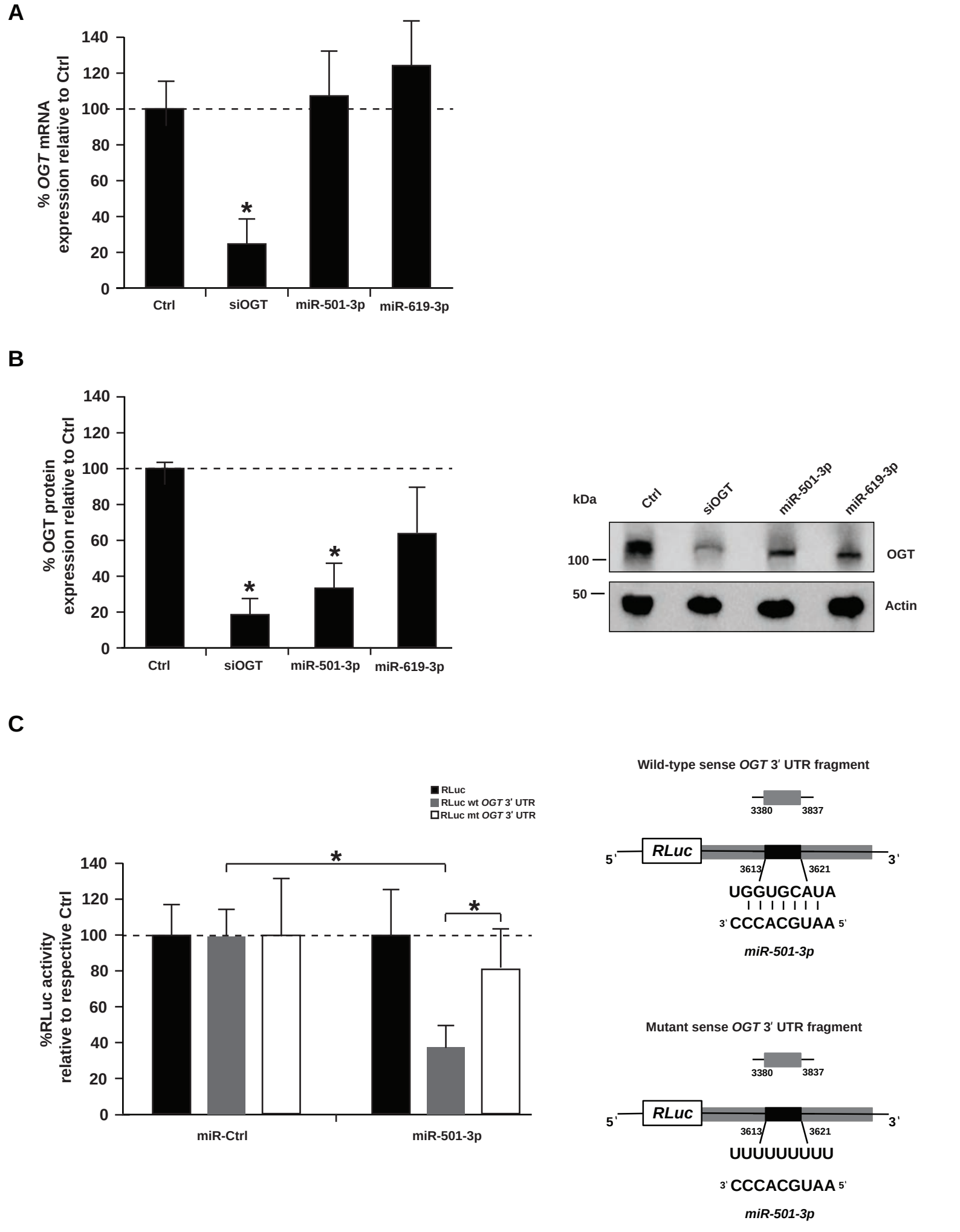

A
% OGT mRNA expression relative to Ctrl
140
120
100
80
60
40
20
0
*
Ctrl
siOGT
miR-501-3p
miR-619-3p
B
% OGT protein expression relative to Ctrl
140
120
100
80
60
40
20
0
*
*
Ctrl
siOGT
miR-501-3p
miR-619-3p
kDa
Ctrl
siOGT
miR-501-3p
miR-619-3p
100
50
OGT
Actin
C
RLuc
RLuc wt OGT 3′ UTR
RLuc mt OGT 3′ UTR
%RLuc activity relative to respective Ctrl
140
120
100
80
60
40
20
0
*
*
miR-Ctrl
miR-501-3p
Wild-type sense OGT 3′ UTR fragment
3380
3837
5′
RLuc
3′
3613
3621
UGGUGCAUA
3′ CCCACGUAA 5′
miR-501-3p
Mutant sense OGT 3′ UTR fragment
3380
3837
5′
RLuc
3′
3613
3621
UUUUUUUUU
3′ CCCACGUAA 5′
miR-501-3p

Figure 3

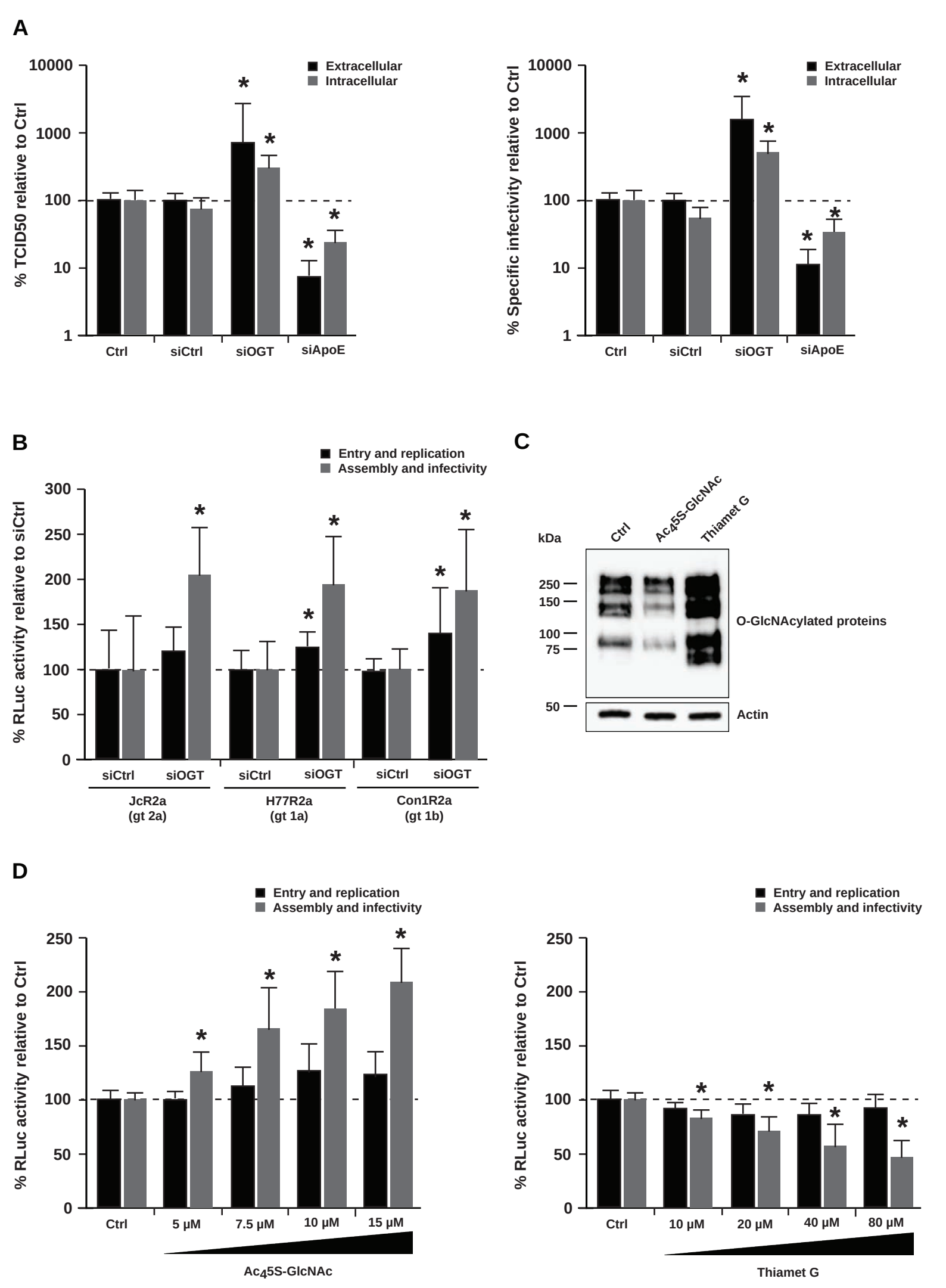

A
% TCID50 relative to Ctrl
Extracellular
Intracellular
Ctrl
siCtrl
siOGT
siApoE
% Specific infectivity relative to Ctrl
B
% RLuc activity relative to siCtrl
Entry and replication
Assembly and infectivity
JcR2a (gt 2a)
H77R2a (gt 1a)
Con1R2a (gt 1b)
C
kDa
Ctrl
Ac4 5S-GlcNAc
Thiamet G
O-GlcNAcylated proteins
Actin
D
% RLuc activity relative to Ctrl
5 µM
7.5 µM
10 µM
15 µM
Ac4 5S-GlcNAc
20 µM
40 µM
80 µM
Thiamet G

Figure 4

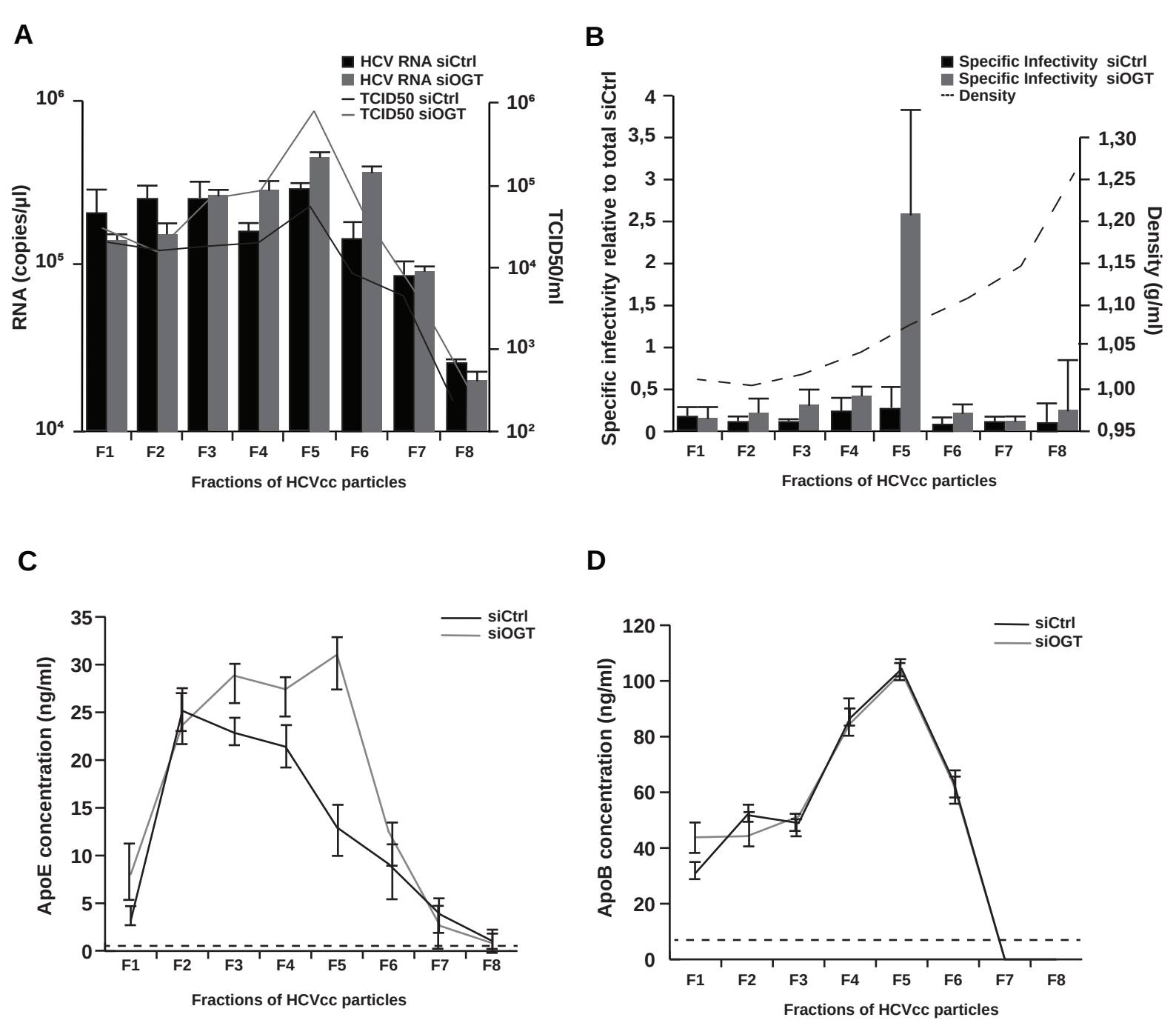

A
HCV RNA siCtrl
HCV RNA siOGT
TCID50 siCtrl
TCID50 siOGT
RNA (copies/µl)
TCID50/ml
Fractions of HCVcc particles
B
Specific Infectivity siCtrl
Specific Infectivity siOGT
Density
Specific infectivity relative to total siCtrl
Density (g/ml)
Fractions of HCVcc particles
C
siCtrl
siOGT
ApoE concentration (ng/ml)
Fractions of HCVcc particles
D
siCtrl
siOGT
ApoB concentration (ng/ml)
Fractions of HCVcc particles

Figure 5

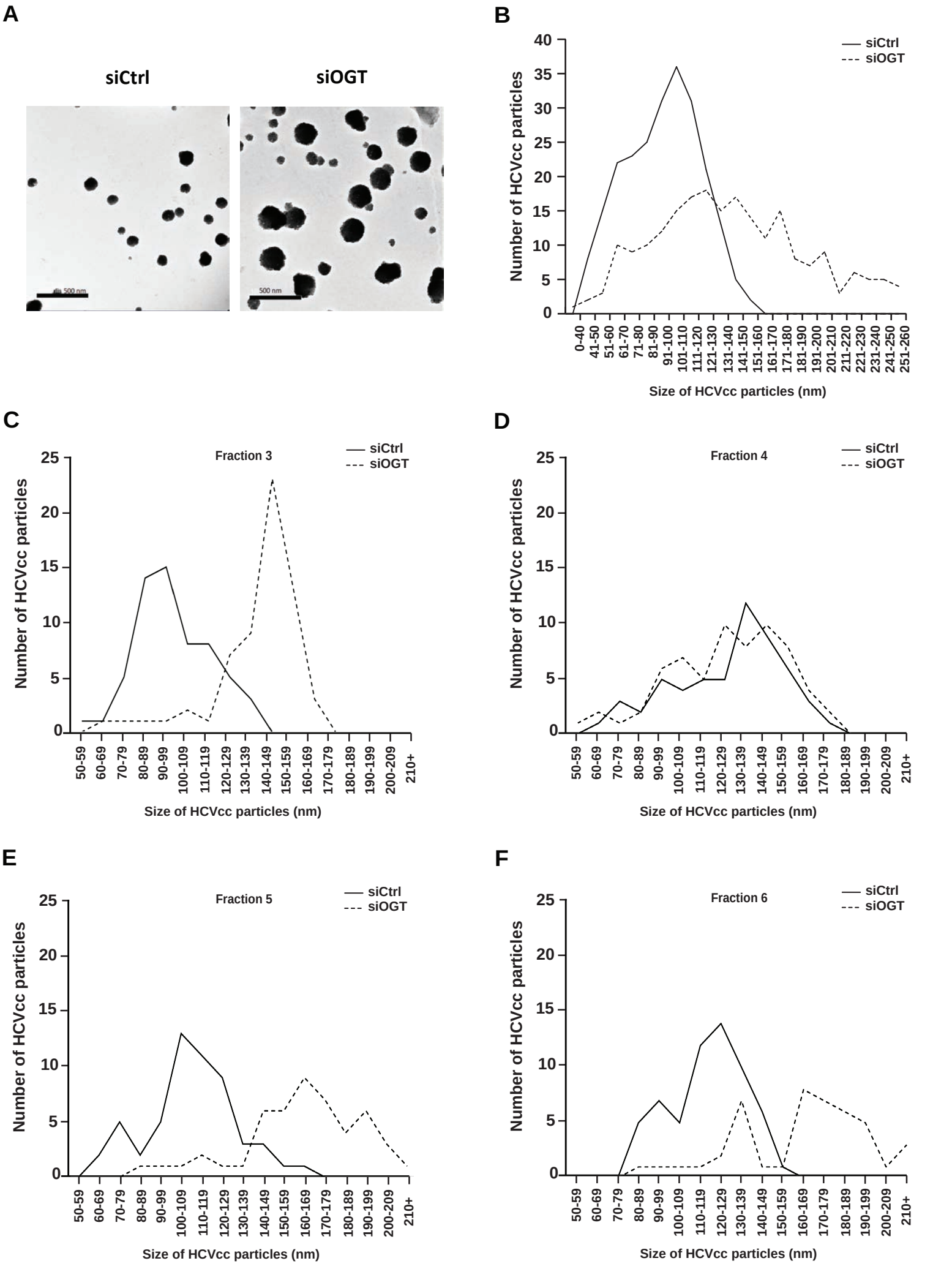

A
siCtrl
siOGT
500 nm
500 nm
B
siCtrl
siOGT
Number of HCVcc particles
Size of HCVcc particles (nm)
C
Fraction 3
siCtrl
siOGT
Number of HCVcc particles
Size of HCVcc particles (nm)
D
Fraction 4
siCtrl
siOGT
Number of HCVcc particles
Size of HCVcc particles (nm)
E
Fraction 5
siCtrl
siOGT
Number of HCVcc particles
Size of HCVcc particles (nm)
F
Fraction 6
siCtrl
siOGT
Number of HCVcc particles
Size of HCVcc particles (nm)

Figure 6

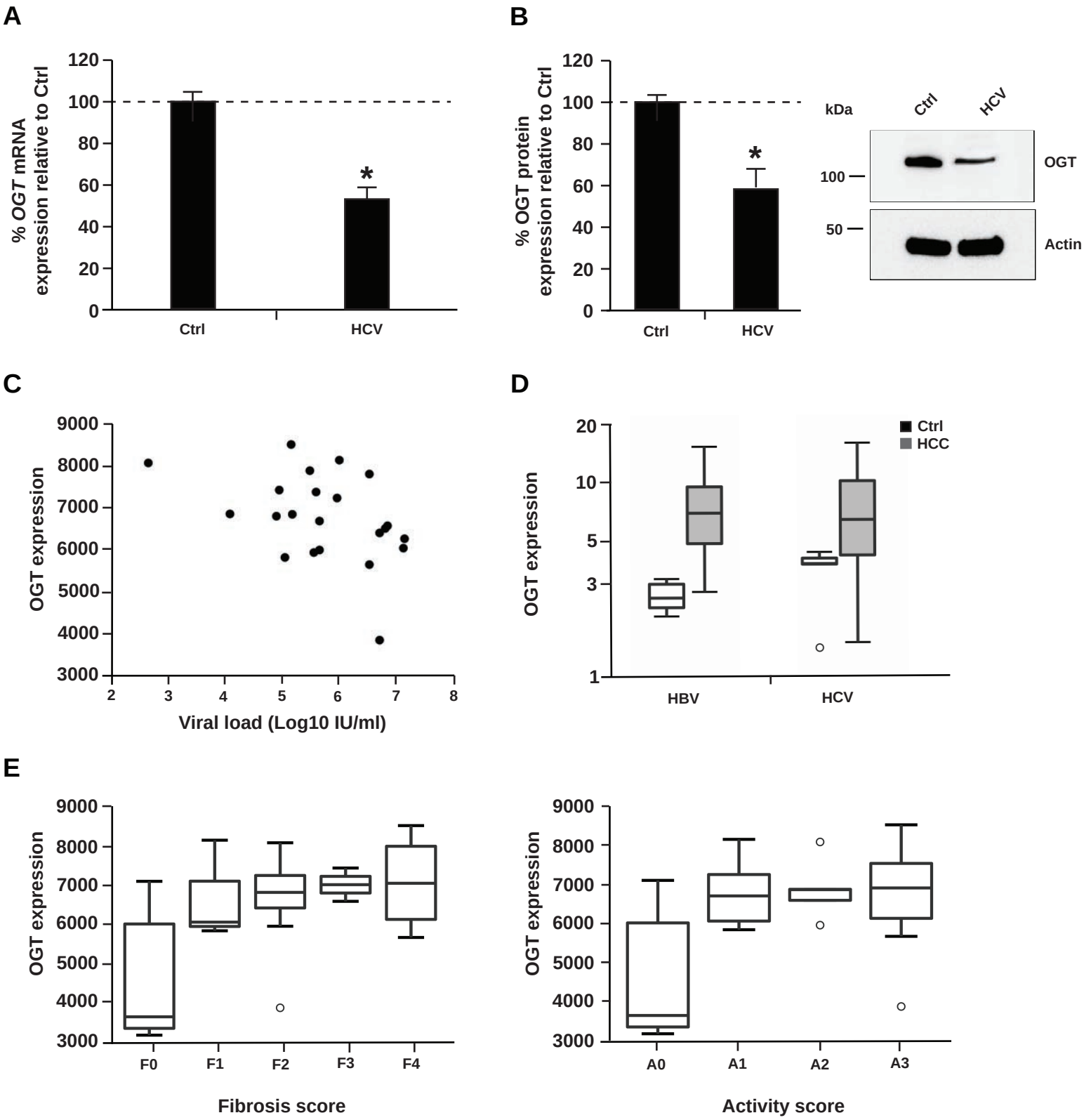

A
% OGT mRNA expression relative to Ctrl
120
100
80
60
40
20
0
*
Ctrl
HCV
B
% OGT protein expression relative to Ctrl
120
100
80
60
40
20
0
*
Ctrl
HCV
kDa
Ctrl
HCV
100
50
OGT
Actin
C
OGT expression
9000
8000
7000
6000
5000
4000
3000
2
3
4
5
6
7
8
Viral load (Log10 IU/ml)
D
OGT expression
20
10
5
3
1
Ctrl
HCC
HBV
HCV
E
OGT expression
9000
8000
7000
6000
5000
4000
3000
F0
F1
F2
F3
F4
Fibrosis score
OGT expression
9000
8000
7000
6000
5000
4000
3000
A0
A1
A2
A3
Activity score

Figure 7

Supplementary Information

A functional microRNA screen uncovers O-linked N-acetylglucosamine transferase as a host factor modulating hepatitis C virus morphogenesis

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*Authors contributed equally to this work

Supplementary Material and methods

Cells and cell culture conditions. The source and culture conditions of Huh7.5.1 cells have been described[1]. HeLa cells were purchased from ATCC and cultured in Dulbecco´s modified Eagle medium (Gibco® DMEM GlutaMAX™, ThermoFisher Scientific) containing 1% sodium pyruvate as described for Huh7.5.1 cells[1].

Viruses and infectivity assays. Cell culture-derived recombinant cell culture-derived hepatitis C virus (HCVcc) Jc1 (genotype 2a/2a chimera), H77R2a (genotype 1a/2a chimera engineered for *Renilla* luciferase expression), Con1R2a (genotype 1b/2b chimera engineered for *Renilla* luciferase expression), and JcR2a (genotype 2a/2a chimera engineered for *Renilla* luciferase expression) were generated in Huh7.5.1 cells as described[1, 2, 3, 4]. HCVcc infectivity was determined by calculating the 50% tissue culture infectious dose (TCID50) using anti-NS5A antibody as described[5, 6] or by assessing luciferase activity. HCVcc were used at 10^5-10^6 TCID50/mL throughout the study. HCV RNA was purified using a QIAmp viral RNA minikit (Qiagen) and analyzed by one-step RT-qPCR using a Sensi Fast NO ROX kit (Bioline) according to the manufacturer's instructions. Standard curves were performed using 10-fold dilution series of HCV RNA.

