Novel Anti-viral Strategies for Hepatitis C

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ISBN: 978-90-8570-966-4

The studies presented in this thesis were performed at the Laboratory of Gastroenterology and Hepatology, Erasmus MC-University Medical Center Rotterdam, The Netherlands.

The research was funded by:
The Foundation for Liver and Gastrointestinal Research (SLO)
The Department of of Gastroenterology and Hepatology, Erasmus MC Rotterdam, The Netherlands.

Financial support for printing of this thesis was provided by:
Nederlandse Vereniging voor Hepatologie, J.E. Jurriaanse Stichting, Astellas Pharma B.V., Novartis Pharma B.V., MSD B.V., Roche Nederland B.V.

Printing: Wöhrmann Print Service, Zutphen, the Netherlands

Novel Anti-viral Strategies for Hepatitis C

Innovatieve antivirale strategieën voor Hepatitis C

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

Prof.dr. H.G. Schmidt

and in accordance with the decision of the Doctorate Board
The public defense shall be held on

Wednesday 22nd February 2012 at 13:30

by

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born in Changxing, Zhejiang, China

ERASMUS UNIVERSITEIT ROTTERDAM

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Cover:

Confocal microscopic imaging of uptake of rhodamine-labeled exosomes (red) by live hepatic cells. Secretion and uptake of exosomes have been shown to mediate cell-to-cell transmission of small RNAs (chapter 8) and hepatitis C viruses (chapter 11).

Chapter 1

General introduction

Hepatitis C virus (HCV) infection

HCV is a single-stranded positive-sense RNA virus that was first identified in 1989[1]. HCV belongs to the Flaviviridae family and has six major genotypes[2]. According to the estimation of the World Health Organization[3], approximately 170 million people, 3% of the world population, are HCV positive with 3 to 4 million de novo infections each year. Of the newly infected individuals only approx. 15-40% will effectively clear the virus and those who fail to do so will develop a chronic and progressive infection. The prevalence is high in Egypt (>10%), Asia (5-10%) and Southern Europe (1-2.5%) and is low in the Netherlands (<1%)[4]. Chronic hepatitis C is a slowly progressive disease causing no or few symptoms in the initial phase, but 10 to 20% of the patients develop liver cirrhosis over a period of 10 to 30 years. Patients with liver cirrhosis have an annual risk of 1 to 5% to develop liver cancer, in particular hepatocellular carcinoma (HCC)[5]. Unfortunately, there are still no effective vaccines or antibodies available for the prevention of infection.

Molecular biology of HCV

HCV particles contain a single positive-stranded RNA genome of approximately 9,600 nucleotides in length. To date, six major genotypes and over 100 subtypes of HCV have been identified that differ in their nucleotide sequence up to 34% between genotypes and up to 25% between subtypes [6]. The whole genome composes of a single open reading frame, encoding a polyprotein precursor of approximately 3,000 amino acids, flanked by 5' and 3' non-coding regions (NCR). The life cycle of HCV consists of binding and entry host cells, uncoating viral proteins, genome translation, polyprotein processing, viral replication, particle assembly and release.

Viral entry involves HCV envelope protein interaction with membrane proteins expressed on the surface of the host cell. The exact nature of the host factors contributing to viral binding and entry is still not completely known and is currently the focus of much research in the field. The viral factors involved in binding and entry, namely the envelope glycoproteins E1 and E2, are well established in several model systems[7-9]. Over the past decade, several host cell surface molecules have been identified as potential binding partners for the HCV envelope proteins E1/E2 and are considered important for viral binding and entry. These include CD81[10], glycosaminoglycan[11], scavenger receptor class B type-1(SR-BI)[12], low-density lipoprotein receptor[13], DC-SIGN[14], L-SIGN[15], Claudin members[16-18] and occludin (OCLN)[19]. A recent study suggested that direct cell-to-cell transmission may represent another route of HCV infection that can evade neutralizing antibodies[20].

Upon entry, HCV genomic RNA is released and acts as both a template for the production of the negative-stranded RNA replication intermediate and an mRNA template for the synthesis of the viral polyprotein. Translation of the viral polyprotein is mediated by an internal ribosome entry site (IRES) located within the highly conserved 5' NCR. The synthesized polyprotein is subsequently cleaved into four structural (core, E1, E2 and p7) and six non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins[21]. Processing of structural proteins, including the p7/NS2 junction, is carried out by at least two host signal peptidases and non-structural proteins are matured by two viral proteases, NS2 and NS3/4A[21-22].

Similarly, HCV replication also requires both viral and cellular factors. The 5’ NCR, in particular the IRES sequence, plays an important function in ribosomal assembly. The non-structural proteins, NS3, NS4A, NS4B, NS5A and NS5B, appear to be essential for replication[23]. NS5B is the active subunit of the replication complex known as an RNA-dependent RNA polymerase for the *de novo* generation of positive and negative stranded RNA[24]. The early knowledge of host factors involved in viral replication actually came from studies with immunosuppressive drugs, such as cyclosporine A (CsA). The anti-HCV activity of CsA was suggested as disrupting the functional association of cyclophilin B with NS5B, which participated in the initiation of replication by facilitating the interaction of NS5B and the 3' NCR of the viral RNA[25]. Further investigation revealed that likely other cyclophilins, including cyclophilin A or C are also involved[26]. Recent development of high-throughput technologies, in particular RNA interference (RNAi) libraries as loss-of-function approaches, allows

genome-wide screening of HCV host factors. Using subgenomic and infectious models, host factors involved in different phases of the viral life cycle can be further characterized[27-30].

In addition, new light has been shed on the interplay between cellular membranes, lipid metabolism and HCV infection. The primary location for the assembly of HCV virions in hepatocytes are shown to be the small lipid droplets located in close proximity of the endoplasmatic reticulum. The HCV core protein initiates the recruitment of nonstructural proteins and replication complexes to lipid droplet-associated membranes[31]. Autophagy ("self eating"), a process for catabolizing cytoplasmic components through the lysosomal machinery, has been implicated in the modulation of interactions between RNA viruses and their host. HCV infection has been shown to induce autophagy[32] and facilitate the viral infection by damping the host innate immunity[33], such as interferon response[34].

Therapy for chronic HCV

Interferons are of vital importance for the innate defense against viral infection and involve the induction of a multitude of interferon-stimulated genes (ISGs) to establish an antiviral status[35]. Despite the fact that the exact antiviral mechanism-of-action of most ISG members remains poorly defined, the clinical application of interferon-alpha (IFN-α) has led to the landmark for the treatment of chronic HCV. The further modification of interferon through PEGylation and the combination with ribavirin, the current standard of care, has dramatically improved the outcome that approx. half of the patients can develop a sustained virologic response (SVR)[36-37]. The translation of molecular biology has recently fuelled a rapid progress in HCV drug development. A range of directly acting antivirals (DAA), including protease and polymerase inhibitors, are at various stages of clinical development[38]. These compounds not only have potent antiviral capacities, but also dramatically potentiate the efficacy of the current standard of care[39-40]. Therefore, the next generation of standard care is expected to be interferon, ribavirin plus DAAs[41].

However, given the large infected population, accumulated non-responders, poor tolerability to interferon or DAAs, and special populations (human immunodeficiency virus coinfected patients, liver transplanted patients, etc.), novel antivirals remain urgently required, which ideally should act on distinct mechanisms and be applicable in the current non-responders and special populations with less side effects.

Liver transplantation, immunosuppressants and HCV recurrence

HCV patients who failed to clear the virus will have high risk of developing end-stage liver disease and HCC. Then, liver transplantation is the only treatment option and HCV has now become the major indication for liver transplantation worldwide. However, HCV universally re-infects the liver graft after transplantation causing an accelerated recurrent liver disease, resulting in cirrhosis of the donor liver in 10-41% of the patients after five to ten years[42-43] Moreover, the current standard therapy in transplant patients is much less efficient, with only 20-30% of SVR rate[44].

The more aggravated course of HCV infection after transplantation and relative resistance to antiviral therapy have been attributed to several host and viral factors including genotype, donor age and in particular the application of specific immunosuppressive medication[45]. However, the arguments regarding the choice of immunosuppressants for HCV-related transplantation patients have come from clear evidence in basic research, rather than from clinical observation. The first evidence that the calcineurin inhibitor (CNI) cyclosporine A (CsA), but not FK506 (tacrolimus, Tac), can inhibit HCV replication in vitro[46], sparked a clinical debate on the possible differential impact of Tac and CsA on HCV recurrence. So far, the actions and mechanisms of glucocorticosteroids[47-48], CNIs[49-50] and mycophenolic acid (MPA)[51] on HCV infection and the antiviral effects of interferon have been extensively studied in cell culture models. Retrospective clinical studies have recently debated on the impact of the newer generation immunosuppressant rapamycin on the recurrence of HCV after liver transplantation[52-53], whereas to personal knowledge no basic study has reported on this rising issue.

RNAi and microRNA

RNAi, discovered a decade ago[54], is a sequence-specific inhibition of gene expression at posttranscriptional level. It is triggered by small interfering RNA (siRNA), which can be introduced into cells directly as synthetic siRNA or indirectly as vector expressed short-hairpin RNA (shRNA) precursor[55]. Encoded shRNA can be exported to the cytoplasm and cleaved into active siRNA by a cellular enzyme, Dicer. These siRNAs are assembled into a multicomponent complex, known as the RNA-induced silencing complex (RISC), which

incorporates a single strand of the siRNA serving as a guide sequence to target and silence homologous messenger RNA (mRNA)[56]. As an innovative loss-of-function technology, RNAi has been widely used for studying gene functions in experimental biology but also fuelled the development of novel therapies for various diseases. Up to date (Dec., 2011), over 20 clinical trials of assessing therapeutic RNAi have been registered at ClinicalTrials.gov.

RNAi has been greatly facilitated the identification of host factors involved in the HCV life cycle but also emerged as a new avenue for therapeutic application against HCV infection. Since HCV is a single-stranded RNA molecule, both the viral genome and host cellular factors involved in the viral life cycle, such as viral receptor CD81, can be targeted by RNAi and convey protection against infection[57]. In the context of treating chronic HCV or preventing recurrence in HCV-positive transplants, a single dose administration with long-lasting therapeutic effects would be ideal. Therefore, viral vectors, such as integrating lentiviral vectors[58] or adeno-associated viral vectors [59], represent suitable tools for in vivo delivery of RNAi.

MicroRNAs (miRNAs) are endogenous noncoding RNAs with approximately 22 nucleotides in length that can broadly regulate gene expression by using the RNAi machinery to trigger either cleavage or translational suppression of the target mRNA[60]. MiR-122 is a liver-abundant miRNA that consists of approx. 70% of the total miRNA population in adult liver[61].Numerous studies have consistently shown that HCV replication is tightly regulated by miR-122[62-63], and treatment of chronically infected chimpanzees with a locked nucleic acid (LNA)-modified oligonucleotide complementary to miR-122 leads to long-lasting suppression of HCV viremia[64]. Moreover, miR-122 has been implicated as a biomarker for liver injury, since liver injury triggered the release of miR-122 into the circulation[65-66].

Mesenchymal stem cells

Mesenchymal stem cells (MSC) are multi-potent stem cells that were originally identified as a heterogeneous population of stromal cells in the bone marrow (BM), but lately studies reported the presence of MSC in other compartments including adult adipose tissue, dermal tissues and spleen as well as umbilical cord blood and various fetal tissues. Notably, the adult human liver also harbors resident MSC that are phenotypically and functionally identical to BM MSC[67].

MSC have been demonstrated to be able to differentiate into various types of cells, including bone, fat, cartilage, muscle cells and hepatocytes[68-70]. Besides its lineage differentiation potential, MSC produced trophic factors have shown to provide paracrine support for hepatocyte proliferation, angiogenesis, tissue repair and immunomodulation[71-73]. Interestingly, a recent study reported that BM MSC could counteract sepsis, a form of serious bacterial infection in blood, by reprogramming of host macrophages to enhance the production of interleukin-10[74]. Based on the compelling results from in vitro and animal models, over 170 clinical trials have been registered (June, 2011, ClinicalTrials.gov) to investigate the therapeutic value of MSC for various diseases.

Aims of the thesis

The general aims of this thesis are: (1) to investigate the effects and mechanisims of different immunosuppressants on HCV infection and antiviral interferon response, (2) to evaluate the biotechnological and therapeutic utility of RNAi for HCV infection and (3) to explore the anti-HCV potential of mesenchymal stem cells.

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Part I.

Drug-based strategies

Chapter 2

Virus-drug interactions: mechanistic insights on the impact of immunosuppressants on hepatitis C infection

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Nature Reviews Gastroenterology & Hepatology, 2012, conditionally accepted

Abstract

Immune suppression considerably affects hepatitis C virus (HCV) recurrence and outcome of antiviral treatment after liver transplantation. Recent findings suggest that the calcineurin inhibitor FK506 (tacrolimus, Tac) but not cyclosporine A (CsA) interfere with the antiviral activity of interferon-α (IFN-α *in vitro*. The aim of our study is to more extensively investigate the effects of calcineurin inhibitors on IFN-α signaling and antiviral activity in subgenomic and infectious HCV models. Treatment with both Tac and CsA did not affect Huh7 cell proliferation at 10 to 500 ng/ml doses, however, completely inhibited T cell proliferation. In contrast to previous reports, Tac had no effect on IFN-α stimulated reporter gene expression, even at 5 μg/ml dose. Furthermore, in Huh7 subgenomic HCV replicon cells, treatment with Tac had no significant effect on the suppression of viral replication by IFN-α. In the infectious HCV model, treatment with IFN-α effectively inhibited both viral RNA replication and de novo production of virus particles and both were not attenuated at any concentration of Tac. CsA had no significant effect on IFN-α stimulated reporter gene expression, however as shown previously, a combination of CsA (at 500 ng/ml and higher) and IFN-α resulted in an enhanced inhibition of viral replication both in the subgenomic and infectious HCV models. In conclusion, our study shows no evidence that Tac or CsA interfere with IFN-α-mediated inhibition of HCV replication and virion production *in vitro*. Therefore, no mechanistic argument is found to break the clinical controversy about choice of calcineurin inhibitor during post-transplantation antiviral therapy.

Introduction

End-stage liver diseases caused by chronic hepatitis C virus (HCV) infection are currently the leading indications for liver transplantation. [1] In general, HCV-positive patients undergoing transplantation experience a reduction in viral RNA levels within the anhepatic phase, followed by a rapid increase in viral load due to re-infection of the graft. HCV infection of the graft occurs universally and rapidly after transplantation, and results in accelerated recurrence of liver fibrosis and early development of cirrhosis. [2]

The current standard therapy of HCV, pegylated-interferon-α (IFN-α) in combination with ribavirin, has achieved substantial success in the primary infected patients with overall half of the patients developing a sustained virologic response. [3] Nevertheless, treatment of HCV recurrence after liver transplantation is much less effective with approximate sustained virologic response rates of only 20%. [4, 5] The more aggravated course of HCV infection after transplantation and relative resistance to antiviral therapy have been attributed to several host and viral factors including genotype, donor age and particular the application of specific immunosuppressive medication. [6] However, the arguments regarding the choice of immunosuppressants for HCV-related transplantation patients have come from clear evidence in basic research, rather than from clinical observation. The first evidence that the calcineurin inhibitor (CNI) cyclosporine A (CsA), but not FK506 (tacrolimus, Tac), can inhibit HCV replication in vitro, [7, 8] sparked a clinical debate on the possible differential impact of Tac and CsA on HCV recurrence. The primary cellular targets of CNI are immunophilins, with CsA targeting cyclophilins and FK binding proteins being targeted by Tac. Recent studies confirmed that the antiviral effect of CsA is mediated by inhibition of cyclophilins, [9] in particular cyclophilin A. [10-13] Although there is still controversy about the effect of CsA alone on HCV replication in the setting of clinical organ transplantation, [14] more recent studies revealed another aspect of CNI and their impact on the treatment for HCV. [15, 16] Hirano and coworkers reported that CNI have different effects on the molecular pathways involved in IFN signaling, showing that Tac but not CsA could interfere with interferon signaling and thereby reducing the antiviral activity of IFN-α in a subgenomic HCV replicon model. [16] Subsequently, this group reported intentional conversion from Tac to CsA based immune suppression for HCV-positive patients on preemptive interferon therapy after living donor

liver transplantation. [17] Moreover, several in vitro studies showed that co-treatment of CsA with IFN-α results in greater, synergistic, inhibition of HCV replication.[18-20]

Given the importance of these laboratory observations in terms of potential translation into the transplant clinic, the aim of the current study is to more extensively evaluate the effects of CNI on IFN-α signaling and antiviral activity in two state-of-the-art HCV replication and infection models.

Materials & Methods

Reagents and cell culture

IFN-α 2a provided by Roche Ltd (Basel, Switzerland) was dissolved in water. CsA provided by Novartis Pharma AG (Basel, Switzerland) was dissolved in a 1:1 mixture of ethanol with 10% Tween-20 and water. Tacrolimus (as intravenous fluid) was provided by Astellas (Hoofddorp, The Netherlands).

The human hepatoma cell line Huh7 and the human embryonic kidney epithelial cell line HEK293 were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen–Gibco, Breda, The Netherlands) complemented with 10% v/v fetal calf serum (Hyclone, Logan, Utah), 100 IU/ml penicillin, 100 mg/ml streptomycin, and 2mM L-glutamine (Invitrogen–Gibco). Stable luciferase expressing cells were generated by transducing naïve Huh7 or HEK293 cells with a lentiviral vector expressing the firefly luciferase gene under control of the human phosphoglyserate kinase (PGK) promoter (PGK-Luc).

HCV replicon models

The HCV bicistronic replicon (I389/NS3-3V/LucUbiNeo-ET, Huh7-ET), containing the non-structure coding sequence of HCV and the luciferase gene, was used.[21] Huh7-ET cells were cultured in the presence of 250 μg/ml G418 (Sigma, Zwijndrecht, The Netherlands). Cells were treated with IFN-α, CsA, Tac or vehicle control alone or in combination and viral luciferase activity was measured after 24h.

Huh7.5 cells harboring the full-length JFH1-derived genome was used as an infectious HCV model. [22] Both intracellular and secreted HCV RNA was determined as described below.

Interferon reporter assay

The ISRE-Luc reporter system, expressing the firefly luciferase gene controlled by the ISRE interferon response element, was obtained from Stratagene, (La Jolla, CA). HEK293 cells were grown in 96-well multiplates and transfected with 0.2 μg of pISRE-Luc plasmid. After overnight incubation, the cells were incubated in the absence or presence of CsA, Tac and IFN-α (100 IU/ml). After 24 hr culture, the luciferase activity was measured.

Luciferase activity measurement

100 mM luciferin potassium salt (Sigma) was added to cells and incubated for 30 min at 37ºC. Luciferase activity was quantified using a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

Real time RT-PCR

HCV genomic RNA of secreted virions was isolated using QIAamp Viral RNA Mini Kit (Qiagen, Venlo, The Netherlands). cDNA was prepared from total RNA of 350 μl supernatant using the iScript cDNA Synthesis Kit from Bio-Rad (Bio-Rad Laboratories, Stanford, CA, USA).

Total RNA of cultured cells was isolated using a Micro RNeasy Kit (Qiagen, Venlo, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from 1 μg total RNA using the iScript cDNA Synthesis Kit from Bio-Rad (Bio-Rad Laboratories, Stanford, CA, USA). The levels of HCV IRES and human GAPDH RNA were quantified using real-time PCR (MJ Research Opticon, Hercules, CA, USA) performed with SYBRGreen (Sigma-Aldrich) according to manufacturer's instructions. Cyclophilin B (CyB) or GAPDH were used as reference genes to normalize gene expression.

MTT cell viability assay

Huh7 cells were plated in 96-well plates and treated with concentrations of CsA or Tac for 48h. Cell metabolic activity and viability were quantified by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml) assay (Sigma-Aldrich).

T cell proliferation assay

Effect of CsA and Tac on proliferation of T cells was determined by [3H] thymidine incorporation assay. Briefly, peripheral blood mononuclear cells (4×10^5) in the presence or

absence of CsA or Tac were seeded in 96-well round-bottom plates. Phytohemagglutinin (PHA; Murex Biotech, UK) was used for stimulation. After 5 days, proliferation was assessed by determination of the incorporation of 0.5 µCi (0.0185 MBq) [3H] thymidine (Radiochemical Center, Amersham, United Kingdom) for 18 hrs.

Statistical analysis

Statistical analysis was performed by using either matched-pair nonparametric test (Wilcoxon signed-rank test) or the non-paired, nonparametric test (Mann–Whitney test) (GraphPad Prism software). P-values less than 0.05 were considered as statistically significant.

