ORIGINAL RESEARCH

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.

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 >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

Significance of this study
What is already known on this subject?
To establish chronic infection, the 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-acetylglucosaminyltransferase (OGT) at the protein level.
Silencing of OGT expression or inhibition of O-linked N-acetylglucosaminyltransferase (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.

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that contribute to the establishment of chronic HCV infection and represent potential antiviral targets, for example, epidermal growth factor receptor, also play a role in liver disease pathogenesis and represent candidate targets for treatment of advanced liver disease and HCC prevention. Thus, uncovering host factors usurped by HCV contributes to a better understanding of virus-host interactions underlying the HCV life cycle and 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), 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 proviral 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. Other miR-122 antisense oligonucleotides were subsequently developed as host-targeting antivirals. Other miRNAs can indirectly target HCV by regulating host factors that participate in antiviral responses and immune surveillance. 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 hypothesised 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 miRNAome and analysed their impact on HCV infection by combining computational and functional approaches.

MATERIALS AND METHODS

Cells, cell culture conditions, viruses, virus purification, infectivity assays, miRNAs, antagoniRs, small interfering RNAs (siRNAs), antibodies, immunoblot, immunocapture, electron microscopy analysis of viral particles and gene expression analysis in liver tissue are described in the online supplementary information.

Functional miRNA/siRNA screens

Huh7.5.1 cells were transfected with the miRIDIAN human miRNA mimic library (miRBase 19) comprising >2000 mature miRNAs or 28 ON-TARGETplus SMARTpool siRNAs (20 nM, Dharmacon) using Interferin HTS (Polyplus) in a 96-well format. After 48 hours, a viability test (Presto Blue, Thermo Scientific) was performed prior to a two-step infection assay.

During part 1 of the protocol, 50 µL of HCV cell culture-derived particles (HCVcc, JcR2a) were incubated with cells during 4 hours. The inoculum was removed and cells were incubated with 150 µL of medium for 48 hours. 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 intraplating and interplate normalisation. First, intraplatal zonal bias was examined and a model of median effects across the entire screen was constructed using the median-polish algorithm and all plates corrected accordingly. Then the dataset was examined for outlier plates, that is, plates where all individual measurements correlate very poorly with the other remaining replicates. Three and nine 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 correlated improved substantially (>40%). Next, the plates were normalised inter replicates using the particularly robust quantile-quantile approach. Finally, the data were tested using a moderated t-test (empirical Bayes shrinkage, R-package limma) for the null-hypothesis of no change of a given miRNA compared with 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 (Fdr). 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 Fdr threshold of 0.00027 was used. Data from part 2 were subject to increase inherent stochastic noise and for

Inhibitor treatment

Four hours following HCV RNA electroporation, Huh7.5.1 cells were incubated with vehicle or inhibitors of OGT (peracetylated 5-thio-N-acetylglucosamine (Ac₅S-GlcNAc)) or OGA (Thiamet G (Sigma)). After 96 hours, supernatants were transferred onto naïve Huh7.5.1 cells for 72 hours prior to determination of luciferase activity while electroporated cells were lysed to determine luciferase activity.

Gene expression analyses

Total RNA was purified and transcribed into cDNA using Maxima reverse transcriptase (Thermo Scientific). GAPDH and OGT mRNAs 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.

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 GENEX) was cloned between the NolI and XhoI 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. The functionality of the OGT 3'UTR was assessed as described. The miRIDIAN mimic negative control 1 was used as control. Renilla and firefly luciferase activity was assessed 48 hours 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 intraplating and interplate normalisation. First, intraplatal zonal bias was examined and a model of median effects across the entire screen was constructed using the median-polish algorithm and all plates corrected accordingly. Then the dataset was examined for outlier plates, that is, plates where all individual measurements correlate very poorly with the other remaining replicates. Three and nine 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 correlated improved substantially (>40%). Next, the plates were normalised inter replicates using the particularly robust quantile-quantile approach. Finally, the data were tested using a moderated t-test (empirical Bayes shrinkage, R-package limma) for the null-hypothesis of no change of a given miRNA compared with the negative control. The resulting p values for independent testing of each miRNA were corrected for the multiple testing situation and expressed as local false discovery rate (Fdr). 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 Fdr 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 (ie, maximum 15% risk for each of the retained hits).