Purification of HCVcc particles using sucrose cushion or iodixanol density gradient. HCVcc (JcR2a) were concentrated 10-fold using a Vivaspin column (GE Healthcare). For sucrose cushion purification, HCVcc were purified by overlaying 3.5 mL of culture media on 1.5 mL of 20% sucrose, and by ultracentrifuging samples for 4h at 40,000 rpm on a SW-55 rotor (Beckman Coulter). Purified HCVcc were resuspended in 30 µL of PBS for analysis via immunocapture and electron microscopy. Density distributions of infectious HCVcc were determined by overlaying 0.5 mL culture media on a 5 mL, 4%-40% iodixanol step gradient, and ultracentrifuging samples for 16h at 40,000 rpm on a SW-55 rotor (Beckman Coulter): 625 µl fractions were carefully harvested from the top of each tube, and density was determined by weighing. Infectivity of each fraction was quantified by TCID50 using anti-NS5A antibody as described[5, 6], while HCV RNA of fractions was purified and analyzed as described above. ApoB and ApoE concentrations of fractions were determined by enzyme-linked immunosorbent assay (Human Apolipoprotein B or E ELISA^{PRO} kit, Mabtech) undiluted or in a 1:50 dilution, respectively, according to the manufacturer's instructions (Mabtech).

miRNA mimics and siRNAs. Non-targeting control miRNA, miR-501-3p mimic, miR-619-3p mimic, antagomiR-122, antagomiR-501-3p, non-targeting control antagomiR, non-targeting control siRNA, siRNAs targeting *OGT, CD81* or *apoliporotein E* (*ApoE*) and a library of 28 custom ON-TARGETplus smart pool siRNAs were purchased from Dharmacon (GE Healthcare).

miRNA expression analysis. Total RNA (100 ng) was purified from control or HCV-infected Huh7.5.1 cells using Tri reagent® (Thermo Scientific) and Direct-zol™ RNA purification kit (Zymo Research). Total RNA was first polyadenylated and reverse transcribed using a miScript II RT system (Qiagen) according to the manufacturer's instructions. The obtained cDNA was subjected to RT-qPCR using miScript SYBR Green kit (Qiagen). Primers were the mature miRNA sequence for the forward primer (Thermo Scientific) and the universal miScript primer (Qiagen) for the reverse primer. Data were analyzed by the ΔΔCt method using small nucleolar RNA, C/D box 61 (SNORD61) as an endogenous reference and the non-infected samples as a calibrator[7].

Antibodies. Rabbit anti-OGT antibodies DM-17 and AL24 were purchased from Sigma or kindly provided by Dr. G. W. Hart and Dr. S. Hardivillé (Johns Hopkins University School of Medicine, Baltimore, MD)[8], respectively. Mouse anti-b-actin antibody was purchased from Abcam and mouse, rabbit or sheep HRP-conjugated secondary antibodies (A9044, A0545 and A3415, respectively) were purchased from Sigma. Sheep anti-NS5A serum for determination of TCID50 was a kind gift from M. Harris[9]. Human anti-E2 (AR3A) antibody[10] for electron microscopy analysis was kindly provided by Mansun Law (SCRIPPS, California, USA).

Western blotting. OGT and actin protein expression in human cells was assessed by Western blot as described[8] with some modifications. Briefly, cells were lysed in lysis buffer no. 6 (R&D Systems) according to the manufacturer's instructions. Equal amounts of protein

(40 µg) were size-separated through a Mini PROTEAN® TGX Stain-Free™ gel electrophoresis (Bio-Rad) and transferred to PVDF membranes (Bio-Rad). Immunoblots were performed using rabbit anti-OGT (1:2000) and mouse anti-b-actin (1:1000) antibodies[8, 11]. Antigen-antibody complexes were detected by incubating the membrane with the appropriate HRP-conjugated secondary antibodies (1:5000; 1:10,000) and imaged by enhanced chemiluminescence with a ChemiDoc MP imager (Bio-Rad). Quantification of protein expression was performed using ImageLab™ 5.2.1 software (BioRad). For analysis of OGT and GAPDH expression in liver tissue from HCV transgenic (FL-N/35) or wild-type mice[12], crude protein extracts were prepared by homogenization of frozen mouse livers (50–100 µg) in tissue lysis buffer from the Ambion PARIS RNA (Thermo Scientific) and protein isolation kit, supplemented with protease inhibitors (cOmpleteTM EDTA-free protease inhibitor mixture, Sigma-Aldrich) and phosphatase inhibitors (PhosSTOPTM, Sigma-Aldrich), using a tissue homogenizer (MP Fast Prep24, MP Biomedicals, Santa Ana, CA) and MP Lysing Matrix A tubes. Proteins were quantified using the BCA assay (Thermo Fisher Scientific). Western blotting was performed as described above.

Immunocapture and electron microscopy analysis of viral particles. Sucrose-cushion purified or iodixanol gradient fractionated HCVcc (JcR2a) produced in cells transfected with a non-targeting siRNA control or a pool of siRNA against OGT were transferred via anti-E2 antibody AR3A on electron microscopy (EM) grids through immunocapture (IC) as described[13]. Particles were stained with uranyl acetate dihydrate and observed in a JEOL 1230 electron microscope. Series of electron micrographs were acquired at random from IC EM grids. The images were then analyzed with Image-J software, to determine the particle size distribution.

Gene expression analysis in patient-derived liver tissue. For OGT expression analysis in patient's samples, raw data were retrieved from the Gene Expression Omnibus (GSE84346) and re-analyzed by quality-trimming (cutadapt) and mapping (HISAT2) to human genome

assembly hg19. Reads mapping to Gencode v.19 genes were counted using htseq-count and normalized applying DESeq2. Activity and fibrosis scores as well as viral load were taken from the supplemental data of[14] including 22 HCV-infected patients and 6 patients not infected with HCV and normal histology who underwent liver biopsy due to unclear hepatopathy (n=4), metastasis of breast cancer (n=1), or metastasis of lung cancer (n=1) as described in[14]. To analyze OGT expression in liver tissue of patients with chronic liver disease, FPKM values and clinical data were retrieved from The Cancer Genome Atlas (TCGA, https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). This data set includes samples from HCV-infected patients (34 tumor samples including 5 paired tumor/non-tumor samples), hepatitis B virus (HBV)-infected patients (76 tumor samples including 7 paired tumor/non-tumor samples), patients with alcoholic liver disease (ALD) (72 tumor samples including 8 paired tumor/non-tumor samples) and patients with non-alcoholic fatty liver disease (NAFLD) (11 tumor samples including 2 paired tumor/non-tumor samples).

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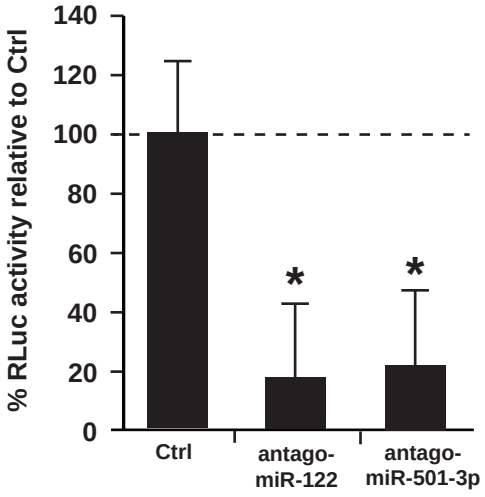
Supplementary figure legends

Figure S1. (A) Effect of miR-501-3p inhibition on HCV infectivity. Huh7.5.1 cells were transfected with control antagomiR (Ctrl), antagomiR-122 as loss-of-function control to perturb

HCV replication and antagomiR-501-3p, prior to infection with HCVcc (JcR2a) according to the two-step protocol depicted in Fig. 1A. After 48h, supernatants were transferred onto naive Huh7.5.1 cells. After 72h, Renilla Luciferase activity of infected Huh7.5.1 cells was determined. Data are expressed as mean percentage as compared to Ctrl ± s.d. Results are from four independent experiments in quadruplicate. The dashed line indicates values from vehicle-treated cells set at 100%. Statistics**: ***, *p*-value < 0.05, Mann-Whitney test. (B) miR-501-3p expression upon HCV infection. Huh7.5.1 cells were infected with HCVcc (JcR2a). After 72h, RNA was purified and miR-501-3p expression analyzed by RT-qPCR. Percentage of miR-501-3p expression relative to uninfected Huh7.5.1 cells (Ctrl). Results are presented as mean ± s.d. from three independent experiments in duplicate. The dashed line indicates values from uninfected Huh7.5.1 cells set at 100%. Statistics: *, *p*-value < 0.05, Mann-Whitney test.

Supplementary Table 1. A genome-wide miRNA mimic screen identifies cellular miRNAs modulating HCV infection. Log2(FC), lfdr and effect on HCV infection in part 1 and part 2 of the screen are shown for the individual miRNAs of the miRNA mimic library. In red: proviral effect, in blue: antiviral effect. FC: fold change, lfdr: local false discovery rate

A


% RLuc activity relative to Ctrl
140
120
100
80
60
40
20
0
*
*
Ctrl
antago-
miR-122
antago-
miR-501-3p

B

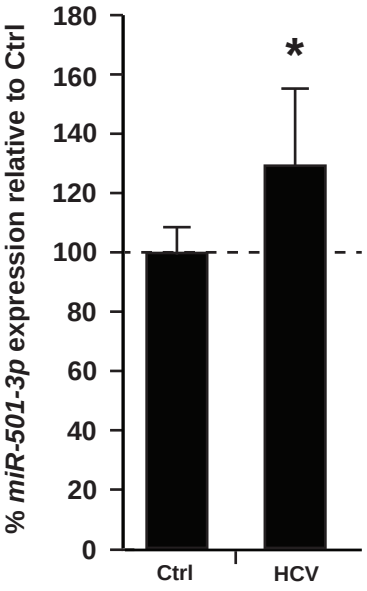

% miR-501-3p expression relative to Ctrl
180
160
140
120
100
80
60
40
20
0
*
Ctrl
HCV

Figure S1

miRNA ID	Mature Sanger ID	Library ID	Mature Sequence	Part 1			Part 2		
				Log2(FC)	lfdr	Effect on HCV	Log2(FC)	lfdr	Effect on HCV
hsa-let-7a	MIMAT0000062	C-300473-05	UGAGGUAGUAGGUUGUAUAGUU	-1.70171935969617	3.9917e-05	TRUE	-3.28400225976063	0.0018396	TRUE
hsa-let-7b	MIMAT0000063	C-300476-05	UGAGGUAGUAGGUUGUGUGGUU	-1.74684091191792	8.3715e-05	TRUE	-3.61453853652595	0.009387	TRUE
hsa-let-7d	MIMAT0000065	C-300478-07	AGAGGUAGUAGGUUGCAUAGUU	-1.53075932976348	0.00023985	TRUE	-2.74632167025162	0.057617	TRUE
hsa-let-7e	MIMAT0000066	C-300479-05	UGAGGUAGGAGGUUGUAUAGUU	-1.30271875995011	0.0014577	FALSE	-1.90993819018051	0.081661	TRUE
hsa-let-7f	MIMAT0000067	C-300480-05	UGAGGUAGUAGAUUGUAUAGUU	-2.22178435619614	6.7884e-06	TRUE	-3.76872186786763	0.014453	TRUE
hsa-let-7g	MIMAT0000414	C-300583-05	UGAGGUAGUAGUUUGUACAGUU	-1.91269526615093	6.7884e-06	TRUE	-2.71878759599202	0.057617	TRUE
hsa-miR-101	MIMAT0000099	C-300518-07	UACAGUACUGUGAUAACUGAA	-0.886376348041933	0.0030028	FALSE	-1.9796810124946	0.11887	TRUE
hsa-miR-103-as	MIMAT0007402	C-301453-00	UCAUAGCCCUGUACAAUGCUGCU	-1.11208100417218	0.0074787	FALSE	-2.91287954813545	0.057617	TRUE
hsa-miR-106a*	MIMAT0004517	C-301159-01	CUGCAAUGUAAGCACUUCUUAC	-1.32520114049511	3.9917e-05	TRUE	-2.3773966141336	0.11887	TRUE
hsa-miR-1178	MIMAT0005823	C-301319-00	UUGCUCACUGUUCUUCCCUAG	-1.22556728667978	0.00061109	FALSE	-2.3126959903038	0.08542	TRUE
hsa-miR-1178-5p	MIMAT0022940	C-301922-00	CAGGGUCAGCUGAGCAUG	-1.16672245017752	0.00026644	TRUE	-0.780775920183507	0.72813	FALSE
hsa-miR-1185	MIMAT0005798	C-301317-00	AGAGGAUACCCUUUGUAUGUU	-1.2315368528283	8.3715e-05	TRUE	-0.721316088104541	0.76975	FALSE
hsa-miR-1200	MIMAT0005863	C-301326-00	CUCCUGAGCCAUUCUGAGCCUC	-1.24866404694154	0.00054279	FALSE	-2.09711908610931	0.08542	TRUE
hsa-miR-1205	MIMAT0005869	C-301331-00	UCUGCAGGGUUUGCUUUGAG	-0.801511472736558	0.0053924	FALSE	-3.77846481724539	0.0018396	TRUE
hsa-miR-1207-3p	MIMAT0005872	C-301334-00	UCAGCUGGCCCUCAUUUC	-1.10040811819955	0.00013963	TRUE	-1.69117584901668	0.26604	FALSE
hsa-miR-122	MIMAT0000421	C-300591-05	UGGAGUGUGACAAUGGUGUUUG	-0.70369537171691	0.040577	FALSE	-3.13425305602985	0.028781	TRUE
hsa-miR-122*	MIMAT0004590	C-301046-01	AACGCCAUUAUCACACUAAAUA	-0.557131436052541	0.037103	FALSE	-1.83404561163316	0.11887	TRUE
hsa-miR-1226	MIMAT0005577	C-301284-01	UCACCAGCCCUGUGUUCCCUAG	-0.741744822716559	0.0099219	FALSE	-1.89688153334356	0.11887	TRUE
hsa-miR-1228*	MIMAT0005582	C-301287-01	GUGGGCGGGGGCAGGUGUGUG	-0.947568053691086	0.0049772	FALSE	-2.29154418859234	0.11887	TRUE
hsa-miR-1237-5p	MIMAT0022946	C-302618-00	CGGGGGCGGGGCCGAAGCGCG	-0.956259568604456	0.021994	FALSE	-2.34546898601693	0.057617	TRUE
hsa-miR-1244	MIMAT0005896	C-301865-00	AAGUAGUUGGUUUGUAUGAGAUGGUU	-1.17945761818024	6.1693e-05	TRUE	-0.989861012597079	0.5811	FALSE
hsa-miR-1245b-3p	MIMAT0019951	C-302405-00	UCAGAUGAUCUAAAGGCCUAUA	-0.414846848981959	0.32544	FALSE	-2.27819747964432	0.081661	TRUE
hsa-miR-1255b-2-3p	MIMAT0022725	C-301882-00	AACCACUUUCUUUGCUCAUCCA	-0.427268737714942	0.061384	FALSE	-2.58892190360625	0.023579	TRUE
hsa-miR-1258	MIMAT0005909	C-301384-00	AGUUAGGAUUAGGUCGUGGAA	-1.57210875214914	1.5894e-05	TRUE	-1.63979276673705	0.36109	FALSE
hsa-miR-125b-2*	MIMAT0004603	C-301061-01	UCACAAGUCAGGCUCUUGGGAC	-0.858902068537019	0.0052694	FALSE	-1.87406855538924	0.11887	TRUE
hsa-miR-1260b	MIMAT0015041	C-301716-00	AUCCCACCACUGCCACCAU	-0.476317862532308	0.10991	FALSE	2.46099391534969	0.057617	TRUE
hsa-miR-1270	MIMAT0005924	C-301867-00	CUGGAGAUAUGGAAGAGCUGUGU	-1.23553336541613	3.9917e-05	TRUE	-2.24160380684469	0.11887	TRUE
hsa-miR-1277	MIMAT0005933	C-301411-00	UACGUAGAUAUAUAUGUAUUUU	-0.745198002045239	0.0074787	FALSE	-3.58615793633136	0.014453	TRUE
hsa-miR-1278	MIMAT0005936	C-301417-00	UAGUACUGUGCAUAUCAUCUAU	-0.494528345361641	0.089234	FALSE	1.60846287059147	0.12226	TRUE
hsa-miR-1283	MIMAT0005799	C-301315-00	UCUACAAAGGAAAGCGCUUUCU	-0.538161112092319	0.061384	FALSE	-2.67520055202511	0.08542	TRUE
hsa-miR-1284	MIMAT0005941	C-301423-00	UCUAUACAGACCCUGGCUUUUC	-0.412436737654723	0.099257	FALSE	-2.28002191497783	0.11527	TRUE
hsa-miR-1287	MIMAT0005878	C-301341-00	UGCUGGAUCAGUGGUUCGAGUC	-1.20683701032783	0.00023985	TRUE	-1.98393331688063	0.08542	TRUE
hsa-miR-1288	MIMAT0005942	C-301424-00	UGGACUGCCCUGAUCUGGAGA	-0.704889827052788	0.0021076	FALSE	-2.89790974514386	0.057617	TRUE
hsa-miR-129-3p	MIMAT0004605	C-301063-01	AAGCCCUUACCCCAAAAAGCAU	-0.999602920325089	0.0021076	FALSE	-1.88681635888787	0.11887	TRUE
hsa-miR-1291	MIMAT0005881	C-301345-00	UGGCCCUGACUGAAGACCAGCAGU	-1.21284220826454	0.00061109	FALSE	-2.02374608043906	0.11887	TRUE
hsa-miR-1293	MIMAT0005883	C-301347-00	UGGGUGGUCUGGAGAUUUGUGC	-1.39687224389743	0.00026644	TRUE	-2.31295737423138	0.057617	TRUE
hsa-miR-1294	MIMAT0005884	C-301348-00	UGUGAGGUUGGCAUUGUUGUCU	-1.44051810095446	8.3715e-05	TRUE	-1.95652126826796	0.057617	TRUE
hsa-miR-1295	MIMAT0005885	C-301349-00	UUAGGCCGCAGAUCUGGGUGA	-1.2640855905457	0.00087976	FALSE	-2.34227893604624	0.11887	TRUE
hsa-miR-1295b-5p	MIMAT0022293	C-302563-00	CACCCAGAUCUGCGGCCUAAU	-0.454374224389677	0.17522	FALSE	-2.73444344828677	0.023579	TRUE
hsa-miR-1298	MIMAT0005800	C-301318-00	UUCAUUCGGCUGUCCAGAUGUA	-0.26688230187125	0.32544	FALSE	-2.44553337713544	0.11887	TRUE
hsa-miR-1302	MIMAT0005890	C-301869-00	UUGGGACAUACUUAUGCUAAA	-1.35138412635794	3.6715e-05	TRUE	-2.60359528922327	0.057617	TRUE
hsa-miR-1302	MIMAT0005890	C-301354-00	UUGGGACAUACUUAUGCUAAA	-1.11168868347783	0.00013793	TRUE	-1.29913904583381	0.51048	FALSE
hsa-miR-1307	MIMAT0005951	C-301434-00	ACUCGGCGUGGCGUCGGUCGUG	-1.12000717587297	0.00054279	FALSE	-2.10295176202445	0.057617	TRUE
hsa-miR-1307-5p	MIMAT0022727	C-301875-00	UCGACCGGACCUCGACCGGCU	-0.803481003211417	0.0030028	FALSE	-1.76491161834359	0.11887	TRUE
hsa-miR-1322	MIMAT0005953	C-301436-00	GAUGAUGCUGCUGAUGCUG	-1.23817643374983	6.1693e-05	TRUE	-1.29920754317295	0.46329	FALSE
hsa-miR-1323	MIMAT0005795	C-301311-00	UCAAAACUGAGGGGCAUUUUCU	-0.756897047236657	0.096607	FALSE	-2.43084213259459	0.11887	TRUE
hsa-miR-135b*	MIMAT0004698	C-301200-01	AUGUAGGGCUAAAAGCCAUGGG	-1.49530939618539	0.0018747	FALSE	-2.98188718020305	0.050122	TRUE
hsa-miR-139-3p	MIMAT0004552	C-301036-03	UGGAGACGCGGCCCUGUUGGAGU	-1.02065377094153	0.00079918	FALSE	-2.77458369079406	0.019699	TRUE
hsa-miR-140-3p	MIMAT0004597	C-301055-01	UACCACAGGGUAGAACCACGG	-0.172675601522775	1	FALSE	2.56894688372309	0.11887	TRUE
hsa-miR-142-3p	MIMAT0000434	C-300610-03	UGUAGUGUUUCCUACUUUAUGGA	-1.00474764620935	0.00023985	TRUE	-1.7577126946261	0.22566	FALSE
hsa-miR-146a	MIMAT0000449	C-300630-03	UGAGAACUGAAUUCCAUGGGUU	0.279820319960687	0.17522	FALSE	1.66479155066337	0.11887	TRUE
hsa-miR-150*	MIMAT0004610	C-301067-01	CUGGUACAGGCCUGGGGGACAG	-1.46501526099695	0.00023985	TRUE	-1.19620229448666	0.40677	FALSE