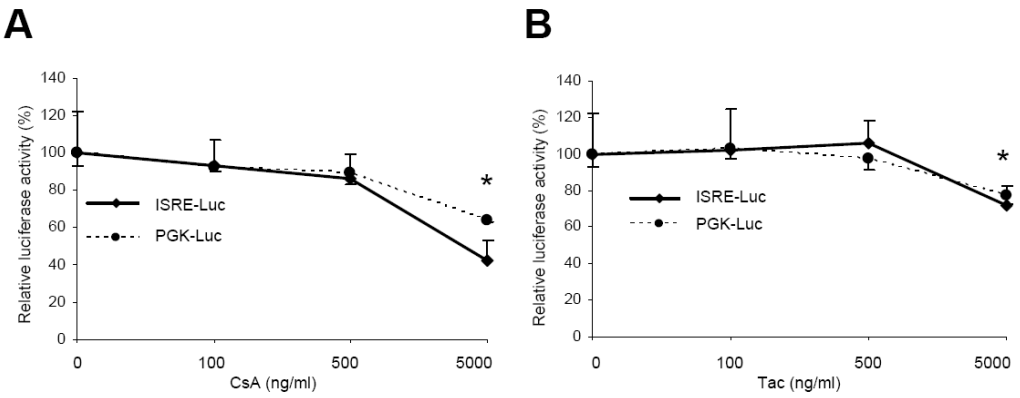

Figure 1. Effect of CsA and Tac on interferon-stimulated reporter gene expression. HEK293 cells were transfected with interferon reporter plasmid, ISRE-Luc. After overnight culture, cells were stimulated with 100 IU/ml IFN-α in the absence or presence of CsA (A) or Tac (B). HEK293 cells stably expressing luciferase driven by the PGK promoter element (PGK-Luc) served as control to determine potential non-specific effect of drugs. After 24 hr treatment, luciferase activity was measured. Shown is the mean ± SD relative luciferase activity of ISRE-Luc and PGK-Luc as percentage of the IFN-α alone group from six experiments. At highest concentration both CsA and Tac inhibited luminescence activity of ISRE-Luc and PGK-Luc, suggesting non-specific effects rather than specific effects on interferon signaling (* $P < 0.05$).

Results

Tac and CsA do not interfere with interferon-induced gene expression

Type I interferons, IFN-α and IFN-β, are known to stimulate the expression of genes containing interferon-stimulated response elements (ISRE) in the promoter region. Using the reporter gene expression assay, the effect of CNI Tac and CsA on IFN-α stimulated gene expression was investigated. A plasmid, containing the luciferase gene driven by a basic

TATA-box promoter containing five ISRE repeats (ISRE-Luc), was transiently transduced in HEK293 cells. To exclude the possible non-specific effects of the calcineurin inhibitors on the host cell and bioluminescence, a control cell line was generated that constitutively expresses the luciferase gene independent of IFN stimulation (PGK-Luc).

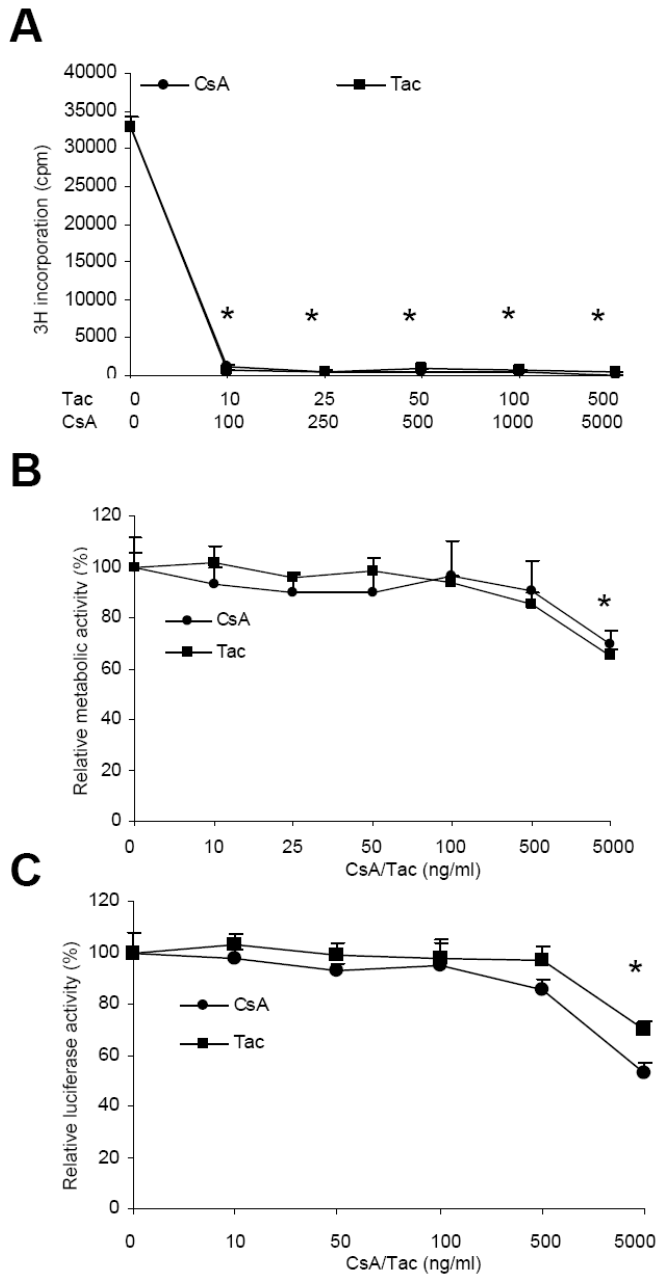

Figure 2. Effect of CsA and Tac on T cells proliferation, Huh7 cell proliferation and control luciferase activity. (A) Both CsA and Tac effectively inhibited PHA-stimulated T cell proliferation (n=6, *P < 0.01), confirming intact biological activity of both CNI. (B) Treatment with CsA or Tac at concentrations up to 500 ng/ml did not affect the number of viable, metabolically active, Huh7 cells as measured by MTT-conversion assay. However at the highest dose of 5000 ng/ml both CNI significantly reduced the number of active cells (mean ± SD, n=6, *P < 0.01). (C) CsA and Tac up to a concentration of 500 ng/ml did not effect control luciferase activity in Huh7 cells. Only a high concentration of 5000 ng/ml CsA or Tac resulted in a significant reduction of 47.1% ± 4.1 and 30.9% ± 3.2 , respectively (mean ± SD, n=4, *P < 0.01).

HEK293 cells containing ISRE-Luc or PGK-Luc were stimulated with IFN-α in the presence or absence of different concentrations of CsA or Tac. Relative luciferase activity is shown in Figure 1. After 24h IFN-α stimulation, a robust luminescence signal was detected in ISRE-Luc

cells, whereas the luminescence in PGK-Luc cells was unaltered. Treatment with CsA or Tac alone did not induce a luminescence signal at any concentration tested (Data not shown). Combined treatment of IFN-α with 100 or 500 ng/ml CsA or Tac did not alter the stimulation of ISRE-Luc (Fig. 1A and B). However at a higher dose of 5 μg/ml CsA or Tac, significant inhibition of the luminescence activity was seen both in ISRE-Luc and PGK-Luc HEK293 cells. This suggests that high dose CsA nonspecifically affects luciferase activity rather than specifically affecting interferon signaling (Fig. 1A and B). These results contradict to the previous studies, [15, 16] which suggested that treatment with Tac attenuated interferon response.

To confirm the biological activity of Tac and CsA, we tested their ability to inhibit T cell proliferation. As shown in Figure 2A, potent inhibition of PHA-stimulated T cell proliferation was observed with both Tac and CsA. The inhibition was dose dependent, with Tac being approximately ten-fold more potent then CsA, consistent with a previous report. [23] The fact that CsA and Tac have the expected potency on T cell proliferation, indicate that the lack of effect on IFN-α signaling is not due to degradation or impaired function of these CNI.

Tac and CsA do not attenuate inhibition of HCV replication by IFN-α

To evaluate whether Tac or CsA interfere with the antiviral action of interferon, a subgenomic HCV replicon model, Huh7 ET, was used. This viral replicon lacks the HCV structural protein coding-sequence and instead contains the luciferase reporter gene allowing sensitive monitoring of viral replication by measurement of luminescence. [24] As shown in Figure 2B, both CsA and Tac did not affect the number of viable Huh7 cells, though at the highest dose of 5000 ng/ml a significant reduction of metabolic active cells was seen by 31% ± 5 and 34% ± 2 (mean ± SD, n=6, $P < 0.01$), respectively. To further distinguish potential non-specific effects of CNI on luciferase activity, we generated a Huh7 cell line that stably expressed luciferase under control of the human PGK promotor (Huh7-PGK-Luc). Using this cell line, no effect of CNI on luciferease activtity was observed at concentrations up to 500 ng/ml (Figure 2C). Only at the highest dose of 5000 ng/ml, both CsA and Tac significantly inhibited luciferase activity by mean 47% ± 4 SD and 31% ± 3 SD, respectively (n=4, $P < 0.01$). These findings indicate that only at the highest dose used, CNI attenuate cell growth of Huh7 cells without further non-specific effects on bioluminescence.

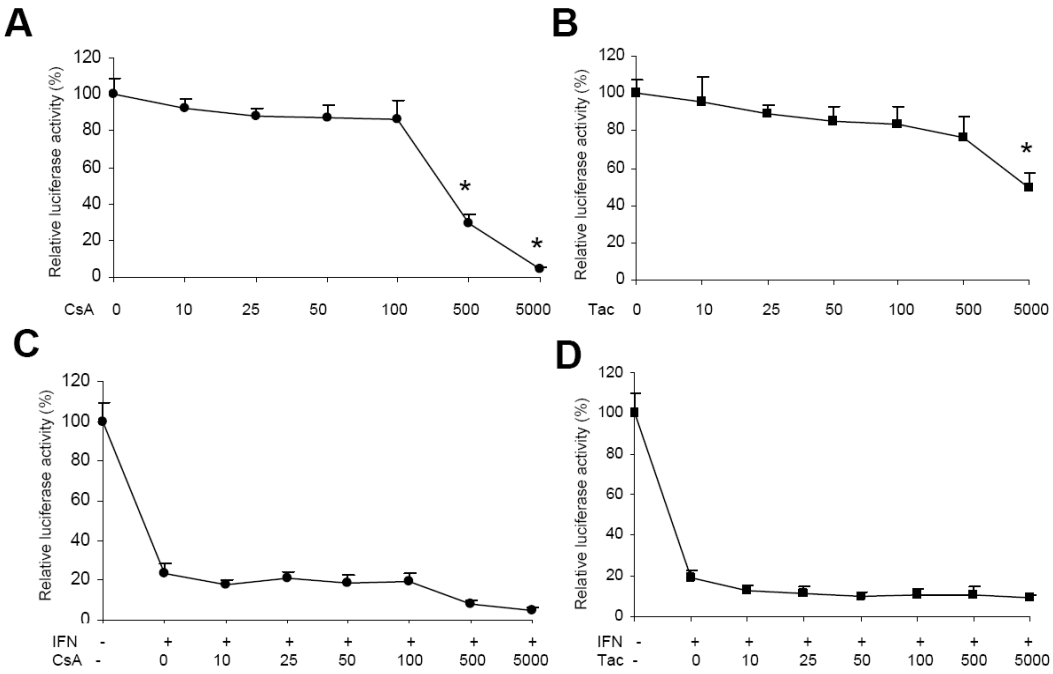

Figure 3. Effect of CsA and Tac on IFN-α mediated suppression of viral replication in subgenomic HCV replicon cells. A) Treatment with CsA alone significantly reduced viral luciferase activity at 500 ng/ml dose by 71.6% ± 5.1. In contrast, Tac alone had a much smaller effect on HCV replication, although at 5000 ng/ml dose significantly inhibited viral luciferase activity. (C) Treatment of low dose of IFN-α (1 IU/ml) resulted in a significant inhibition of viral replication ($P < 0.01$). Combined treatment with CsA (10-100ng/ml) had no effect on IFN-α mediated suppression of viral replication, while at higher dose (500 or 5000ng/ml) an enhanced inhibition of viral replication was observed. (D) Combined treatment of IFN-α with Tac at any dose did not interfere the antiviral activity of interferon. Data are presented as mean ± SD of six independent experiments.

In Huh7 ET cells, treatment with CsA alone significantly reduced viral luciferase activity (mean inhibition of 71.6% ± 5.1 SD at 500 ng/ml dose, n=6, $P < 0.01$) (Fig. 3A) without strong effect on control luciferase activity in Huh7-PGK-Luc cells (Fig. 2C). In contrast, Tac alone had a much smaller effect on HCV replication, although at 5000 ng/ml dose significantly reduced viral luciferase activity (51% ± 8, mean ± SD, n=5, $P < 0.01$) (Fig. 3B) but with clear effect on control luciferase (Fig. 2C). As shown in Figure 3C, treatment with low dose IFN-α (1 IU/ml) resulted in inhibition of HCV replication by 73.2% ± 4.5 (mean ± SD, n=6, $P < 0.01$). Combined treatment of IFN-α with CsA had no effect on suppression of viral replication at low doses of CsA (10-100 ng/ml) where as, consistent with previous studies, [7, 18-20] at higher dose of CsA an enhanced inhibition of HCV replication was observed (Fig. 3C). However, combined treatment of IFN-α with Tac did not attenuate the suppression of viral replication at any dose tested (Fig. 3D). Similar results were obtained when Huh7 ET cells were pre-treated

with Tac or CsA for 16 hrs before IFN-α stimulation (data not shown). Overall, no evidence was found that Tac or CsA interfere with the inhibition of HCV replication by IFN-α.

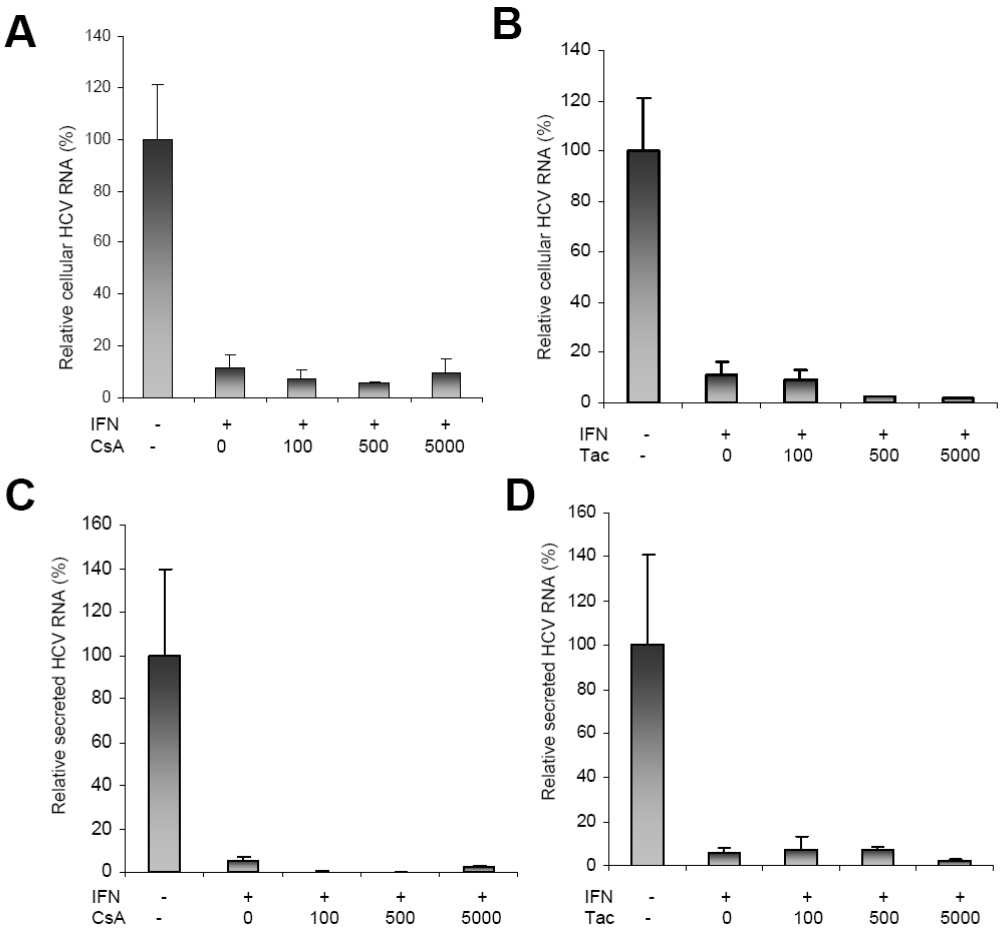

Figure 4. IFN-α mediated suppression of viral replication and virion production in the JFH1-derived infectious HCV model is not affected by Tac. Four days treatment of IFN-α (10 IU/ml) profoundly inhibited production of both cellular (A, B) and secreted (C, D) viral RNA levels. Combined treatment of IFN-α with different concentrations of CsA (A) or Tac (B) did not affect the inhibition of viral RNA replication in the cells. Furthermore, no effect was observed on the secretion of virus particles after combined treatment with different concentrations of CsA (C) or Tac (D). Data are presented as mean ± SD of three independent experiments.

Effect of Tac and CsA on antiviral action of IFN-α in the infectious HCV model

The obvious limitation of subgenomic HCV models is the lack of a productive infection. With the identification of a genotype 2a isolate, JFH1, an infectious HCV model was established which allows the study of the complete viral lifecycle in culture, including the assembly and release of infectious particles. [22] Using this model, we evaluated the effect of CNI on the inhibition of both viral replication and the secretion of de novo viral particles. Four days treatment of IFN-α (10 IU/ml) profoundly inhibited the production of cellular and secreted

viral RNA levels by 91.7% ± 1.7 and 93.5% ± 2.4 (n=3, P < 0.01), respectively (Figure 4). Consistent with the results in the subgenomic replicon model, combination of IFN-α and CsA (Fig. 4A) or Tac (Fig. 4B) did not interfere with the inhibition of viral RNA levels. Moreover no interfere was observed on the secretion of virus particles after combined treatment with CsA (Fig. 4C) or Tac (Fig. 4D). Although the highest dose of Tac accentuated the inhibitory effect of IFN, it is most likely due to the non-specific effect of on host cells. These results in the infectious HCV model again did not show evidence that Tac or CsA interfere with IFN-α signaling.

Discussion

In the current study, we found no evidence that either CsA or Tac interferes with IFN-α induced gene expression (Fig. 1) or IFN-α mediated antiviral activity against HCV (Fig. 3-4). For our study we used two well-established HCV culture models, [22, 24] which allowed determination of both viral replication and the production and release of infectious HCV particles. We confirmed results from earlier studies, [7, 8, 18-20] showing that CsA has antiviral activity alone and in combination with IFN-α (Fig. 3A and 3C, Fig. 4A and Fig. 4C). Treatment with Tac alone showed limited antiviral activity, as shown earlier. [7, 19, 25] Importantly, however, we did not observe any inhibitory effect of Tac on IFN-α signaling (Fig 1B) or its antiviral activity (Fig. 3D, Fig. 4B and Fig. 4D), in contrast to previous reports. [15, 16] In both the interferon reporter assay and HCV culture models, clinically relevant concentrations of Tac reached in serum of organ transplant recipients (5-30 ng/ml) [26] had no negative effect on IFN-α activity.

Of note, there are several clear differences between our study and that of Hirano et al, [16] including drug dosing, HCV models and experimental cell lines. In the earlier study, effects on IFN-α signaling were only observed at extremely high concentrations of Tac (5-10 μM), which is equivalent to 4000-8000 ng/ml and more than two log higher than serum concentration in Tac-treated patients. In our study, we applied a proper internal luciferase control to distinguish and normalize for non-specific effects of drugs on cell viability, cell proliferation and bioluminescence activity. Indeed, as shown in Figure 2, 5000 ng/ml Tac significantly affected the number of viable cells and total luciferase activity of Huh7-PGK-Luc.

Moreover, we have tested the effects of Tac in both subgenomic (genotype 1b) and infectious (genotype 2a) HCV models, while Hirano et al only used 1b subgenomic model. Although Huh7.5 cells, the host of infectious HCV, is defective in RIG-I pathway, an important pathway in IFN stimulation, 293T cells which we used for the IFN reporter array have been reported to be competent in this pathway. [27] Therefore, RIG-I is olso unlikely an explanation for the differences between our study and that of Hirano et al, although the exact factors determining the different conclusions remain unclear.

To investigate the effects of CNI on IFN-α signaling bears significant clinical relevance. Although a multitude of studies have demonstrated the potent antiviral effects of CsA in vitro, the clinical evidence that CsA has an impact on HCV infection after transplantation is rather limited. In contrast to its limited potency in monotherapy, substantial clinical studies has suggested its benefit when combined with pegylated IFN-α [25, 28-30] However, this superiority of CsA over Tac was failed to observe in chronic HCV patients by other studies. [31, 32] Since lacking of proper randomized clinical trial, basic experimental data appears to be important in the debate of this controversial issue.

In this study, we show no evidence that Tac interferes with IFN-α-mediated inhibition of HCV replication and virion production *in vitro*, suggesting no mechanistic argument is found to break the clinical controversy about choice of calcineurin inhibitor during post-transplantation antiviral therapy.