Other datasets were analysed using the two-tailed Mann-Whitney U test, Wilcoxon test, Spearman’s 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 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 (figure 1A). Silencing of CD81 and ApoE, two essential host factors required for HCV entry or assembly, respectively, was performed in parallel using 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 (figure 1B). 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 (figure 1B). The screen identified 427 miRNAs (corresponding to about 16% of the library) that significantly modulated HCV infection (fdr <threshold, online supplementary table 1 and figure 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 230 miRNAs decreased HCV infection by at least 2.7-fold. Hits from the screen included siRNA pools exhibiting strong silencing without cytotoxicity (figure 2). Silencing of CD81 and antagoniR-122 served as controls for part 1; knock-down of ApoE served as control for part 2 (figure 2). Hits were defined as genes whose knock-down modulated HCV infection in at least one part of the screen with high significance (figure 2, p<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/inf ectivity 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 associated with an enhancement of HCV assembly/release/inf ectivity (p=0.0002), while that of the other hits was associated with reduced HCV infection (figure 2). These results indicate that the downregulation of OGT phenocopies the effect of miR-501-3p and miR-619-3p on HCV infection (figure 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 analysed 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 (figure 3A), upregulation of miR-501-3p significantly decreased OGT protein expression by ~65% (figure 3B, p<0.05, t-test). miR-619-3p also decreased OGT expression but less robustly than miR-501-3p (figure 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 harbours 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 with the empty vector (figure 3C, p<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 (figure 3C). These data are consistent in indicating that miR-501-3p mediates post-transcriptional regulation of OGT.
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) 2 days later (part 1). Cell supernatants (SN) 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 postinfection, respectively) to determine luciferase activity. (B) Modulation of HCV entry and replication (part 1) and/or assembly and infectivity (part 2) following 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 SD. 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) for the null-hypothesis of no change of a given miRNA compared with the negative control. The resulting p values for independent testing of each miRNA were corrected for the multiple testing situation and expressed as local false discovery rate (lfdr, R-package fdrtool). 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±SD from three independent experiments in triplicate. The dashed line indicates values from control-transfected cells set at 100%. *P<0.05, Mann-Whitney U test.
O-GlcNAcylation modulates HCVcc infectivity

To investigate whether OGT modulates HCV assembly and/or infectivity, we detected infectious virus titre (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 (figure 4A, p<0.05, Mann-Whitney U 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 following OGT-silencing (figure 4B). We next sought to investigate how OGT could modulate HCVcc infectivity. OGT is the only enzyme that catalyses 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. To assess whether the enzymatic activity of OGT modulates HCVcc infectivity, we used pharmacological inhibitors of OGT (Ac45S-GlcNAc) or O-GlcNAcase (OGA) (Thiamet G), the OGT counterpart that removes O-GlcNAc (figure 4C). Ac45S-GlcNAc led to a significant enhancement of HCVcc infectivity in a dose-dependent manner, while the opposite effect was observed with Thiamet G (figure 4D, p<0.05, Mann-Whitney U 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 analysed the structural and biophysical properties of HCVcc produced in siCtrl-transfected 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 (figure 5A–B) as well as higher ApoE concentrations (figure 5C, Spearman’s correlation: 0.06004019, p=0.7661) suggesting that HCV proteins do not directly modulate OGT expression. In Huh7.5.1 cells, HCV infection led to small but significant increase of miR-501-3p and decrease of OGT levels (figure 7A–B and online supplementary figure 1B; p<0.05, Mann-Whitney U test), which may promote viral infection given the proviral and antiviral roles of miR-501-3p and O-GlcNAcylation, respectively (figures 1C–D and 4D). In contrast, no significant difference of OGT expression was observed between the livers of HCV transgenic and wild-type mice (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 (figure 7C, Spearman’s correlation: 0.06004019, p=0.7661) suggesting that in patients there is likely no direct effect of HCV on OGT expression.