hsa-miR-151b	MIMAT0010214	C-301973-00	UCGAGGAGCUCACAGUCU	-1.03668648961171	3.9917e-05	TRUE	-2.8985532020104	0.057617	TRUE
hsa-miR-182	MIMAT0000259	C-300557-07	UUUGGCAAUGGUAGAACUCACACU	-0.581360116993454	0.03164	FALSE	-1.81305061189634	0.11887	TRUE
hsa-miR-184	MIMAT0000454	C-300635-03	UGGACGGAGAACUGAUAAGGGU	-1.31700777618768	3.9917e-05	TRUE	-1.52851322717993	0.36109	FALSE
hsa-miR-185	MIMAT0000455	C-300636-07	UGGAGAGAAAGGCAGUUCCUGA	-1.59673044023595	0.00013963	TRUE	-1.74454670174015	0.1633	FALSE
hsa-miR-18b*	MIMAT0004751	C-301187-01	UGCCCUAAAUGCCCCUUCUGGC	-0.825568919117559	0.0027607	FALSE	-2.30862554668178	0.023579	TRUE
hsa-miR-1909	MIMAT0007883	C-301456-00	CGCAGGGGCCGGGUGCUCACCG	-0.511863313449074	0.17522	FALSE	2.49877552994666	0.11887	TRUE
hsa-miR-1915*	MIMAT0007891	C-301466-00	ACCUUGCCUUGCUGCCCGGGCC	-1.71935071114424	8.3715e-05	TRUE	-1.13532112712663	0.46329	FALSE
hsa-miR-196b*	MIMAT0009201	C-301305-00	UCGACAGCACGACACUGCCUUC	-0.949306838412525	0.00054279	FALSE	-3.1358874675771	0.023579	TRUE
hsa-miR-19b	MIMAT0000074	C-300489-03	UGUGCAAAUCCAUGCAAAACUGA	-1.01461439258753	0.00013793	TRUE	-2.75192926226938	0.014453	TRUE
hsa-miR-19b-1*	MIMAT0004491	C-301021-01	AGUUUUGCAGGUUUGCAUCCAGC	-1.68388148726454	0.0030028	FALSE	-2.85695043305243	0.014453	TRUE
hsa-miR-19b-2*	MIMAT0004492	C-301139-01	AGUUUUGCAGGUUUGCAUUUCA	-0.594068837054281	0.037103	FALSE	-2.06967686748099	0.11887	TRUE
hsa-miR-200b*	MIMAT0004571	C-301144-01	CAUCUUACUGGGCAGCAUUGGA	-1.05721574165849	0.0074787	FALSE	-4.59657299807562	0.0018396	TRUE
hsa-miR-203b-5p	MIMAT0019813	C-302277-00	UAGUGGUCCUAAACAUUUCACA	-1.0335612225008	0.00016067	TRUE	-2.13526669981558	0.08542	TRUE
hsa-miR-208a	MIMAT0000241	C-300537-03	AUAAGACGAGCAAAAAGCUUGU	-0.823665097293502	0.0030028	FALSE	-1.54639877799123	0.11887	TRUE
hsa-miR-21	MIMAT0000076	C-300492-03	UAGCUUAUCAGACUGAUGUUGA	-0.768228630205262	0.096607	FALSE	3.29607415881261	0.057617	TRUE
hsa-miR-211-3p	MIMAT0022694	C-301905-00	GCAGGGACAGCAAAGGGGUGC	-0.812107428438748	0.0027607	FALSE	-2.21028883028492	0.08542	TRUE
hsa-miR-2114	MIMAT0011156	C-301489-00	UAGUCCCUUCCUUGAAGCGGUC	-0.958796902783202	0.00016326	TRUE	-1.5768286317563	0.26604	FALSE
hsa-miR-2114*	MIMAT0011157	C-301490-00	CGAGCCUCAAGCAAGGGACUU	-1.02419077915881	0.00013793	TRUE	-2.57866858794879	0.057617	TRUE
hsa-miR-2117	MIMAT0011162	C-301496-00	UGUUCUCUUUGCCAAGGACAG	-0.806177347742896	0.00054279	FALSE	-2.55943646917884	0.057617	TRUE
hsa-miR-216a-3p	MIMAT0022844	C-301886-00	UCACAGUGGUCUCUGGGAUUAU	-1.4780620737632	8.3715e-05	TRUE	-1.62257411616963	0.1633	FALSE
hsa-miR-22	MIMAT0000077	C-300493-03	AAGCUGCCAGUUGAAGAACUGU	-1.14812626998085	0.00054279	FALSE	-2.8254651928315	0.009387	TRUE
hsa-miR-220b	MI0005529	C-301218-01	CCACCACCGUGUCUGACACUU	-1.10856445415353	0.12653	FALSE	-2.50799687656707	0.11887	TRUE
hsa-miR-221*	MIMAT0004568	C-301163-01	ACCUGGCAUACAAUGUAGAUUU	-1.0647285020049	0.00016067	TRUE	0.493351763855846	0.86349	FALSE
hsa-miR-223*	MIMAT0004570	C-301197-01	CGUGUAUUUGACAAGCUGAGUU	-0.596047980424722	0.0074787	FALSE	-2.64001675119474	0.057617	TRUE
hsa-miR-2276	MIMAT0011775	C-301481-00	UCUGCAAGUGUCAGAGGCGAGG	-1.23563628890583	3.9917e-05	TRUE	-0.719842576977581	0.76975	FALSE
hsa-miR-2277-3p	MIMAT0011777	C-301482-00	UGACAGCGCCCUGCCUGGCUC	-1.22725149342576	3.9917e-05	TRUE	-2.2780178572373	0.11887	TRUE
hsa-miR-23a*	MIMAT0004496	C-301025-01	GGGGUUCCUGGGGAUGGGAUUU	-0.773715976814457	0.0018747	FALSE	-2.22574058493535	0.11887	TRUE
hsa-miR-25*	MIMAT0004498	C-301183-01	AGGCGGAGACUUGGGCAAUUG	-1.14410824968417	0.0018747	FALSE	-2.13159960818718	0.057617	TRUE
hsa-miR-2681-3p	MIMAT0013516	C-301978-00	UAUCAUGGAGUUGGUAAAGCAC	-0.84154241088718	0.00023985	TRUE	-0.564927523090931	0.86349	FALSE
hsa-miR-2681-5p	MIMAT0013515	C-301977-00	GUUUUACCACCUCCAGGAGACU	-1.20496402288836	3.6715e-05	TRUE	-2.40988448605506	0.057617	TRUE
hsa-miR-2682-3p	MIMAT0013518	C-301980-00	CGCCUCUUCAGCGCUGUCUUCC	-0.908772237664288	0.00013793	TRUE	-1.43184476418853	0.29187	FALSE
hsa-miR-2682-5p	MIMAT0013517	C-301979-00	CAGGCAGUGACUGUUCAGACGUC	-1.24830345350196	1.5894e-05	TRUE	-2.80779774369284	0.014453	TRUE
hsa-miR-26b	MIMAT0000083	C-300501-07	UUCAAGUAAUUCAGGAUAGGU	-0.405072630482104	0.12653	FALSE	-2.50877510131769	0.081661	TRUE
hsa-miR-27a	MIMAT0000084	C-300502-03	UUCACAGUGGCUAAGUUCCGC	-1.32790971119818	3.9917e-05	TRUE	-1.95676458087438	0.1633	FALSE
hsa-miR-27a*	MIMAT0004501	C-301028-01	AGGGCUUAGCUGCUUGUGAGCA	-0.887009503478049	0.0052694	FALSE	-3.44308089121935	0.028781	TRUE
hsa-miR-27b*	MIMAT0004588	C-301154-01	AGAGCUUAGCUGAUUGGUGAAC	-1.00848877755825	0.0014577	FALSE	-2.34115081043794	0.050122	TRUE
hsa-miR-28-5p	MIMAT0000085	C-300503-05	AAGGAGCUCACAGUCUAUUGAG	-0.358344656239635	0.17522	FALSE	-1.5918060993224	0.11887	TRUE
hsa-miR-2861	MIMAT0013802	C-301642-00	GGGGCCUGGCGGUGGGCGG	-0.615484847088555	0.0046172	FALSE	-2.44784331747309	0.081661	TRUE
hsa-miR-2964a-3p	MIMAT0019748	C-302218-00	AGAAUUGCGUUUGGACAAUCAGU	-1.13977573653624	6.1693e-05	TRUE	-0.558827938583115	0.76975	FALSE
hsa-miR-298	MIMAT0004901	C-301212-01	AGCAGAAGCAGGGAGGUUCUCCCA	-0.456122353506126	0.54004	FALSE	-2.83068032162142	0.11887	TRUE
hsa-miR-299-5p	MIMAT0002890	C-300854-03	UGGUUUACCGUCCCACAUACAU	-1.25864926851518	0.00023985	TRUE	-0.317867194423722	1	FALSE
hsa-miR-29a*	MIMAT0004503	C-301178-01	ACUGAUUUCUUUUGGUGUUCAG	-1.35357089379682	0.0021076	FALSE	-2.75349311494331	0.057617	TRUE
hsa-miR-29b-1*	MIMAT0004514	C-301150-01	GCUGGUUUCAUAUGGUGGUUUAGA	-0.970652939446982	0.015067	FALSE	-2.38342736203068	0.11887	TRUE
hsa-miR-301b	MIMAT0004958	C-301252-01	CAGUGCAAUGAUAUUGUCAAAGC	-0.837318501823261	0.0014577	FALSE	-2.28547978225747	0.11887	TRUE
hsa-miR-302b*	MIMAT0000714	C-300668-07	ACUUUAACAUGGAAGUGCUUUC	-0.550910910384927	0.037103	FALSE	-1.76045353694163	0.12226	TRUE
hsa-miR-302f	MIMAT0005932	C-301410-00	UAAUUGCUUCCAUGUUU	-1.5960005623826	1.5894e-05	TRUE	-3.17802223827817	0.014453	TRUE
hsa-miR-30b	MIMAT0000420	C-300590-03	UGUAAACAUCCUACACUCAGCU	-0.759206041294187	0.0018747	FALSE	-2.5817157170778	0.057617	TRUE
hsa-miR-30c-1*	MIMAT0004674	C-301199-01	CUGGGAGAGGGUUGUUUACUCC	-1.43787786865803	8.3715e-05	TRUE	0.291968781990601	1	FALSE
hsa-miR-30c-2*	MIMAT0004550	C-301034-01	CUGGGAGAAGGCUGUUUACUCU	-1.18964958597662	8.3715e-05	TRUE	-0.203715081689554	1	FALSE
hsa-miR-30d	MIMAT0000245	C-300543-03	UGUAAACAUCCCCGACUGGAAG	-0.469794284958227	0.061384	FALSE	-2.24325814749711	0.08542	TRUE
hsa-miR-3115	MIMAT0014977	C-301644-00	AUAUGGGUUUACUAGUUGGU	-0.595634685516391	0.0052694	FALSE	-2.22531374934429	0.11887	TRUE
hsa-miR-3116	MIMAT0014978	C-301645-00	UGCCUGGAACAUAGUAGGGACU	-1.61818516940984	6.7884e-06	TRUE	-1.27627977004857	0.51048	FALSE
hsa-miR-3117	MIMAT0014979	C-301647-00	AUAGGACUCAUAUAGUGCCAG	-1.07944692693698	6.1693e-05	TRUE	0.0583708702116637	1	FALSE
hsa-miR-3119	MIMAT0014981	C-301651-00	UGGCUUUUAACUUUGAUGGC	-0.77136494055742	0.00087976	FALSE	-2.20041353445194	0.11887	TRUE

hsa-miR-3121	MIMAT0014983	C-301654-00	UAAAUAGAGUAGGCAAAGGACA	-0.550473953423974	0.0074787	FALSE	-2.06892978205979	0.11887	TRUE
hsa-miR-3124	MIMAT0014986	C-301657-00	UUCGCGGGCGAAGGCAAAGUC	-1.13549064733399	3.9917e-05	TRUE	-0.910461837996433	0.5811	FALSE
hsa-miR-3126-5p	MIMAT0014989	C-301661-00	UGAGGGACAGAUGCCAGAAGCA	-1.43373948465677	1.5894e-05	TRUE	-0.744651597941774	0.76975	FALSE
hsa-miR-3127	MIMAT0014990	C-301662-00	AUCAGGGCUUGUGGAAUGGGAAG	-0.992180086498039	0.00013793	TRUE	-1.20167440176899	0.51048	FALSE
hsa-miR-3130-3p	MIMAT0014994	C-301665-00	GCUGCACCGGAGACUGGGUAA	-0.645251817092445	0.0021076	FALSE	-3.33421355039417	0.023579	TRUE
hsa-miR-3130-5p	MIMAT0014995	C-301666-00	UACCCAGUCUCCGGUGCAGCC	-1.12058237373742	6.1693e-05	TRUE	-2.44166606880781	0.11887	TRUE
hsa-miR-3131	MIMAT0014996	C-301669-00	UCGAGGACUGGUGGAAGGGCCUU	-1.10475333868087	6.1693e-05	TRUE	-0.776623344642163	0.76864	FALSE
hsa-miR-3132	MIMAT0014997	C-301670-00	UGGGUAGAGAAGGAGCUCAGAGGA	-1.0649683988313	8.3715e-05	TRUE	-2.09592053963675	0.11887	TRUE
hsa-miR-3136	MIMAT0015003	C-301676-00	CUGACUGAAUAGGUAGGGUCAUU	-0.837973854149906	0.00054279	FALSE	-2.76623087444917	0.057617	TRUE
hsa-miR-3137	MIMAT0015005	C-301678-00	UCUGUAGCCUGGGAGCAAUGGGGU	-1.05179643968079	8.3715e-05	TRUE	-1.1088570611196	0.51579	FALSE
hsa-miR-3138	MIMAT0015006	C-301679-00	UGUGGACAGUGAGGUAGAGGGAGU	-1.09246960431895	6.1693e-05	TRUE	-1.78790357269758	0.22566	FALSE
hsa-miR-3139	MIMAT0015007	C-301680-00	UAGGAGCUCAACAGAUGCCUGUU	-1.37716815069908	1.5894e-05	TRUE	-1.31106163706907	0.46329	FALSE
hsa-miR-3140	MIMAT0015008	C-301681-00	AGCUUUUGGGAAUUCAGGUAGU	-1.58079760272847	6.7884e-06	TRUE	-3.18144016432131	0.014453	TRUE
hsa-miR-3142	MIMAT0015011	C-301684-00	AAGGCCUUUCUGAACCUUCAGA	-0.977053358967313	0.00013963	TRUE	-1.89446739540295	0.1633	FALSE
hsa-miR-3144-5p	MIMAT0015014	C-301687-00	AGGGGACCAAAGAGAUAUAUAG	-1.13947775501217	3.9917e-05	TRUE	-0.414321660104908	1	FALSE
hsa-miR-3147	MIMAT0015019	C-301692-00	GGUUGGGCAGUGAGGAGGGUGUGA	-0.942186557775687	0.00023985	TRUE	-0.157149408887514	1	FALSE
hsa-miR-3148	MIMAT0015021	C-301694-00	UGGAAAAAACUGGUGUGUGCUU	-1.14553360648851	3.9917e-05	TRUE	-1.08300314433575	0.51579	FALSE
hsa-miR-3150	MIMAT0015023	C-301696-00	CUGGGGAGAUCCUCGAGGUUGG	-1.4167699283789	1.5894e-05	TRUE	-0.986527775853387	0.5811	FALSE
hsa-miR-3150b-5p	MIMAT0019226	C-301961-00	CAACCUCGAGGAUCUCCCCAGC	-1.04471163915449	3.9917e-05	TRUE	-2.12053729635218	0.08542	TRUE
hsa-miR-3151	MIMAT0015024	C-301697-00	GGUGGGGCAAUGGGAUCAGGU	-1.02537680303558	0.00013793	TRUE	-1.56671822128811	0.26604	FALSE
hsa-miR-3152	MIMAT0015025	C-301698-00	UGUGUUAGAAUAGGGGCAAUAA	-1.13601969432149	6.1693e-05	TRUE	-2.17593163296179	0.11887	TRUE
hsa-miR-3157	MIMAT0015031	C-301704-00	UUCAGCCAGGCUAGUGCAGUCU	-1.81838879899633	0.00023985	TRUE	-0.798984655509925	0.76975	FALSE
hsa-miR-3180	MIMAT0018178	C-301583-00	UGGGGCGGAGCUUCCGGAG	-1.37633415637047	0.00013793	TRUE	-1.9747378577519	0.050122	TRUE
hsa-miR-3186-5p	MIMAT0015067	C-301749-00	CAGGCGUCUGUCUACGUGGCUU	-0.536494894531891	0.061384	FALSE	-2.19613374331388	0.11887	TRUE
hsa-miR-3190-5p	MIMAT0015073	C-301756-02	UCUGGCCAGCUACGUCCCCA	-1.56898213610615	3.9917e-05	TRUE	-3.02592555730969	0.014453	TRUE
hsa-miR-3191-5p	MIMAT0022732	C-302750-00	CUCUCUGGCCGUCUACCUUCCA	-0.896644736818671	0.01741	FALSE	-2.93191055011036	0.057617	TRUE
hsa-miR-32*	MIMAT0004505	C-301181-01	CAAUUUAGUGUGUGUGAUAUUU	-0.561723514860645	0.37761	FALSE	-2.47247129599692	0.11887	TRUE
hsa-miR-323-5p	MIMAT0004696	C-301085-01	AGGUGGUCCGUGGCGCGUUCGC	-0.997451491983143	0.0049772	FALSE	-2.55198466781697	0.014453	TRUE
hsa-miR-323b-5p	MIMAT0001630	C-301724-00	AGGUUGUCCGUGGUGAGUUCGCA	-0.870040751918234	0.00054279	FALSE	-3.51049237466915	0.014453	TRUE
hsa-miR-324-3p	MIMAT0000762	C-300705-05	ACUGCCCCAGGUGCUGCUGG	-0.930125097729152	0.0021076	FALSE	-2.18712046531723	0.081661	TRUE
hsa-miR-328	MIMAT0000752	C-300695-03	CUGGCCCUCUCUGCCCUUCCGU	-1.1639709975505	8.3715e-05	TRUE	-1.57395137899233	0.26604	FALSE
hsa-miR-339-3p	MIMAT0004702	C-301185-01	UGAGCGCCUCGACGACAGAGCCG	-0.424124048504717	0.061384	FALSE	-2.06515171637643	0.08542	TRUE
hsa-miR-342-5p	MIMAT0004694	C-301083-01	AGGGGUGCUAUCUGUGAUUGA	-1.66163157467999	0.00016326	TRUE	-2.01939161434937	0.12226	TRUE
hsa-miR-345-3p	MIMAT0022698	C-301887-00	GCCCUGAACGAGGGGUCUGGAG	-0.839605975652715	0.0021076	FALSE	-2.80466918793858	0.023579	TRUE
hsa-miR-346	MIMAT0000773	C-300712-03	UGUCUGCCCGCAUGCCUGCCUCU	-1.22592404544756	0.00023985	TRUE	-1.35203588028221	0.20114	FALSE
hsa-miR-34a*	MIMAT0004557	C-301145-01	CAAUCAGCAAGUAUACUGCCCU	-1.27197605437233	6.1693e-05	TRUE	-0.378051811558674	1	FALSE
hsa-miR-3529-5p	MIMAT0019828	C-302292-00	AGGUAGACUGGGAUUUGUUGUU	-0.674576387471472	0.034257	FALSE	-1.99747275957492	0.11887	TRUE
hsa-miR-3612	MIMAT0017989	C-301505-00	AGGAGGCAUCUUGAGAAAUGGA	-1.26286494709099	0.00026644	TRUE	-1.44238772362423	0.51048	FALSE
hsa-miR-3616-3p	MIMAT0017996	C-301512-00	CGAGGGCAUUUCAUGAUGCAGGC	-1.10327166715049	0.00054279	FALSE	-1.69108153949499	0.11887	TRUE
hsa-miR-3619-3p	MIMAT0019219	C-301952-00	GGGACCAUCCUGCCUGCUGUGG	-0.361591800679582	0.037103	FALSE	-2.1201206119135	0.11887	TRUE
hsa-miR-3620-5p	MIMAT0022967	C-302619-00	GUGGGCUGGGCUGGGCUGGGCC	-0.963210303847923	0.01741	FALSE	-2.95077111022971	0.014453	TRUE
hsa-miR-3622b-5p	MIMAT0018005	C-301522-00	AGGCAUGGGAGGUCAGGUGA	-1.03124833680421	0.00087976	FALSE	-1.86140566900932	0.08542	TRUE
hsa-miR-365	MIMAT0000710	C-300666-03	UAAUGCCCCUAAAAAUCCUUAU	-1.07713706038737	0.03164	FALSE	-2.565586989697	0.12226	TRUE
hsa-miR-3654	MIMAT0018074	C-301532-00	GACUGGACAAGCUGAGGAA	-0.394926641879625	0.096607	FALSE	-1.99274206927806	0.057617	TRUE
hsa-miR-365b-5p	MIMAT0022833	C-301900-00	AGGGACUUUCAGGGGCAGCUGU	-1.17934943470937	0.00026644	TRUE	-1.48034463468101	0.22566	FALSE
hsa-miR-3666	MIMAT0018088	C-301546-00	CAGUGCAAGUGUAGAUGCCGA	-0.8322031427779	0.0027607	FALSE	-2.76031104596189	0.009387	TRUE
hsa-miR-3667-3p	MIMAT0018090	C-301547-00	ACCUUCCUCUCCAUGGGUCUUU	-1.53737106921222	6.1693e-05	TRUE	-0.189890808664278	1	FALSE
hsa-miR-3674	MIMAT0018097	C-301555-00	AUUGUAGAACCUAAGAUUGGCC	-0.686422173208126	0.0099219	FALSE	-1.66427885423157	0.08542	TRUE
hsa-miR-3675-5p	MIMAT0018098	C-301557-00	UAUGGGGCUUCUGUAGAGAUUUC	-0.704414688397948	0.0074787	FALSE	-1.47755963621301	0.11887	TRUE
hsa-miR-3676-5p	MIMAT0022734	C-301954-00	AGGAGAUCCUGGGUU	-0.876412168433536	0.00016326	TRUE	-2.81631813362903	0.028781	TRUE
hsa-miR-3680	MIMAT0018106	C-301565-00	GACUCACUCACAGGAUUGUGCA	-0.710055072199479	0.0074787	FALSE	-1.48818626629073	0.11887	TRUE
hsa-miR-3680*	MIMAT0018107	C-301564-00	UUUUGCAUGACCCUGGGAGUAGG	-0.68553099516054	0.0099219	FALSE	-1.8440688012842	0.057617	TRUE
hsa-miR-3681	MIMAT0018108	C-301566-00	UAGUGGAUGAUGCACUCUGUGC	-1.41416859913926	0.00013793	TRUE	-1.60964747472672	0.1633	FALSE
hsa-miR-3681*	MIMAT0018109	C-301567-00	ACACAGUGCUUCAUCCACUACU	-1.39472879333958	0.00013793	TRUE	-1.15667754722324	0.26604	FALSE