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Chapter 3

Calcineurin inhibitor FK506 does not interfere with the suppression of hepatitis C virus infection by interferon-α

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Liver Transplantation, 2010, 16(4):520-6

Abstract

Immune suppression considerably affects hepatitis C virus (HCV) recurrence and outcome of antiviral treatment after liver transplantation. Recent findings suggest that the calcineurin inhibitor FK506 (tacrolimus, Tac) but not cyclosporine A (CsA) interfere with the antiviral activity of interferon-α (IFN-α *in vitro*. The aim of our study is to more extensively investigate the effects of calcineurin inhibitors on IFN-α signaling and antiviral activity in subgenomic and infectious HCV models. Treatment with both Tac and CsA did not affect Huh7 cell proliferation at 10 to 500 ng/ml doses, however, completely inhibited T cell proliferation. In contrast to previous reports, Tac had no effect on IFN-α stimulated reporter gene expression, even at 5 μg/ml dose. Furthermore, in Huh7 subgenomic HCV replicon cells, treatment with Tac had no significant effect on the suppression of viral replication by IFN-α. In the infectious HCV model, treatment with IFN-α effectively inhibited both viral RNA replication and de novo production of virus particles and both were not attenuated at any concentration of Tac. CsA had no significant effect on IFN-α stimulated reporter gene expression, however as shown previously, a combination of CsA (at 500 ng/ml and higher) and IFN-α resulted in an enhanced inhibition of viral replication both in the subgenomic and infectious HCV models. In conclusion, our study shows no evidence that Tac or CsA interfere with IFN-α-mediated inhibition of HCV replication and virion production *in vitro*. Therefore, no mechanistic argument is found to break the clinical controversy about choice of calcineurin inhibitor during post-transplantation antiviral therapy.

Introduction

End-stage liver diseases caused by chronic hepatitis C virus (HCV) infection are currently the leading indications for liver transplantation. [1] In general, HCV-positive patients undergoing transplantation experience a reduction in viral RNA levels within the anhepatic phase, followed by a rapid increase in viral load due to re-infection of the graft. HCV infection of the graft occurs universally and rapidly after transplantation, and results in accelerated recurrence of liver fibrosis and early development of cirrhosis. [2]

The current standard therapy of HCV, pegylated-interferon-α (IFN-α) in combination with ribavirin, has achieved substantial success in the primary infected patients with overall half of the patients developing a sustained virologic response. [3] Nevertheless, treatment of HCV recurrence after liver transplantation is much less effective with approximate sustained virologic response rates of only 20%. [4, 5] The more aggravated course of HCV infection after transplantation and relative resistance to antiviral therapy have been attributed to several host and viral factors including genotype, donor age and particular the application of specific immunosuppressive medication. [6] However, the arguments regarding the choice of immunosuppressants for HCV-related transplantation patients have come from clear evidence in basic research, rather than from clinical observation. The first evidence that the calcineurin inhibitor (CNI) cyclosporine A (CsA), but not FK506 (tacrolimus, Tac), can inhibit HCV replication in vitro, [7, 8] sparked a clinical debate on the possible differential impact of Tac and CsA on HCV recurrence. The primary cellular targets of CNI are immunophilins, with CsA targeting cyclophilins and FK binding proteins being targeted by Tac. Recent studies confirmed that the antiviral effect of CsA is mediated by inhibition of cyclophilins, [9] in particular cyclophilin A. [10-13] Although there is still controversy about the effect of CsA alone on HCV replication in the setting of clinical organ transplantation, [14] more recent studies revealed another aspect of CNI and their impact on the treatment for HCV. [15, 16] Hirano and coworkers reported that CNI have different effects on the molecular pathways involved in IFN signaling, showing that Tac but not CsA could interfere with interferon signaling and thereby reducing the antiviral activity of IFN-α in a subgenomic HCV replicon model. [16] Subsequently, this group reported intentional conversion from Tac to CsA based immune suppression for HCV-positive patients on preemptive interferon therapy after living donor

liver transplantation. [17] Moreover, several in vitro studies showed that co-treatment of CsA with IFN-α results in greater, synergistic, inhibition of HCV replication.[18-20]

Given the importance of these laboratory observations in terms of potential translation into the transplant clinic, the aim of the current study is to more extensively evaluate the effects of CNI on IFN-α signaling and antiviral activity in two state-of-the-art HCV replication and infection models.

Materials & Methods

Reagents and cell culture

IFN-α 2a provided by Roche Ltd (Basel, Switzerland) was dissolved in water. CsA provided by Novartis Pharma AG (Basel, Switzerland) was dissolved in a 1:1 mixture of ethanol with 10% Tween-20 and water. Tacrolimus (as intravenous fluid) was provided by Astellas (Hoofddorp, The Netherlands).

The human hepatoma cell line Huh7 and the human embryonic kidney epithelial cell line HEK293 were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen–Gibco, Breda, The Netherlands) complemented with 10% v/v fetal calf serum (Hyclone, Logan, Utah), 100 IU/ml penicillin, 100 mg/ml streptomycin, and 2mM L-glutamine (Invitrogen–Gibco). Stable luciferase expressing cells were generated by transducing naïve Huh7 or HEK293 cells with a lentiviral vector expressing the firefly luciferase gene under control of the human phosphoglyserate kinase (PGK) promoter (PGK-Luc).

HCV replicon models

The HCV bicistronic replicon (I389/NS3-3V/LucUbiNeo-ET, Huh7-ET), containing the non-structure coding sequence of HCV and the luciferase gene, was used.[21] Huh7-ET cells were cultured in the presence of 250 μg/ml G418 (Sigma, Zwijndrecht, The Netherlands). Cells were treated with IFN-α, CsA, Tac or vehicle control alone or in combination and viral luciferase activity was measured after 24h.

Huh7.5 cells harboring the full-length JFH1-derived genome was used as an infectious HCV model. [22] Both intracellular and secreted HCV RNA was determined as described below.

Interferon reporter assay

The ISRE-Luc reporter system, expressing the firefly luciferase gene controlled by the ISRE interferon response element, was obtained from Stratagene, (La Jolla, CA). HEK293 cells were grown in 96-well multiplates and transfected with 0.2 μg of pISRE-Luc plasmid. After overnight incubation, the cells were incubated in the absence or presence of CsA, Tac and IFN-α (100 IU/ml). After 24 hr culture, the luciferase activity was measured.

Luciferase activity measurement

100 mM luciferin potassium salt (Sigma) was added to cells and incubated for 30 min at 37ºC. Luciferase activity was quantified using a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

Real time RT-PCR

HCV genomic RNA of secreted virions was isolated using QIAamp Viral RNA Mini Kit (Qiagen, Venlo, The Netherlands). cDNA was prepared from total RNA of 350 μl supernatant using the iScript cDNA Synthesis Kit from Bio-Rad (Bio-Rad Laboratories, Stanford, CA, USA).

Total RNA of cultured cells was isolated using a Micro RNeasy Kit (Qiagen, Venlo, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from 1 μg total RNA using the iScript cDNA Synthesis Kit from Bio-Rad (Bio-Rad Laboratories, Stanford, CA, USA). The levels of HCV IRES and human GAPDH RNA were quantified using real-time PCR (MJ Research Opticon, Hercules, CA, USA) performed with SYBRGreen (Sigma-Aldrich) according to manufacturer's instructions. Cyclophilin B (CyB) or GAPDH were used as reference genes to normalize gene expression.

MTT cell viability assay

Huh7 cells were plated in 96-well plates and treated with concentrations of CsA or Tac for 48h. Cell metabolic activity and viability were quantified by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml) assay (Sigma-Aldrich).

T cell proliferation assay

Effect of CsA and Tac on proliferation of T cells was determined by [3H] thymidine incorporation assay. Briefly, peripheral blood mononuclear cells (4×10^5) in the presence or

absence of CsA or Tac were seeded in 96-well round-bottom plates. Phytohemagglutinin (PHA; Murex Biotech, UK) was used for stimulation. After 5 days, proliferation was assessed by determination of the incorporation of 0.5 µCi (0.0185 MBq) [3H] thymidine (Radiochemical Center, Amersham, United Kingdom) for 18 hrs.

Statistical analysis

Statistical analysis was performed by using either matched-pair nonparametric test (Wilcoxon signed-rank test) or the non-paired, nonparametric test (Mann–Whitney test) (GraphPad Prism software). P-values less than 0.05 were considered as statistically significant.

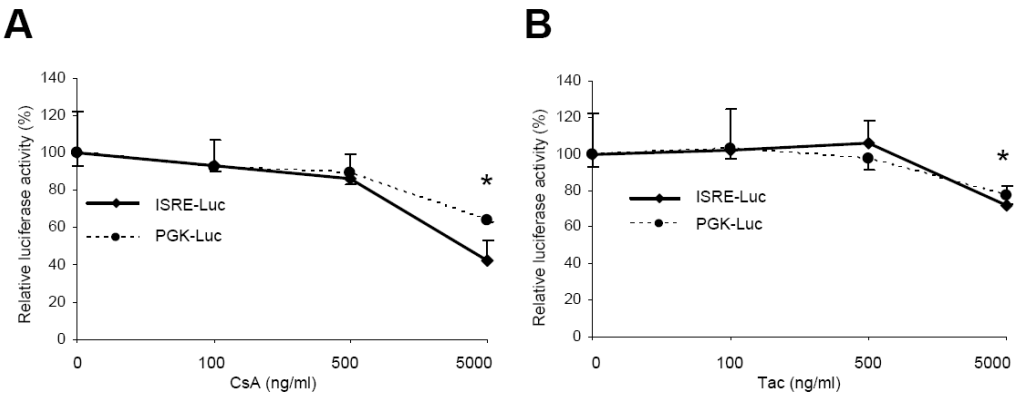

Figure 1. Effect of CsA and Tac on interferon-stimulated reporter gene expression. HEK293 cells were transfected with interferon reporter plasmid, ISRE-Luc. After overnight culture, cells were stimulated with 100 IU/ml IFN-α in the absence or presence of CsA (A) or Tac (B). HEK293 cells stably expressing luciferase driven by the PGK promoter element (PGK-Luc) served as control to determine potential non-specific effect of drugs. After 24 hr treatment, luciferase activity was measured. Shown is the mean ± SD relative luciferase activity of ISRE-Luc and PGK-Luc as percentage of the IFN-α alone group from six experiments. At highest concentration both CsA and Tac inhibited luminescence activity of ISRE-Luc and PGK-Luc, suggesting non-specific effects rather than specific effects on interferon signaling (* $P < 0.05$).

Results

Tac and CsA do not interfere with interferon-induced gene expression

Type I interferons, IFN-α and IFN-β, are known to stimulate the expression of genes containing interferon-stimulated response elements (ISRE) in the promoter region. Using the reporter gene expression assay, the effect of CNI Tac and CsA on IFN-α stimulated gene expression was investigated. A plasmid, containing the luciferase gene driven by a basic

TATA-box promoter containing five ISRE repeats (ISRE-Luc), was transiently transduced in HEK293 cells. To exclude the possible non-specific effects of the calcineurin inhibitors on the host cell and bioluminescence, a control cell line was generated that constitutively expresses the luciferase gene independent of IFN stimulation (PGK-Luc).

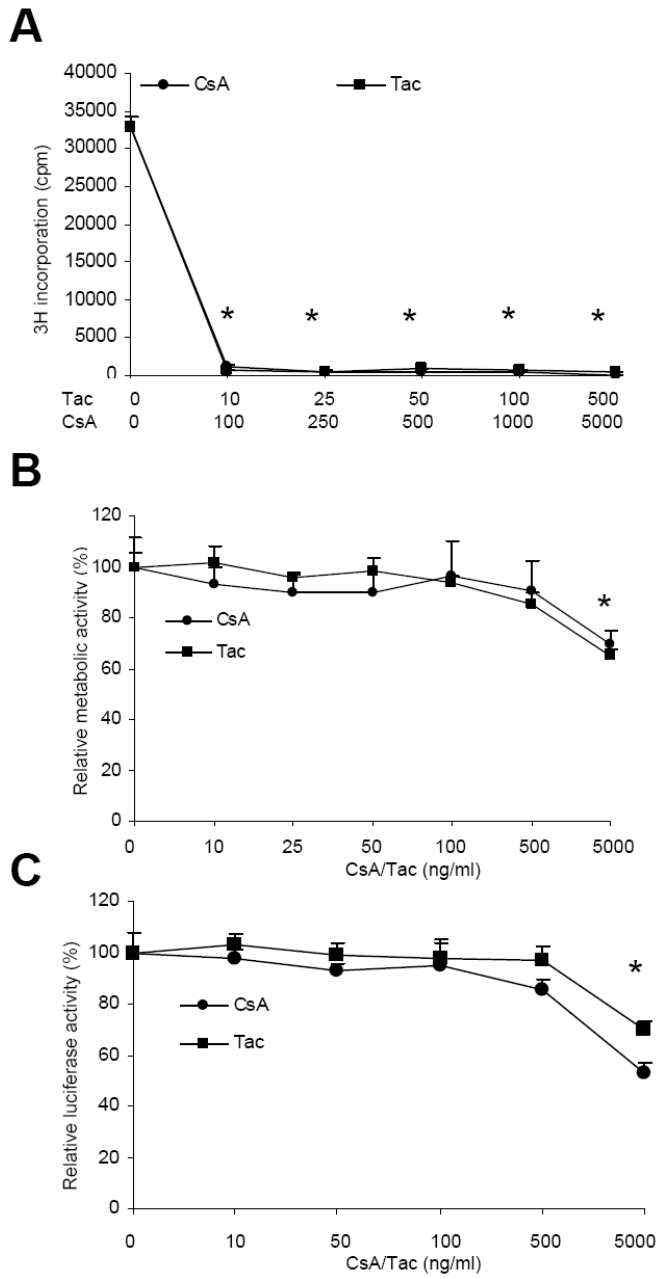

Figure 2. Effect of CsA and Tac on T cells proliferation, Huh7 cell proliferation and control luciferase activity. (A) Both CsA and Tac effectively inhibited PHA-stimulated T cell proliferation (n=6, *P < 0.01), confirming intact biological activity of both CNI. (B) Treatment with CsA or Tac at concentrations up to 500 ng/ml did not affect the number of viable, metabolically active, Huh7 cells as measured by MTT-conversion assay. However at the highest dose of 5000 ng/ml both CNI significantly reduced the number of active cells (mean ± SD, n=6, *P < 0.01). (C) CsA and Tac up to a concentration of 500 ng/ml did not effect control luciferase activity in Huh7 cells. Only a high concentration of 5000 ng/ml CsA or Tac resulted in a significant reduction of 47.1% ± 4.1 and 30.9% ± 3.2 , respectively (mean ± SD, n=4, *P < 0.01).

HEK293 cells containing ISRE-Luc or PGK-Luc were stimulated with IFN-α in the presence or absence of different concentrations of CsA or Tac. Relative luciferase activity is shown in Figure 1. After 24h IFN-α stimulation, a robust luminescence signal was detected in ISRE-Luc

cells, whereas the luminescence in PGK-Luc cells was unaltered. Treatment with CsA or Tac alone did not induce a luminescence signal at any concentration tested (Data not shown). Combined treatment of IFN-α with 100 or 500 ng/ml CsA or Tac did not alter the stimulation of ISRE-Luc (Fig. 1A and B). However at a higher dose of 5 μg/ml CsA or Tac, significant inhibition of the luminescence activity was seen both in ISRE-Luc and PGK-Luc HEK293 cells. This suggests that high dose CsA nonspecifically affects luciferase activity rather than specifically affecting interferon signaling (Fig. 1A and B). These results contradict to the previous studies, [15, 16] which suggested that treatment with Tac attenuated interferon response.

To confirm the biological activity of Tac and CsA, we tested their ability to inhibit T cell proliferation. As shown in Figure 2A, potent inhibition of PHA-stimulated T cell proliferation was observed with both Tac and CsA. The inhibition was dose dependent, with Tac being approximately ten-fold more potent then CsA, consistent with a previous report. [23] The fact that CsA and Tac have the expected potency on T cell proliferation, indicate that the lack of effect on IFN-α signaling is not due to degradation or impaired function of these CNI.

Tac and CsA do not attenuate inhibition of HCV replication by IFN-α

To evaluate whether Tac or CsA interfere with the antiviral action of interferon, a subgenomic HCV replicon model, Huh7 ET, was used. This viral replicon lacks the HCV structural protein coding-sequence and instead contains the luciferase reporter gene allowing sensitive monitoring of viral replication by measurement of luminescence. [24] As shown in Figure 2B, both CsA and Tac did not affect the number of viable Huh7 cells, though at the highest dose of 5000 ng/ml a significant reduction of metabolic active cells was seen by 31% ± 5 and 34% ± 2 (mean ± SD, n=6, $P < 0.01$), respectively. To further distinguish potential non-specific effects of CNI on luciferase activity, we generated a Huh7 cell line that stably expressed luciferase under control of the human PGK promotor (Huh7-PGK-Luc). Using this cell line, no effect of CNI on luciferease activtity was observed at concentrations up to 500 ng/ml (Figure 2C). Only at the highest dose of 5000 ng/ml, both CsA and Tac significantly inhibited luciferase activity by mean 47% ± 4 SD and 31% ± 3 SD, respectively (n=4, $P < 0.01$). These findings indicate that only at the highest dose used, CNI attenuate cell growth of Huh7 cells without further non-specific effects on bioluminescence.

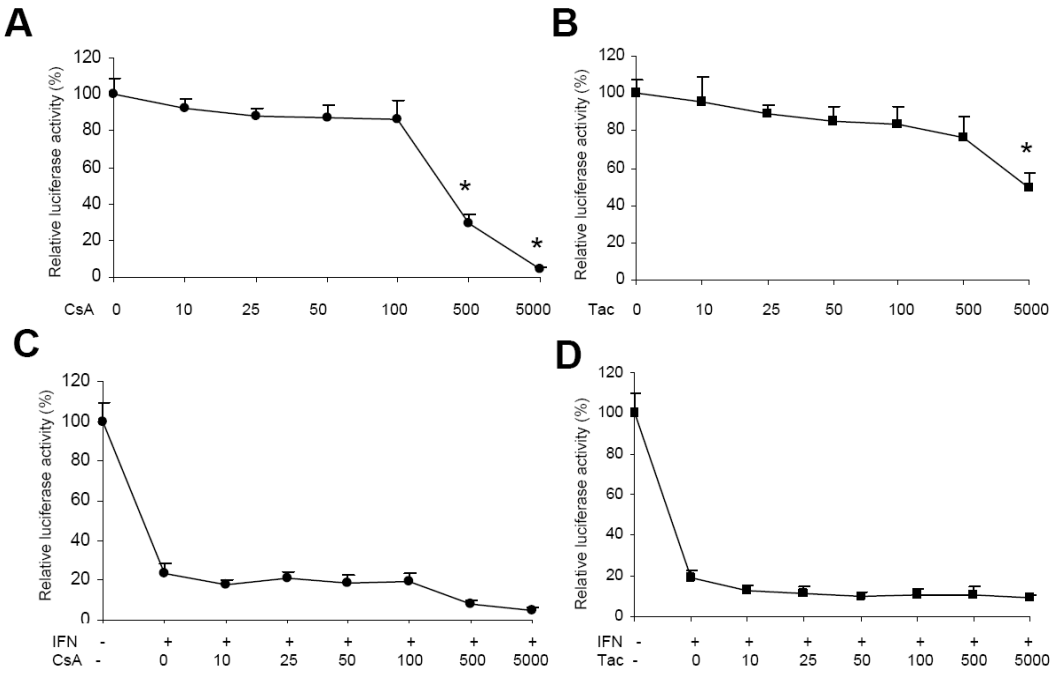

Figure 3. Effect of CsA and Tac on IFN-α mediated suppression of viral replication in subgenomic HCV replicon cells. A) Treatment with CsA alone significantly reduced viral luciferase activity at 500 ng/ml dose by 71.6% ± 5.1. In contrast, Tac alone had a much smaller effect on HCV replication, although at 5000 ng/ml dose significantly inhibited viral luciferase activity. (C) Treatment of low dose of IFN-α (1 IU/ml) resulted in a significant inhibition of viral replication ($P < 0.01$). Combined treatment with CsA (10-100ng/ml) had no effect on IFN-α mediated suppression of viral replication, while at higher dose (500 or 5000ng/ml) an enhanced inhibition of viral replication was observed. (D) Combined treatment of IFN-α with Tac at any dose did not interfere the antiviral activity of interferon. Data are presented as mean ± SD of six independent experiments.