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 led to small but significant increase of miR-501-3p and decrease of OGT levels (figure 7A–B and online supplementary figure 1B; p<0.05, Mann-Whitney U test), which may promote viral infection given the proviral and antiviral roles of miR-501-3p and O-GlcNAcylation, respectively (figures 1C–D and 4D). In contrast, no significant difference of OGT expression was observed between the livers of HCV transgenic and wild-type mice (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 (figure 7C, Spearman’s correlation: 0.06004019, p=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. 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 (figure 7D–E), OGT levels were markedly and significantly elevated in the tumour liver tissue of patients chronically infected with HCV or HBV and patients with alcoholic liver disease or non-alcoholic fatty liver disease as compared with non-tumour tissue (figure 7F, p<0.05, Wilcoxon test). These data suggest that OGT expression increases in HCC in an aetiology-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 characterising the role of OGT/O-GlcNAcylation for HCV morphogenesis, Li et al published their functional genomics study of HCV-miRNA interactions. By conducting genome-wide miRNA mimic and hairpin inhibitor screens, they identified a set of miRNAs exhibiting a proviral or antiviral effect on HCV. Characterisation 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. Despite similarities in the cell type and HCV infection models used here and by Li et al, 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 and is likely due (i) to the different sizes of miRNA mimic libraries as the library used here was more than two times larger than the one used by Li et al 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 and the global multistep inhibitory effects of the let-7 family on HCV infection, 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