hsa-miR-3682	MIMAT0018110	C-301568-00	UGAUGAUACAGGUGGAGGUAG	-1.46673360047121	8.3715e-05	TRUE	-0.643125216570606	0.72813	FALSE
hsa-miR-3682-5p	MIMAT0019222	C-301956-00	CUACUUCUACCUGUGUUAUCAU	-1.23125674549655	1.5894e-05	TRUE	-2.81908923583428	0.023579	TRUE
hsa-miR-3684	MIMAT0018112	C-301570-00	UUAGACCUAGUACACGUCCUU	-0.580463595828849	0.021994	FALSE	-1.45573759159026	0.11887	TRUE
hsa-miR-3688	MIMAT0018116	C-301574-00	UAUGGAAAGACUUUGCCACUCU	-0.832441806479729	0.0027607	FALSE	-2.02033498247755	0.057617	TRUE
hsa-miR-3688-5p	MIMAT0019223	C-301957-00	AGUGGCAAAGUCUUUCCAUAU	-0.588424467468018	0.0030028	FALSE	-2.09619814608759	0.11887	TRUE
hsa-miR-3689b*	MIMAT0018181	C-301587-00	CUGGGAGGUGUGAUAUUGUGGU	-1.45567876271802	9.6971e-05	TRUE	-1.73246672882077	0.081661	TRUE
hsa-miR-3691	MIMAT0018120	C-301578-00	AGUGGAUGAUGGAGACUCGGUAC	-1.18761474653793	0.00054279	FALSE	-1.75547134579776	0.057617	TRUE
hsa-miR-3691-3p	MIMAT0019224	C-301958-00	ACCAAGUCUGCGUCAUCCUCUC	-0.810629756562153	0.00026644	TRUE	-1.96328717500158	0.11887	TRUE
hsa-miR-374a	MIMAT0000727	C-300681-05	UUAUAAUACAACCUGAUAAGUG	-0.718874319759212	0.0021076	FALSE	-2.60836689817691	0.081661	TRUE
hsa-miR-374c	MIMAT0018443	C-301630-00	AUAAUACAACCUGCUAAGUGCU	-0.337432743025632	0.32544	FALSE	-1.98175619738517	0.11887	TRUE
hsa-miR-374c-3p	MIMAT0022735	C-301969-00	CACUUAGCAGGUUGUAUUAUAU	-0.646260597978354	0.0018747	FALSE	-2.63477891317834	0.028781	TRUE
hsa-miR-376c	MIMAT0000720	C-300674-05	AACAUAGAGGAAAUUCCACGU	-0.624380737173221	0.0053924	FALSE	-2.68639877338995	0.057617	TRUE
hsa-miR-378b	MIMAT0014999	C-301672-00	ACUGGACUUGGAGGCAGAA	-0.950272981825821	0.00016326	TRUE	-0.901403427172712	0.72813	FALSE
hsa-miR-378g	MIMAT0018937	C-302000-00	ACUGGGCUUGGAGUCAGAAG	-0.836483927856313	0.00023985	TRUE	-1.88595985918819	0.11887	TRUE
hsa-miR-380*	MIMAT0000734	C-300688-03	UGGUUGACCAUAGAACAUGCGC	-1.15838836623363	0.0018747	FALSE	-2.13916483631805	0.12226	TRUE
hsa-miR-381-5p	MIMAT0022862	C-301920-00	AGCGAGGUUGCCCUUUGUAUAU	-0.569567310635211	0.021994	FALSE	-2.14982823754668	0.08542	TRUE
hsa-miR-383	MIMAT0000738	C-300692-03	AGAUCAGAAGGUGAUUGUGGCU	-0.925079491666264	0.0052694	FALSE	-2.8317437192294	0.050122	TRUE
hsa-miR-3907	MIMAT0018179	C-301585-00	AGGUGCUCCAGGCUGGCUCACA	-1.38465900005315	0.00013793	TRUE	-0.257773760992354	1	FALSE
hsa-miR-3910	MIMAT0018184	C-301590-00	AAAGGCAUAAAACCAAGACA	-0.501095287980396	0.037103	FALSE	-1.43931285152292	0.11887	TRUE
hsa-miR-3913-3p	MIMAT0019225	C-301959-00	AGACAUCAAGAUCAGUCCCAAA	-2.00645940017379	2.9753e-06	TRUE	-3.02362886477847	0.014453	TRUE
hsa-miR-3918	MIMAT0018192	C-301600-00	ACAGGGCCGCAGAUGGAGACU	-0.975422481909615	0.015067	FALSE	-2.44273172350024	0.081661	TRUE
hsa-miR-3922-5p	MIMAT0019227	C-301962-00	UCAAGGCCAGAGGUCCCACAGCA	-1.00561107859174	6.1693e-05	TRUE	-1.71505157929257	0.1633	FALSE
hsa-miR-3935	MIMAT0018350	C-301618-00	UGUAGAUACGAGCACCAGCCAC	-0.43578505271152	0.17522	FALSE	-2.47251139472301	0.057617	TRUE
hsa-miR-3972	MIMAT0019357	C-302158-00	CUGCCAGCCCCGUUCCAGGGCA	-1.16965458786124	0.00023985	TRUE	-1.70649157359084	0.40677	FALSE
hsa-miR-3975	MIMAT0019360	C-302161-00	UGAGGCUAAUGCACUACUUCAC	-1.37543164339877	8.3715e-05	TRUE	-1.26480301471027	0.51579	FALSE
hsa-miR-425*	MIMAT0001343	C-300718-07	AUCGGGAAUGUCGUGUCCGCCC	-0.937175749542432	0.00054279	FALSE	-3.15498966014918	0.028781	TRUE
hsa-miR-4253	MIMAT0016882	C-301816-00	AGGGCAUGUCCAGGGGGU	-1.35070000422013	0.00016326	TRUE	-2.47892017845828	0.11887	TRUE
hsa-miR-4290	MIMAT0016921	C-301856-00	UGCCCUCCUUUCUUCCCUC	-1.2579851622971	0.00026644	TRUE	-0.780428141336904	0.86349	FALSE
hsa-miR-4306	MIMAT0016858	C-301792-00	UGGAGAGAAAGGCAGUA	-1.10160128889539	0.0046172	FALSE	-2.08580333486915	0.11887	TRUE
hsa-miR-4312	MIMAT0016864	C-301798-00	GGCCUUGUUCCUGUCCCCA	-0.866321092589685	0.0030028	FALSE	-2.71345507691741	0.11887	TRUE
hsa-miR-4314	MIMAT0016868	C-301802-00	CUCUGGGAAAUGGGACAG	-1.5364651282067	8.3715e-05	TRUE	-1.29173007577176	0.5811	FALSE
hsa-miR-4417	MIMAT0018929	C-301991-00	GGUGGGCUUCCCGGAGGG	-0.611560680456615	0.0021076	FALSE	-2.09593331520836	0.11887	TRUE
hsa-miR-4418	MIMAT0018930	C-301992-00	CACUGCAGGACUCAGCAG	-0.946166316606651	0.00013793	TRUE	-1.12537501301133	0.46329	FALSE
hsa-miR-4419a	MIMAT0018931	C-301993-00	UGAGGGAGGAGACUGCA	-1.57297371821019	6.7884e-06	TRUE	-1.73343971226014	0.1633	FALSE
hsa-miR-4420	MIMAT0018933	C-301995-00	GUCACUGAUGUCUGUAGCUGAG	-0.924563865566564	0.00013963	TRUE	-0.533919074584704	0.86349	FALSE
hsa-miR-4422	MIMAT0018935	C-301997-00	AAAAGCAUCAGGAAGUACCCA	-0.890570506779186	0.00023985	TRUE	-0.609244483333412	0.86349	FALSE
hsa-miR-4423-3p	MIMAT0018936	C-301999-00	AUAGGCACCAAAAAGCAACAA	-0.908201173118296	0.00013793	TRUE	-0.922983857808818	0.5811	FALSE
hsa-miR-4423-5p	MIMAT0019232	C-301998-00	AGUUGCCUUUUUGUUCCCAUGC	-0.973577427508824	8.3715e-05	TRUE	-1.75215013385816	0.1633	FALSE
hsa-miR-4425	MIMAT0018940	C-302003-00	UGUUGGGAUUCAGCAGGACCAU	-2.09487999782656	6.0932e-07	TRUE	-4.02209575968718	0.0018396	TRUE
hsa-miR-4426	MIMAT0018941	C-302004-00	GAAGAUGGACGUACUUU	-1.51937100953274	6.7884e-06	TRUE	-1.79705743821345	0.12226	TRUE
hsa-miR-4427	MIMAT0018942	C-302005-00	UCUGAAUAGAGUCUGAAGAGU	-0.853522749406319	0.00023985	TRUE	-1.87772807758284	0.1633	FALSE
hsa-miR-4428	MIMAT0018943	C-302006-00	CAAGGAGACGGGAACAUGGAGC	-1.00869386291556	6.1693e-05	TRUE	-2.00933698314586	0.12226	TRUE
hsa-miR-4430	MIMAT0018945	C-302008-00	AGGCUGGAGUGAGCGGAG	-0.738546756609574	0.00054279	FALSE	-2.96189732903016	0.014453	TRUE
hsa-miR-4431	MIMAT0018947	C-302010-00	GCGACUCUGAAAACUAGAAGGU	-0.605956299781477	0.0021076	FALSE	-1.84934532554394	0.11887	TRUE
hsa-miR-4432	MIMAT0018948	C-302011-00	AAAGACUCUGCAAGAUGCCU	-1.01744360848873	6.1693e-05	TRUE	-3.10500591742549	0.023579	TRUE
hsa-miR-4433-3p	MIMAT0018949	C-302012-00	ACAGGAGUGGGGGUGGGACAU	-0.842019427210466	0.00023985	TRUE	-1.23537194391469	0.40677	FALSE
hsa-miR-4433-5p	MIMAT0020956	C-302013-00	CGUCCCACCCCCCACUCCUGU	-1.14337035396948	3.6715e-05	TRUE	-1.97664293733303	0.11887	TRUE
hsa-miR-4434	MIMAT0018950	C-302014-00	AGGAGAAGUAAAGUAGAA	-0.883254828802553	0.00013963	TRUE	-1.1657175196267	0.46329	FALSE
hsa-miR-4435	MIMAT0018951	C-302015-00	AUGGCCAGAGCUCACACAGAGG	-0.883497505941514	0.00023985	TRUE	-0.615152700112339	0.76975	FALSE
hsa-miR-4436a	MIMAT0018952	C-302016-00	GCAGGACAGGCAGAAGUGGAU	-1.04036408328386	3.9917e-05	TRUE	-1.59208296058719	0.22566	FALSE
hsa-miR-4437	MIMAT0018953	C-302018-00	UGGGCUCAGGGUACAAAGGUU	-1.08972765547642	3.9917e-05	TRUE	-1.15841014023857	0.51048	FALSE
hsa-miR-4438	MIMAT0018956	C-302021-00	CACAGGCUUAGAAAAGACAGU	-1.17220321847248	3.6715e-05	TRUE	-1.92817351938145	0.11887	TRUE
hsa-miR-4440	MIMAT0018958	C-302023-00	UGUCGUGGGGCUUGCUGGCUUG	-1.11752167173499	3.9917e-05	TRUE	-2.33357733512522	0.08542	TRUE
hsa-miR-4441	MIMAT0018959	C-302024-00	ACAGGGAGGAGAUUGUA	-1.2984552451679	1.5894e-05	TRUE	-0.431268961949208	0.86349	FALSE

hsa-miR-4442	MIMAT0018960	C-302025-00	GCCGGACAAGAGGGAGG	-0.97262245143818	8.3715e-05	TRUE	-1.15721358729306	0.46329	FALSE
hsa-miR-4443	MIMAT0018961	C-302026-00	UUGGAGGCGUGGGUUUU	-1.75283027769511	3.4785e-06	TRUE	-2.08359341608655	0.11887	TRUE
hsa-miR-4445-5p	MIMAT0018963	C-301976-00	AGAUUGUUUCUUUUGCCGUGCA	-1.26374145331415	1.5894e-05	TRUE	-4.01626597303824	0.0018396	TRUE
hsa-miR-4447	MIMAT0018966	C-302029-00	GGUGGGGGCUGUUGUUU	-0.841752979683909	0.00023985	TRUE	-2.88111133299715	0.028781	TRUE
hsa-miR-4449	MIMAT0018968	C-302031-00	CGUCCCGGGGCUGCGCGAGGCA	-0.853462598263724	0.00023985	TRUE	-1.80522351536343	0.1633	FALSE
hsa-miR-4450	MIMAT0018971	C-302034-00	UGGGGAUUUGGAGAAGUGGUGA	-1.06492940641686	4.5608e-05	TRUE	-2.95948317827462	0.014453	TRUE
hsa-miR-4451	MIMAT0018973	C-302037-00	UGGUAGAGCUGAGGACA	-1.13976267989773	3.6715e-05	TRUE	-0.526465448512174	0.86349	FALSE
hsa-miR-4452	MIMAT0018974	C-302038-00	UUGAAUUCUUGGCCUUAAGUGAU	-0.813274313439393	0.00026644	TRUE	-0.166636659928165	1	FALSE
hsa-miR-4453	MIMAT0018975	C-302039-00	GAGCUUGGUCUGUAGCGGUU	-1.18208500406966	3.9917e-05	TRUE	-2.62562177516204	0.050122	TRUE
hsa-miR-448	MIMAT0001532	C-300721-05	UUGCAUAUGUAGGAUGUCCCAU	-1.39737830033137	0.00054279	FALSE	-2.29949072457764	0.11887	TRUE
hsa-miR-449c	MIMAT0010251	C-301488-00	UAGGCAGUGUAUUGCUAGCGGCUGU	-0.977360548104279	0.00013963	TRUE	-1.65073035658006	0.26604	FALSE
hsa-miR-4507	MIMAT0019044	C-302113-00	CUGGGUUGGGCUGGGCUGGG	-1.08254852754089	0.015067	FALSE	-2.63018823024416	0.11887	TRUE
hsa-miR-450a-3p	MIMAT0022700	C-301907-00	AUUGGGGACAUUUUGCAUUCAU	-1.3171609377358	0.00013793	TRUE	-1.14626230328136	0.46329	FALSE
hsa-miR-451	MIMAT0001631	C-300734-05	AAACCGUUACCAUUACUGAGUU	-1.43368456108961	8.3715e-05	TRUE	-0.268670734386333	1	FALSE
hsa-miR-452	MIMAT0001635	C-300735-07	AACUGUUUGCAGAGGAAACUGA	-0.355271404072044	0.12653	FALSE	-2.45598627080639	0.050122	TRUE
hsa-miR-4525	MIMAT0019064	C-302136-00	GGGGGGAUGUGCAUGCUGGUU	-1.29162472134399	0.00013793	TRUE	-1.82359755370572	0.26604	FALSE
hsa-miR-4526	MIMAT0019065	C-302137-00	GCUGACAGCAGGGCUGGCCGCU	-0.819545705994525	0.0021076	FALSE	-3.08101976925394	0.057617	TRUE
hsa-miR-4527	MIMAT0019066	C-302138-00	UGGUCUGCAAAGAGAUGACUGU	-1.48851753125946	3.9917e-05	TRUE	-1.1928606551261	0.5811	FALSE
hsa-miR-4533	MIMAT0019072	C-302145-00	UGGAAGGAGGUUGCCGGACGCU	-1.18624537355721	0.00016326	TRUE	-0.593158084514742	0.86349	FALSE
hsa-miR-4536-3p	MIMAT0020959	C-301930-00	UCGUGCAUAUAUCUACCACAU	-1.07232143191926	0.00054279	FALSE	-3.00264840143944	0.014453	TRUE
hsa-miR-4536-5p	MIMAT0019078	C-301929-00	UGUGGUAGAUAUAUGCACGAU	-1.31712298567513	0.00013963	TRUE	-1.61271864964865	0.20114	FALSE
hsa-miR-4538	MIMAT0019081	C-302154-00	GAGCUUGGAUGAGCUGGGCUGA	-1.22689177781842	0.00013963	TRUE	-1.39982249594569	0.5811	FALSE
hsa-miR-4539	MIMAT0019082	C-302155-00	GCUGAACUGGGCUGAGCUGGGC	-0.662063245722664	0.0074787	FALSE	-2.55330760069272	0.11887	TRUE
hsa-miR-4632-5p	MIMAT0022977	C-302499-00	GAGGGCAGCGUGGGUGUGGCGGA	-0.597712246032446	0.015067	FALSE	-2.79546040686595	0.057617	TRUE
hsa-miR-4642	MIMAT0019702	C-302175-00	AUGGCAUCGUCCCCUGGUGGCU	-1.24506307785847	0.00013963	TRUE	-1.87231348016783	0.26604	FALSE
hsa-miR-4645-3p	MIMAT0019706	C-302179-00	AGACAGUAGUUCUUGCCUGGUU	-1.12025038846698	0.00026644	TRUE	-1.08522858184346	0.5811	FALSE
hsa-miR-4650-3p	MIMAT0019714	C-302184-00	AGGUAGAAUGAGGCCUGACAU	-1.52761158686618	3.9917e-05	TRUE	-2.47248261479947	0.11887	TRUE
hsa-miR-4651	MIMAT0019715	C-302187-00	CGGGGUGGGUGAGGUCGGGC	-1.12142324138952	0.00023985	TRUE	-1.06587157649805	0.5811	FALSE
hsa-miR-4652-5p	MIMAT0019716	C-302188-00	AGGGGACUGGUUAAUAGAACUA	-1.12850660881293	0.00023985	TRUE	-0.668591975163071	0.86349	FALSE
hsa-miR-4655-5p	MIMAT0019721	C-302194-00	CACCGGGGAUGGCAGAGGGUCG	-1.21402640640325	0.00016326	TRUE	-1.71020871463468	0.40677	FALSE
hsa-miR-466	MIMAT0015002	C-301675-00	AUACACAUACACGCAACACACAU	-1.57268812194273	1.5894e-05	TRUE	-1.36589716672211	0.46329	FALSE
hsa-miR-4664-3p	MIMAT0019738	C-302210-00	CUUCCGGUCUGUGAGCCCCGUC	-1.12808833805518	8.3715e-05	TRUE	-1.3144620182497	0.26604	FALSE
hsa-miR-4664-5p	MIMAT0019737	C-302209-00	UGGGGUGCCCACUCCGCAAGUU	-1.35541980608348	3.6715e-05	TRUE	-0.623887404733994	0.76864	FALSE
hsa-miR-4665-5p	MIMAT0019739	C-302211-00	CUGGGGGACGCGUGAGCGCGAGC	-1.14650290801351	6.1693e-05	TRUE	-2.55003959556736	0.014453	TRUE
hsa-miR-4666b	MIMAT0022485	C-302595-00	UUGCAUGUCAGAUUGUAAUUCCC	-1.25134263280118	0.0053924	FALSE	-1.96334534104703	0.11887	TRUE
hsa-miR-4668-5p	MIMAT0019745	C-302216-00	AGGGAAAAAAAAAAGGAUUUGUC	-1.06833102929774	0.00013793	TRUE	-1.46510395349218	0.1633	FALSE
hsa-miR-4672	MIMAT0019754	C-302224-00	UUACACAGCUGGACAGAGGCA	-1.69132941190395	1.5655e-05	TRUE	-1.82970618393803	0.11887	TRUE
hsa-miR-4674	MIMAT0019756	C-302226-00	CUGGGCUCGGGACGCGCGGCU	-1.30671632029371	3.9917e-05	TRUE	-1.87257075861882	0.08542	TRUE
hsa-miR-4675	MIMAT0019757	C-302227-00	GGGGCUGUGAUUGACCAGCAGG	-0.49750400822998	0.015067	FALSE	-1.65778479186002	0.11887	TRUE
hsa-miR-4677-5p	MIMAT0019760	C-302231-00	UUGUUCUUUGGUCUUUCAGCCA	-1.01207284849144	0.00013963	TRUE	-2.02433296093174	0.1633	FALSE
hsa-miR-4678	MIMAT0019762	C-302232-00	AAGGUAUUGUUCAGACUUAUGA	-0.678062145099685	0.0021076	FALSE	-1.8265679594388	0.11887	TRUE
hsa-miR-4682	MIMAT0019767	C-302236-00	UCUGAGUUCCUGGAGCCUGGUCU	-0.95136903371094	0.00023985	TRUE	-0.279156377102257	1	FALSE
hsa-miR-4683	MIMAT0019768	C-302237-00	UGGAGAUCCAGUGCUCGCCCGAU	-1.03845589118353	0.00013793	TRUE	-2.39905380190628	0.023579	TRUE
hsa-miR-4684-3p	MIMAT0019770	C-302239-00	UGUUGCAAGUCGGUGGAGACGU	-0.997924294092217	0.00016326	TRUE	-1.70225287410888	0.1633	FALSE
hsa-miR-4687-3p	MIMAT0019775	C-302242-00	UGGCUGUUGGAGGGGGCAGGC	-1.08006757648135	9.6971e-05	TRUE	-2.49518358442824	0.023579	TRUE
hsa-miR-4688	MIMAT0019777	C-302244-00	UAGGGGCAGCAGAGGACCUGGG	-0.933026739975212	0.00023985	TRUE	-2.48777268680185	0.023579	TRUE
hsa-miR-4689	MIMAT0019778	C-302245-00	UUGAGGAGACAUGGUGGGGGCC	-1.02151466268037	0.00023985	TRUE	-1.54092925086873	0.1633	FALSE
hsa-miR-4692	MIMAT0019783	C-302250-00	UCAGGCAGUGUGGGUAUCAGAU	-0.988783938058944	0.00016326	TRUE	-1.1486808400672	0.40677	FALSE
hsa-miR-4693-3p	MIMAT0019785	C-302252-00	UGAGAGUGGAAUUCACAGUAUUU	-1.44962435716591	3.6715e-05	TRUE	-1.73885084830262	0.11887	TRUE
hsa-miR-4694-3p	MIMAT0019787	C-302253-00	CAAAUGGACAGGAUAACACCU	-0.93855714822229	0.00023985	TRUE	-1.50626462839686	0.1633	FALSE
hsa-miR-4694-5p	MIMAT0019786	C-302254-00	AGGUGUUAUCCUAUCCAUUUGC	-0.952993076462483	0.00023985	TRUE	-2.88415743782109	0.014453	TRUE
hsa-miR-4695-3p	MIMAT0019789	C-302458-00	UGAUCUCACCGCUGCCUCCUUC	-1.73712530730441	3.6715e-05	TRUE	-2.61115419781241	0.11887	TRUE
hsa-miR-4696	MIMAT0019790	C-302255-00	UGCAAGACGGAUACUGUCAUCU	-1.05967205505661	0.00013793	TRUE	-3.0267965783108	0.009387	TRUE
hsa-miR-4697-5p	MIMAT0019791	C-302257-00	AGGGGGCGCAGUCACUGACGUG	-1.49838261147195	1.5894e-05	TRUE	-1.93121149945073	0.08542	TRUE