In Huh7 ET cells, treatment with CsA alone significantly reduced viral luciferase activity (mean inhibition of 71.6% ± 5.1 SD at 500 ng/ml dose, n=6, $P < 0.01$) (Fig. 3A) without strong effect on control luciferase activity in Huh7-PGK-Luc cells (Fig. 2C). In contrast, Tac alone had a much smaller effect on HCV replication, although at 5000 ng/ml dose significantly reduced viral luciferase activity (51% ± 8, mean ± SD, n=5, $P < 0.01$) (Fig. 3B) but with clear effect on control luciferase (Fig. 2C). As shown in Figure 3C, treatment with low dose IFN-α (1 IU/ml) resulted in inhibition of HCV replication by 73.2% ± 4.5 (mean ± SD, n=6, $P < 0.01$). Combined treatment of IFN-α with CsA had no effect on suppression of viral replication at low doses of CsA (10-100 ng/ml) where as, consistent with previous studies, [7, 18-20] at higher dose of CsA an enhanced inhibition of HCV replication was observed (Fig. 3C). However, combined treatment of IFN-α with Tac did not attenuate the suppression of viral replication at any dose tested (Fig. 3D). Similar results were obtained when Huh7 ET cells were pre-treated

with Tac or CsA for 16 hrs before IFN-α stimulation (data not shown). Overall, no evidence was found that Tac or CsA interfere with the inhibition of HCV replication by IFN-α.

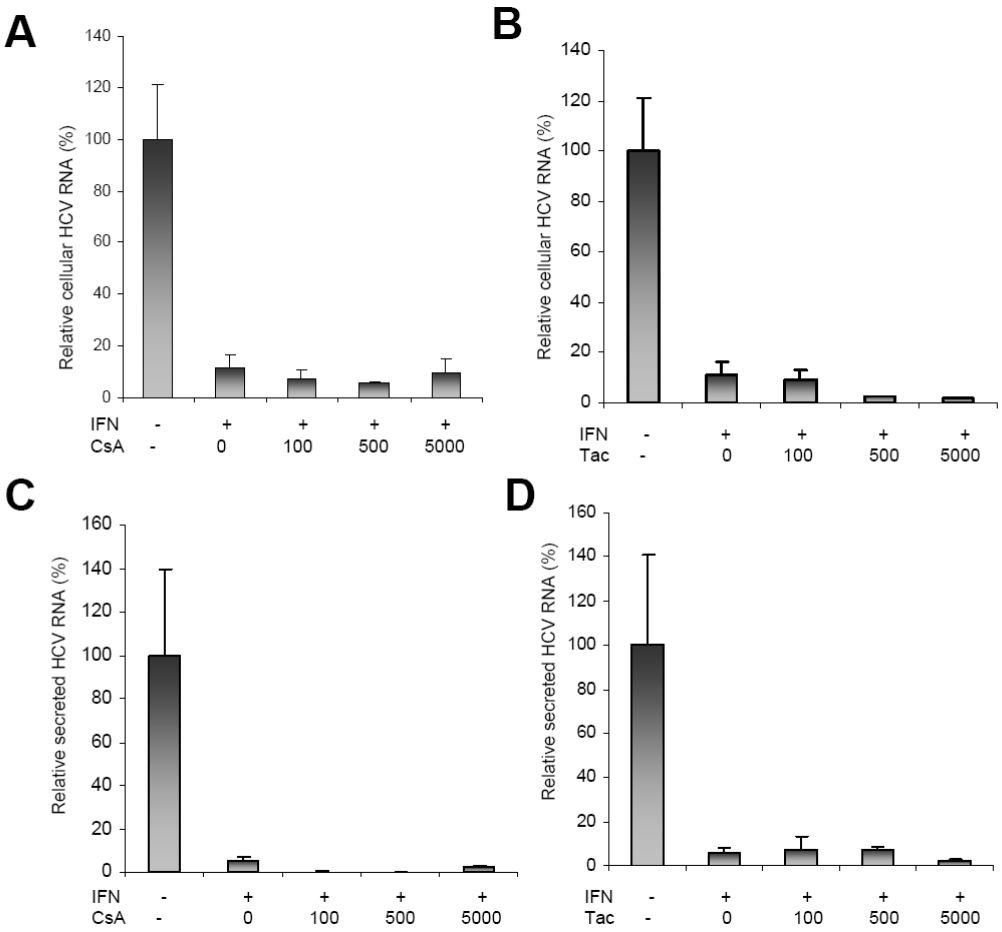

Figure 4. IFN-α mediated suppression of viral replication and virion production in the JFH1-derived infectious HCV model is not affected by Tac. Four days treatment of IFN-α (10 IU/ml) profoundly inhibited production of both cellular (A, B) and secreted (C, D) viral RNA levels. Combined treatment of IFN-α with different concentrations of CsA (A) or Tac (B) did not affect the inhibition of viral RNA replication in the cells. Furthermore, no effect was observed on the secretion of virus particles after combined treatment with different concentrations of CsA (C) or Tac (D). Data are presented as mean ± SD of three independent experiments.

Effect of Tac and CsA on antiviral action of IFN-α in the infectious HCV model

The obvious limitation of subgenomic HCV models is the lack of a productive infection. With the identification of a genotype 2a isolate, JFH1, an infectious HCV model was established which allows the study of the complete viral lifecycle in culture, including the assembly and release of infectious particles. [22] Using this model, we evaluated the effect of CNI on the inhibition of both viral replication and the secretion of de novo viral particles. Four days treatment of IFN-α (10 IU/ml) profoundly inhibited the production of cellular and secreted

viral RNA levels by 91.7% ± 1.7 and 93.5% ± 2.4 (n=3, P < 0.01), respectively (Figure 4). Consistent with the results in the subgenomic replicon model, combination of IFN-α and CsA (Fig. 4A) or Tac (Fig. 4B) did not interfere with the inhibition of viral RNA levels. Moreover no interfere was observed on the secretion of virus particles after combined treatment with CsA (Fig. 4C) or Tac (Fig. 4D). Although the highest dose of Tac accentuated the inhibitory effect of IFN, it is most likely due to the non-specific effect of on host cells. These results in the infectious HCV model again did not show evidence that Tac or CsA interfere with IFN-α signaling.

Discussion

In the current study, we found no evidence that either CsA or Tac interferes with IFN-α induced gene expression (Fig. 1) or IFN-α mediated antiviral activity against HCV (Fig. 3-4). For our study we used two well-established HCV culture models, [22, 24] which allowed determination of both viral replication and the production and release of infectious HCV particles. We confirmed results from earlier studies, [7, 8, 18-20] showing that CsA has antiviral activity alone and in combination with IFN-α (Fig. 3A and 3C, Fig. 4A and Fig. 4C). Treatment with Tac alone showed limited antiviral activity, as shown earlier. [7, 19, 25] Importantly, however, we did not observe any inhibitory effect of Tac on IFN-α signaling (Fig 1B) or its antiviral activity (Fig. 3D, Fig. 4B and Fig. 4D), in contrast to previous reports. [15, 16] In both the interferon reporter assay and HCV culture models, clinically relevant concentrations of Tac reached in serum of organ transplant recipients (5-30 ng/ml) [26] had no negative effect on IFN-α activity.

Of note, there are several clear differences between our study and that of Hirano et al, [16] including drug dosing, HCV models and experimental cell lines. In the earlier study, effects on IFN-α signaling were only observed at extremely high concentrations of Tac (5-10 μM), which is equivalent to 4000-8000 ng/ml and more than two log higher than serum concentration in Tac-treated patients. In our study, we applied a proper internal luciferase control to distinguish and normalize for non-specific effects of drugs on cell viability, cell proliferation and bioluminescence activity. Indeed, as shown in Figure 2, 5000 ng/ml Tac significantly affected the number of viable cells and total luciferase activity of Huh7-PGK-Luc.

Moreover, we have tested the effects of Tac in both subgenomic (genotype 1b) and infectious (genotype 2a) HCV models, while Hirano et al only used 1b subgenomic model. Although Huh7.5 cells, the host of infectious HCV, is defective in RIG-I pathway, an important pathway in IFN stimulation, 293T cells which we used for the IFN reporter array have been reported to be competent in this pathway. [27] Therefore, RIG-I is olso unlikely an explanation for the differences between our study and that of Hirano et al, although the exact factors determining the different conclusions remain unclear.

To investigate the effects of CNI on IFN-α signaling bears significant clinical relevance. Although a multitude of studies have demonstrated the potent antiviral effects of CsA in vitro, the clinical evidence that CsA has an impact on HCV infection after transplantation is rather limited. In contrast to its limited potency in monotherapy, substantial clinical studies has suggested its benefit when combined with pegylated IFN-α [25, 28-30] However, this superiority of CsA over Tac was failed to observe in chronic HCV patients by other studies. [31, 32] Since lacking of proper randomized clinical trial, basic experimental data appears to be important in the debate of this controversial issue.

In this study, we show no evidence that Tac interferes with IFN-α-mediated inhibition of HCV replication and virion production *in vitro*, suggesting no mechanistic argument is found to break the clinical controversy about choice of calcineurin inhibitor during post-transplantation antiviral therapy.

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Chapter 4

Mycophenolic acid augments interferon-stimulated gene expression and inhibits hepatitis C virus infection *in vitro* and *in vivo*

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Hepatology, 2011, Epub

Abstract

Previously, we demonstrated that mycophenolic acid (MPA), an immunosuppressant commonly used in organ transplantation, effectively inhibited hepatitis C virus (HCV) replication in synergy with interferon-α (IFN-α). MPA is a potent IMPDH inhibitor but its anti-viral mechanism is less understood. The aim of this study is to investigate the anti-HCV mechanism of MPA and the molecular basis for it's synergy with IFN-α. The role of IMPDH and interferon-stimulated genes (ISGs) was investigated in two HCV models using gain or loss-of-function approaches. *In vivo* effects of MPA treatment was studies in NOD/SCID mice engrafted with HCV replicon cells. Potent anti-viral effects of MPA at clinically relevant concentrations were observed in both the subgenomic and JFH1-derived infectious HCV models. In mice, MPA treatment led to robust inhibition of HCV replication by 76% ± 12 SD ($P < .05$). Ectopic expression of a MPA-resistant IMPDH2 mutant in HCV host cells completely reversed the anti-proliferative effect of MPA but only partially affected the anti-viral potency. However, similar to ribavirin, MPA induced expression of multiple anti-viral ISGs, including IRF1. Co-treatment of MPA with IFN-α resulted in additive effects on ISG expression and enhanced IFN-induced luciferase reporter activity. Knockdown of IRF1, but not IFITM3, significantly attenuated the inhibition of HCV replication by MPA. In conclusion, MPA exerts a potent anti-HCV effect *in vitro* and in mice and acts in synergy with IFN-α. MPA's anti-HCV activity is partially dependent of IMPDH but also involves stimulation of ISGs, providing a molecular basis for it's synergy with IFN-α.

Introduction

End-stage liver diseases caused by chronic hepatitis C virus (HCV) infection has become the leading indication for liver transplantation worldwide.[1] However, HCV re-infection of the graft occurs universally and rapidly after transplantation with much poorer response rate to the current standard therapy of pegylated interferon-alpha (IFN-α) in combination with ribavirin,[2] which subsequently results in accelerated recurrence of liver fibrosis and early development of cirrhosis.[3] The more aggravated course of HCV recurrence and relative resistance to anti-viral therapy have been attributed to several host and viral factors including genotype, donor age and in particular the application of specific immunosuppressive medication.

Mycophenolic acid (MPA) is the activated component of the pro-drug mycophenolate mofetil (MMF), a highly effective immunosuppressant that lacks the nephrotoxicity associated with CNI-based immunosuppression. Therefore, MMF is commonly used in kidney transplantation, but it is also approved for the prophylaxis of allograft rejection after cardiac or liver transplantation. Recently, MMF-based CNI-free or CNI-reduction strategies gain more interest in liver transplantation to prevent renal dysfunction.[4-6] Previous work from our group has shown that MPA, which is known to have anti-viral activity against many viruses,[7-10] also has potent inhibitory effects on HCV replication and further act in synergy with IFN-α.[11]

MPA acts as an uncompetitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), in particular the isoform IMPDH2. Inhibition of IMPDH decreases intracellular levels of guanosine nucleotide pools resulting in inadequate quantities for nominal DNA duplication.[12] Guanosine depletion was thought to be responsible for the anti-proliferative and immunosuppressive effects of MPA.[13] This mechanism likely is also responsible for the anti-viral effects of MPA against the west nile,[8, 11] yellow fever[9] and chikungunya[10] viruses, as supplementation by exogenous guanosine near-completely overcomes these inhibitory effects. To the contrary, supplementation of guanosine had only little effect on the inhibition of HCV replication by MPA,[11, 14] suggesting also other mechanisms are involved.

Recent evidence suggests that ribavirin, which is used with pegylated IFN-α for treatment of HCV, has the previously unrecognized ability to induce the expression of

interferon-stimulates genes (ISGs).[15] Ribavirin is a synthetic nucleoside analogue which is a known inhibitor of the IMPDH enzyme, be it with less potency than MPA. Though ribavirin monotherapy appears to have only limited clinical efficacy, *in vitro* studies showed that ribavirin has a remarkable broad anti-viral activity against a spectrum of RNA and DNA viruses.[16] Thomas et al.[15] showed that ribavirin potentiated the anti-viral action of IFN-α by inducing the expression of multiple ISGs, including IRF7 and IRF9, known to be critical for defenses against HCV. These findings are consistent with an earlier clinical study showing ribavirin combination treatment results in more rapid and higher interferon-induced cytokine production as compared to patients receiving IFN- monotherapy.[17] The exact mechanism how ribavirin induces the expression of ISGs remains enigmatic but seems to involve the direct activation of the so-called interferon-stimulated response element (ISRE), known to promote transcription of most ISGs.[18] Whether other IMPDH inhibitors, like MPA, have similar effects on ISG expression as ribavirin remains unknown.

The aim of the current study was to gain more insight in the anti-viral mechanism of action of MPA. We investigated the effects of MPA *in vitro* using the JFH1-derived infectious HCV model and in a mouse model using subgenomic HCV replicon cells. Using a specific MPA-resistant IMPDH2 mutant, we demonstrated the anti-HCV effect of MPA is largely independent of the IMPDH enzymatic activity. However, similar to ribavirin, MPA treatment alone or combined with IFN-α augmented the expression of multiple ISGs by activation of the ISRE promoter element. These results provide new mechanistic insights into the anti-viral effects of MPA and it's synergy with interferon.

Materials & Methods

Reagents

Stock of MPA (Novartis Pharma AG, Basel, Switzerland) was dissolved in DMSO and IFN-α 2a (Roche Ltd, Basel, Switzerland) was dissolved in water. All agents were stored in 15 μl aliquots and frozen at –20ºC. Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin and L-glutamine were obtained from Invitrogen–Gibco (Breda, The Netherlands). Fetal calf serum was obtained from Hyclone (Logan, Utah). Geneticin (G418),

luciferin potassium salt, guanosine (diluted to 1 mM in PBS and stored at -20ºC) and SYBRGreen were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Cell culture

Cell monolayers of the human embryonic kidney epithelial cell line 293T and human hepatoma cell lines Huh7, Huh7-ET, Huh7.5.1 and Huh6 were maintained in complete DMEM (cDMEM) containing 10% v/v fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Stable luciferase expressing cells were generated by transducing naïve Huh7 cells with a lentiviral vector expressing the firefly luciferase gene (LV-PGK-Luc). Transduced cells were expanded for at least 10 days before use in experiments.

HCV culture models

Huh7-ET replicon was based on Huh7 cells containing a subgenomic HCV bicistronic replicon (I389/NS3-3V/LucUbiNeo-ET).[19] The viral replication of Huh7-ET can be monitored by measuring luciferase activity. The Huh6 replicon contains Con1 replicon without reporter gene.[20] Both replicons were maintained with 250 μg/ml G418. Huh7 cells harboring the full-length JFH1-derived genome was used as an infectious HCV model.[21] Infected Huh7 cells were treated with MPA for six days. In order to normalize cell numbers as a consequence of inhibition of cell proliferation by MPA, cells were harvested at day five and re-seeded at same density. 24hrs after re-seeding, cells and conditioned cultured medium were harvested and analyzed for HCV viral RNA content by RT-PCR quantification. Conditioned cultured medium was centrifugated at 4000 rpm for 10 min to remove cell debris. For secondary infection, naïve Huh7 cells were incubated with conditioned medium diluted 1:1 with fresh cDMEM medium (not containing MPA) and, after 24 hrs, analysed for cellular HCV RNA.

HCV mouse model

Huh7-ET HCV replicon cells and control Huh7 cells constitutively expressing luciferase gene (1 x 10^6 cells in 200 L PBS) were simultaneously engrafted into the right and left flanks of 8-week-old female NOD/LtSz-scid/scid (NOD/SCID) mice. After overnight recovery, mice were intraperitoneally injected with 100 μl of 50 mg/ml D-luciferin potassium salt dissolved

in PBS (Caliper Life Sciences, Hopkinton, MA). After 10 minutes, the pre-treatment luciferase activity was measured in living mice using an IVIS camera under anaesthesia by 1.5% isoflurane inhalation. Immediately after measurement, 50 mg/kg body weight of MPA (in 200 µl) was intraperitoneally injected. Control animals were intraperitoneally injected with PBS/10%DMSO as vehicle control. The dose of MPA used was based on previous studies of preclinical efficacy and toxicology of MPA in experimental mice models.[22-23] 24 hrs after injection, luciferase activity was measured again as a post-treatment value.

Lentiviral vector (LV) production and transduction

The LV backbone plasmid for expression of mutated IMPDH2 (LV-mutIMPDH2) was gifted by Dr. Jiing-Kuan Yee (City of Hope National Medical Center, USA). The LV-ISRE transcriptional reporter system was obtained from SBI Systems Biosciences (Mountain View, CA, USA). The LV expressing the firefly luciferase gene under control of the human phosphoglycerate kinase (PGK) promoter (LV-PGK-Luc) was a kind gift of Dr. Pascal van der Wegen (Erasmus MC, Rotterdam, The Netherlands). Plasmids (Sigma-Aldrich) for expression of interfering short hairpin RNA (shRNA) targeting IRF1, IFITM3 or GFP were obtained from Erasmus Center for Biomics (Rotterdam, The Netherlands). LV were prepared as previously published.[24] In brief, a third-generation lentiviral packaging system was used to produce high-titer VSV-G-pseudotyped LV in 293T cells. Packaging cell supernatants were removed 36 and 48 hr post-transfection, passed through a 0.45 µM filter, and concentrated 1000-fold by ultracentrifugation. LV pellet was resuspended in PBS.

Since the LV shRNA vectors contain puromycin resistance gene, stably transduced Huh7 cells can be selected by culturing in 1 µg/ml puromycin to clear the non-transduced cells.[25]

Cell number counting

Huh7 cell transduced with or without LV-mutIMPDH2 were treated with MPA for seven days. The number of viable cells was counted by staining with trypan blue solution, after trypsinization.

Interferon signaling reporter assay

It is known that the expression of most ISGs is regulated by the ISRE promoter element. Type I interferon-stimulation will trigger the binding of particular transcription factors to the ISRE, thereby enhancing the transcription of the ISGs.[26] To mimic this biological process, we used a LV transcriptional reporter system expressing the firefly luciferase gene driven by a minimal CMV basal promoter containing multiple ISREs.

The HCV permissive Huh7 cells were transduced with LV-ISRE-Luc to create a stable reporter cell line. Transduced cells were plated in 96-well multiplates and treated with IFN-α, MPA or a combination. After 24 hr of culture, the luciferase activity was measured. For this, 100 mM luciferin potassium salt was added to cells and incubated for 30 min at 37ºC. Luciferase activity was quantified using a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

Real-time RT-PCR

RNA was isolated using a Machery-Nagel NucleoSpin RNA II kit (Bioké, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using an iScript cDNA Synthesis Kit from Bio-Rad (Bio-Rad Laboratories, Stanford, CA, USA). The cDNA of IRF1, IRF7, IRF9, IFITM3, HCV IRES and GAPDH were quantified using real-time PCR (MJ Research Opticon, Hercules, CA, USA) performed with SYBRGreen according to manufacturer's instructions. GAPDH was used as reference gene to normalize gene expression.

Statistical analysis

Statistical analysis was performed by using either the matched-pair nonparametric test (Wilcoxon signed-rank test) or the non-paired, nonparametric test (Mann–Whitney test) using Graphpad Prism software. P-values less than 0.05 were considered as statistically significant.