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 figure 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 siCtrl (Ctrl). Results for different replicates are shown as individual points. For each gene, median fold change of luciferase activity±SD 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.
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), siOGT, miR-501-3p or miR-619-3p. After 96 hours, RNA and proteins were purified, and OGT expression assessed by RT-qPCR and western blot analysis. (A) Percentage of OGT mRNA expression in miRNA-transfected cells as compared with negative control. Results are presented as mean±SD and are from three independent experiments in triplicate. The dashed line indicates values from control-transfected cells set at 100%. *P<0.05, t-test. (B) OGT protein expression. Left: percentage of OGT protein expression in siRNA-transfected or miRNA-transfected cells as assessed by quantification of western blot analysis. OGT levels were normalised to actin levels using ImageLab 5.2.1 software (Bio-Rad). Results are presented as mean±SD and are from three independent experiments. The dashed line indicates values from control-transfected cells set at 100%. *P<0.05, Mann-Whitney U-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±SD normalised 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%. *P<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, siOGT or siApoE as a loss-of-function control to perturb HCV assembly, prior to infection with HCVcc (Jc1) 2 days later (entry and replication). Mock-transfected cells were used as control (Ctrl). After another 48 hours, intracellular and extracellular HCVcc particles were used to infect naïve Huh7.5.1 cells (infectious virus production). Virus supernatants of Huh7.5.1 cells were assayed by (left) end point dilution assay (TCID50). Intracellular and extracellular HCV RNA was purified and analysed by RT-qPCR to calculate (right) the specific infectivity (TCID50/RNA). Data are expressed as mean percentage as compared with control±SD. Results are from four independent experiments in triplicate. The dashed line indicates values from control-transfected cells set at 100%. *P<0.05, Mann-Whitney U 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 analysed as described in A. Data are expressed as mean percentage of Renilla luciferase activity as compared with control±SD. Results are from three independent experiments in quadruplicate. The dashed line indicates values from control-transfected cells set at 100%. *P<0.05, Mann-Whitney U test. (C) Activity of OGT/OGA inhibitors on O-GlcNAcylation. The activity of Ac45S-GlcNAc (OGT inhibitor) or Thiamet G (OGA inhibitor) on O-GlcNAcylation of proteins in Huh7.5.1 cells was demonstrated by western blot analysis as described in online 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 Ac45S-GlcNAc (OGT inhibitor, left) or Thiamet G (OGA inhibitor, right) 4 hours later. After 96 hours, supernatants were transferred onto naïve Huh7.5.1 cells and electroporated cells were lysed to determine luciferase activity (entry and replication). Luciferase activity in infected Huh7.5.1 cells was assessed 72 hours later (infectious virus production). Data are expressed as mean percentage as compared with control±SD. Results are from three independent experiments in quadruplicate. The dashed line indicates values from vehicle-treated cells set at 100%. *P<0.05, Mann-Whitney U test.
infection. Since none of these miRNAs except miR-185 has been previously associated with HCV infection, it might be interesting to further characterise the involvement of these miRNAs in HCV-host interactions. Interestingly, an overall proviral effect of miR-501-3p was also observed by Li et al.; however, the mechanism of action was not studied. By characterising 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-neutralising antibodies, 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 localised in the nucleus, cytoplasm or mitochondria. This O-glycosylation/deglycosylation of proteins is catalysed by a single pair of nucleocytoplasmic enzymes, OGT/
Figure 6  Silencing of OGT increases the size of HCVcc. (A) Representative pictures of HCV particles generated in Huh7.5.1 cells transfected with siCtrl or siOGT. (B–F) Comparative analysis of particle size distribution for immunocapture (IC) from HCV particles (JcR2a) produced in Huh7.5.1 cells transfected with siCtrl or siOGT 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 ImageJ 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 72 hours, RNA and proteins were purified, and OGT expression assessed by RT-qPCR and western blot analysis. (A) Percentage of OGT mRNA expression relative to uninfected Huh7.5.1 cells (Ctrl). Results are presented as mean±SD from three independent experiments in duplicate. The dashed line indicates values from uninfected Huh7.5.1 cells set at 100%. *P<0.05, Mann-Whitney U test. (B) OGT protein expression. Left: percentage of OGT protein expression relative to uninfected Huh7.5.1 cells (Ctrl) following quantification of western blot analysis as described in online supplementary methods. Results are presented as mean±SD from three independent experiments. The dashed line indicates values from uninfected Huh7.5.1 cells set at 100%. *P<0.05, Mann-Whitney U 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 online supplementary material and methods). Spearman’s correlation: rho=0.06004019, p=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 online supplementary material and methods). Wilcoxon test: F1 vs F0 p=0.38; F2 vs F0 p=0.18; F3 vs F0 p=0.43; F4 vs F0 p=0.17; A1 vs A0 p=0.28; A2 vs A0 p=0.23; A3 vs A0 p=0.09. (F) OGT expression in tumour (HCC) and non-tumour (Ctrl) liver tissue from HCV-infected patients (34 tumour samples including 5 paired tumour/non-tumour samples), HBV-infected patients (76 tumour samples including 7 paired tumour/non-tumour samples), patients with alcoholic liver disease (ALD) (72 tumour samples including 8 paired tumour/non-tumour samples) and patients with non-alcoholic fatty liver disease (NAFLD) (11 tumour samples including 2 paired tumour/non-tumour samples) as described in online supplementary methods. *P<0.05, Wilcoxon test.
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 signalling. O-GlcNAcylation and phosphorylation on the same or neighbouring serine or threonine residue is known as yin yang site.

O-GlcNAcylation plays a major role in the regulation of metabolic pathways in the liver, including insulin signalling, bile acid metabolism and lipogenesis. The large number of OGT/OGA substrates and cellular pathways regulated by O-GlcNAcylation hampers a detailed characterisation 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 localise in the ER lumen and (iii) O-GlcNAcylation of extracellular proteins containing EGF-like domains is catalysed by EGF domain-specific OGT in the ER lumen in an OGT-independent manner, 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 and OCLN at serine sites that can also be phosphorylated and this has been suggested to potentially play a role for HCV entry. 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. 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 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. 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. Interestingly, while OGT expression modulates the levels of human papillomavirus 16 (HPV16) oncoproteins E6 and E7, E6 in turn can upregulate OGT to increase O-GlcNAcylation and the oncogetic activities of HPV, 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 oncogetic signalling pathways in non-alcoholic steatohepatitis-related HCC and O-GlcNAcylation has been associated with HCC recurrence linked to increased O-GlcNAcylation after liver transplantation, 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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