hsa-miR-4699-5p	MIMAT0019794	C-302259-00	AGAAGAUUGCAGAGUAAGUUCC	-0.321333385517737	0.096607	FALSE	-1.70896571432969	0.11887	TRUE
hsa-miR-4700-5p	MIMAT0019796	C-302262-00	UCUGGGGAUGAGGACAGUGUGU	-0.63878129737828	0.0046172	FALSE	-2.49301512707352	0.023579	TRUE
hsa-miR-4701-5p	MIMAT0019798	C-302460-00	UUGGCCACCACACCUACCCCUU	-1.25407592715591	0.00016326	TRUE	-2.10511844533172	0.1633	FALSE
hsa-miR-4706	MIMAT0019806	C-302269-00	AGCGGGGAGGAAGUGGGCGCUGCUU	-0.640574202002897	0.0030028	FALSE	-1.80378336355061	0.11887	TRUE
hsa-miR-4707-3p	MIMAT0019808	C-302270-00	AGCCCGCCCCAGCCGAGGUUCU	-0.833524657548794	0.00079918	FALSE	-2.32407172887442	0.057617	TRUE
hsa-miR-4708-3p	MIMAT0019810	C-302273-00	AGCAAGGCGGCAUCUCUCUGAU	-0.969695371193823	0.00023985	TRUE	-0.525873276306624	0.86349	FALSE
hsa-miR-4708-5p	MIMAT0019809	C-302272-00	AGAGAUGCCGCCUUGCUCCUU	-0.822684020528361	0.00079918	FALSE	-2.10220660879154	0.08542	TRUE
hsa-miR-4709-3p	MIMAT0019812	C-302275-00	UUGAAGAGGAGGUGCUCUGUAGC	-0.66432740058703	0.0027607	FALSE	-1.68675985049984	0.11887	TRUE
hsa-miR-4709-5p	MIMAT0019811	C-302274-00	ACAACAGUGACUUGCUCUCCAA	-1.08969801660567	9.6971e-05	TRUE	-0.80626334250579	0.5811	FALSE
hsa-miR-4711-5p	MIMAT0019816	C-302279-00	UGCAUCAGGCCAGAAGACAUGAG	-0.502094584622719	0.015067	FALSE	-2.22945919207469	0.057617	TRUE
hsa-miR-4712-3p	MIMAT0019819	C-302282-00	AAUGAGAGACCUGUACUGUAU	-1.26003058356888	3.9917e-05	TRUE	-2.5282291027878	0.028781	TRUE
hsa-miR-4713-5p	MIMAT0019820	C-302283-00	UUCUCCCACUACCAGGCUCCCA	-1.41056492413791	3.6715e-05	TRUE	0.152837140204165	1	FALSE
hsa-miR-4714-5p	MIMAT0019822	C-302286-00	AACUCUGACCCCUUAGGUUGAU	-0.971641326370144	0.00023985	TRUE	-1.9820676345786	0.08542	TRUE
hsa-miR-4715-3p	MIMAT0019825	C-302288-00	GUGCCACCUUAACUGCAGCCAAU	-0.831711497711227	0.0014577	FALSE	-1.64639738941666	0.11887	TRUE
hsa-miR-4720-3p	MIMAT0019834	C-302297-00	UGCUUAAGUUGUACCAAGUAU	-0.267039361511682	0.37761	FALSE	-2.41942301448004	0.057617	TRUE
hsa-miR-4726-3p	MIMAT0019846	C-302309-00	ACCCAGGUUCCCUCUGGCCGCA	-1.05457626001572	0.0046172	FALSE	-1.96757030993053	0.11887	TRUE
hsa-miR-4726-5p	MIMAT0019845	C-302310-00	AGGGCCAGAGGAGCCUGGAGUGG	-0.548731137329315	0.061384	FALSE	-2.20367296738285	0.11887	TRUE
hsa-miR-4730	MIMAT0019852	C-302314-00	CUGGCGGAGCCCAUUCCAUGCCA	-1.14323623131763	0.0021076	FALSE	-3.22072847594229	0.014453	TRUE
hsa-miR-4731-3p	MIMAT0019854	C-302316-00	CACACAAGUGGCCCCCAACACU	-0.715286408900071	0.021994	FALSE	-2.11972721193743	0.11887	TRUE
hsa-miR-4733-5p	MIMAT0019857	C-302319-00	AAUCCCAAUGCUAGACCCGGUG	-0.918486094585803	0.0074787	FALSE	-2.80327741332156	0.023579	TRUE
hsa-miR-4740-3p	MIMAT0019870	C-302329-00	GCCCGAGAGGAUCCGUCCCUGC	-0.824911201381423	0.015067	FALSE	-3.46216635615516	0.014453	TRUE
hsa-miR-4743-5p	MIMAT0019874	C-302462-00	UGGCCGGAUGGGACAGGAGGCAU	-1.02760222969445	0.00061109	FALSE	-2.22597648616421	0.11887	TRUE
hsa-miR-4745-3p	MIMAT0019879	C-302464-00	UGGCCCGGCGACGUCUCACGGUC	-1.23742515486855	0.00016326	TRUE	-2.78131955234324	0.08542	TRUE
hsa-miR-4747-3p	MIMAT0019883	C-302339-00	AAGGCCCGGGCUUUCCUCCCAG	-0.465231590374108	0.12653	FALSE	-2.90430878110698	0.023579	TRUE
hsa-miR-4747-5p	MIMAT0019882	C-302340-00	AGGGAAGGAGGCUUGGUCUUAG	-0.431504547805173	0.17522	FALSE	-1.91877072137694	0.12226	TRUE
hsa-miR-4748	MIMAT0019884	C-302341-00	GAGGUUUGGGGAGGAUUUGCU	-0.555957276676544	0.061384	FALSE	-2.01827086334852	0.11887	TRUE
hsa-miR-4750-3p	MIMAT0022979	C-302491-00	CCUGACCCACCCCCUCCCGCAG	-0.714754040609532	0.0074787	FALSE	-2.39203721905178	0.11887	TRUE
hsa-miR-4755-5p	MIMAT0019895	C-302349-00	UUUCCCUUCAGAGCCUGGCUUU	-0.539113669488979	0.061384	FALSE	-2.80807175968132	0.057617	TRUE
hsa-miR-4760-5p	MIMAT0019906	C-302359-00	UUUAGAUUGAACAUGAAGUUAG	-0.823743933447296	0.015067	FALSE	-2.78675399181231	0.028781	TRUE
hsa-miR-4768-5p	MIMAT0019920	C-302372-00	AUUCUCUCUGGAUCCCAUGGAU	-0.784576616178355	0.061384	FALSE	-2.06931455868509	0.11887	TRUE
hsa-miR-4774-3p	MIMAT0019930	C-302383-00	AUUGCCUAACAUGUGCCAGAA	-0.980156502965659	0.03164	FALSE	-1.92072766628618	0.12226	TRUE
hsa-miR-4778-3p	MIMAT0019937	C-302393-00	UCUUCUUCCUUUGCAGAGUUGA	-0.468380465892249	0.17522	FALSE	-2.12161516444605	0.11887	TRUE
hsa-miR-4778-5p	MIMAT0019936	C-302392-00	AAUUCUGUAAAGGAAGAAGAGG	-0.108428651324849	1	FALSE	2.20881646867654	0.08542	TRUE
hsa-miR-4782-3p	MIMAT0019945	C-302398-00	UGAUUGUCUUCAUAUCUAGAAC	-0.415227659905038	0.32544	FALSE	-1.91949188729733	0.11887	TRUE
hsa-miR-4792	MIMAT0019964	C-302418-00	CGGUGAGCGCUCGCUGGC	-0.681007711978918	0.096607	FALSE	-2.3586952181417	0.057617	TRUE
hsa-miR-4799-5p	MIMAT0019976	C-302431-00	AUCUAAAUGCAGCAUGCCAGUC	-0.362222805879938	0.32544	FALSE	-1.88523388837028	0.11887	TRUE
hsa-miR-4800-5p	MIMAT0019978	C-302435-00	AGUGGACCGAGGAAGGAAGGA	-1.32275981315377	0.0074787	FALSE	-2.73500556024618	0.028781	TRUE
hsa-miR-4804-3p	MIMAT0019985	C-302441-00	UGCUUAACCUUGCCCUCGAAA	-0.336610566496397	0.37761	FALSE	-2.24438580605872	0.11887	TRUE
hsa-miR-486-3p	MIMAT0004762	C-301211-01	CGGGGCAGCUCAGUACAGGAU	-0.915928947716196	0.0027607	FALSE	-1.90219636227184	0.11887	TRUE
hsa-miR-487a	MIMAT0002178	C-300747-03	AAUCAUACAGGGACAUCCAGUU	-0.747164545262154	0.015067	FALSE	-2.42832566173827	0.040079	TRUE
hsa-miR-488	MIMAT0004763	C-301189-01	UUGAAAGGCUAUUUCUUGGUC	-0.908290335869122	0.01741	FALSE	-2.18110128296813	0.11887	TRUE
hsa-miR-491-3p	MIMAT0004765	C-301091-01	CUUAUGCAAGAUUCCCUUCUAC	-1.62232250510605	0.00023985	TRUE	-1.07582371390113	0.46329	FALSE
hsa-miR-492	MIMAT0002812	C-300757-05	AGGACCUGCGGGACAAGAUUCUU	-0.468584149908068	0.061384	FALSE	-2.72511166766836	0.014453	TRUE
hsa-miR-494	MIMAT0002816	C-300761-05	UGAAACAUACACGGGAAACCUC	-0.970219284972552	0.0021076	FALSE	-3.24114885056762	0.014453	TRUE
hsa-miR-499b-3p	MIMAT0019898	C-302351-00	AACAUCACUGCAAGUCUUAACA	-0.450448811655319	0.10991	FALSE	-2.12877293491975	0.11887	TRUE
hsa-miR-5001-3p	MIMAT0021022	C-302470-00	UUCUGCCUCUGUCCAGGUCCUU	-1.33428639345599	0.00013793	TRUE	-1.54637406738355	0.40677	FALSE
hsa-miR-5003-5p	MIMAT0021025	C-302474-00	UCACAACAACCUUGCAGGGUAGA	-1.32073825957867	0.00013793	TRUE	-1.18738346839705	0.51579	FALSE
hsa-miR-5004-5p	MIMAT0021027	C-302477-00	UGAGGACAGGGCAAAUUCACGA	-1.00354080968358	0.00079918	FALSE	-3.14572597959306	0.057617	TRUE
hsa-miR-5006-5p	MIMAT0021033	C-302479-00	UUGCCAGGGCAGGAGGUGGAA	-1.29090326952253	0.00013963	TRUE	-1.67173993218432	0.40677	FALSE
hsa-miR-501-3p	MIMAT0004774	C-301167-01	AAUGCACCCGGGCAAGGAUUCU	-0.0731774010060508	1	FALSE	1.96439959148781	0.057617	TRUE
hsa-miR-5087	MIMAT0021079	C-301942-00	GGGUUUGUAGCUUUGCUGGCAUG	-0.359393924285983	0.10991	FALSE	-1.78188598696404	0.11887	TRUE
hsa-miR-509-5p	MIMAT0004779	C-301166-01	UACUGCAGACAGUGGCAAUCA	-1.74945283553728	0.00013793	TRUE	-2.47315672999895	0.081661	TRUE
hsa-miR-5092	MIMAT0021084	C-302456-00	AAUCCACGCUGAGCUUGGCAUC	-1.18967239061038	0.00023985	TRUE	-1.511756387148	0.46329	FALSE
hsa-miR-513a-5p	MIMAT0002877	C-300844-07	UUCACAGGGAGGUGUCAU	-1.05997050839063	0.0074787	FALSE	-2.37670537648159	0.11887	TRUE

hsa-miR-513c-3p	MIMAT0022728	C-301908-00	UAAAUUUCACCUUUCUGAGAAGA	-0.803025559359186	0.0046172	FALSE	-2.73190794276724	0.014453	TRUE
hsa-miR-514	MIMAT0002883	C-300851-07	AUUGACACUUCUGUGAGUAGA	-0.348236527420602	0.12653	FALSE	-1.56224654603507	0.11887	TRUE
hsa-miR-516a-5p	MIMAT0004770	C-301104-01	UUCUCGAGGAAAGAAGCACUUUC	-0.681407959396855	0.0046172	FALSE	-2.18673664105892	0.11887	TRUE
hsa-miR-517a	MIMAT0002852	C-300811-05	AUCGUGCAUCCCUUUAGAGUGU	-1.09820612826732	0.0018747	FALSE	-2.32535259864992	0.08542	TRUE
hsa-miR-517b-3p	MIMAT0002857	C-300817-06	AUCGUGCAUCCCUUUAGAGUGU	-1.68014170163936	3.9917e-05	TRUE	-1.45577777456063	0.26604	FALSE
hsa-miR-517c	MIMAT0002866	C-300832-03	AUCGUGCAUCCUUUUAGAGUGU	-1.39967029576568	0.00054279	FALSE	-3.92792989935189	0.009387	TRUE
hsa-miR-518a-5p	MIMAT0005457	C-301099-01	CUGCAAAGGGAAGCCCUUUC	-1.69701165144962	0.00013963	TRUE	-3.84851299481868	0.014453	TRUE
hsa-miR-518b	MIMAT0002844	C-300798-03	CAAAGCGCUCCCCUUUAGAGGU	-1.15359484149699	0.00087976	FALSE	-2.22045683232246	0.08542	TRUE
hsa-miR-518c*	MIMAT0002847	C-300804-03	UCUCUGGAGGGAAGCACUUUCUG	-1.15870379627599	0.0014577	FALSE	-3.20841244988613	0.014453	TRUE
hsa-miR-518d-5p	MIMAT0005456	C-301100-01	CUCUAGAGGGAAGCACUUUCUG	-0.509493211764305	0.54004	FALSE	-2.82765759572384	0.081661	TRUE
hsa-miR-5192	MIMAT0021123	C-302496-00	AGGAGAGUGGAUUCCAGGUGGU	-0.929717635688366	0.0014577	FALSE	-4.01918995190962	0.014453	TRUE
hsa-miR-5196-5p	MIMAT0021128	C-301981-00	AGGGAAGGGGACGAGGGUUGGG	-1.16761124143466	3.6715e-05	TRUE	-2.56840698714633	0.040079	TRUE
hsa-miR-520a-3p	MIMAT0002834	C-300788-03	AAAGUGCUUCCCUUUGGACUGU	0.0374069999290813	1	FALSE	2.98674734617433	0.08542	TRUE
hsa-miR-520c-3p	MIMAT0002846	C-300803-05	AAAGUGCUUCCUUUUAGAGGGU	-0.199389168688778	0.37761	FALSE	-1.76230191681356	0.11887	TRUE
hsa-miR-520h	MIMAT0002867	C-300833-03	ACAAAGUGCUUCCCUUUAGAGU	-0.694676795373559	0.015067	FALSE	-2.26960151079303	0.08542	TRUE
hsa-miR-532-5p	MIMAT0002888	C-300867-01	CAUGCCUUGAGUGUAGGACCGU	-0.687783861572295	0.021994	FALSE	-3.38518185188287	0.014453	TRUE
hsa-miR-539-3p	MIMAT0022705	C-301909-00	AUCAUACAAGGACAAUUUCUUU	-0.712513192949551	0.0074787	FALSE	-1.7777539713116	0.12226	TRUE
hsa-miR-544b	MIMAT0015004	C-301677-00	ACCUGAGGUUGUGCAUUUCUAA	-1.14820342901038	3.9917e-05	TRUE	-0.449549813604399	0.91039	FALSE
hsa-miR-548ab	MIMAT0018928	C-301990-00	AAAAGUAAUUGUGGAUUUUGCU	-0.602579302030159	0.0021076	FALSE	-2.46432616915756	0.057617	TRUE
hsa-miR-548ac	MIMAT0018938	C-302001-00	CAAAAACCGGCAAUUACUUUUG	-0.979633570213558	6.1693e-05	TRUE	-2.0217602235335	0.11887	TRUE
hsa-miR-548ae	MIMAT0018954	C-302019-00	CAAAAACUGCAAUUACUUUCA	-0.606375099488669	0.0030028	FALSE	-2.77725501966519	0.057617	TRUE
hsa-miR-548ah-3p	MIMAT0020957	C-302036-00	CAAAAACUGCAGUUACUUUUGC	-0.668405768437197	0.0014577	FALSE	-2.22611084098903	0.081661	TRUE
hsa-miR-548at-5p	MIMAT0022277	C-302544-00	AAAAGUUAUUGCGGUUUUGGCU	-0.0453519642557928	1	FALSE	-1.8924808826669	0.11887	TRUE
hsa-miR-548au-3p	MIMAT0022292	C-302561-00	UGGCAGUUACUUUUGCACCAG	-1.52595082150064	0.0021076	FALSE	-3.17672563043804	0.014453	TRUE
hsa-miR-548ax	MIMAT0022474	C-302582-00	AGAAGUAAUUGCGGUUUUGCCA	-0.564257743083232	0.10991	FALSE	-2.34784280477006	0.057617	TRUE
hsa-miR-548ay-5p	MIMAT0025452	C-302660-00	AAAAGUAAUUGUGGUUUUUGC	-0.653127591660778	0.021994	FALSE	-3.23819702467989	0.014453	TRUE
hsa-miR-548h-3p	MIMAT0022723	C-301888-00	CAAAAACCGCAAUUACUUUUGCA	-1.06047561824622	0.00054279	FALSE	-2.88715465104665	0.014453	TRUE
hsa-miR-548l	MIMAT0005889	C-301353-00	AAAAGUAUUUGCGGGUUUUGUC	-0.59227174574884	0.061384	FALSE	-2.37472439626248	0.11887	TRUE
hsa-miR-548s	MIMAT0014987	C-301658-00	AUGGCCAAAACUGCAGUUAUUUU	-0.871664637647317	0.00054279	FALSE	-2.25194852231406	0.11887	TRUE
hsa-miR-549	MIMAT0003333	C-300991-01	UGACAACUAUGGAUGAGCUCU	-0.71851748320711	0.01741	FALSE	-2.02535291589875	0.12226	TRUE
hsa-miR-550b-2-5p	MIMAT0022737	C-301971-00	AUGUGCCUGAGGGAGUAAGACA	-0.509912734915377	0.0074787	FALSE	-1.84020688668008	0.12226	TRUE
hsa-miR-5580-5p	MIMAT0022273	C-302541-00	UGCUGGCUCAUUUCAUAUGUGU	-0.250725616752794	0.54004	FALSE	-2.09116428366529	0.08542	TRUE
hsa-miR-5583-3p	MIMAT0022282	C-302549-00	GAAUAUGGGUAUAUUAGUUUGG	-0.580364168150873	0.10991	FALSE	-2.828220915146	0.014453	TRUE
hsa-miR-5583-5p	MIMAT0022281	C-302548-00	AAACUAAUAUACCCAUAUUCUG	-0.74119839633839	0.050037	FALSE	-2.77350973293048	0.014453	TRUE
hsa-miR-568	MIMAT0003232	C-300886-01	AUGUAUAAAUGUAUACACAC	-0.546512240064326	0.050037	FALSE	-2.16900241945777	0.11887	TRUE
hsa-miR-5681a	MIMAT0022469	C-302576-00	AGAAAGGGUGGCAAUACCUCUU	-0.554451480617061	0.10991	FALSE	-2.10313927081946	0.11887	TRUE
hsa-miR-5681b	MIMAT0022480	C-302589-00	AGGUAUUGCCACCCUUUCUAGU	-0.990407574357977	0.015067	FALSE	-2.24966625727449	0.11887	TRUE
hsa-miR-5685	MIMAT0022475	C-302583-00	ACAGCCCAGCAGUUAUCACGGG	-1.10866668617995	0.0099219	FALSE	-1.81151890217456	0.11887	TRUE
hsa-miR-5686	MIMAT0022477	C-302586-00	UAUCGUAUCGUAUUGUAUUGU	-0.618603205233964	0.096607	FALSE	-3.26787707054355	0.014453	TRUE
hsa-miR-5687	MIMAT0022478	C-302587-00	UUAGAACGUUUUAGGGUCAAAU	-0.4136100982672	0.25618	FALSE	-1.96140285293105	0.11887	TRUE
hsa-miR-5688	MIMAT0022479	C-302588-00	UAACAAACACCUGUAAAACAGC	-0.496708860788786	0.17522	FALSE	-2.54610797665859	0.028781	TRUE
hsa-miR-5689	MIMAT0022481	C-302590-00	AGCAUACACCUGUAGUCCUAGA	-0.623295340978942	0.089234	FALSE	-2.01329320718309	0.11887	TRUE
hsa-miR-5692a	MIMAT0022484	C-302593-00	CAAAUAAUACCACAGUGGGUGU	-0.71714659454192	0.061384	FALSE	-2.47331051365896	0.057617	TRUE
hsa-miR-5692c	MIMAT0022476	C-302584-00	AAUAAUAUCACAGUAGGUGUAC	-0.930027776637268	0.021994	FALSE	-2.41733401849822	0.057617	TRUE
hsa-miR-5697	MIMAT0022490	C-302600-00	UCAAGUAGUUUCAUGAUAAAGG	-0.509179181511475	0.17522	FALSE	-1.87679966081257	0.11887	TRUE
hsa-miR-5701	MIMAT0022494	C-302604-00	UUAUUGUCACGUUCUGAUU	-0.713050083304961	0.061384	FALSE	-2.19998791399188	0.081661	TRUE
hsa-miR-5702	MIMAT0022495	C-302605-00	UGAGUCAGCAACAUAUCCCAUG	-0.634695457873147	0.089234	FALSE	-2.91461280705682	0.014453	TRUE
hsa-miR-571	MIMAT0003236	C-300890-01	UGAGUUGGCCAUCUGAGUGAG	-1.04012448788134	0.00016326	TRUE	-1.0680220266461	0.5811	FALSE
hsa-miR-574-3p	MIMAT0003239	C-300893-03	CACGCUCAUGCACACACCCACA	-1.4521414321912	0.0018747	FALSE	-2.74091004364483	0.057617	TRUE
hsa-miR-578	MIMAT0003243	C-300897-01	CUUCUUGUGCUCUAGGAUUGU	-0.525376007672939	0.038627	FALSE	-2.14162436977285	0.081661	TRUE
hsa-miR-579	MIMAT0003244	C-300898-03	UUCAUUUGGUAUAAACCGCGAUU	-1.2654167775587	0.0030028	FALSE	-3.8111485398612	0.014453	TRUE
hsa-miR-582-5p	MIMAT0003247	C-300901-01	UUACAGUUGUUCAACCAGUUACU	-1.2010641323251	0.00070025	FALSE	-2.60372535467359	0.040079	TRUE
hsa-miR-586	MIMAT0003252	C-300906-01	UAUGCAUUGUAUUUUUAGGUCC	-0.445099473295375	0.10991	FALSE	-3.97498411008623	0.014453	TRUE
hsa-miR-593*	MIMAT0003261	C-300917-01	AGGCACCAGCCAGGCAUUGCUCAGC	-1.46368716545378	0.00023985	TRUE	-1.69498877920483	0.1633	FALSE

hsa-miR-606	MIMAT0003274	C-300931-01	AAACUACUGAAAAUCAAAGAU	-0.637631782545375	0.32544	FALSE	-2.73649791406254	0.11887	TRUE
hsa-miR-607	MIMAT0003275	C-300932-01	GUUCAAAUCCAGAUCUAUAAC	-0.096398469481977	1	FALSE	2.3242183673772	0.11887	TRUE
hsa-miR-6071	MIMAT0023696	C-302625-00	UUCUGCUGCCGGCCAAGGC	-0.528822533996229	0.12653	FALSE	-2.1934583463063	0.11887	TRUE
hsa-miR-6075	MIMAT0023700	C-302629-00	ACGGCCCAGGCGGCAUUGGUG	-0.592038077604373	0.037103	FALSE	-2.63925181780449	0.057617	TRUE
hsa-miR-608	MIMAT0003276	C-300933-01	AGGGGUGGUGUUGGGACAGCUCCGU	-1.41476375494069	9.6971e-05	TRUE	-1.32647981772413	0.22566	FALSE
hsa-miR-6080	MIMAT0023705	C-302634-00	UCUAGUGCGGGCGUUCCCG	-0.377302781648075	0.17522	FALSE	-3.12142545628473	0.023579	TRUE
hsa-miR-6081	MIMAT0023706	C-302635-00	AGGAGCAGUGCCGGCCAAGGCGCC	-1.07161737179969	0.0018747	FALSE	-2.06272320366808	0.11887	TRUE
hsa-miR-6086	MIMAT0023711	C-302640-00	GGAGGUUGGGAAGGGCAGAG	-0.556948350994301	0.040903	FALSE	-2.86501870739423	0.028781	TRUE
hsa-miR-6133	MIMAT0024617	C-302655-00	UGAGGGAGGAGGUUGGGUA	-1.00026187079406	0.0021076	FALSE	-3.11682515905473	0.014453	TRUE
hsa-miR-617	MIMAT0003286	C-300943-01	AGACUUCCCAUUUGAAGGUGGC	-1.41203659250412	0.00023985	TRUE	-0.966712399788375	0.51579	FALSE
hsa-miR-619	MIMAT0003288	C-300945-01	GACCUGGACAUGUUUGUGCCCAGU	0.0866202298806936	1	FALSE	2.48494644624597	0.014453	TRUE
hsa-miR-620	MIMAT0003289	C-300946-01	AUGGAGAUAGAUAUAGAAAU	-0.746644845920626	0.096607	FALSE	-3.35303260797096	0.057617	TRUE
hsa-miR-621	MIMAT0003290	C-300947-01	GGCUAGCAACAGCGCUUACCU	-0.524135944687041	0.49143	FALSE	-3.56178952288145	0.023579	TRUE
hsa-miR-624*	MIMAT0003293	C-300950-01	UAGUACCAGUACCUUGUGUUCA	-1.18323865292578	8.3715e-05	TRUE	-2.08818623024311	0.11887	TRUE
hsa-miR-627	MIMAT0003296	C-300953-01	GUGAGUCUCUAAGAAAAGAGGA	-1.05366486573687	0.00016326	TRUE	-1.71256975426944	0.22566	FALSE
hsa-miR-631	MIMAT0003300	C-300957-01	AGACCUGGCCCAGACCUCAGC	-2.01925344204044	3.9917e-05	TRUE	-1.07267199301577	0.51048	FALSE
hsa-miR-634	MIMAT0003304	C-300961-01	AACCAGCACCCCAACUUUGGAC	-1.30123850604998	0.00016067	TRUE	-0.64623077279342	0.76864	FALSE
hsa-miR-638	MIMAT0003308	C-300965-01	AGGGAUCGCGGGCGGGUGGCGGCCU	-0.639193662554625	0.03164	FALSE	-1.99612853656179	0.12226	TRUE
hsa-miR-642a-3p	MIMAT0020924	C-301902-00	AGACACAUUUGGAGAGGGAACC	-0.669360409839209	0.0099219	FALSE	-2.67786191538987	0.023579	TRUE
hsa-miR-642b-5p	MIMAT0022736	C-301970-00	GGUUCCCUCUCCAAAUGUGUCU	-0.982372985036737	6.1693e-05	TRUE	-2.91448575097456	0.023579	TRUE
hsa-miR-644	MIMAT0003314	C-300971-01	AGUGUGGCUUUCUUAGAGC	-1.1328764594198	0.0014577	FALSE	-2.28201156273226	0.12226	TRUE
hsa-miR-646	MIMAT0003316	C-300973-01	AAGCAGCUGCCUCUGAGGC	-1.37335174868112	3.9917e-05	TRUE	-1.88378259141162	0.1633	FALSE
hsa-miR-647	MIMAT0003317	C-300974-01	GUGGCUGCACUCACUUCCUUC	-0.662202123721489	0.015067	FALSE	-2.34112755480514	0.057617	TRUE
hsa-miR-6499-3p	MIMAT0025451	C-302659-00	AGCAGUGUUUGUUUUGCCCACA	-0.78931105472599	0.0099219	FALSE	-2.42776308682332	0.08542	TRUE
hsa-miR-650	MIMAT0003320	C-300977-01	AGGAGGCAGCGCUCUCAGGAC	-1.32232002772507	0.00054279	FALSE	-2.38532145199764	0.11887	TRUE
hsa-miR-6503-3p	MIMAT0025463	C-302671-00	GGGACUAGGAUGCAGACCUCC	-0.701857748301807	0.01741	FALSE	-2.26797239631951	0.08542	TRUE
hsa-miR-6503-5p	MIMAT0025462	C-302670-00	AGGUCUGCAUUCAAAUCCCCAGA	-1.28829279156107	0.00061109	FALSE	-4.16301032924927	0.009387	TRUE
hsa-miR-6504-3p	MIMAT0025465	C-302672-00	CAUUACAGCACAGCCAUUCU	-0.219268298279075	0.49143	FALSE	-2.3682663259571	0.08542	TRUE
hsa-miR-6505-3p	MIMAT0025467	C-302675-00	UGACUUCUACCUCUUCCAAAG	-1.41016990839787	0.00054279	FALSE	-2.92045878806631	0.040079	TRUE
hsa-miR-6507-5p	MIMAT0025470	C-302679-00	GAAGAAUAGGAGGGACUUUGU	-0.637513482741369	0.03164	FALSE	-2.64696299700191	0.057617	TRUE
hsa-miR-6514-3p	MIMAT0025485	C-302693-00	CUGCCUGUUCUUCCACUCCAG	-1.57253367763337	0.00023985	TRUE	-0.857463656453242	0.72813	FALSE
hsa-miR-652-5p	MIMAT0022709	C-301928-00	CAACCCUAGGAGAGGGUGCCAUUCA	-0.590578760858739	0.01741	FALSE	-2.04113601671663	0.11887	TRUE
hsa-miR-654-5p	MIMAT0003330	C-300988-01	UGGUGGGCCGCAGAACAUGUGC	-1.24102088307823	0.00054279	FALSE	-4.37321715649902	0.0018396	TRUE
hsa-miR-659	MIMAT0003337	C-300994-01	CUUGGUUCAGGGAGGGUCCCCA	-0.865338332757254	0.0053924	FALSE	-2.2970254587848	0.12226	TRUE
hsa-miR-660-3p	MIMAT0022711	C-301897-00	ACCUCCUGUGUGCAUGGAUUA	-0.914364872105563	0.0018747	FALSE	-2.7989676696545	0.028781	TRUE
hsa-miR-661	MIMAT0003324	C-300981-01	UGCCUGGGUCUCUGGCCUGCGCGU	-0.966061272920722	0.0046172	FALSE	-2.04789510522588	0.11887	TRUE
hsa-miR-664b-3p	MIMAT0022272	C-302539-00	UUCAUUUGCCUCCCAGCCUACA	-0.63588937093853	0.061384	FALSE	-2.38779084523873	0.057617	TRUE
hsa-miR-671-5p	MIMAT0003880	C-301000-03	AGGAAGCCCUGGAGGGGCUGGAG	-1.01268622344544	0.00023985	TRUE	-0.995050180707598	0.5811	FALSE
hsa-miR-6721-5p	MIMAT0025852	C-302707-00	UGGGCAGGGGCUUAUUGUAGGAG	-1.0522716562067	0.0021076	FALSE	-2.86092601806193	0.028781	TRUE
hsa-miR-7	MIMAT0000252	C-300546-07	UGGAAGACUAGUGAUUUUGUUGU	-0.833080606802588	0.0014577	FALSE	-2.34382127982937	0.11887	TRUE
hsa-miR-718	MIMAT0012735	C-301486-00	CUUCCGCCCCGCCGGGCGUCG	-0.712476270533885	0.0014577	FALSE	-2.51689917044679	0.08542	TRUE
hsa-miR-744	MIMAT0004945	C-301242-01	UGCGGGGCUAGGGCUAACAGCA	-1.31292964878657	0.0027607	FALSE	-2.17130853543699	0.057617	TRUE
hsa-miR-744*	MIMAT0004946	C-301241-01	CUGUUGCCACUAACCUCAACCU	-0.909425861300587	3.9917e-05	TRUE	-2.41920375944258	0.11887	TRUE
hsa-miR-764	MIMAT0010367	C-301640-00	GCAGGUGCUCACUUGUCCUCCU	-1.1596052122796	6.1693e-05	TRUE	-0.963991799367289	0.5811	FALSE
hsa-miR-767-3p	MIMAT0003883	C-301003-01	UCUGCUCAUACCCCAUGGUUUCU	-0.875815222995269	0.0021076	FALSE	-2.87852305265755	0.009387	TRUE
hsa-miR-801	MI0005202	C-301014-01	GAUUGCUCUGCGUGCGGAAUCGAC	-1.02838938735747	0.00023985	TRUE	0.128066636696993	1	FALSE
hsa-miR-873-3p	MIMAT0022717	C-301921-00	GGAGACUGAUGAGUUCCCGGGA	-0.45500701580676	0.050037	FALSE	-2.17559760678154	0.08542	TRUE
hsa-miR-888*	MIMAT0004917	C-301227-01	GACUGACACCUCUUUGGGUGAA	-0.798719536763374	0.015067	FALSE	-1.83668949541677	0.11887	TRUE
hsa-miR-940	MIMAT0004983	C-301269-01	AAGGCAGGGCCCCCGCUCCCC	-1.31504996360232	0.00054279	FALSE	-2.52010733193133	0.11887	TRUE
hsa-miR-96	MIMAT0000095	C-300514-07	UUUGGCACUAGCACAUUUUUGCU	-0.788957233607577	0.0074787	FALSE	-2.19234338350236	0.11527	TRUE
hsa-miR-99a	MIMAT0000097	C-300516-03	AACCCGUAGAUCCGAUCUUGUG	-0.986802247020601	0.0030028	FALSE	-2.42723567464877	0.11887	TRUE