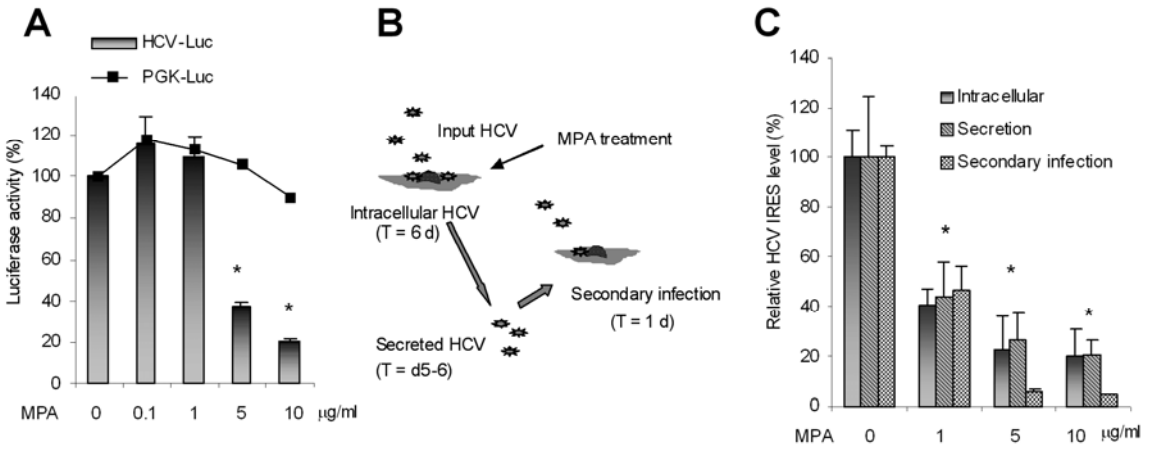

Figure 1. Potent and specific anti-viral activity of MPA in subgenomic and infectious HCV culture models. (A) Huh7-ET cells with the subgenomic HCV replicon containing the luciferase reporter gene and control luciferase expressing Huh7 cells were treated for 24 hrs (short-term) with a dose-range of MPA. At 5 and 10 µg/ml concentration, MPA significantly inhibited HCV luciferase activity by 62% ± 1 and 79% ± 0.5 (* $P < 0.01$, mean ± SD, n=6) but did not significantly affect control luciferase activity (B) Design of experiments in the JFH-1 derived infectious HCV model. Huh7 cells were treated with different concentrations of MPA starting directly after inoculation with JFH-1 HCV. At day five, cells were harvested and re-seeded at same density for all conditions and further cultured for 24 hrs. At day six, cells and conditioned culture medium were analysed for HCV viral RNA by RT-PCR. For secondary infection, naïve Huh7 cells were incubated with 50% diluted conditioned medium and analysed for cellular HCV RNA after 24 hrs. (C) Treatment of MPA (1, 5 or 10 µg/ml) significantly reduced both cellular and secreted HCV RNA, as well as secondary infection. Strongest effects were observed with 10 µg/ml MPA, showing inhibition of cellular HCV RNA by a mean of 80% ± 11, inhibition of secreted HCV RNA by 79% ± 6 and inhibition of secondary infectivity of the supernatants by 96% ± 0.1. Shown are the mean of relative HCV RNA levels of three independent experiments ± SD. * $P < 0.01$

Results

Potent anti-viral activity of MPA in subgenomic and infectious HCV culture models

We and others previously showed in two subgenomic HCV replicon models that treatment with clinical relevant doses of MPA resulted in potent inhibition of viral replication which was not due to loss of cell proliferation or cell viability.[11, 14] To further confirm the specificity of the anti-viral effect of MPA, we repeated experiments with Huh7-ET cells and included Huh7 cells constitutively expressing the luciferase reporter gene not controlled by a viral promoter but the human PGK promoter.[27] As shown in Figure 1A, 24 hrs (short-term) treatment with 5 and 10 µg/ml MPA significantly reduced HCV IRES-driven luciferase activity but did not significantly affect the control, PGK-driven, luciferase activity. This result

confirms that MPA specifically acts on viral replication and does not interfere with luciferase enzymatic activity or cell viability/numbers.

With the identification of a genotype 2a isolate, JFH1, an infectious HCV model was established which allows the study of the complete viral lifecycle in culture, including the assembly and release of infectious particles.[21] Using this model, the effects of MPA on primary and secondary HCV infection were investigated (Figure 1B). As shown in Figure 1C, treatment of 1, 5 or 10 μg/ml MPA significantly reduced both cellular and secreted HCV RNA. Strongest effects were observed with 10 μg/ml MPA, showing inhibition of cellular HCV RNA by a mean of 80% ± 11 SD, inhibition of secreted HCV by 79% ± 6 SD as well as inhibition secondary infectivity of secreted HCV by 96% ± 0.1.

MPA inhibits HCV replication in mice

To further evaluate the anti-viral potency of MPA *in vivo*, we used an HCV replicon based mouse model by engrafting Huh7-ET cells subcutaneously in the flank of immunodeficient NOD/SCID mice. As a control for potential non-specific effects on injected cells or luciferase activity, Huh7 cells stably expressing luciferase under control of the PGK promotor were engrafted in the opposite flank of the same animal. After overnight engraftment of Huh7 cells, mice were treated for 24 hrs with MPA (50 mg/kg body weight) or vehicle control. Luciferase activity was measured in live animals 24 hrs after the treatment. As shown in Figure 2A, MPA treatment resulted in a robust inhibition of HCV replicon luciferase as compared to the vehicle control treatment. Both control and MPA treatments did not affect signals of the control luciferase Huh7 cells. Quantification of luciferase activities in all mice showed that MPA treatment did not significantly alter the control luciferase activity (Figure 2*B*), but resulted in a 76% ± 12 SD reduction in HCV replication (n=7) compared to the vehicle control treated group (n=7, $P < 0.05$; Figure 2C). These experiments clearly showed that systemic treatment with MPA in mice resulted in strong local inhibition of HCV replication in ectopically engrafted human hepatoma cells.

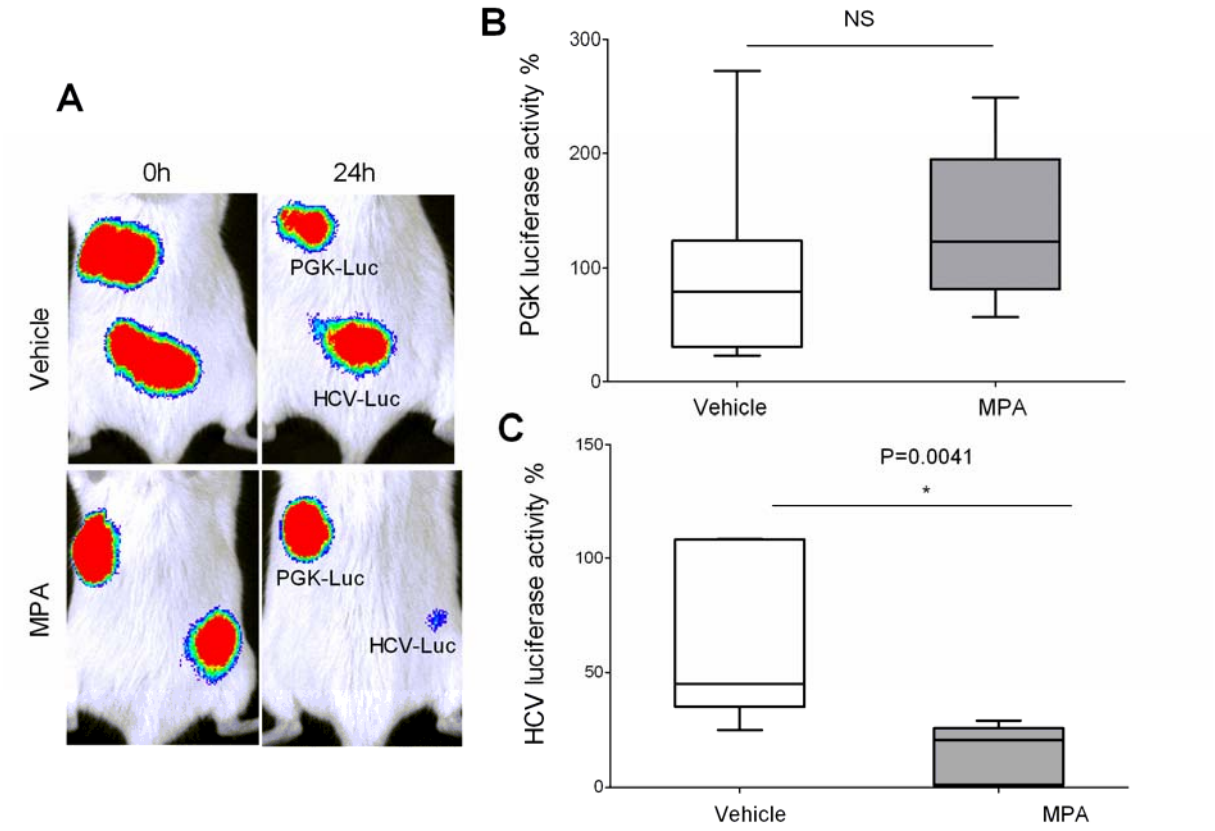

Figure 2. MPA treatment of mice significantly inhibited HCV replication in engrafted human replicon cells. NOD/SCID mice were subcutaneously engrafted with Huh7-ET HCV replication cells (HCV-Luc) at the right flank and with control luciferase Huh7 cells (PGK-Luc) left flank of the back. After 18 hrs recovery, mice were treated with MPA or vehicle control administered intraperitoneally. (A) Before treatment (0h) and 24 hrs after treatment (24h) luciferase activity was non-invasively measured using a highly sensitive photocamera. Baseline luciferase activity of HCV-Luc and PGK-Luc cells were similar (left panels), but after 24 hrs MPA treatment dramatically reduced the luciferase activity of HCV-Luc cells as compared to PBS treatment (right panels). MPA did not affect signals of control PGK-Luc cells. (B) Quantification of PGK-Luc luciferase activity of mice treated with vehicle (n=7) or MPA (n=7). MPA or control treatments did not significantly reduced luciferase activity as compared to baseline values. (C) Quantification of HCV-Luc luciferase activity. MPA treatment resulted in a 76% ± 12 SD reduction in HCV replication (n=7) as compared to the control group (n=7).

IMPDH2 only partially contributes to the anti-HCV effect of MPA

MPA can effectively bind to IMPDH, leading to the inhibition of the enzymatic activity of *de novo* guanosine nucleotide biosynthesis. However, supplementation of exogenous guanosine only had minor effects on the restoration of the inhibitory effects of MPA on HCV replication (Figure 3A). MPA has a five-fold higher potency for inhibition of the type II isoform (IMPDH2) than the type I isoform (IMPDH1).[28] Proliferating lymphocytes depend, more than other cell types, on *de novo* synthesis of purines. Therefore MPA sensitively inhibits lymphocyte proliferation, thereby conferring immunosuppressive effects.[12] Recently,

an experimentally mutated IMPDH2 coding sequence fused to the green fluorescent protein (GFP) reporter gene was generated and expressed by a lentiviral vector (LV-mutIMPDH2). It has normal IMP hydrogenase activity but lacks the binding site for MPA. Recently, an experimental mutant of IMPDH2 has been generated, which has normal IMP hydrogenase activity but lacks the binding site for MPA. Ectopic expression of this IMPDH2 variant leads to significantly increased resistance to MPA in lymphocytes, monocytes and hematopoietic stem cells.[13]

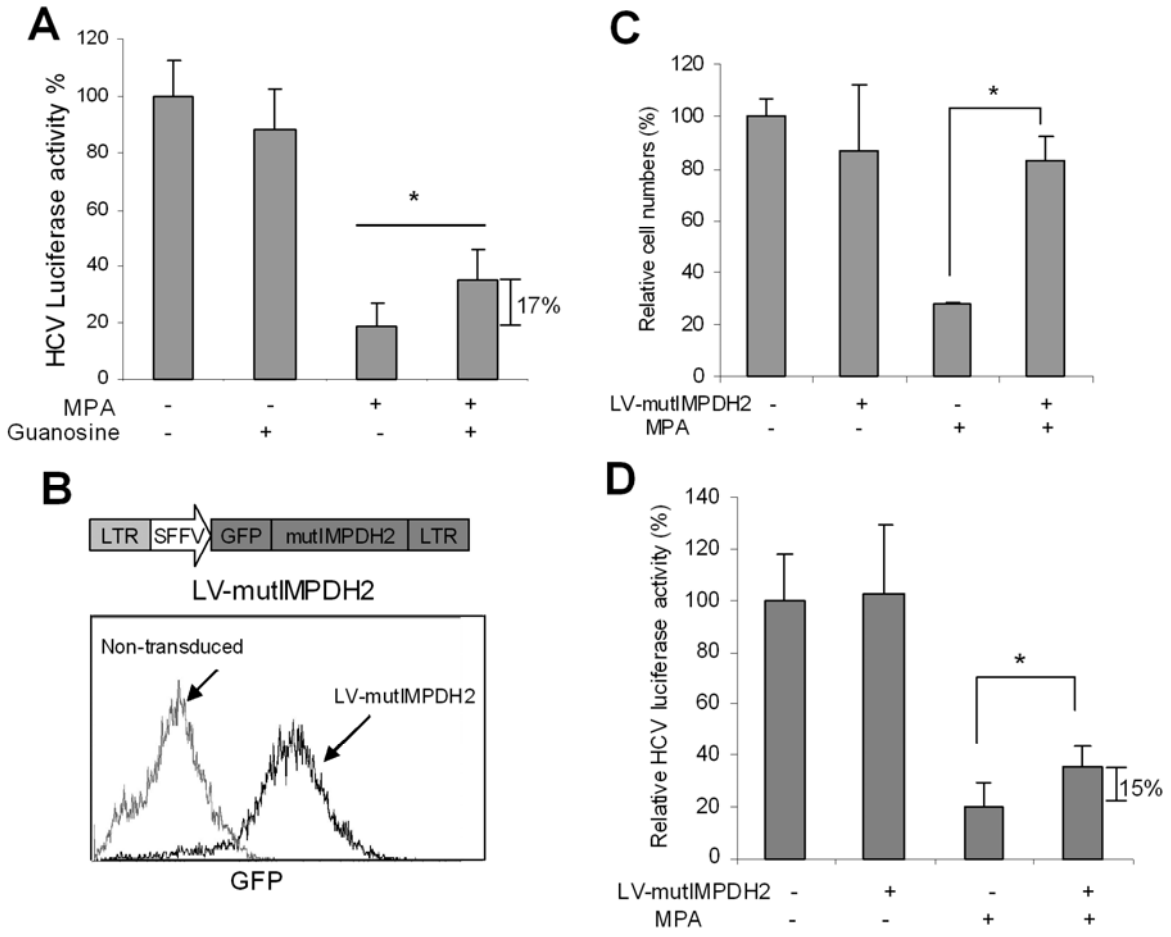

Figure 3. Ectopic expression of MPA-resistant IMPDH2 completely restored cell proliferation but hardly affected the inhibition of HCV replication by MPA. (A) Supplementation of exogenous guanosine slightly affected the anti-HCV effect of MPA in Huh7-ET replicon cells. (B) Expression of the IMPDH2-GFP fusion protein in LV-mutIMPDH2 transduced Huh7 cells could be detected by flowcytometric analysis of green fluorescence. Overexpression of IMPDH2 alone did not affect cell growth (C) and viral replication (D). (C) Upon treatment of MPA, overexpression of IMPDH2 almost completely restored the anti-proliferative effect on Huh7 cells (n=3). (D) However, HCV replication was only slightly affected, although it is significant (n=6). Data are presented as the mean ± SD. * $P < 0.01$

Previously we showed that the anti-viral activity of MPA appeared largely independent of guanosine depletion. To further investigate the role of IMPDH2 in HCV inhibition by MPA, we used a lentiviral vector expressing MPA-resistant IMPDH2 (LV-mutIMPDH2) (Figure 3B). As shown in Figure 3C, transduction of Huh7 cells with LV-mutIMPDH2 alone did not affect cell proliferation during a seven day (long-term) culture. However, these cells became almost completely resistant against the anti-proliferative effects of MPA (mean 72% ± 1 SD inhibition in control cells and 5% ± 9 in LV-mutIMPDH2 cells). Contrary, as shown in Figure 3D, expression of MPA-resistant IMPDH2 in Huh7-ET replicon cells had only minor effects on the inhibition of HCV replication (mean 80% ± 9 SD inhibition in control cells and 66% ± 8 in LV-mutIMPDH2 cells n=6, $P < 0.01$), which is consistent with the observation in Figure 3A. These findings suggest that other pathways are involved in the anti-viral action of MPA, besides the IMPDH enzymatic inhibition.

MPA induces expression of interferon-stimulated genes

Earlier *in vitro* and *in vivo* studies showed that MPA synergizes with IFN-α in inhibiting HCV.[11, 29] To further explore the mechanism of the anti-viral synergy with IFN-α, the effect of MPA on interferon induced gene expression was investigated in Huh7 and in a T-lymphocyte cell line. It is known that expression of most ISGs by interferons is regulated by the ISRE promoter element.[26] To investigate whether MPA can stimulate ISRE-driven transcription, we used a lentiviral transcriptional reporter system expressing the firefly luciferase gene driven by a promoter containing multiple ISREs (LV-IRES-Luc). Huh7 cells were transduced with the LV-IRES-Luc or control vector, LV-PGK-Luc, to create stable reporter cell lines (Figure 4A). As shown in Figure 4B, stimulation with IFN-α resulted in a dose-dependent induction of ISRE regulated luciferase activity (up to 230% of baseline activity with 100 IU/ml), without stimulation of control luciferase activity. Surprisingly, treatment with MPA, at clinically achievable doses, also resulted in a dose-dependent induction of ISRE-related luciferase activity without affecting the control luciferase activity (Figure 4C). At 5 μg/ml of MPA the ISRE luciferase activity was 131% ± 6 (mean ± SEM, n=7, $P < 0.01$) of baseline activity, which is equivalent to stimulation with approx. 2-4 IU/ml IFN-α.

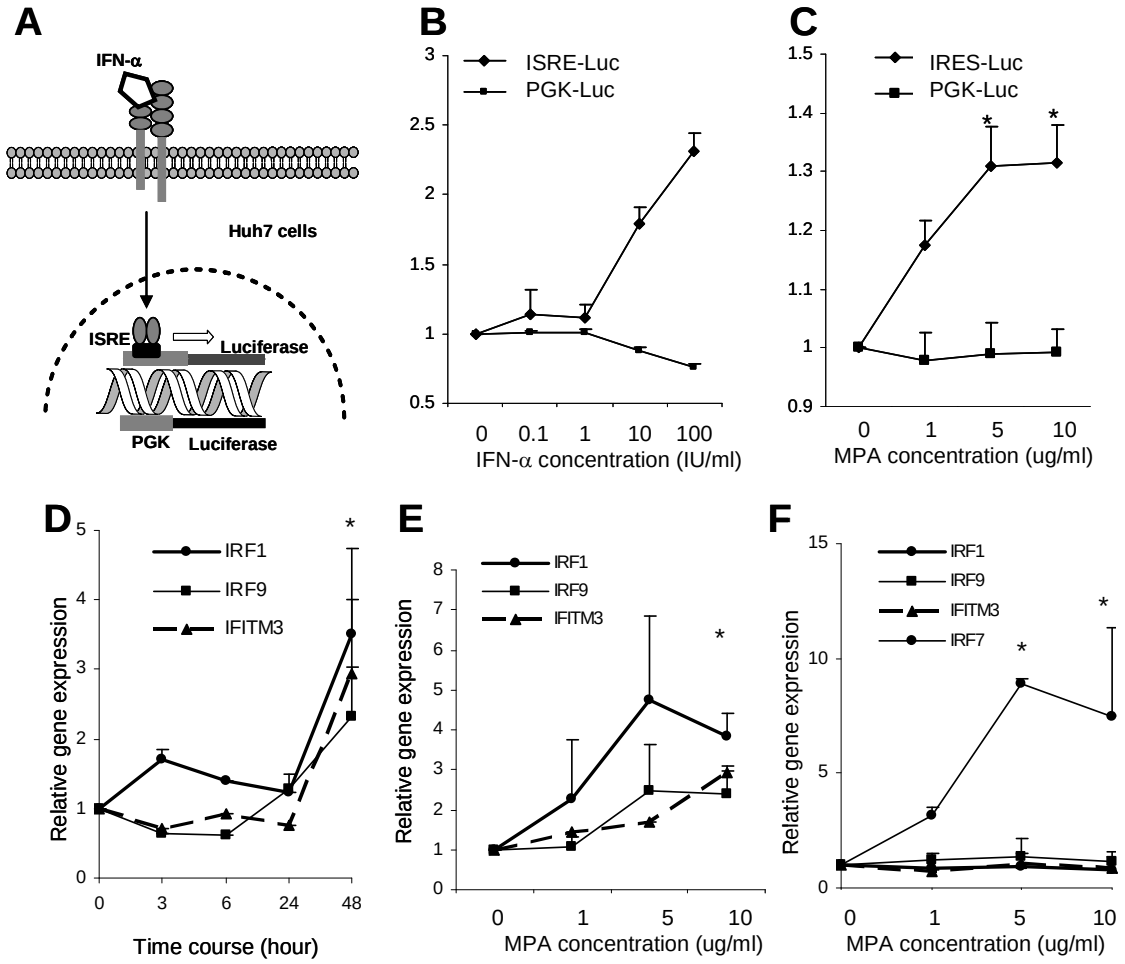

Figure 4. MPA induced the expression of interferon-stimulated genes. (A) To study IFN-α stimulated gene expression, a stable Huh7 reporter cell line was used containing the firefly luciferase gene under the control of multiple ISRE promoter elements (ISRE-Luc). As control, Huh7 cells constitutively expressing luciferase under control of human PGK promoter (PGK-Luc) were used, to rule out non-specific effects on host cells or bioluminescence. (B) Stimulation with IFN-α for 24 hours resulted in dose-dependent enhancement of ISRE-regulated luciferase activity, up to 2.5-times the baseline level at 100 IU/ml concentrations. No stimulation of control luciferase activity was observed in PGK-Luc cells. (C) Treatment of the Huh7 reporter cells with different doses of MPA (1, 5 or 10 μg/ml for 24 hrs) resulted in a dose-dependent induction of ISRE regulated luciferase activity without affecting the control PGK luciferase activity. (D) Treatment of naïve Huh7 cells with 10 μg/ml MPA up to 48 hrs results in a significant upregulation of IRF1 (n=6), IRF9 (n=9) and IFITM3 (n=5) expression. The induction of IRF1 gene expression occurred as early as 3 hrs after treatment, whereas highest expression of all ISGs was observed after 48 hrs. (E) Induction of IRF1, IRF9 and IFITM3 gene expression by MPA is dose-dependent. Cells were treated with MPA for 48 hrs and shown is the mean of four independent experiments. (F) The induction of these three ISGs was not observed in the T lymphocyte cell line SupT1, treated with MPA for 48 hrs. Instead, IRF7 was significantly induced. All gene expression was normalized to the reference gene, GAPDH. Data are presented as the mean ± SD. * $P < 0.01$

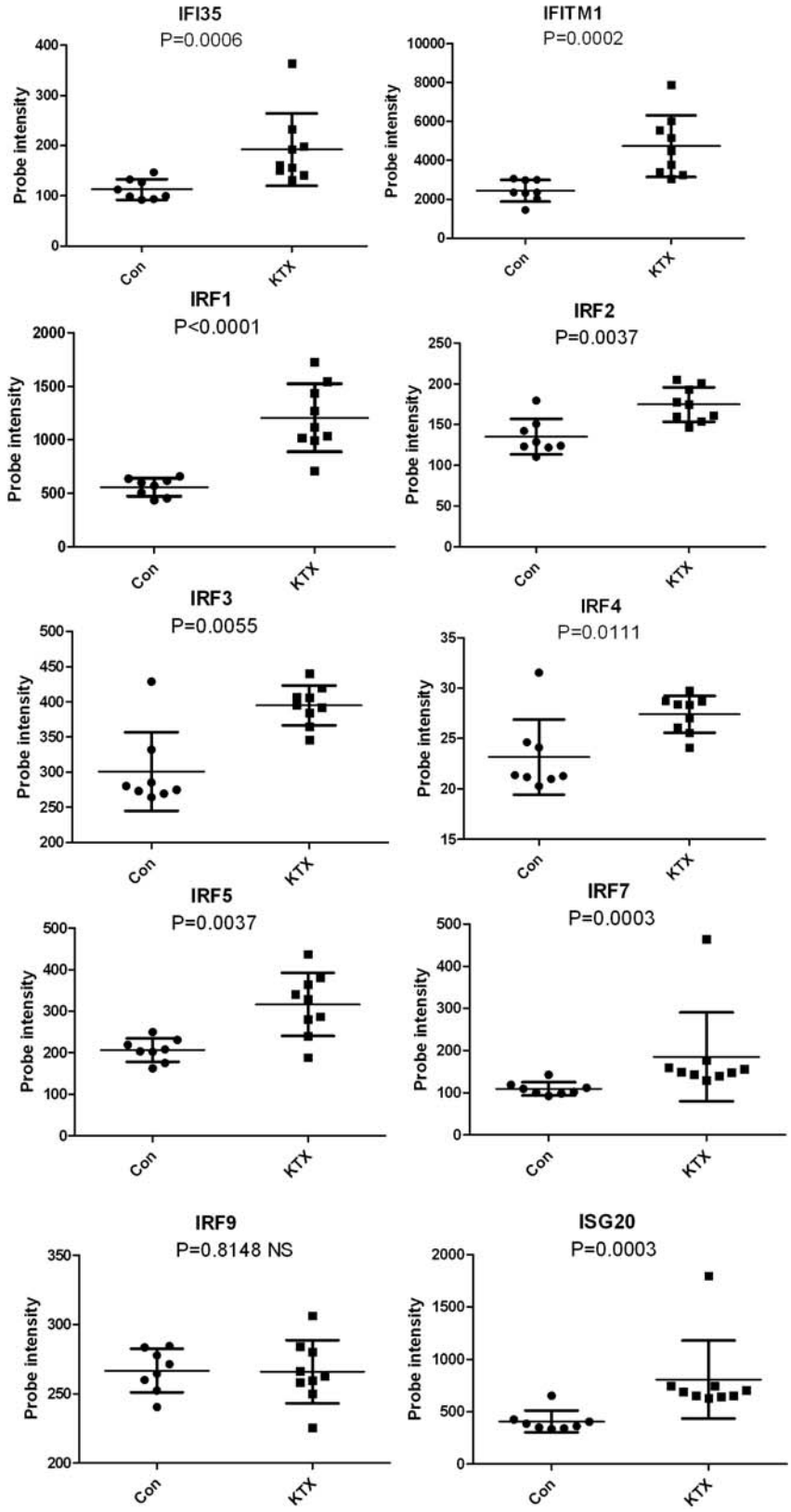

Figure 5. Analysis of genome-wide expression profile of peripheral blood mononuclear cells (PBMC) from kidney transplantation patients, who were treated with MMF for induction of immunosuppression (GEO accession: GSE1563). Compared with PBMC from healthy volunteers (red bar, n=8), several Interferon-Induced Genes (ISGs) were significantly upregulated in MMF treated patients (blue bar, n=9). These ISGs include interferon regulatory factor (IRF) 1, 2, 3, 4, 5, 7, interferon-induced transmembrane protein 1 (IFITM1), interferon-induced protein 35 (IFI35), interferon-stimulated exonuclease gene 20 KDa (ISG20).

To test whether activation of ISRE-induced transcription indeed resulted in induction of ISGs, we analyzed the expression of IRF1, IRF7, IRF9 and IFITM3 in Huh7 cells treated with

MPA. In particular, IRF1, IRF7 and IFITM3 are well-known for their broad anti-viral effects.[30-32] Consistent with the enhanced ISRE activity, we observed significant upregulation of IRF1, IRF9 and IFITM3 (Figure 4D and E), but IRF7 was below detection level (data not shown). Highest expression levels were observed at 48 hrs of treatment reaching up to 4-times the base line expression for IRF1 (Figure 4D). Remarkably, induction of IRF1 was already observed after 3 hrs of MPA treatment (approx. 70% increase). In contrast, the induction of IRF9 and IFITM3 did not occur in the first 24 hrs but was significantly upregulated at 48 hrs (Figure 4D). The induction of IRF1, IRF9 and IFITM3 by MPA in Huh7 cells showed clear dose-dependency (Figure 4E), but the induction was not observed in the T cell line, SupT1 (Figure 4F). Although IRF7 was not detectable in Huh7 cells, it was significantly upregulated in SupT1 cells after MPA treatment (Figure 4F). These findings suggest that the induction of ISGs by MPA may be a general effect, but the type of ISGs may vary per cell type. Consistently, genome-wide gene expression profiling of peripheral blood mononuclear cells (PBMC) from kidney transplantation patients on MMF immunosuppression (n=9) showed that several ISGs were significantly upregulated as compared to PBMC of healthy controls (n=8). As shown in Figure 5, these ISGs included interferon regulatory factors (IRF) 1, 2, 3, 4, 5, 7, interferon-induced transmembrane protein 1 (IFITM1), interferon-induced protein 35 (IFI35) and interferon-stimulated exonuclease gene 20 KDa (ISG20). Distinct from the anti-proliferative mechanism, ectopic expression of mutated IMPDH2 or addition of exogenous guanosine had no effect on the induction of ISGs by MPA (data not shown), suggesting that inhibition of IMPDH activity and induction of ISGs are two independent pathways.

MPA augments IFN-α-induced ISG expression

To address whether MPA can further potentiate the interferon-induced ISRE transcription activity, we treated the Huh7 reporter cells with a combination of IFN and MPA. As shown in Figure 6A, combining stimulation with IFN-α at different concentrations (1, 10 or 100 IU/ml) with different doses of MPA (1, 5 or 10 μg/ml) resulted in a general increase of ISRE-driven luciferase activity as compared to IFN-α treatment alone. For instance, combination of 1 μg/ml MPA with 10 IU of IFN-α significantly increased the ISRE-driven luciferase actvitivity by a mean 36% ± 14 SEM (n=6, $P < 0.05$) compared to IFN-α alone. Consistent with enhanced ISRE stimulation, combining MPA with IFN-α significantly enhanced the induction

of IRF1 (Figure 6B), IRF9 (Figure 6C) and IFITM3 (Figure 6D) expression (n=3, *P* <0.01). For IRF1 and IFITM3, the combined effect of IFN-α and MPA seems additive, whereas for the combined induction of IRF9 appeared to be synergistic (upregulation of IRF9 mRNA levels was 1.7 fold ± 0.3 SD with 5 μg/ml MPA alone, 9.3 fold ± 2.2 SD with 10 IU/ml IFN-α alone and 31.8 fold ± 12.5 SD with combination of MPA and IFN-α (Figure 6C). Taken together, these results showed that the treatment of hepatocyte-like cells with MPA induced the expression of ISGs and, in combination with IFN-α, further augmented the expression of these anti-viral effectors in an either additive or synergistic fashion. These results support our previous finding showing synergistic anti-HCV effects of MPA and IFN-α.[11]

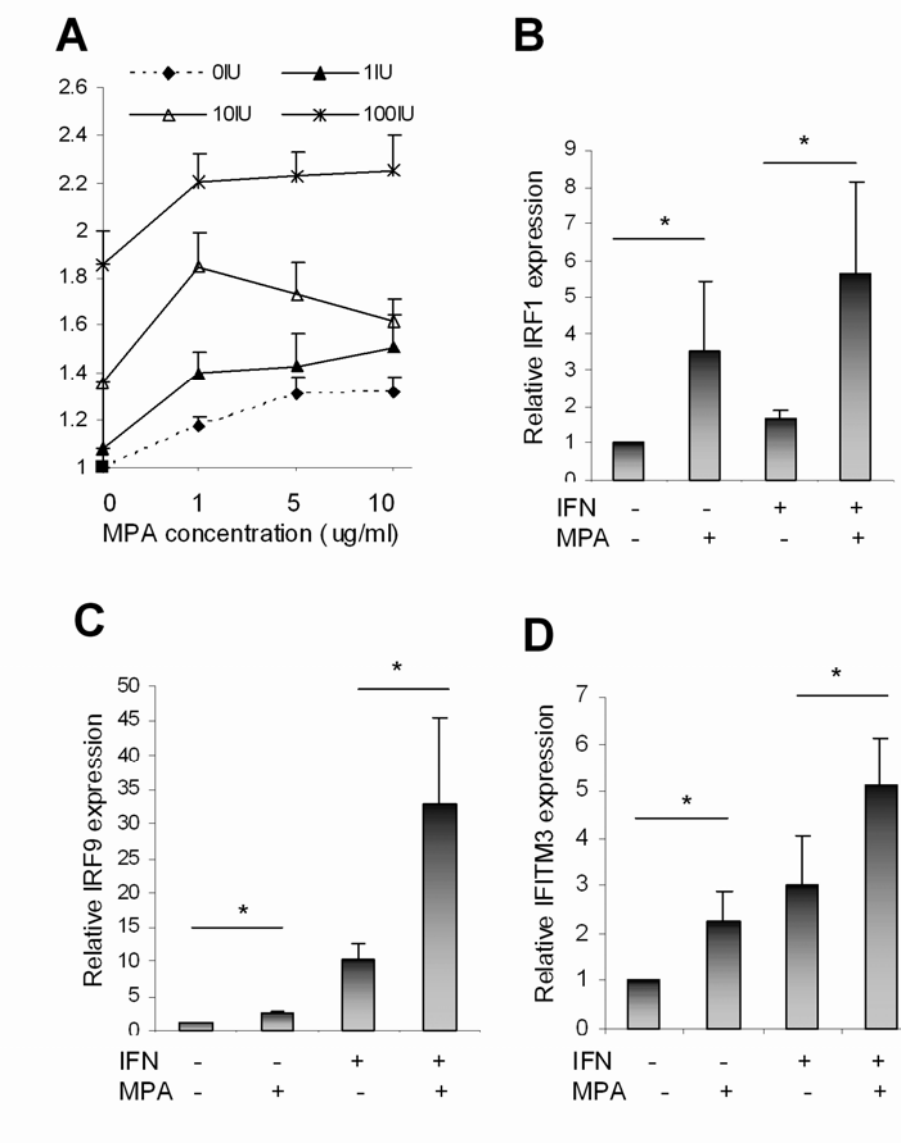

Figure 6. MPA potentiated IFN-α-induced ISG expression. (A) The stable Huh7 reporter cell line containing the firefly luciferase gene under the control of multiple ISRE promoter elements (ISRE-Luc) was used to investigate the combined effects of IFN-α and MPA. Cells were stimulated with 1, 10 or 100 IU/ml IFN-α alone or in combination with different doses of MPA (1, 5 or 10 μg/ml) for 24 hrs. At all concentrations of IFN, MPA resulted in a significant increase of ISRE-driven luciferase activity as compared to IFN-α alone (P < 0.05). Consistent with enhanced ISRE stimulation, 48 hrs treatment of naïve Huh7 cells with IFN-α (10 IU/ml) combined with MPA (5 μg/ml) enhanced the induction of ISGs. Gene expression of (B) IRF1, (C) IRF9 and (D) IFITM3 was significantly higher compared to treatment with IFN or MPA alone. Shown is the mean of three independent experiments and gene expression was normalized to GAPDH. *P < 0.01

Anti-HCV activity of MPA is partially mediated by IRF1 but not IFITM3

A recent study by the group of Rice has identified IRF1 out of 380 human ISGs as the most potent ISG to inhibit HCV replication.[32] To investigate the role of IRF1 in the inhibition of HCV by MPA, a Huh7 cell line was generated stably expressing shRNA directed against IRF1. For this, Huh7cells were transduced with a vector expressing shRNA complementary to the human IRF1 RNA transcript (shIRF1). Vectors containing shRNA targeting IFITM3 (shIFITM3) or GFP (shCon) served as controls. As shown in Figure 7A, shIRF1 significantly reduced IRF1 gene expression as compared to shCon control cells. Treatment with MPA resulted in upregulation of IRF1 in both shIRF1 and shGFP cells, but was significantly lower in the shIRF1 compared to shCon cells (1.5 fold ± 0.6 SD versus 3.2 fold ± 1.1 SD, $P < 0.05$, Figure 7A). Similar results were obtained with shIFITM3, resulting in an over 70% reduction in IFITM3 gene expression in MPA or untreated cells (Figure 7B). Remarkably, given the relative low potency of shIRF1 to reduce IRF1 gene expression (approx. 50% reduction), a significant loss of anti-viral effect of MPA was observed in shIRF1 cells challenged with JFH1 HCV (Figure 7C). Treatment with MPA resulted in an inhibition of HCV infection by 83% and 85% in control cells containing shCon or shIFITM3, respectively. However, in cells expressing shIRF1 the observed inhibition of HCV infection was only 67%, which is approx. 20% less inhibition than in shIFITM3 and shCon controls (n=4, $P < 0.05$). Likely, the loss of inhibition by MPA would have been higher if a more potent shRNAs against IRF1 would have been available. Of note, we screened five different IRF1 shRNA sequences, but none resulted in greater knockdown than 50% of IRF1 expression, which unfortunately is not unusual for shRNAs. Overall these findings clearly indicated that IRF1 is involved in the anti-viral effects of MPA.

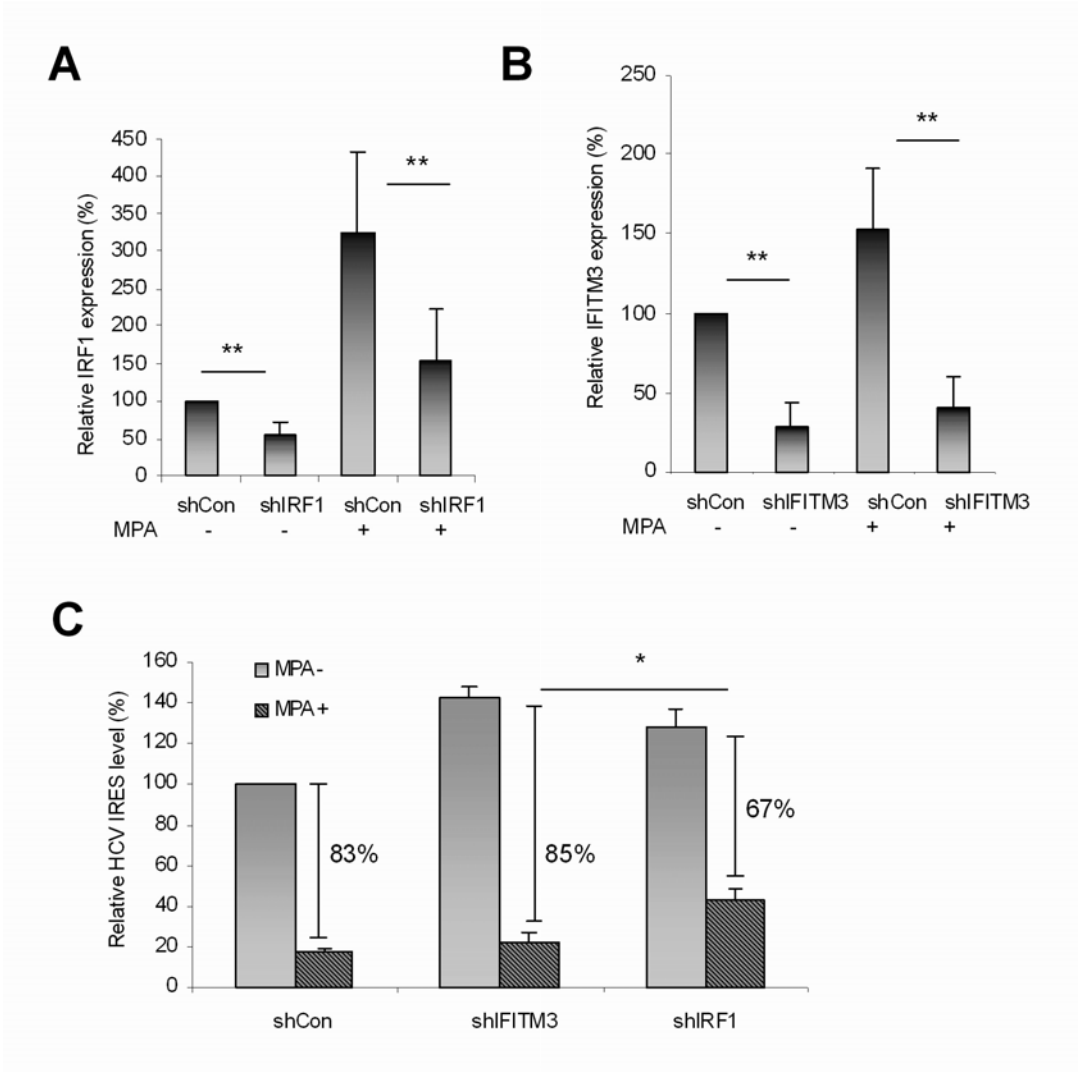

Figure 7. IRF1 but not IFITM3 contributed to the anti-HCV activity of MPA. Naïve Huh7 cells were stably transduced with lentiviral vectors expressing shRNA targeting IRF1 (shIRF1), IFITM3 (shIFITM3) or negative control GFP (shCon). (A) IRF1 gene expression. IRF1 mRNA levels were approx. 50% reduced by shIRF1, as compared to shCon, in Huh7 cells with or without MPA treatment (10 µg/ml). (B). IFITM3 gene expression. ShIFITM3 resulted in an over 70% reduction in IFITM3 gene expression both in MPA or untreated cells. (C) Effect of shRNA on HCV infection and inhibition by MPA. Huh7 cells were inoculated with infectious HCV particles and subsequently treated with10 µg/ml MPA for four days. HCV RNA levels were determined by IRES-specific qRT-PCR. Both shIRF1 and shIFITM3 resulted in higher HCV infection levels compared to shCon by 28% and 42%, respectively. Treatment by MPA resulted in a mean inhibition of HCV infection by 83% ± 2 and 85% ± 5 for shCon and shIFITM3 expressing cells, respectively, similar to non-transduced Huh7 cells (Fig. 1C). However, MPA treatment in shIRF1 expressing cells only resulted in a mean inhibition of HCV infection by 67% ± 6. Shown is the mean ± SD of three or four independent experiments.* $P < 0.05$, **$P < 0.01$

Discussion

In the current study we demonstrated that MPA significantly inhibited HCV replication and infection, both in subgenomic and infectious culture models (Figure 1). This anti-viral effect was confirmed *in vivo* after systemic treatment with MPA in mice bearing subgenomic HCV replicon cells (Figure 2). The anti-viral activity of MPA was found to be only partially dependent on IMPDH2 (Figure 3) but also involved the upregulation and action of ISGs like IRF1 (Figure 4 and 6). When combined with IFN-α, MPA significantly promoted the induction of ISGs (Figure 6). These findings provided mechanistic supports for previously reported synergic effects of MPA and IFN-α on HCV infection.[11, 29] Effects of MPA on HCV and ISGs were observed at doses from 1 to 10 μg/ml, which are clinically achievable concentrations. In liver transplant recipients receiving MMF or MPA, serum peak levels range from 0.6 to 11.5 μg/ml and trough levels average around 3 μg/ml.[33] Moreover, animal studies have indicated that MPA accelerates in the liver and that drug levels in hepatocytes will exceed those observed in serum.[34]

We found that clinical relevant doses of MPA could directly induce the expression of the important anti-viral ISGs, IRF1, IRF9 and IFITM3 (Figure 4). The induction of these ISGs by MPA was comparable to the levels observed after stimulation with 1 to 5 IU/ml IFN-α. Combining MPA and IFN-α resulted in additive effects on the induction of IRF1 and IFITM3 and even synergistic effects on IRF9 expression (Figure 6). In particular IRF1, but also IRF7 and IRF9 have been shown to have potent anti-viral effects on HCV.[30, 32] IFITM3 was recently identified as an important ISG for the innate cellular defense against H1N1 influenza, West Nile and dengue viruses.[31] The role of IFITM3 in HCV infection is less well established, though we did observe elevated HCV infectivity in Huh7 cells with reduced IFITM3 expression (Figure 7C). However, knockdown of IFITM3 gene expression did not interfere with the inhibition of HCV infection by MPA. In contrast, a mere 50% knockdown of IRF1 gene expression by shRNA resulted in significant loss of HCV inhibition by MPA of approx. 20% (Figure 7C). These findings suggest that IRF1, but not IFITM3, mediate part of the anti-viral effect of MPA, but likely other ISGs or other effector molecules may be also involved in this process. In human, approximately 400 ISGs have been identified, many of which have individually no or limited anti-viral effects, but in combined action with other ISGs have an important contribution to

the anti-viral effects of interferons.[32] Therefore it is tempting to hypothesize that, in addition to IRF1, the anti-viral action of MPA involves the concerted action of multiple other ISGs.