Legend

Antiviral effect

Proviral effect

Note: Hits were selected using the following Local False Discovery Rate (lfdr) thresholds: 0.00027 in screen part 1 and 0.1226 in screen part 2. Positive log2 fold change values indicated a proviral effect of the miRNA on the HCV life cycle, while negative log2 fold change values indicated an antiviral effect.

V. DISCUSSION

A genome-wide miRNA mimic screen in Huh7.5.1 cells to systematically uncover human miRNAs affecting the HCV replication cycle, especially HCV assembly and release of infectious virions, provided the basis of the present thesis. To this end, new achievements of virus-host interactions could be obtained: (i) miR-501-3p and miR-619-3p were found to enhance late steps of HCV infection (ii) using endogenous overexpression of miR-501-3p in human hepatoma cells, we have demonstrated that this miRNA specifically targets OGT and regulates its expression at the protein level, (iii) increased infectivity of HCV particles through silencing or inhibition of OGT's enzymatic activity, and (iv) release of bigger HCV particles after knock-down of OGT proposing that *O*-GlcNAcylation mediated by OGT affects HCV morphology. Whether miR-501-3p contributes to regulate the physiological expression of OGT in human hepatocytes remains elusive. Interestingly both miR-501-3p and *OGT* are located on the X chromosome. Overall, our data and data from the literature suggest an involvement of OGT and *O*-GlcNAcylation for HCV infection as well as for HCC progression, even if their role in each disease may be completely different.

Enveloped viruses consist of an host-derived envelope membrane with viral proteins more or less heavily glycosylated by the host glycosylation machinery as viruses pass through the secretory pathway protecting from the host immune system (e.g. HCV) or playing a crucial role for virus entry into host cells (e.g. Influenza virus) (Air 2014). While *N*-glycosylation of HCV E1 and E2 glycoproteins is well-defined as it serves as a protective glycan shield from the humoral immune response (reviewed in Helle, Duverlie et al. 2011), a link between *O*-GlcNAcylation and HCV infection had not been described before. *O*-GlcNAcylation is a crucial regulator for metabolic pathways within the liver (e.g. insulin signaling, bile acid metabolism and lipogenesis) (Yang and Qian 2017), however, due to the enormous number of *O*-GlcNAcylated substrates, detailed characterization of their role in HCV infection is lacking. Interestingly, proteins can either be *O*-GlcNAcylated or phosphorylated on the same residues resulting in adjusted cellular signaling (Hart, Slawson et al. 2011) (see p.28). Potential O-GlcNAcylation and phosphorylation at the same serine or threonine residue are defined as Yin Yang sites (Hart, Greis et al. 1995).

When we think retrospectively on the present results, since silencing of *OGT* through siRNAs or inhibition of its enzymatic activity by small molecules both lead to an enhancement of HCV infection as well as bigger particles proposing a potential role of *O*-GlcNAcylation for HCV morphology, one hypothesis could be that HCV envelope glycoproteins or LVP-associated host proteins are *O*-GlcNAcylated. However, since HCV assembly takes place in the ER lumen, and OGT/OGA are found in nucleocytoplasmic and mitochondrial compartments and so far, not known to localize to the ER lumen, a direct modulation of HCV viral proteins through *O*-GlcNAcylation seems unlikely, even if

E1 and E2 both contain putative *O*-GlcNAcylation sites (Bandiera and Zeisel, unpublished data and reviewed in Lavie, Hanoulle et al. 2018).

Another important aspect could have been the potential O-GlcNAcylation of host-derived proteins associated with HCV, such as ApoE and ApoB. However, to our knowledge, although ApoE has been shown to be O-glycosylated, its O-GlcNAcylation by OGT has not been described. Indeed, ApoE which is primarily produced in the liver and associated with HDL and VDL has been shown the be *O*-glycosylated at threonine residue 194 (Thr^{194}) (Wernette-Hammond, Lauer et al. 1989) and serine residue 290 (Ser^{290}) (Lee, Kockx et al. 2010), while he Asn-X-Thr/Ser consensus sequence needed for *N*-glycosylation is lacking. Amino sugar analysis of monosialic apoE peptides revealed the presence of galactosamine, indicating the presence of GalNAc, but absence of glucosamine, indicating absence of GlcNAc (Wernette-Hammond, Lauer et al. 1989). In contrast to monosialic apoE, disialic apoE may exhibit a more complex carbohydrate structure as disialic apoE peptides contained both galactosamine and glucosamine (Wernette-Hammond, Lauer et al. 1989). It could be demonstrated that the *O*-glycosylation site Thr^{194} is not important for secretion (Wernette-Hammond, Lauer et al. 1989) but Ser^{290} *O*-glycosylation that is situated at the C-terminus of ApoE, is important for ApoE stability, solubility and lipid binding (Lee, Kockx et al. 2010). In line with this, another study could reveal that long-term ethanol treatment decreased *O*-glycosylation of ApoE resulting in a shift of ApoE association from HDL to VLDL; indeed under alcoholic control decreased ApoE levels were found in association with VLDL (Ghosh, Liu et al. 1995). In contrast, ApoB molecules are highly N-glycosylated with 16 of 19 *N*-glycosylation sites found to be glycosylation (Fujioka, Taniguchi et al. 1994; Wong and Torbati 1994), while no *O*-glycosylation sites have been reported yet.

Another possibility than directly affecting the viral particle-associated proteins, could be the post-translational modulation of one or several HCV host factors required for HCV morphogenesis via OGT/OGA. *OGT*-silencing in the experimental settings used in the present paper showed no significant effect on early steps of HCV infection, even if some *O*-GlcNAcylation or Yin Yang sites of CLDN1 (Ahmad, Shabbiri et al. 2011; Butt, Khan et al. 2012) and OCLN (Butt, Feng et al. 2012) could be predicted suggesting that *O*-GlcNAcylation of CLDN1 and OCLN is not relevant for HCV infection. Ribosomal receptor or activated C-kinase 1 (RACK1), an adaptor protein and component of the 40S subunit of ribosomes, is a cellular factor required for IRES-dependent translation and replication of HCV in human hepatocytes without interacting with the miRNA pathway (Majzoub, Hafirassou et al. 2014). Interestingly, RACK1 is highly *O*-GlcNAcylated at Serine position 122 resulting in its stability and ribosome binding. Indeed, *O*-GlcNAcylation of RACK1 increases phosphorylation of elF4E and translation of oncogenes in human hepatoma cells (Duan, Wu et al. 2018). Since increased O-GlcNAcylation is associated with tumorigenesis, angiogenesis and

metastasis, RACK1 has been reported as one host factor whose *O*-GlcNAcylation contributes to these processes in HCC patients and is correlated to tumor progression and recurrence after chemotherapy (Duan, Wu et al. 2018). Of note, Cao et al demonstrated that O-GlcNAcylation of elF4E resulted in a stem-like cell potential of hepatoma cells contributing to HCC progression and tumorigenesis proposing two host *O*-GlcNAcylated host factors, elF4E and RACK1, that built a bridge between O-GlcNAc metabolism and tumorigenesis (Duan, Wu et al. 2018; Cao, Duan et al. 2019).

Among the host factors involved in the HCV replication cycle, nuclear pore complexes (NPCs) consistent of nucleoporins (Nups) were also observed to be *O*-GlcNAcylated (Zhu, Liu et al. 2016). NPCs are hijacked by HCV and recruited to the membranous web during viral replication where they associate with viral proteins (core. NS5A, NS2 and NS3) contributing to the architecture of the membranous web and facilitating transport of viral and host proteins into the membranous web (Neufeldt, Joyce et al. 2013; Levin, Neufeldt et al. 2014). Depletion of Nup98, Nup153 and Nup155 appeared to inhibit HCV replication and assembly resulting in reduced intracellular levels of viral RNA and secreted virus, however, without affecting the specific infectivity of secreted virions (Neufeldt, Joyce et al. 2013). However, we could demonstrate here that knock-down of OGT resulted in an enhanced viral infection and an alteration of specific infectivity of viral particles leading to the conclusion that *O*-GlcNAcylation level of Nups does not contribute to the effects of OGT-silencing on HCVcc infectivity.

Besides HCV, *O*-GlcNAcylation and the action of OGT and OGA could be demonstrated to be involved in other viral infections: (i) Human immunodeficiency virus type 1 (HIV-1), Human T-cell leukemia virus type-1 (HTVL-1), Herpes simplex virus (HSV) and Kaposi's sarcoma-associated herpesvirus (KSHV) (Jochmann, Thurau et al. 2009; Jochmann, Pfannstiel et al. 2013; Groussaud, Khair et al. 2017; Angelova, Ortiz-Meoz et al. 2015). It can be assumed that the involvement of O-GlcNAcylation is as different as the viruses itself: (i) Overexpression of OGT as well as increased O-GlcNAcylation led to an repressed HIV-1 transcription as well as an decreased KSHV replication highlighting the link between viral replication and glucose metabolism (Jochmann, Thurau et al. 2009; Jochmann, Pfannstiel et al. 2013), (ii) Inhibition of OGA by HTLV-1 protein Tax led to an increased O-GlcNAcylation resulting in an increased viral transcription (Groussaud, Khair et al. 2017), and (iii) Inhibition of OGT resulted in a decreased HSV replication (Angelova, Ortiz-Meoz et al. 2015). Decreased OGT levels could be observed *in vitro* upon HCV infection (Herzog, Bandiera et al. 2019).

Of note, deregulation of O-GlcNAcylation is associated with a variety of cancers. Increased OGT expression as well as an enhanced *O*-GlcNAcylation could be observed in HCC tissue of HCV-infected patients. It was reported that OGT activates oncogenic signaling pathways in liver-derived cells via regulation of palmitic acid and the induction of ER stress thereby promoting tumor growth

and metastasis (Xu, Zhang et al. 2017), and additionally a link between *O*-GlcNAcylation and HCC recurrence after LT could be determined (Zhu, Zhou et al. 2012) proposing a potential role of *O*-GlcNAcylation and its enzymes, OGT and OGA, in the contribution to HCV-induced liver disease and hepatocarcinogenesis. As described in 2.3, an increased flux through glycolysis is observed in cancer cells, additionally, it has been described that cancer cells show altered lipogenesis pathways supporting membrane synthesis and generating signaling molecules resulting in rapid cell growth (Baenke, Peck et al. 2013); indeed, cancer cells utilize *de novo* lipogenesis instead of obtaining lipids from the bloodstream (Swinnen, Brusselmans et al. 2006) involving activation and expression of enzymes able to generate lipids such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), ATP-citrate lyase (ACLY) as well as the transcription factor that regulates these enzymes, termed sterol regulatory element binding protein (SREBP-1) (Santos and Schulze 2012). Recently could be demonstrated that *O*-GlcNAcylation is a crucial factor in directly regulating SREBP-1 phosphorylation and stability as well as controlling ACLY and FAS (Baldini, Wavelet et al. 2016) resulting in the regulation of lipid metabolism and cancer cell growth and survival (Sodi, Bacigalupa et al. 2018). Moreover, liver X receptor (LXR), another key transcriptional factor to regulate *de novo* lipogenesis, has been shown to be *O*-GlcNAcylated in response to glucose and to directly transactivate SREBP-1 (Bindesboll, Fan et al. 2015). These results highlight that in addition to its role in *O*-GlcNAcylation of oncogenic proteins, OGT is directly linked to the lipid metabolism in (cancer) cells and might serve as a therapeutic approach against highly metabolic cancer.

Of note, in addition to HCV, another oncogenic virus, Human papillomavirus 16 (HPV16), has been linked to OGT as well. Interestingly, it could be demonstrated that levels of HVP16 oncoproteins E6 and E7 are modulated through OGT expression (Kim, Kim et al. 2016), while in turn upregulation of OGT through E6 resulted in an increase of O-GlcNAcylation and an activation of oncogenic activity of HPV (Zeng, Zhao et al. 2016) proposing a general involvement of OGT and *O*-GlcNAcylation in virus-induced cancer.

Apart from O-GlcNAcylation and to close the circle of the present work, miR-501-3p who can functionally target OGT (Herzog, Bandiera et al. 2019), has been shown to be involved in the formation of metastasis in HCC (Luo, Yin et al. 2018). Indeed, it could be demonstrated that a downregulation of miR-501-3p is associated with tumor progression and poor prognosis in patients with HCC, while in contrast overexpression of this miRNA could inhibit cancer cell proliferation, migration and evasion (Luo, Yin et al. 2018). This would be in line with the finding that O-GlcNAcylation is increased in HCC and possibly contribute to hepatocarcinogenesis.

VI. RÉSUMÉ DE LA THÈSE DE DOCTORAT

Etude du rôle de l'OGT dans les étapes tardives du cycle de réplication du virus de l'hépatite C

6.1 Introduction

L'infection chronique par le virus de l'hépatite C (HCV) est un facteur de risque majeur de maladies hépatiques et de carcinome hépatocellulaire (CHC), la seconde cause de mortalité par cancer dans le monde. Depuis la mise sur le marché d'antiviraux à action directe qui ciblent tous les génotypes du HCV, l'hépatite C chronique peut être guérie chez plus de 90% des patients traités (Baumert, Juhling et al. 2017). Cependant, il est estimé que 71 millions d'individus sont toujours infectés par le HCV dans le monde et la guérison virale n'élimine pas le risque de développer un CHC lorsque la maladie hépatique est avancée (Baumert, Juhling et al. 2017; Hamdane, Juhling et al. 2019). Les traitements curatifs du CHC sont limités à la résection chirurgicale et la transplantation hépatique. De plus, pour prévenir la transmission globale de l'infection par le HCV, il sera primordial de développer un vaccin préventif.

L'infection des hépatocytes humains par le HCV est un processus complexe qui fait intervenir des facteurs viraux et cellulaires. L'identification de facteurs de l'hôte détournés par le HCV contribue à une meilleure compréhension des interactions virus-hôte sous-tendant le cycle de réplication du HCV et le développement d'une infection chronique ainsi qu'à l'identification de cibles potentielles pour le traitement des maladies hépatiques et la prévention du CHC (Zeisel and Baumert 2017; Zeisel, Crouchet et al. 2015). Le cycle de réplication du HCV peut être divisé en étapes précoces (entrée, traduction et réplication virale) et tardives (assemblage et relargage de nouveaux virions). Chacune de ces étapes dépend d'interactions virus-hôte qui mettent en jeu des protéines et microARN (miR) de l'hôte (Zeisel, Crouchet et al. 2015). Ces derniers constituent une classe de petits ARN non-codants qui régulent l'expression de gènes au niveau post-transcriptionnel. Ainsi ils contrôlent l'expression d'environ 60% de gènes codant des protéines et sont impliqués dans tous les processus biologiques. Les miR ciblent spécifiquement des ARN messagers (ARNm) par appariement de bases avec un site complémentaire qui est habituellement localisé dans la région 3' non-traduite (3´UTR). Ceci conduit généralement à la dégradation de l'ARNm ou à la répression de sa traduction en protéine. De récentes études indiquent que les miR contribuent à la réplication du HCV en exerçant des effets pro- ou antiviraux. La découverte du ciblage direct du HCV par miR-122, un miR fortement exprimé dans le foie, a permis de mettre en lumière son rôle essentiel dans la traduction et la réplication du HCV en stabilisant l'ARN HCV (Jopling, Yi et al. 2005). miR-122 est un facteur proviral qui joue un rôle

dans l'infection chronique par le HCV, la progression de la maladie hépatique et le CHC (Jopling, Yi et al. 2005). D'autres miR ont un effet indirect sur le HCV en modulant l'expression de protéines de l'hôte qui régulent les réponses antivirales et la surveillance immunitaire (Li, Lowey et al. 2017; Bandiera, Pfeffer et al. 2015; Li, Jiang et al. 2016). Par leur capacité à réguler l'expression génique, les miR représentent des outils pour des études de perte de fonction visant à découvrir de nouveaux facteurs de l'hôte impliqués dans le cycle de réplication du HCV.

Afin d'identifier de manière systématique les miR impliqués dans le cycle de réplication du HCV, le laboratoire a précédemment conduit un criblage dans les cellules hépatocytaires Huh7.5.1 en utilisant une librairie génomique de miR et un virus portant un gène rapporteur luciférase (JcR2a). Ce criblage a permis d'identifier 495 miR qui modulent significativement l'infection par le HCV. Parmi ces miR, 186 miR sont impliqués dans les étapes précoces du cycle viral et 309 miR jouent un rôle dans l'assemblage et le relargage, deux étapes encore peu caractérisées du cycle viral. Le laboratoire a ainsi décidé d'étudier plus particulièrement les miR qui modulent les étapes tardives du cycle de réplication du HCV. Parmi ces miR, miR-501-3p et miR-619-3p augmentent l'infection par le HCV, ce qui suggère qu'ils régulent des facteurs de l'hôte qui jouent un rôle dans la morphogenèse ou la sécrétion de virions. Par une approche combinant outils bioinformatiques et études fonctionnelles, le laboratoire a identifié l'OGT (*O-linked N-acetylglucosamine transferase* ou UDP-N-acétylglucosamine-peptide N-acétylglucosaminyltransférase ou O-GlcNAc transférase) comme cible de miR-501-3p et miR-619-3p impliquée dans l'assemblage et l'infectivité du HCV (Herzog, Bandiera et al. 2019). L'OGT est une enzyme qui catalyse l'addition de N-acétylglucosamine (O-GlcNAc) à des résidus sérine et thréonine de protéines nucléaires, cytoplasmiques et mitochondriales (O-GlcNAcylation) (Levine and Walker 2016). En plus de sa fonction enzymatique, l'OGT contribue à la stabilisation de protéines dans des complexes multiprotéines (fonction d'échaffaudage ou *scaffold*). Cette modification post-traductionnelle est hydrolisée par l'OGA (N-acétyl-b-D glucosaminidase ou O-GlcNAcase). L'OGT et l'OGA sont les deux seules enzymes responsables de la modulation de l'O-GlcNAcylation des protéines (Levine and Walker 2016).

6.2 Objectifs

Le but de ma thèse a été d'étudier le rôle de l'OGT dans les interactions HCV-hôte. Les objectifs spécifiques étaient i) de caractériser le ciblage prédit de l'OGT par miR-501-3p et miR-619-3p, ii) de déterminer comment l'OGT pouvait contribuer à la morphogenèse du HCV et iii) d'étudier le rôle de miR-501-3p et de l'OGT dans la maladie hépatique induite par HCV et le CHC.

6.3 Résultats

6.3.1 miR-501-3p régule l'expression de l'OGT au niveau post-transcriptionnel

Pour caractériser la régulation de l'OGT par miR-501-3p et miR-619-3p, l'expression de l'OGT a été analysée au niveau de l'ARNm et au niveau protéique dans les cellules Huh7.5.1 après surexpression de miR-501-3p et miR-619-3p. Tandis qu'aucun miR n'avait d'impact sur les niveaux d'ARNm de l'OGT, la surexpression de miR-501-3p a significativement diminué l'expression de la protéine OGT. miR-619-3p a également diminué l'expression protéique de l'OGT mais de manière moins robuste que miR-501-3p. Ainsi nous avons concentré nos travaux sur miR-501-3p.

Pour déterminer si l'OGT est une cible de miR-501-3p, un fragment de la région 3′UTR de l'ARNm de l'OGT contenant un site potentiel de fixation pour miR-501-3p a été sous-cloné dans une cassette d'expression de luciférase *Renilla* (RLuc) d'un vecteur double rapporteur exprimant également la luciférase *Firefly*. La co-transfection de miR-501-3p avec le vecteur rapporteur contenant la séquence 3'UTR sauvage (RLuc wt *OGT* 3'UTR) a significativement diminué l'activité luciférase comparé à la co-transfection avec le vecteur vide. Par contre, cette répression de l'activité luciférase n'a pas été observée lorsqu'un vecteur contenant un site de fixation pour miR-501-3p muté a été co-transfecté (RLuc mt *OGT* 3'UTR). L'ensemble de ces résultats indique que miR-501-3p régule l'expression post-transcriptionnelle de l'OGT en diminuant son expression protéique.