Our experiments with the T-lymphocyte cell line suggest that the type of ISGs induced by MPA may be very different between different cell lines. In the hepatocyte-like cell line Huh7, both ribavirin[15] and MPA (Figure 4) induced the expression of IRF9, but this was not observed in T cells. Contrary, MPA treatment of T cells induced the expression of IRF7, which was not detectable by in Huh7 cells (Figure 4). MPA's potent induction of IRF1 expression in Huh7 cells was not observed in T cells or macrophages,[35] and even reduced IRF1 expression has been observed in rat fibroblasts[35] and astrocytes.[36] Though further study is required, these findings indicate that the transcriptional regulation of ISGs by MPA is cell type-specific. Furthermore, it will be interesting to investigate whether some of the tissue type-specific adverse effects of MPA treatment, like observed in the intestine or bone marrow compartments, are related to the induction of particular ISGs.

The transcriptional activity of most ISGs is regulated by the ISRE promoter element.[26] Interferon-stimulation will trigger the binding of IFN-stimulated gene factor 3 (ISGF-3) to the ISRE, thereby enhancing the transcription of the ISGs. In the present study we found that MPA, like IFN-α, significantly induced ISRE-driven transcription in a luciferase reporter assay. When MPA was used in combination with IFN-α a significant increase of ISRE activation was observed (Figure 6A). Recently, we reported that also ribavirin has the ability to enhance ISRE-driven transcription activity,[18] consistent with the study of Thomas et al.[15] who showed that ribavirin induced expression of multiple ISGs. Further study is required to determine the exact molecular mechanism of ISG induction and understand the interplay between inteferons, ribavirin and MPA. This may help advancing our knowledge on immune-suppressive and anti-viral strategies for HCV recurrence after liver transplantation.

Despite the safety and efficacy of MMF as immunosuppressive medication in HCV transplant patents has been demonstrated,[6, 37] the exact effects on HCV infection has not been clearly studied in prospective, randomized and double-blinded trials. In the organ transplantation setting, MMF has been reported to reduce the incidence of HCV recurrence used at optimal doses and for a minimal period of one year after liver transplantation.[38] Several studies, including two prospective non-randomized studies, showed a significant viral load reduction by MMF treatment,[39-41] whereas three other studies reported no effect of MPA on HCV infection.[42-44] However, the effects of MMF on HCV-related liver fibrosis

progression appears less controversial with several prospective/randomized studies[43-44] and retrospective studies[38, 40, 45] showing a significant benefit of MMF. Interestingly, no clear anti-viral effect was observed with MPA monotherapy in chronic HCV patients outside the transplant setting.[46] However, similar effects were observed for ribavirin, showing substantial anti-HCV activity *in vitro*[15, 47] but is generally considered to have little or no detectable anti-viral activity as monotherapy in patients.[48] By analyzing the early viral kinetics during ribavirin monotherapy, minor and transient effects were observed on HCV viral load.[49] However, when combined with IFN-α, ribavirin clearly increases the sustained virologic response by 3-fold.[50] *In vitro*, we found that MPA, like ribavirin, acts in synergy with IFN-α on HCV replication.[11] Consistent with this synergistic action, an earlier clinical study showed that combining MPA with pegylated IFN-α in chronic HCV patients resulted in a significantly better end-of-therapy response rate (72%) over the amantadine/pegIFN-α group (42%) or standard treatment group (ribavirin/pegIFN-α, 59%) in a difficult-to-treat population of previous non-responders to standard therapy.[29] This effect of MPA on the end-of-therapy response rate was not observed in another study using ineffective anti-viral therapy with non-pegylated IFN-α.[51] In contrast to ribavirin, both studies showed no benefit of MPA on the sustained virological response rate of the IFN-α therapy.[29, 51] It is known that most anti-viral compounds can cause a good end-of-therapy response rate that is often not sufficient to result in an effective, immune-mediated, elimination of the virus which is required for a curative sustained virologic response. It is conceivable that the potent immunosuppressive activity of MPA in patients (more potent than ribavirin) interferes with the anti-viral immune responses and ultimate clearance of the virus by the immune system. This may explain the discrepancy between the clear and broad anti-viral effects of MPA observed in culture models,[7-10] and in immunodeficient mice (Figure 2) and the less clear anti-viral effects of MPA treatment in immune-competent patients. Thus, the suppressive effects on host immune responses could mask changes in viral replication (i.e. viral load) caused by the ant-viral activity of immunosuppressive drugs such as cyclosporine A (CsA) and MPA. Consistent with this theory, the non-immunosuppressive derivative of CsA, Debio 025, was shown to have much more anti-viral activity in animals and patients than immunosuppressive CsA.[52-55] It will therefore be interesting to further study non-

immunosuppressive derivatives for MPA for their anti-viral properties *in vitro* and in patients.

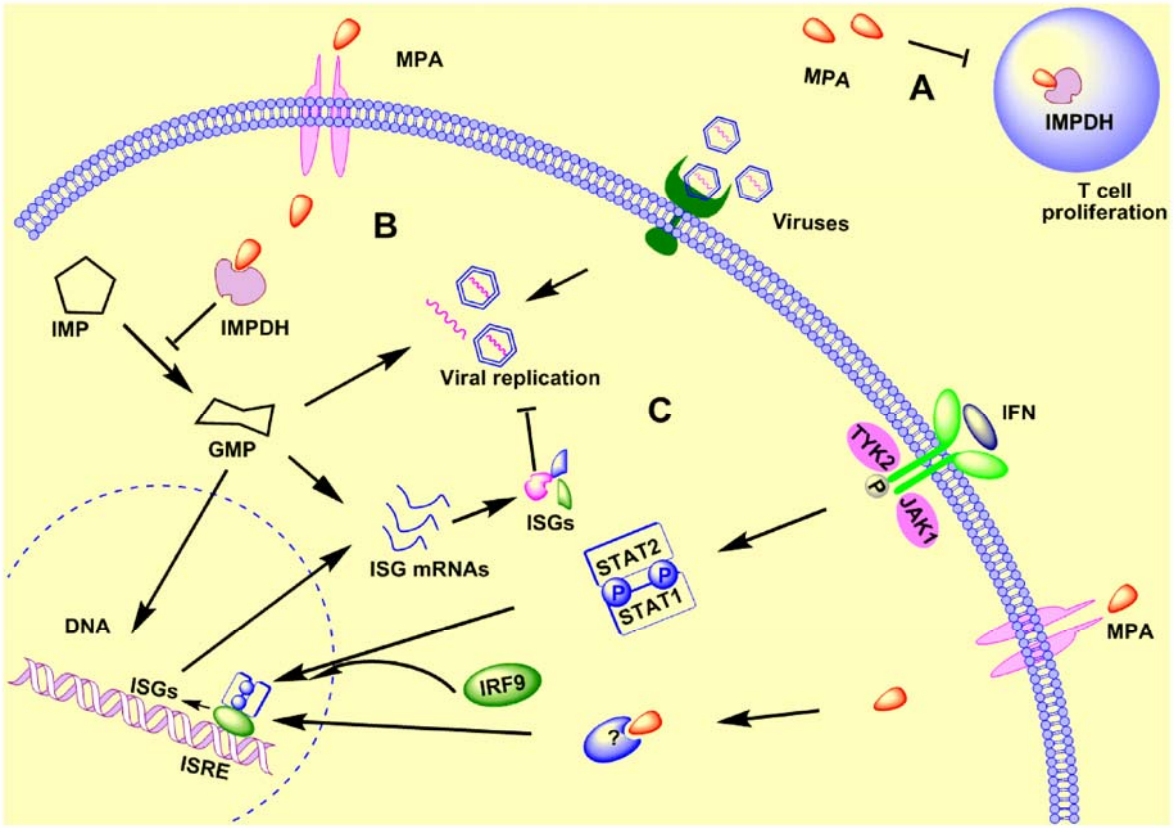

Figure 8. Proposed anti-viral mechanisms of MPA. The uptake of MPA by cells involves organic anion transporting polypeptides (AOTPs) and potential other transporters. Intracellular, MPA acts as an uncompetitive inhibitor of IMPDH, in particular the isoform IMPDH2, and results in the inhibition of the enzymatic activity of de novo nucleotide biosynthesis. (A) Lymphocytes are highly dependent on de novo synthesis of purines for cell proliferation and therefore are most sensitive to the inhibition by MPA. In addition, the present study provided evidence that MPA induces expression of the ISG, IRF7 (Fig. 4). (B) In epithelial cells like hepatocytes, persistent attenuation of nucleotide biosynthesis by MPA leads to GTP depletion and potential inhibition of viral replication. In addition to this IMPDH-dependent anti-viral pathway, MPA also conveys a rapid anti-viral effect mediated by induction of ISG expression (C). Trough an unknown mechanism, MPA directly potentiates the activity of the ISRE promoter element both in the presence or absence of IFN-stimulation. In particular, the induction of IRF1 expression by MPA in hepatocyte-like cells seems to contribute to the IMPDH2-independent inhibition of HCV infection. The sensitivity to either or both of these anti-viral pathways depends on the type of host cell, the type of virus and dosing/duration of treatment by MPA.

As proposed in Figure 8, the general anti-viral mechanism of MPA involves two distinct pathways, an IMPDH dependent pathway and an IMPDH-independent ISG pathway.

Inhibition of IMPDH by MPA reduces the *de novo* production of GTP nucleotides which are required for DNA and RNA synthesis. The inhibition of many viruses by MPA, including west nile virus,[8, 11] yellow fever virus,[9] and chikungunya virus[10] seem to predominately depend on this pathway as replication is fully restored by supplementation with exogenous guanosine. In case of HCV, however, IMPDH inhibition and ISG induction simultaneously contributed to the anti-viral action of MPA. Conceivably, MPA may be beneficial in reducing HCV recurrence after liver transplantation.

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Chapter 5

Ribavirin enhances interferon-stimulated gene transcription by activation of the interferon-stimulated response element

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Modified from:

Hepatology, 2011, 53(4):1400-1

Introduction

Ribavirin, a synthetic nucleoside analogue, is used in combination with pegylated interferon-α (IFN-α) as the standard of care for the treatment of patients with chronic hepatitis C. The combination of ribavirin significantly improves the sustained virologic response of IFN-α therapy, but the exact mechanism remains enigmatic.[1] Though ribavirin monotherapy appears to have only limited clinical efficacy,[1-2] *in vitro* studies showed that ribavirin by itself has a remarkable broad antiviral activity against a spectrum RNA and DNA viruses, equivalent to interferons.[3] Now, an exciting new study by Thomas et al. in Hepatology[4] shows that ribavirin treatment induces the expression of particular interferon-stimulates genes (ISGs), including IRF7 and IRF9, and thereby potentiates the antiviral action of IFN-α in hepatitis C virus (HCV) cell culture models. As the transcription factors IRF7 and IRF9 are known to be critical for antiviral defenses, including against HCV infection, the authors conclude that antiviral action of ribavirin alone[1-2] and in particular in combination with IFN-α[4] acts via the induction of ISGs. This study supports earlier clinical evidence by the same group that patients receiving ribavirin in addition to IFN-α had a more rapid and higher elevation of interferon-induced cytokine, IP-10/CXCL10.[5] The authors put great afford in further unraveling the signal transduction events leading to induction of ISGs and ruled out the involvement of NF-κB, IPS-1 and STAT1 signaling pathways. However, the direct mechanism how ribavirin induces gene expression of ISGs was not resolved. Studying the antiviral action of another IMPDH inhibitor mycophenolic acid,[6] we now have found new further evidence on how ribavirin can promote the transcription of a broad range of ISGs.

Materials & Methods

Cell culture and HCV model

Huh7-ET replicon was based on Huh7 cells containing a subgenomic HCV bicistronic replicon (I389/NS3-3V/LucUbiNeo-ET). Cell monolayers of the human embryonic kidney epithelial cell line 293T and human hepatoma cell lines Huh7 and Huh7-ET were maintained in complete DMEM (cDMEM) containing 10% v/v fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Stable luciferase expressing cells were generated by

transducing naïve Huh7 cells with a lentiviral vector expressing the firefly luciferase gene (LV-PGK-Luc). Transduced cells were expanded for at least 10 days before use in experiments

Interferon signaling reporter assay

It is known that the expression of most ISGs is regulated by the ISRE promoter element. Type I interferon-stimulation will trigger the binding of particular transcription factors to the ISRE, thereby enhancing the transcription of the ISGs. To mimic this biological process, we used a LV transcriptional reporter system expressing the firefly luciferase gene driven by a minimal CMV basal promoter containing multiple ISREs.

The HCV permissive Huh7 cells were transduced with LV-ISRE-Luc to create a stable reporter cell line. Transduced cells were plated in 96-well multiplates and treated with IFN-α, ribavirin or a combination. After 24 hr of culture, the luciferase activity was measured. For this, 100 mM luciferin potassium salt was added to cells and incubated for 30 min at 37ºC. Luciferase activity was quantified using a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

Real-time RT-PCR

RNA was isolated using a Machery-Nagel NucleoSpin RNA II kit (Bioké, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using an iScript cDNA Synthesis Kit from Bio-Rad (Bio-Rad Laboratories, Stanford, CA, USA). The cDNA of IRF1, IRF9, IFITM3, HCV IRES and GAPDH were amplified by 40 cycles and quantified using real-time PCR (MJ Research Opticon, Hercules, CA, USA) performed with SYBRGreen according to manufacturer's instructions. GAPDH was used as reference gene to normalize gene expression.

Statistical analysis

Statistical analysis was performed by using either the matched-pair nonparametric test (Wilcoxon signed-rank test) or the non-paired, nonparametric test (Mann–Whitney test) using Graphpad Prism software. P-values less than 0.05 were considered as statistically significant.

Results & Discussion

To further investigate the specificity of the anti-viral effect of ribavirin, we tested in Huh7-ET subgenomic HCV replicon cells expressing luciferase reporter related to HCV replication. As a control, Huh7 cells constitutively expressing the luciferase reporter gene not controlled by a viral promoter but the human PGK promoter were included. As shown in Figure 1A, the specific anti-HCV effects were observed with 25 to 100 μm ribavirin treatment. We further confirmed that ribavirin were able to induce multiple ISG expression, including IRF1, IRF9 and IFITM3 (Fig. 1B).

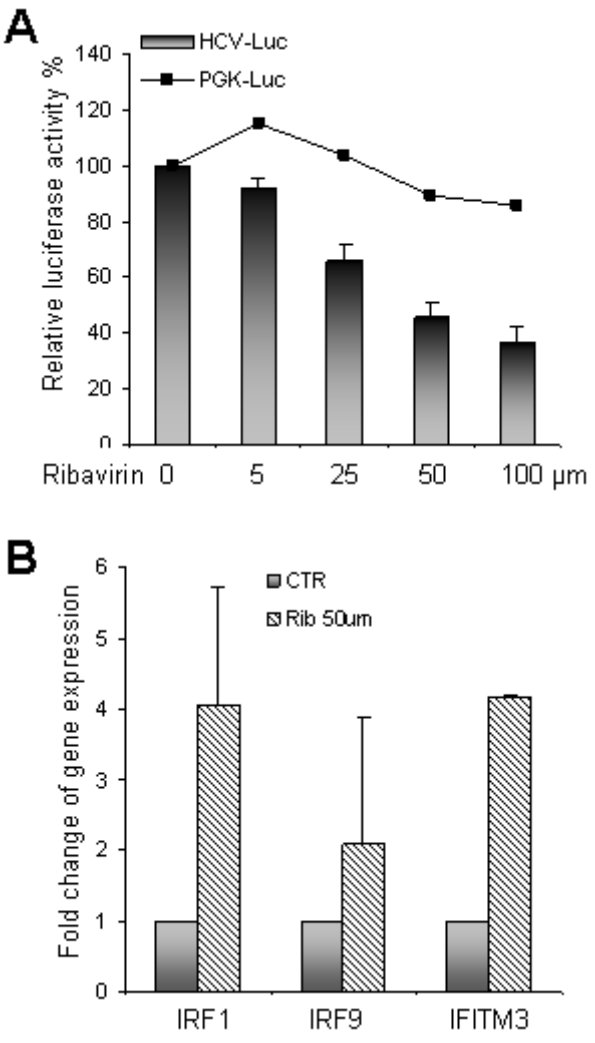

Figure 1. (A) The effects of ribavirin on HCV replication was tested in Huh7-ET replicon cells (HCV-Luc) (mean±SD). PGK-Luc indicates the effects of ribavirin on the constitutively expressed control luciferase gene in Huh7 cells. (B) The induction of IRF1, IRF9 and IFITM3 by ribavirin in Huh7 cells (mean±SD).

It is known that gene expression of many ISGs is regulated by the interferon stimulated response element (ISRE) in the promoter region. Upon exposure to type I interferon, transcriptional activity of ISGs will be enhanced by binding of different transcription factors to the ISRE.[7] To mimic this biological process, we used a lentiviral transcriptional reporter system expressing the firefly luciferase gene driven by multiple ISREs (SBI Systems Biosciences, Mountain View, CA). Huh7 cells were transduced with this vector to create a stable reporter cell line (Fig. 2A). As expected, stimulation with IFN-α resulted in a dose-dependent induction of luciferase activity (Fig. 2B), over 3-times the baseline activity. Remarkably, also treatment with clinical achievable doses of ribavirin[8] resulted in a dose-dependent induction of ISRE-related

luciferase activity (Fig. 2C). For instance, 2 ug/ml of ribavirin has significantly increased luciferase activity by 23%±2 (mean±SEM, n=5, Mann–Whitney test $p<0.05$) and 20 ug/ml increased luciferase activity by 45%±12 ($p<0.05$), compared with basal luciferase expression. No effect on cell viability or control luciferase activity was observed (not shown), suggesting ribavirin directly augment the ISRE-mediated transcription activity. To address whether ribavirin can potentiate the interferon-induced ISRE transcription activity, we treated the Huh7 reporter cells with a combination of IFN-α and ribavirin. As shown in figure 2C, combining a lowest dose of ribavirin (0.2 ug/ml) has already significantly increased the luciferase activity by 25%±7 (mean±SEM, n=8, Wilcoxon matched pairs test $P<0.05$), compared with interferon treatment alone. At the highest dose (100 ug/ml), ribavirin enhanced the luciferase activity by 65%±17 (mean±SEM, n=6, $P<0.01$).