6.3.2 La O-GlcNAcylation module l'infectivité des HCVcc

Pour déterminer l'effet de l'OGT sur l'assemblage et le relargage du HCV, nous avons utilisé de petits ARN interférants (siARN) pour diminuer l'expression de l'OGT dans les cellules Huh7.5.1. Les siARN sont des molécules ARN double brin d'environ 20-25 paires de bases qui, comme les miR, régulent l'expression de gènes en dégradant spécifiquement certains ARNm pour empêcher leur traduction. Nous avons ensuite déterminé les titres infectieux HCV (TCID50) et les taux d'ARN HCV pour calculer l'infectivité spécifique des particules HCVcc produites dans les cellules Huh7.5.1 dans lesquelles l'expression de l'OGT a été diminuée. De manière intéressante, la diminution de l'expression de l'OGT a conduit à une diminution significative des TCID50 et l'infectivité spécifique des HCVcc. Cet effet de l'OGT sur l'infectivité des HCVcc n'est pas dépendent du génotype HCV étant donné qu'une augmentation de l'infectivité des HCVcc comportant les glycoprotéines d'enveloppe des génotypes 1a, 1b et 2a a été observée dans les conditions où l'expression de l'OGT a été diminuée. Afin de déterminer si l'activité enzymatique de l'OGT module l'infectivité des HCVcc, nous avons incubé des cellules Huh7.5.1 infectées par des HCVcc avec deux inhibiteurs pharmacologiques ciblant l'OGT ($Ac_4$5S-GlcNAc) ou l'OGA (Thiamet G). Le traitement par l'$Ac_4$5S-

GlcNAc a augmenté significativement l'infectivité des HCVcc de manière dose-dépendante, tandis que l'effet opposé a été observé après le traitement par le Thiamet G. L'ensemble de ces résultats montre que la O-GlcNAcylation module l'infectivité des HCVcc.

6.3.3 La diminution de l'expression de l'OGT module les propriétés biophysiques et la taille des HCVcc

Au cours de la morphogenèse virale, les glycoprotéines d'enveloppe du HCV interagissent étroitement avec les apolipoprotéines B et E (ApoB et ApoE) de l'hôte, ce qui contribue à masquer les épitopes viraux à la surface des lipo-viro-particules (LVPs) (Bartenschlager, Penin et al. 2011). Afin de déterminer l'effet de l'OGT sur la morphogenèse virale, nous avons analysé les propriétés structurales et biophysiques des HCVcc produits dans des cellules Huh7.5.1 transfectées avec des siOGT ou des siARN contrôle après ultracentrifugation en gradients d'iodixanol. La diminution de l'expression de l'OGT a conduit à une production d'HCVcc plus infectieux, d'une densité plus élevée et avec des concentrations supérieures en ApoE. Ces résultats indiquent que l'OGT a un impact sur les propriétés biophysiques des HCVcc. A l'inverse, aucun changement dans les concentrations en ApoB n'a été observé, en accord avec le modèle selon lequel les HCV LVPs contiendraient plusieurs molécules échangeables d'ApoE mais une seule molécule d'ApoB. Nous avons également purifié les HCVcc pour les visualiser par microscopie électronique après immunocapture par un anticorps dirigé contre la protéine d'enveloppe E2 du HCV (Piver, Boyer et al. 2017) pour déterminer si la diminution de l'expression de l'OGT avait un impact sur la taille des HCVcc. En comparaison avec les particules virales produites dans les conditions contrôles, les particules virales produites dans des cellules Huh7.5.1 qui avaient été préalablement transfectées avec les siOGT présentaient des tailles plus grandes. Il est intéressant de noter que des particules plus grandes ont été observées dans plusieurs fractions du gradient d'iodixanol dans lesquels les HCVcc présentaient une infectivité et une concentration en ApoE plus élevées. Ces résultats suggèrent que la diminution de l'expression de l'OGT a un impact sur la lipidation des HCVcc.

6.3.4 L'expression de l'OGT augmente dans les maladies hépatiques

Etant donné que la diminution de l'expression de l'OGT conduit à la production d'HCVcc plus infectieuses, nous nous sommes interrogés si l'infection par le HCV pouvait avoir un effet sur l'expression de l'OGT afin de promouvoir le cycle viral. Dans les cellules Huh7.5.1, l'infection par le HCV a conduit à une diminution de l'expression de l'ARNm et des protéines OGT qui pourraient ainsi augmenter l'infection par le HCV, en accord avec le rôle antiviral de la O-GlcNAcylation que nous avions observé. Cependant, dans les tissus hépatiques de patients atteints d'hépatite C chronique, les taux d'ARN HCV n'étaient pas corrélés avec l'expression de l'OGT, suggérant que chez les patients il n'y a pas d'effet direct du HCV sur l'expression de l'OGT. Ensuite, étant donné que la O-

GlcNAcylation a été associée à de nombreux cancers, nous avons également analysé l'expression de l'OGT dans des tissus hépatiques de patients atteints de maladies hépatiques chroniques et de CHC. Alors que nous n'avons observé qu'une tendance à l'augmentation de l'expression de l'OGT dans des tissus hépatiques de patients atteints d'hépatite C et présentant une fibrose et une inflammation hépatique, les taux d'expression de l'OGT étaient significativement augmentés dans les tissus tumoraux de patients chroniquement infectés par le HCV ou par le virus de l'hépatite B ainsi que de patients atteints de maladie hépatique alcoolique ou non-alcoolique en comparaison avec des tissus hépatiques non tumoraux. Ces résultats suggèrent que l'expression de l'OGT augmente dans le CHC de manière indépendante de l'étiologie du cancer. L'ensemble de ces résultats suggère que l'augmentation de l'expression de l'OGT est liée à l'inflammation et à la fibrose hépatique au cours de la maladie viro-induite plutôt qu'à un effet direct du virus..

6.4 Conclusions et perspectives

Au cours de ma thèse, nous avons découvert un rôle pour l'OGT dans l'infection par le HCV. Nous avons montré que i) miR-501-3p régule l'expression de l'OGT au niveau protéique; ii) la diminution de l'expression de l'OGT par des siARN ou l'inhibition de son activité enzymatique augmente l'infectivité des particules virales; iii) la diminution de l'expression de l'OGT conduit à la production de particules virales présentant des tailles plus grandes suggérant que la O-GlcNAcylation a un impact sur la morphogenèse et l'infectivité du HCV; et iv) l'expression de l'OGT augmente au cours des maladies hépatiques chroniques et du CHC.

En conclusion, en identifiant l'OGT comme nouveau facteur de l'hôte régulant la morphogenèse du HCV, notre étude permet de mieux comprendre les interactions HCV-hôte modulant l'infection virale. Etant donné qu'il a été précédemment montré que l'OGT jouait un rôle dans les modifications métaboliques au cours du cancer (Jopling, Yi et al. 2005), nos résultats suggèrent que l'OGT pourrait contribuer au développement de la maladie hépatique induite par le HCV et du CHC.

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VIII. ANNEX

Review: Plissonnier ML, **Herzog K**, Levrero M, Zeisel MB. Non-coding RNA and HCV-induced HCC. *Viruses 2018, 10 (11): 591*

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Review

Non-Coding RNAs and Hepatitis C Virus-Induced Hepatocellular Carcinoma

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Abstract: Hepatitis C virus (HCV) infection is a worldwide health problem and is one of the main causes of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). Despite recent improvements, effective treatments for HCC are still missing and new tools for early detection are needed. Non-coding RNAs (ncRNAs) have emerged as important regulators of gene expression and key players in human carcinogenesis, including HCC. Aberrant expression of ncRNAs is associated with HCC metastasis, invasion, dissemination, and recurrence. This review will focus on the recent advances in ncRNA expression profiles, their dysregulation in HCV-related HCC, and the clinical perspective of ncRNA signatures for the early detection of HCC.

Keywords: hepatocellular carcinoma; hepatitis C virus; microRNA; long non-coding RNA

1. Introduction

Despite an overall drop in the incidence of hepatitis C virus (HCV) infection within the past years due to direct-acting antivirals (DAAs), which enable chronic hepatitis C (CHC) to be cured, an estimated 71 million individuals are still chronically infected by HCV worldwide [1]. HCV infection is a major risk factor of HCC and associated with 20% of cases of liver cirrhosis, which is functional decompensation leading to hepatocellular carcinoma (HCC) [2]. Other major HCC etiologies include chronic hepatitis B virus (HBV) infection, alcohol abuse, and metabolic causes. In HCV-infected patients, HCC development is a consequence of fibrosis progression and occurs after the establishment of cirrhosis [3]. According to the GLOBOCAN series of the International Agency for Research on Cancer in 2012 [4], HCC is the fifth most common cancer and the second cause of cancer death worldwide.

HCC is a poor prognosis disease. Although the risk of developing HCC can be reduced in patients by treatment of the underlying cause, e.g., by viral clearance or abstinence from alcohol, strategies to prevent cancer development in patients with advanced fibrosis and/or established cirrhosis are still lacking. Early diagnosis increases the chance of effective therapy, but the detection of small liver tumours by ultrasound is challenging and currently there is no reliable serum biomarker that can be used in surveillance programmes. Patients with advanced HCC carry a very poor prognosis, with an expected survival of four to six months and despite recent improvements, treatment options for HCC remain scarce. According to the clinical practice guidelines from the European Association for the Study of the liver (EASL), patients eligible for curative treatment can undergo surgical resection, radiofrequency ablation, or liver transplantation [5]. Tumour recurrence remains the major cause of

death in HCC patients following loco-regional ablation and liver resection. Intermediate (stage B according to the Barcelona-Clínic Liver Cancer (BCLC) classification [5]) HCCs have been shown to benefit from trans-arterial chemoembolization (TACE) [5]. The first-line treatments for patients with advanced HCC (BCLC stage C) [5], which are not eligible for curative treatment, are the multikinase inhibitors sorafenib and lenvatinib, which increase survival by approximately three months [6]. Phase III clinical trials with regorafenib or cabozantinib as second-line treatment for HCC patients undergoing tumour progression after sorafenib (RESORCE, CELESTIAL) showed an extension of overall survival by 10 months [7,8]. Novel treatment options and prognostic tools are required to improve the management of patients with HCC.

Among the risk factors of HCC, HCV is a positive-strand RNA virus [9,10] that does not integrate into the host genome. It encodes a large polyprotein of about 3000 amino acids from a single open reading frame which is processed into three structural (core, E1, and E2) and seven non-structural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins [11]. HCV proteins interact with many host-cell factors well beyond their roles in the viral life cycle and are notably involved in cell signalling, transcription, cell proliferation, apoptosis, vesicular trafficking, and translational regulation [12]. HCV proteins contribute to HCC by modulating pathways that promote the malignant transformation of hepatocytes through the accumulation of genetic damages and epigenetic dysregulation [13–15]. For instance, the HCV core protein sensitizes host cells to TRAIL-induced cell apoptosis by activating the CK1α-p53-Bid dependent pathway in human hepatoma cells [16] or is able to suppress the p53-dependent apoptosis induced by the all-*trans* retinoic acid (ATRA), the most biologically active metabolite of vitamin A [17]. Moreover, the HCV core protein binds to several tumour suppressor proteins, including p53, p73, and pRb [18,19]. Despite the development of DAAs enabling an HCV cure and reducing the risk of liver complications [20,21], a long-term risk of HCC remains in cirrhotic patients, even after achieving a sustained virological response (SVR) [22]. Of note, while some reports published within the past two years have raised concerns about a potential higher risk of post-SVR HCC in patients treated with DAAs [23–25] compared to patients treated with the previous standard-of-care interferon and ribavirin, several subsequent reports—including large multicenter studies—have failed to confirm this [21,26–33].

Liver carcinogenesis is a multistep process driven by chronic inflammation, DNA damage, senescence and telomerase reactivation, chromosomal instability, and epigenetic modifications. All etiologic factors seem to act through similar mechanisms (i.e., point mutations, chromosomal aberrations, epigenetic changes) that converge to affect common pathways. Notably, mutations and chromosomal aberrations have been predominantly found in malignant tumour tissues, whereas the dysregulation of signalling pathways and epigenetic changes are also detected earlier in the natural history of HCC development, at the stage of cirrhosis [34]. Epigenetic changes that include DNA methylation, post-translational histone modifications, and ncRNA-mediated silencing pathways occur early in the development of HCC. Several studies have also identified mutations in a group of chromatin regulators (*ARID1A*, *ARID1B*, *ARID2*, *MLL*, and *MLL3*) in approximately 20% of all tumours, including virus- and alcohol-related HCCs (reviewed in [34]). Modulation of the methylation status of DNA CpG islands and lysines in the histone tails in gene promoters represents important epigenetic alterations in human cancer, including HCC. Next-generation sequencing technology has revealed that many ncRNAs play important roles in biological processes, such as differentiation, proliferation, and cell death [35–37], as well as in cancer [38,39]. Based on their length, ncRNAs are classified into small ncRNAs (sncRNAs, less than 200 nucleotides) and long ncRNAs (lncRNAs, more than 200 nucleotides), according to the RNA purification protocol that excludes small RNAs described by Kapranov and colleagues [40]. Aberrant expression of ncRNAs is associated with HCC metastasis, invasion, dissemination, and recurrence [41–45], suggesting that ncRNAs are key players of human carcinogenesis, including HCC. This review will focus on sncRNAs and lncRNAs in HCV-induced HCC.

2. Expression and Functions of ncRNAs in HCV-Related HCC

2.1. LncRNAs in HCV-Related HCC

LncRNAs are a heterogeneous group of non-coding transcripts more than 200 nucleotides long that are transcribed by RNA polymerase II, 5′ capped, spliced, and polyadenylated [46,47]. The FANTOM project [48] and the GENCODE consortium in the framework of the ENCODE project [49] led to the identification of over 20,000 lncRNAs. In 2015, an analysis of 7256 RNA-seq libraries from tumours, normal tissues, and cell lines reported the identification of 58,648 lncRNA genes [46].

LncRNAs are involved in many biological processes, such as proliferation, differentiation and development [47,50]. The dysregulation of lncRNAs significantly contributes to numerous human diseases, especially cancers [51,52], including HCC [47,53]. The structural complexity of lncRNAs offers multiple possibilities for interactions with DNA, RNA, and/or proteins that depend on secondary and tertiary structures. Several lncRNAs are localized to specific cellular compartments, nuclear or cytosolic, in accordance with their biological function. Nuclear lncRNAs can act as guides for chromatin-modifying-complexes or transcription factors. The majority of lncRNAs are in the cytoplasm and often function as regulators of protein levels, either by directly controlling mRNA stability or by acting as competing endogenous RNA [54]. Depending on their genomic location and context, lncRNAs are classified into five categories [50,55]: (1) intergenic lnRNAs, the so-called lincRNAs are transcribed in the intergenic region between two protein-coding genes; (2) sense/intronic lncRNAs, which arise from intronic regions within protein-coding genes; (3) antisense lncRNAs (NATs), when overlapping one or more exons of another transcript on the opposite strand; (4) bidirectional lncRNAs, such as promoter upstream transcripts (PROMPTSs), which are transcribed in the promoter regions of protein-coding genes in a bidirectional manner; and (5) enhancer lncRNAs which are transcribed in the enhancer regions of the genome. Two other ncRNAs can be considered as lncRNAs: circular RNAs (circRNAs), which are created from protein-coding mRNAs or linear ncRNAs that join an upstream 3′ splice site and downstream 5′ splice site to form a covalently closed continuous loop [56]; and pseudogenes, which originate from gene duplication and have acquired various mutations, inducing a loss of their protein-coding capacity [57,58]. Most of these pseudogenes serve as competitive endogenous RNAs (ceRNA) to sequester miRNAs. The *PTEN* pseudogene, *PTENP1*, was the first pseudogene shown to regulate the expression of its parental gene by binding and sequestering *PTEN*-targeting miR-17, miR-19, miR-20a, and miR-21 in prostate cancer [57].

LncRNAs regulate gene expression through different mechanisms, such as epigenetic silencing, splicing regulation, lncRNA-miRNA interaction by sequestering miRNAs, lncRNA-protein interaction, and genetic variation [53,59,60]. The molecular functions of lncRNAs are not well-characterized, but four categories of mode of action have been proposed [39,53]. LncRNAs can function as decoys by binding and titrating away proteins or RNA targets such as miRNAs. This family of lncRNAs can negatively regulate the expression of their target or bind to the transcription binding sites and avoid the fixation of the transcription factor. LncRNAs can also have a guide function: these lncRNAs bind proteins and direct their localization to a specific target. They act, for example, as epigenetic repressors, i.e., HOX transcript antisense intergenic RNA (HOTAIR), or epigenetic activators, such as HOTTIP or H19. Moreover, signal lncRNAs are expressed in a cell-type specific and stage-specific manner and can regulate transcriptional activity or biological pathways by interacting with transcription factors or chromatin-modifying enzymes. Finally, lncRNAs can have a scaffold function. This class of lncRNAs serves as a central platform to bind different molecular components and facilitates their intermolecular interactions.

Many studies have described the role of lncRNAs in HCC (for review see [47,53,61–63]). HCC-related lncRNAs participate in diverse biological processes involved in HCC progression, such as cell proliferation, apoptosis, invasion, metastasis, and angiogenesis. In the last decade, hundreds of dysregulated lncRNAs have been characterized in HCC tissues compared with normal tissues [64]. For example, the lncRNA HOTAIR is highly expressed in HCC and is associated with

poor prognosis [65] and an increased risk of recurrence and metastasis [66,67]. Recently, lncRNA HULC polymorphisms have been associated with HCC risk and prognosis [68]. Furthermore, Guo and colleagues demonstrated that the lncRNA PVT1 is upregulated in HCC, promoting HCC cell propagation and inhibiting apoptotic cells by recruiting EZH2 [69].

LncRNAs can be differentially expressed depending on the HCC etiology. In 2015, Zhang et al. explored lncRNA expression profiles of 73 tissue samples at different stages of HCV-induced HCC: cirrhotic tissue, dysplastic nodules, and HCC samples compared to healthy liver tissue [70]. The expression of seven lncRNAs (LINC01419, BC014579, AK021443, RP11-401P9.4, RP11-304 L19.5, AF070632, CTB-167B5.2) in preneoplastic lesions and HCC was significantly different. Among these lncRNAs, the lncRNA LINC01419 transcripts were expressed at higher levels in early stage HCC compared to dysplasia and early stage HCC, and were overexpressed in HCV-related HCC when compared with matched non-tumour liver tissues. LncRNA AK021443 levels increase in advanced stage HCC, while lncRNA AF070632 levels decrease in advanced stage HCC. Moreover, computational analysis suggested that LINC01419 and AK021443 regulate cell cycle genes, whereas AF070632 is associated with cofactor binding, oxidation-reduction, and the carboxylic acid catabolic process. LINC01419 and AK021443 could thus promote HCV-related HCC development by modulating the cell cycle progression. In another study conducted by Zhang et al. in 2016 [71], lncRNA hypoxia-inducing factor a (aHIF), Prader Willi/Angelman region RNA 5 (PAR5), and human downregulated expression by HBx (hDREH) were associated with HCV-related HCC since their expressions were significantly downregulated (aHIF and PAR5) or upregulated (hDREH) in tumour vs. non-tumour tissues, but these observations have to be confirmed in a larger patient cohort. Two additional lncRNAs, urothelial carcinoma associated-1 (UCA1) and WD repeat containing antisense to TP53 (WRAP53), have been found to be upregulated in HCC patients with chronic HCV infection [72]. UCA1 upregulation has been shown to increase epithelial-to-mesenchymal transition in HCC via sponging miR-203, thereby activating the expression of transcription factor Snail2 [73] (Table 1).

Table 1. Role of lncRNAs in HCV-induced HCC. The expression of lncRNAs that have been associated with HCV-induced HCC, as well as their biological function, are shown. lncRNAs that have been reported to be uniquely modulated in HCC induced by HCV, but not in HCC induced by another etiological factor, are highlighted in bold.

lncRNA	Expression in HCV-Induced HCC	Molecular Mechanism for HCV-Induced HCC	References
HOTAIR	↑	Epigenetic repression	[66,67]
HULC	↑	Polymorphism	[68]
PVT1	↑	Cell cycle progression	[69]
LINC01419	↑	**Regulation of cell cycle genes**	**[70]**
BC014579	↑	**unknown**	**[70]**
AK021443	↑	**Regulation of cell cycle genes**	**[70]**
RP11-401P9.4	↑	**unknown**	**[70]**
RP11-304 L19.5	↑	**unknown**	**[70]**
AF070632	↓	**Cofactor binding and catabolic processes**	**[70]**
CTB-167B5.2	↓	**unknown**	**[70]**
aHIF	↓	**unknown**	**[71]**
PAR5	↓	**unknown**	**[71]**
LINC01152	↓	unknown	[71]
TMEVPG1	↓	unknown	[71]
BC017743	↑	unknown	[71]
BC043430	↑	unknown	[71]
PCNA-AS1	↑	unknown	[71]
UFC1	↑	unknown	[71]
ZEB1-AS1	↑	unknown	[71]
hDREH	↑	unknown	[71]
UCA1	↑	Control of gene expression (target: miR-203)	[72]
WRAP53	↑	unknown	[72]
MALAT1	↑	Regulation of splicing processes	[73]
HEIH	↑	Cell proliferation	[74]

2.2. SncRNA in HCV-Related HCC

High-throughput sequencing technologies have enabled researchers to uncover different types of sncRNA: small nucleolar RNAs (snoRNAs), piwi-interacting RNAs (piRNAs), and miRNAs. Within the last decade, the role of sncRNAs—and particularly the one of miRNAs—in physiological and pathological processes, including HCC, has been extensively studied [38].

2.2.1. Small Nucleolar RNAs (snoRNAs)

SnoRNAs are a class of intermediate-sized ncRNAs of 60 to 300 nucleotides discovered in the nucleolus and able to regulate ribosome maturation and function [75], as well as alternative splicing and gene silencing [76,77]. SnoRNAs are divided into two major highly conserved families based on their structure and main function: box C/D snoRNAs and box H/ACA snoRNAs. Box C/D snoRNAs are responsible for 2′-*O*-methylation of ribosomal RNAs [78], while the second family of snoRNAs guides the pseudouridylation of nucleotides [79]. A third less represented class includes the small Cajal body-specific RNAs (scaRNAs) that are associated with Cajal bodies, which are small membrane-less sub-compartments of the nucleus [80]. SnoRNAs are located in introns. They are components of small nucleolar ribonucleoprotein (snoRNPs) complexes along with specific proteins and function as a guide for the post-transcriptional modification of ribosomal RNAs by facilitating rRNA folding and stability [81,82]. The sequences of snoRNAs are responsible for targeting the assembled snoRNPs to a specific target.

Alterations in snoRNA expression in human cells can affect numerous biological processes, leading to tumorigenesis. Six snoRNAs are well-described to be dysregulated in HCC [83], regardless of the etiological factor. For example, SNORD113-1 is significantly downregulated in HCC-tumour tissues compared with non-tumour tissues; furthermore, a statistically significant association between low-level expression of SNORD113-1 and relapse-free survival was observed, which suggests that downregulation of SNORD113-1 is associated with HCC aggressiveness [84]. Furthermore, a study performed by Fang et al. [85] demonstrated that SNORD126—located within the intron of the *cyclin B1-interacting protein 1 (CCNB1IP1)* gene—was upregulated to a high level in HCC compared with non-tumour tissues. Overexpression of SNORD126 was associated with a shorter survival rate in HCC patients and promoted HCC growth through upregulation of the PI3K-AKT pathway.

2.2.2. Piwi-Interacting RNAs (piRNAs)

PiRNAs are ncRNAs of 24–30 nucleotides in length that bind to the piwi subfamily of argonaute proteins to form a piRNA-induced silencing complex (piRISC), which inhibits transposon mobilization by both epigenetic and post-transcriptional silencing [86,87]. They are transcribed from regions in the genome that contain transcribed transposable elements and other repetitive elements. Three major PIWI-class proteins (PIWIL1, PIWIL2, and PIWIL4) are involved in a so-called 'ping-pong' amplification cycle, creating antisense piRNAs that are capable of repressing the transcript of origin [87].

PiRNAs are abundant in the human liver, but no data is available on specific piRNA expression profiles in HCV-related HCC. However, it has been shown by small RNA-seq that an expression pattern of 125 piRNAs clearly differentiates cirrhotic liver from HCC tissues. Interestingly, 24 piRNAs dysregulated in advanced HCC also showed distinctive expression patterns in earlier hepatic lesions, suggesting that these ncRNAs may participate in the carcinogenic process in the liver and could represent new markers of early hepatocarcinogenic lesions [87,88]. The accumulation of piR-LLi-30552 and has-piR-020498 is associated with progression from the dysplasia stage to HCC [88]. Furthermore, Law et al. showed that piR-Hep1 is upregulated in 46.6% of HCC tumours compared to the corresponding adjacent non-tumour liver. piR-Hep1 could play a functional role in hepatocarcinogenesis as it has been shown to promote cell viability, motility, and invasiveness, with a concomitant increase in the level of active AKT phosphorylation [89].

2.2.3. MicroRNAs (miRNAs)

MiRNAs are an important class of ncRNAs of 18–25 nucleotides that regulate gene expression at the post-transcriptional level. MiRNAs bind to the 3′ untranslated region (3′UTR) of complementary sequences of mRNAs to mediate mRNA deadenylation or translation blockage [90]. MiRNAs are estimated to regulate the translation of more than 60% of protein-coding genes: a single miRNA can target hundreds of mRNAs, thereby affecting a broad network of genes [91].

Biogenesis of miRNAs takes place through a multistep process [92]. miRNAs are most commonly transcribed in the nucleus by the RNA polymerase II (Pol II). Monocistronic or polycistronic primary miRNA transcripts (pri-miRNAs) are processed into precursor miRNAs (pre-miRNAs) by the DGCR8-Drosha complex and exported to the cytoplasm by exportin 5. These pre-miRNAs undergo cleavage by the endoribonuclease called Dicer that produces a miRNA duplex. These molecules are loaded by the Dicer–TARBP2 (TAR RNA-binding protein 2; also known as TRBP) complex into a member of the argonaute protein subfamily to form the RNA-induced silencing complex (RISC), of which argonaute proteins are the catalytic endonuclease components. RISC directs the regulation of mRNAs by recognizing a complementary sequence in the targeted mRNAs. Translation of mRNAs into proteins is repressed by miRNAs by two main means: mRNA degradation and the inhibition of translation initiation [93].

Several studies have investigated the role of miRNAs in various biological processes [38,39,94–96], including proliferation, differentiation, angiogenesis, apoptosis, and development. Abnormal expression levels of miRNAs have been described in inflammation, Alzheimer's disease, cardiovascular disease, cancer, and viral infection, including HCV infection [38,39,42,54,97]. Of note, several studies revealed an association between the dysregulation of miRNAs and HCC carcinogenesis, including HCV-related HCC [42,98,99]. For example, miR-221 that modulates different pathways involved in the proliferation of tumour cells, survival, and metastasis [100,101] is frequently upregulated in HCC with advanced tumour stages and associated with poor prognosis, irrespective of the HCC etiology. In contrast, other miRNAs have been shown to be specifically deregulated in HCV-induced HCC. Using a microarray analysis of liver tissue samples, Diaz et al. identified 18 miRNAs specifically expressed in HCV-related HCC, including 15 miRNAs that had already been reported in previous studies and three miRNAs (miR-497, miR-1269, and miR-424-3p) which had not been previously described to be modulated in HCV-related HCC (Table 2) [42].

Table 2. miRNA-specific signature of HCV-induced HCC. The expression of miRNAs that have been associated with HCV-induced HCC [42], as well as their biological function, are shown.

miRNA	Expression in HCV-Induced HCC	Molecular Mechanism for HCV-Induced HCC
mir-1269	↑	Increase of proliferation
mir-224	↑	Increase of proliferation
mir-452	↑	Increase of proliferation, migration and invasion
mir-224-3p	↑	unknown
mir-224-5p	↑	unknown
mir-221	↑	Increase of proliferation and invasion
mir-497	↓	Inhibition of proliferation, induction of apoptosis
mir-214	↓	Inhibition of proliferation, migration and invasion
mir-195	↓	Inhibition of proliferation and EMT
mir-130a	↓	Inhibition of proliferation, migration and invasion
mir-125a-5p	↓	Inhibition of proliferation
mir-125b-5p	↓	Inhibition of proliferation
mir-424-3p	↓	unknown
mir-139-3p	↓	Inhibition of proliferation and metastasis
mir-139-5p	↓	Inhibition of EMT, migration and invasion
mir-199b-3p	↓	unknown
mir-199a-3p	↓	Inhibition of proliferation, migration, invasion and angiogenesis
mir-199a-5p	↓	Inhibition of proliferation, migration and invasion

Furthermore, it has been shown that the expression of oncogenic miR-155 is increased in patients infected with HCV and this promotes hepatocyte proliferation and tumorigenesis by activating the Wnt signalling pathway [102]. Likewise, miR-135a-5p was shown to be upregulated in HCV-infected patients and to promote the HCV-induced STAT3 transcriptional program in the liver of patients by suppressing its regulator protein tyrosine phosphatase receptor delta (PTPRD), resulting in the malignant progression of liver disease [103]. These studies underscore the functional role that miRNAs may play in the pathogenesis of HCV-induced HCC.

The study of the role of miRNAs in HCV-induced HCC is particularly interesting since there is a tight interplay between HCV, miRNAs, and hepatocyte metabolic pathways that contributes to liver disease development. One of the hallmarks of HCV replication is its dependency on miR-122, the most abundant miRNA in the liver. miR-122 plays a major role in liver physiology by regulating metabolic pathways and as a tumour suppressor (for review see [104]). It has been shown that HCV sequesters miR-122 from its endogenous mRNA targets, thereby leading to their derepression and liver carcinogenesis [105]. Several other miRNAs that have a dual role in both the HCV replication cycle and in liver disease development have been reported, underscoring the tight interplay between HCV and miRNAs in the liver (Figure 1).

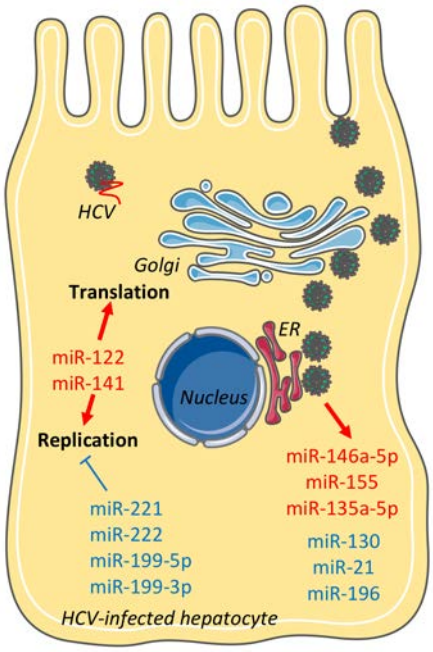

Figure 1. Impact of cellular miRNAs on the HCV life cycle and their contribution to HCV-induced HCC. Impact of individual miRNAs whose expression is modulated upon HCV infection on steps of the HCV life cycle (entry, translation, replication, assembly, and LVP release) are shown. miRNAs in blue display a proviral effect; miRNAs in red have an antiviral effect. HCV: hepatitis C virus. LVP: lipoviral particle. ER: endoplasmic reticulum. Golgi: Golgi apparatus. Images were adapted from SMART (Servier Medical Art).

A recent study that comprehensively analysed miRNAs modulated upon HCV infection showed that several of these miRNAs were known to regulate inflammation, fibrosis, and cancer development [106]. Among the miRNAs whose expression increased during HCV infection was miR-146a-5p, which has been associated with a modulation of the proteasome pathway, fatty acid, and anaerobic energetic metabolism. These regulatory pathways are both in favour of HCV infection (Figure 1) and liver disease development by promoting liver inflammation and HCC [106]. Furthermore, it has been shown that HCV infection modulates miR-196, miR-130, and miR-21 that are able to regulate type I IFN signalling pathways to overcome their antiviral activity [45] (Figure 1). These data are in line with miRNA expression profiles (differential expression of 19 miRNAs, including

miR-124b, miR-34c, and miR-23a) associated with HCV infection [41]. Analysis of targeted genes using infection-associated miRNAs revealed that the pathways related to the immune response, antigen presentation, the cell cycle, proteasome, and lipid metabolism, are activated in the HCV-infected liver, suggesting their implication in HCV-induced liver disease pathogenesis. Interestingly, some of these miRNAs also contribute to the modulation of HCV infection. For example, miR-141 that is upregulated in HCV-infected cells has been shown to downregulate the DLC-1 (Rho GTPase) tumour suppressor and to be required for HCV replication [44] (Figure 1). Finally, miR-199-5p/miR-199-3p, miR-221, and miR-222, whose expression is correlated with fibrosis in HCV-infected patients, have been shown to reduce HCV RNA replication by binding the stem loop II region of HCV 5′ UTR [107]. Of note, while DAA therapy has been reported to modulate hepatic miRNA expression, no significant changes in the expression of miRNAs that have been previously associated with a pro- or antiviral effect on HCV were shown, except an increase in miR-122 expression [108].

3. Conclusions and Perspectives

3.1. ncRNAs as Novel Biomarkers for Detection of HCV-Induced HCC

By contributing to the regulation of the HCV life cycle, liver disease development, and carcinogenesis, ncRNAs play a major role in CHC. Most CHC patients are asymptomatic for many years, and HCC usually develops after several decades of HCV infection [5]. The long latency period between initial HCV infection and HCC development provides an important time window of opportunity for individuals to be monitored for disease progression and intervention. Since early diagnosis increases the chance of effective therapy, patients with cirrhosis are enrolled in periodic ultrasound-based surveillance programmes [109]. However, given the challenge of detecting small liver tumours using ultrasound and the absence of a robust HCC serum marker, reliable non-invasive biomarker(s) would be most helpful for determining HCC risk and/or detecting HCC at early stages.

Molecular signatures using ncRNA expression profiles have been described as potential predictive or prognostic biomarkers of HCC and circulating ncRNAs hold promise as biomarkers for the (early) detection of HCC. In association with alpha-fetoprotein (AFP, the currently most widely used diagnostic HCC serum marker), UCA1 and WRAP53 have been suggested as diagnostic and prognostic markers of HCV-induced HCC [72] (Figure 2). Indeed, a high expression of serum UCA1 was significantly associated with a high tumour grade, large tumour size, positive vascular invasion, and advanced TNM stage in HCC patients [110]. Furthermore, two other lncRNAs might hold promise as potential biomarkers for HCV-related HCC: MALAT1 and HEIH (Figure 2). Indeed, it has been reported that MALAT1 in combination with AFP serum levels might indicate a worse liver failure score in HCV-related HCC patients [111] and lncRNA HEIH expression in serum and exosomes appeared to be increased in HCV-related HCC patients in contrast to patients with CHC or HCV-induced cirrhosis [74]. Further studies using different patient cohorts are needed to assess the potential of lncRNAs as HCC biomarkers. Several panels of miRNAs have also been suggested as potential HCC biomarkers in HCV-infected patients. For example, Zekri et al. identified an miRNA panel composed of miR-122, miR-885-5p, and miR-29b in association with AFP as a new biomarker for the early detection of HCC in a normal population, while another miRNA panel composed of miR-122, miR-885-5p, miR-221, and miR-22 in association with AFP provided a high diagnostic accuracy for the early detection of HCC in cirrhotic patients [112]. Furthermore, the potential of circulating miR-15b and miR-122, as well as miR-182 and miR-150, have been suggested as biomarkers to assess HCC risk in cirrhotic HCV-infected patients [113] and for the detection of cirrhosis progression and HCC in a cohort of HCV-infected Egyptian patients [114], respectively. Moreover, Okajima et al. [115] showed that circulating miR-224 could be a novel biomarker for the detection of primary and recurrent HCC. Finally, a panel of nine liver-associated miRNAs (miR-21, miR-30c, miR-93, miR-122, miR-125b, miR-126, miR-130a, miR-193b, and miR-222) could discriminate healthy individuals from patients with HCV-related HCC [116]

(Figure 2). Further patient cohort studies are required to assess the utility of these miRNAs for CHC patient surveillance programmes.

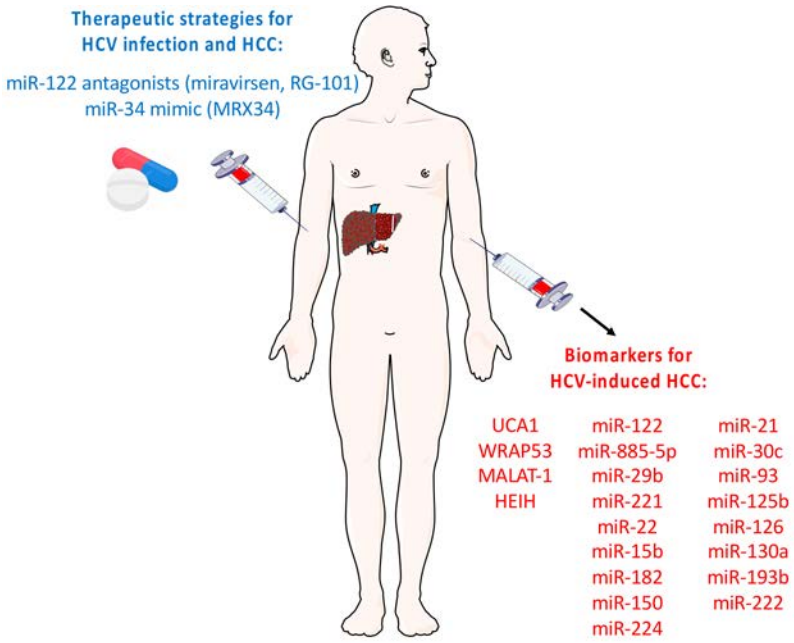

Figure 2. Schematic representation of the potential use of ncRNAs as therapeutic targets for HCV infection and HCC or biomarkers for HCV-induced HCC. MiR-122 antagonists miravirsen and RG-101 have been shown to lead to a dose-dependent reduction of viral RNA in CHC patients [108,109]. miR-34 mimic (MRX34, Mirna Therapeutics) has been administred to patients with primary liver cancer, but this trial was abrogated due to immune-related adverse effects. LncRNAs and miRNAs that have been suggested as biomarkers for HCV-induced HCC are also indicated [42,72,74,111]. HCV: hepatitis C virus. HCC: hepatocellular carcinoma. Images were adapted from SMART (Servier Medical Art).

3.2. ncRNAs as Novel Therapeutic Targets of HCV-Induced HCC

In addition to their potential role as biomarkers, ncRNAs may also represent therapeutic targets (Figure 2). Several studies demonstrated in vivo proof-of-concept of using ncRNA/ncRNA antagonists to modulate gene expression, including in the liver [117–119]. LncRNAs that act as transcriptional enhancers might be used to increase gene expression, as demonstrated by the use of "antagoNATs" (oligonucleotides or siRNA designed to inhibit NATs) [119]. Another therapeutic option is to target miRNAs by using miRNA antagonists/antisense oligonucleotides to sequester miRNAs. The feasibility of such strategies for targeting the human liver is demonstrated by clinical trials in CHC patients having shown a dose-dependent prolonged reduction of viral RNA in patients treated with miR-122 antagonists miravirsen or RG-101 [117,118] (Figure 2). Furthermore, another clinical trial tested a nanoliposome-incorporated miR-34 mimic (MRX34, Mirna Therapeutics) in patients with primary liver cancer (Figure 2). However, this trial was abrogated due to immune-related adverse effects. Further investigations are required to fully characterize the biological functions of ncRNAs and to understand the impact of ncRNA modulation in liver carcinogenesis in order to develop new clinical therapies.

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IX. CURRICULUM VITAE

General information

Full Name: Katharina HERZOG

Date of birth: September 1st 1989

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Personalia

Katharina Herzog was born in Lahr/Schwarzwald, Germany on September 1st, 1989. She completed her secondary education at the Scheffel-Gymnasium in Lahr/Schwarzwald with the “general qualification for university entrance". She continued to study Biology, obtaining her degree of Bachelor and Master of Science (Biology) in 2014 and 2016, respectively. Captivated by the subject of virology, she spent a large part of her last year at the university to work as a scientific assistant at the university hospital in Freiburg i.Br., Germany in the group of Prof. Robert Thimme obtaining technical and practical insides.

In an effort to increase her knowledge and acquire international experience, she then moved to Strasbourg where she obtained a competitive IDEX scholarship of the University of Strasbourg for a PhD fellowship. At INSERM UMR1110, the Institute of Viral and Liver Disease headed by Prof. Dr. Thomas Baumert, she uncovered a host factor that is modulating hepatitis C virus (HCV) morphogenesis and infectivity. She further characterized changes in the circadian clock upon HCV infection and identified cellular proteins and host factors bound to the HCV genome.

Scientific output

Original publications

2019 **Herzog K***, Bandiera S*, Pernot S, Fauvelle C, Jühling F, Weiss A, Bull A, Durand SC, Chane-Woon-Ming B, Pfeffer S, Mercey M, Lerat H, Meunier JC, Raffelsberger W, Brino L, Baumert TF, Zeisel MB (*contributed equally). Functional microRNA screen uncovers O-linked N-acetylglucosamine transferase as a host factor modulating hepatitis C virus morphogenesis and infectivity. Original Article. *Gut* 2019. pii: gutjnl-2018-317423. doi: 10.1136/gutjnl-2018-317423. Epub ahead of print May 10. *Authors contributed equally. Impact factor 2018: 17,016.

Review article

2018 Plissonnier ML, **Herzog K**, Levrero M, Zeisel MB. Non-coding RNA and HCV-induced HCC. *Viruses 2018, 10 (11): 591*.

Oral communications

2019 **Herzog K**[+], Bandiera S, Pernot S, Fauvelle C, Jühling F, Weiss A, Bull A, Durand SC, Chane-Woon-Ming B, Pfeffer S, Mercey M, Lerat H, Meunier JC, Raffelsberger W, Brino L, Baumert TF, Zeisel MB ([+]presenter). Functional microRNA screen uncovers O-linked N-acetylglucosamine transferase as a host factor modulating hepatitis C virus morphogenesis and infectivity. *Réunion du réseau national hépatites de l'ANRS (AC 42 groupe « métabolisme lipidique »), April 3, 2019, Paris, France.*

2018 Bandiera S, Pernot S, **Herzog K**[+]**,** Fauvelle C, Weiss A, Durand SC, Raffelsberger W, Brino L, Baumert TF, Zeisel MB ([+]presenter). A microRNA Screen uncovers O-Linked N-Acetylglucosamine Transferase as a Host Factor involved in Hepatitis C Virus Morphogenesis. *18ème réunion du réseau national hépatites de l'ANRS, March 12-13, 2018, Paris, France.*

2018 Bandiera S, Pernot S, **Herzog K**[+]**,** Fauvelle C, Weiss A, Durand SC, Raffelsberger W, Brino L, Baumert TF, Zeisel MB ([+]presenter). A microRNA Screen uncovers O-Linked N-Acetylglucosamine Transferase as a Host Factor involved in Hepatitis C Virus Morphogenesis. *Génomique fonctionelle du Foie (GFF), March 14-16, 2018, Lyon, France.*

2018 Bandiera S, Pernot S, **Herzog K**[+]**,** Fauvelle C, Weiss A, Durand SC, Raffelsberger W, Brino L, Baumert TF, Zeisel MB ([+]presenter). A microRNA Screen uncovers O-Linked N-Acetylglucosamine Transferase as a Host Factor involved in Hepatitis C Virus Morphogenesis. *International Liver Congress (ILC, EASL), April 11-15, 2018, Paris, France.*

Key skills

Soft skills:

Interpersonal and leadership skills (teamwork):
Written and oral communication:
Self-management and work habits (time management):
Research and information management:
Project management:
Analysis and Problem-solving abilities:

Language skills:

German (Native language)
English (Fluent in spoken and written)
French (Basic knowledge)

Computer literacy:

Microsoft Office 10
Computational tools to analyze and represent scientific data

Scientific Skills:

Molecular biological techniques (e.g. plasmid cloning, sequencing, electroporation, transfection)
Microbiological techniques (e.g. bacterial transformation)
Virologic techniques (e.g. production of recombinant viruses and pseudotyped virus particles, infection experiments (BSL3))
Immunological techniques (e.g. ELISA, Western Blot)
Flow cytometry (BD FACS Canto II, LSR Fortessa)
Cell culture
Routine analytics

Practical experiences

2015-2016 Scientific assistant University hospital Freiburg i.Br., Germany. Group Thimme/Hofmann.

2013-2015 Scientific assistant, IMTEK, Albert-Ludwigs University, Freiburg i.Br., Germany. Group Kerzenmacher.

Education

Higher education

2016-2019 PhD student INSERM U1110, Institute of Viral and Liver Disease, Strasbourg, France. Thesis title: "Impact of O-linked N-acetylglucosamine transferase on late steps of the hepatitis C replication cycle" (Defense scheduled: October 29th 2019).

2014-2016 Master of Science Biology (Immunology, Virology), Albert-Ludwigs-University, Freiburg i.Br., Germany. Thesis title: "TCF1 expression in human virus-specific CD8^{+} T cells" (degree: 1.4).

2009-2014 Bachelor of Science Biology, Albert-Ludwigs-University, Freiburg i.Br., Germany. Thesis title: "Conjugative transfer experiments with Corynebacteria"(degree: 1.2).

Secondary education

2000-2009 General qualification for university entrance, Scheffel-Gymnasium, Lahr/Schwarzwald, Germany (degree: 2.5).

Katharina HERZOG

Impact of OGT on late steps of the hepatitis C virus replication cycle

Hepatitis C is caused by the hepatitis C virus (HCV) leading in most subjects to chronic liver infection resulting in chronic hepatitis and progressive liver disease and thereby to development of lethal complications, i.e. cirrhosis and hepatocellular carcinoma (HCC). Infection of human hepatocytes by HCV is a multistep process involving viral and host factors. microRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate gene expression. A functional high-throughput miRNA mimic screen identified miR-501-3p and miR-619-3p as novel modulators of HCV assembly/release. We discovered that these miRNAs regulate O-linked N-acetylglucosamine (O-GlcNAc) transferase (OGT) protein expression and identified OGT and O-GlcNAcylation as regulators of HCV morphogenesis and infectivity. Furthermore, increased OGT expression in patient-derived liver tissue was associated with HCV liver disease and cancer. In addition to its effect on HCV morphogenesis, OGT may thus play a role in HCV-induced liver disease and hepatocarcinogenesis.

Keywords: Hepatitis C virus, microRNA, OGT, hepatocellular carcinoma

L'hépatite C est causée par le virus de l'hépatite C (HCV) qui est responsable de maladies chroniques du foie et l'une des principales causes de développement du carcinome hépatocellulaire (CHC). L'infection des hépatocytes humains par le HCV est un processus en plusieurs étapes impliquant des facteurs viraux et des facteurs de l'hôte. Les microARN (miR) sont de petits ARN non codants qui régulent l'expression des gènes au niveau post-transcriptionnel. En utilisant un criblage génomique de miR, nous avons identifié miR-501-3p et miR-619-3p comme modulateurs de l'assemblage et l'export du HCV. Nous avons découvert que ces miR régulent l'expression de l'OGT (UDP-N-acétylglucosamine-peptide N-acétylglucosaminyltransférase) et identifié l'OGT et la O-GlcNAcylation comme régulateurs de la morphogénèse et de l'infectiosité du HCV. De plus, l'expression de l'OGT dans les tissus hépatiques de patients infectés par le HCV était associée à la maladie hépatique et au cancer. En plus de son effet sur la morphogénèse du HCV, l'OGT peut donc jouer un rôle dans les maladies hépatiques induites par le HCV et l'hépatocarcinogenèse.

Mots-clés : virus de l'hépatite C, microARN, l'OGT, carcinome hépatocellulaire