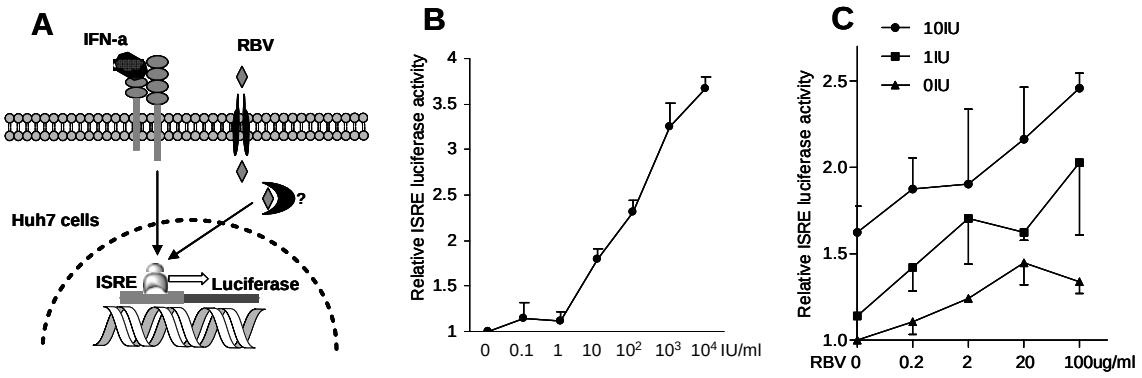

Figure 2. The effects of ribavirin on the transcription activity of ISRE. (A) A stable reporter cell line for modeling interferon response was generated by transducing Huh7 cells with a lentiviral vector expressing luciferase gene under the control of multiple ISREs with a minimal CMV basal promoter. (B) Interferon-alpha (0.1-10^4 IU/ml) treatment (24h) resulted in dose-dependent induction of ISRE-regulated luciferase activity in the reporter cell line. (C) The reporter cell line was treated with 0.2, 2, 20 or 100 ug/ml ribavirin or in combination with IFN-α (1 or 10 IU/ml) for 24 hrs. Ribavirin alone dose-dependently induced luciefrase activity. Data presented the mean and SEM of five replicates of three independent experiments. Furthermore, Ribvirin augments interferon triggered induction of luciferase activity. Data presented the mean and SEM of three independent experiments (2-3 replicates for each). The basal luciferase activity was settled as 1.

Taken together, our findings show that ribavirin potentiates the transcription activity of ISRE and can explain the enhanced expression of ISGs when combined with IFN-α.[4-5] Moreover, it is known that ISRE regulate the expression of the *IP-10/CXCL10* and *PKR* genes, thereby providing a molecular basis for the observed effect of ribavirin on the expression of these genes.[9-10] Further understanding of the interplay between inteferons and ribavirin could be useful in advancing therapeutic strategies for hepatitis C.

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Part II.

RNAi-based strategies

Chapter 6.1

Prospects of RNAi and microRNA-based therapies for hepatitis C

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Modified from:

Expert Opin Biol Ther, 2009, 9(6):713-24

Expert Opin Biol Ther, 2011, 11(3):315-27

Methods Mol Biol, 2011, 721:313-32

Abstract

BACKGROUND: Chronic viral infections have a tremendous impact on global health and therefore new therapeutic options should be urgently explored. RNA interference (RNAi) represents a promising new approach to combat viral infections and recent developments in the field of gene therapy have increased the feasibility of clinical applications. OBJECTIVE: to explore the utility of RNAi for the treatment of the ultimately life-threatening liver disease caused by the hepatitis C virus (HCV), affecting approx. 170 million people worldwide. METHODS: A comprehensive review of current developments in liver-directed gene delivery and the potential application of RNAi for the treatment of HCV is provided. In addition, the involvement of microRNAs (miRNA) in HCV infection and the potential therapeutic implications are emphasized. CONCLUSIONS: RNAi technologies have fuelled a rapid progress in the basic understanding of the HCV biology and revealed numerous new viral and host cell factors as potential targets for therapy. Together with the improvement of gene delivery technology and the discovery of the critical role of miRNA in HCV infection, RNAi and miRNA-based antiviral strategies hold great promise for the future.

1. Introduction

Infection with hepatitis C virus (HCV) is one of the major global health problems. In total, an estimated 170 million people are infected worldwide, with 3 to 4 million new infections per year. A range of 55–85% of those infected individuals fail to clear the virus and progress to a chronic infection state which is associated with a high risk of developing liver cirrhosis, hepatocellular carcinoma (HCC) or liver failure [1]. End-stage liver diseases and HCC caused by chronic HCV infection are currently the major indications for liver transplantation. However, re-infection of HCV occurs universally causing accelerated recurrence of liver disease [2-3]. Unfortunately, there is still no effective vaccine or antibodies available for the prevention of infection.

The current standard therapy of HCV is pegylated-interferon-α (Peg-IFN-α) in combination with ribavirin [4]. However, the success of the therapy is dependent on the genotype and the viral load at the start of therapy and during treatment. Overall, still half of the patients fail to develop a sustained virologic response, i.e. fail to completely eradicate the virus [5-6]. Treatment against HCV recurrence after liver transplantation is even less effective with a sustained virologic response rate of only about 20% [7-8]. Furthermore, both interferon and ribavirin are expensive and often cause severe side effects, limiting their broader use [4, 9-10].

The accumulation of basic knowledge regarding the HCV biology has dramatically speeded up due to the advances of HCV cell culture systems. Recent research has identified several promising targets for the development of novel therapies. This has resulted in the initiation of numerous clinical trials to test new viral enzyme inhibitors, immune modulators, monoclonal and polyclonal antibodies, antisense RNA and therapeutic vaccination [11-12]. Among the new technologies, RNA interference (RNAi) is one of the most promising avenues for the development of anti-viral therapies.

RNAi, only discovered a decade ago [13], is a sequence-specific inhibition of gene expression at posttranscriptional level. It is triggered by 21 nucleotides small interfering RNAs (siRNAs), which can be directly introduced into cells as synthetic siRNAs, or indirectly, as double-stranded RNAs delivered by vectors. Long double-stranded RNAs (dsRNAs) or short-hairpin RNAs (shRNAs) are cleaved into active siRNA by a cellular enzyme, Dicer. These

siRNAs are assembled into a multicomponent complex, known as the RNA-induced silencing complex (RISC), which incorporates a single strand of the siRNA serving as a guide sequence to target and silence homologous messenger RNA (mRNA) [14-15]. Indeed, such a simple, potent and specific gene silencing system has greatly fuelled the identification of novel anti-HCV targets as well as sparked the development of new therapeutic strategies. In addition, the discovery of a regulatory role for endogenous host cell microRNAs (miRNAs) in HCV viral replication have shed new light on HCV biology and represents a promising new avenue for therapeutic intervention [16]. In this article, we will provide a comprehensive overview of current developments and the potential applications of RNAi and miRNA-based antiviral strategies in HCV therapy.

2. Therapeutic application of RNAi

2.1 Viral targets

HCV contains a positive-stranded RNA genome of about 9,600 nucleotides in length, belonged to the *Flaviviridae* family. To date, six major genotypes and over 70 subtypes of HCV have been identified that differ by 31%-34% between genotypes and 20%-25% between subtypes in their nucleotide sequence [17-18]. The whole genome composes of a 5' and 3' non-coding region (NCR) flanking a single open reading frame encoding a polyprotein precursor of approximately 3,000 amino acids. The polyprotein is cleaved into four structural (core, E1, E2 and p7) and six non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins [19-20]. Since HCV is a single positive-strand RNA virus, the genomic replication is achieved by synthesis of a full-length negative-strand RNA intermediate. Negative stranded RNA in turn provides a template for the de novo production of positive-stranded RNA. NS5B, the HCV RNA-dependent RNA-polymerase, is responsible for synthesis of both the positive- and negative-stranded genomes. In general, the positive-stranded template is much more abundant than the negative strand one, because of its utilization of translation, replication, and packaging into progeny virus. Synthesis of HCV viral proteins by the host cell machinery is initiated by binding of the viral 5' NCR internal ribosome entry site (IRES) with the 40S ribosomal subunit.

As a RNA virus that the genome functions both as the viral messenger RNA and a template for viral replication, the HCV genome seems a sensitive target for RNAi. In particular, the property of HCV replication that exclusively occurs in cytoplasm leads to a better susceptibility of the virus to the host cell RNAi machinery. Within the viral genome, the IRES is the most conserved sequence and therefore may represent one of the most ideal target for RNAi [21]. Also conserved domains of HCV structural and non-structural proteins are suitable to RNAi mediated silencing.

In the past five years, a multitude of in vitro studies have been published showing that HCV infection and replication can be effectively targeted by RNAi. First studies showing efficacy used HCV non-structuaral sequences including, NS3, NS4B, NS5A and NS5B [22-25]. After that, several studies also demonstrated efficient inhibition of HCV replication by RNAi targeting the HCV IRES region [26-28]. Reduced production of infectious HCV particles was observed when cells were transduced with the IRES-targeting siRNA in cell culture models [29].

The NS5B RNA-dependent RNA-polymerase lacks proofreading abilities. As a result, HCV has a high mutation rate (10^3 per nucleotide per generation). Due to this and the high replication rate in chronic hepatitis C patients innumerable quasispecies are formed [30]. Considering this extensive genomic variation and the high mutation rate during replication, it is conceivable that the virus will likely escape sequence-restricted silencing effects of RNAi [31]. Indeed, under high pressure of siRNA, is was found that resistant replicons can emerge by the generation of point mutations within the siRNA target sequence [32-33]. Potential strategies to avoid this problem of viral resistance development by mutational escape are being developed and will be discussed in section 5.1. Additionally, the problem of resistance development against RNAi could largely be avoided by targeting host cell factors, rather then viral factors, as will be discussed in the next paragraph.

2.2 Host cell targets

Owing to development of cell culture systems for HCV [34, 35], the progress of understanding the HCV life-cycle and host cell interactions was rapidly accelerated. Several host factors essential in viral entry, i.e. CD81 [36], glycosaminoglycan [37], scavenger receptor class B type-1 (SR-BI) [38], low-density lipoprotein receptor [39], DC-SIGN [40], L-SIGN [41] and Claudin -1, -6 and -9, have been identified [42-44]. Likely, these receptors need to act in concurrence and contribute

to different stages of the entry process like viral binding, clustering, clatherin-mediated endocytosis and endosomal escape of the viral genome to the cytoplasm [45]. Most recently human occludin was identified as a crucial factor for HCV entry in human cells and combined expression with human CD81 could even render mouse cells infectable with HCV psuedotyped particles [46].

In addition to viral entry, many host factors have been identified that contribute to viral replication. The best documented factors so far are lipid and vesicle-associated membrane proteins (FBL-2, VAP-A and VAP-B) [47-49] and the chaperone proteins cyclophilins (CyPA and CyPB) [50], FKBP8 and heat-shock proteins [51]. In particular, the process of understanding HCV biology and identifying novel therapeutic targets has been greatly speeded up by successfull application of RNAi-based single target silencing as well as genome-wide screening of multiple targets. Most of these novel but less established host factors are involved in signal transduction, gene transcription, protein synthesis and RNAi pathway, but most of these are still controversial and require further investigation [52-54].

Due to high-fidelity proof reading ability of mammalian DNA polymerases host-cellular factors are not prone to mutation and, as a consequence, genetic variation is much lower as compared to that of HCV. Host cellular factors targeted by RNAi are therefore not likely to develop resistance by mutational escape. Several studies have shown that host cell factors can be effectively targeted by RNAi to silence HCV infection. For instance, knockdown of CD81 by RNAi can significantly reduce the binding of HCV envlope protein E2 to human hepatoma cells [55-56]. Indeed definitive proof of the therapeutic value of CD81 in vivo was provided by a recent study showing that treatment with anti-CD81 antibodies completely protected humanized liver-uPA-SCID mice from a challenge by different HCV genotype strains [57]. Another potential HCV receptor, SR-BI, is primarily expressed in the liver and steroidogenic tissues. A 90% downregulation of SR-BI expression in Huh7 cells by RNAi caused a 30–90% inhibition of infection by HCV envelope pseudotyped virus particles, depending on the HCV genotype [58]. Also host cell factors involved in supporting the function of HCV non-structural proteins, like NS5A and NS5B, could provide useful targets for silencing viral replication [52].

Though host cell factors offer numerous opportunities for RNAi-based therapeutic intervention, an obvious drawback to this strategy is the potential severe side-effects

silencing may have on homeostasis of hepatocytes. All the host factors identified to date are known to perform important cellular functions. Silencing of these genes could have determent effects on the function and survival of hepatocytes. These effects need to be more extensively investigated before host cell factors could be seriously considered as targets for therapy.

2.3 Gap between basic experimental investigations and clinical applications

Although the discovery of HCV in 1989 was greatly contributed by using chimpanzees model [18], wide scale use of this endangered species as laboratory animals has been limited by ethical concerns and the high financial costs. Engraftment of hepatoma cells harbouring subgenomic HCV replicon or infected virions into mice or rat served as an alternative [59-60], although these types of tumor-based models could be considered artificial. Recently, development of a chimeric mouse model has facilitated the study of HCV virology and antiviral treatment. This immunodeficient mouse model carries the mouse urokinase-type plasminogen activator (uPA) gene, which can induce severe liver toxicity. Following the transplantation of human hepatocytes, a humanized liver can be established in the mice, which supports in vivo infection of HCV [61]. However, this transgenic mouse model remains very challenging due to high mortality of homozygote mice, inconvenience logistics of obtaining fresh human hepatocytes and the required use of newborn animals. Moreover, it is an immunodificient mouse model that is clearly not suitable to study immune responses against HCV or responses against RNAi vehicles and gene therapy vectors.

Although the first proof-of-concept of applying RNAi against HCV in vivo has been provided by co-transduction in mice liver with a plasmid expressing an anti-NS5B shRNA and a plasmid containing luciferase gene fused with the HCV NS5B sequences [62], the therapeutic value of RNAi has to be further validated in better in vivo models. This is dependent on the future development of more robust and appropriate small animal models that will allow studying HCV infection and antiviral therapy in the context of a (humanized) immune system.

3. Liver-targeted delivery of synthetic siRNA or vector-expressed shRNA

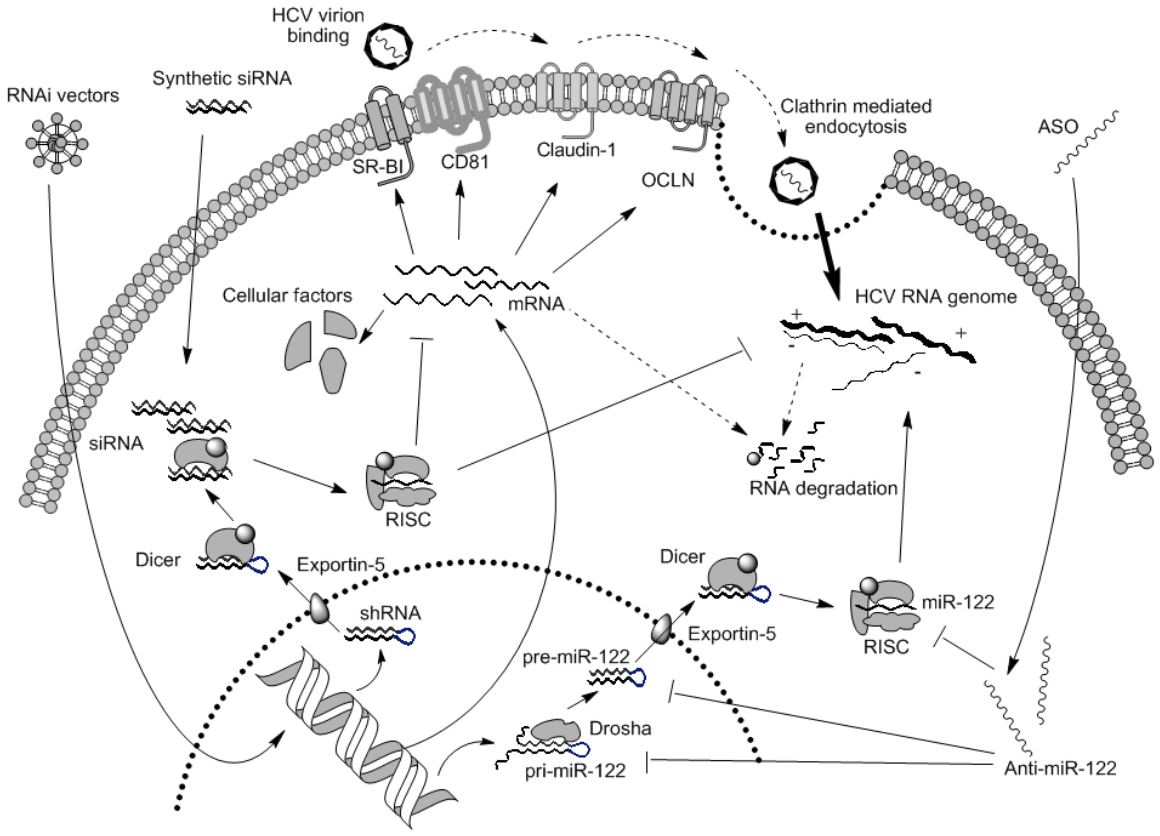

Figure 1. Mechanisms of RNAi and miRNA-based therapies for hepatitis C infection. RNAi effects can be achieved by delivery of chemically synthetic siRNA or by vector-expression of shRNA. ShRNA can be expressed in the host cell using non-integrating, adenoviral or adeno-associated viral vectors, or integrating, lentiviral vectors. ShRNA is cleaved into active siRNA by the cellular enzyme, Dicer. RNAi mediated gene silencing can be applied to treat HCV infection by targeting either viral RNA or mRNA of host cell factors, such as surface receptor CD81. MiR-122, a liver specific microRNA, plays a positive role in HCV replication and therefore silencing of miR-122 by modified antisense oligonucleotides (ASO), such as the locked nucleic acid probes, could be considered as a potential therapeutic modality.

In terms of in vivo application of RNAi, particularly for the treatment of Hepatitis C, the success will greatly depend on the effective delivery systems to target the liver. The first in vivo proof-of-concept that RNAi can be delivered to the liver came from a study by the group of Mark Kay [62]. Using hydrodynamic transduction in mice they demonstrated that a plasmid expressing an anti-NS5B shRNA could effectively silence the expression of a luciferase fusion gene containing the HCV NS5B sequences in the liver. RNAi can be achieved by different

means (Figure 1) and each requiring specific delivery systems for effective targeting of the liver, as will be discussed here.

3.1 Synthetic siRNA

Chemically-synthesized siRNAs have been widely used at the early stages of RNAi research [13, 63]. However, their pharmacological application faces a number of limitations, including restricted cell penetration, short blood persistence due to sensitivity for degradation and detrimental off-target effects [64-67]. Thereby, proper modification and conjugation of "naked" raw siRNA are required to achieve targeted delivery to the liver.

Several chemical modifications have been reported that improve the in vivo properties of siRNA, including phosphorothioate linkages, boranophosphate linkages, locked nucleic acid, 2′-modified RNA, 4′-thio modified RNA, Ribo-difluorotoluyl nucleotide and uncharged nucleic acid mimics [68]. Chemically modified siRNAs have been shown to significantly prolong stability, improve liver targeting ability and enhance gene silencing capacity with reduced toxic and immunostimulatory side effects [69-71]. Another strategy that has been implied is to conjugate siRNAs to small molecules or peptides. Conjugation of siRNA with antibody or vitamin A-coupled liposomes or cationic liposomes has shown to be effective of liver-targeted RNAi delivery in small animals [72-75]. Efficacy of liposome delivery has been shown in HCV gene transgenic mice [72, 76].

Synthetic siRNAs have moved into the clinic at an unprecedented pace [77]. Several of the most advanced clinical trials focus on the treatment of age-related macular degeneration, which is a leading cause of blindness [78]. If synthetic siRNAs would be applicable in treatment of chronic HCV infection, we expect that proper modification or conjugation, like with polymer, lipid or lipidoid-composed nanoparticles, will be required for effective delivery to the liver. The incorporation of liver-specific ligands into these particles may further enhance the delivery efficacy and reduce the side-effects and non-specificity.

3.2 Vector-expressed shRNA

If RNAi is to be utilized as an effective treatment of chronic infectious disease like hepatitis C, long-term and stable silencing needs to be achieved. Gene therapy vector-based encoding of shRNA can produce relative long-term and continuous silencing. Most of gene therapy

vectors are modified viruses which can be applied to deliver a cargo sequence to cells. Currently the most commonly used viral vectors for the delivery of RNAi are derived from the adenoviruses, adeno-associated viruses and lentiviruses and will be discussed.

Adenoviral vectors are the earliest vector system that has been developed and is most effective in transducing proliferating cells**.** First evidence that adenoviral vectors could be used to target the liver was shown in HBV transgenic mice. Using an adenoviral vector expressing an anti-HBV siRNA specific inhibition of HBV gene expression was observed [79]. More recent, intravenous delivery of the adenovirus-vector expressing HCV targeted shRNA was also shown efficient in specific suppression of HCV genomic RNA and protein synthesis in the liver of HCV structural protein transgenic mice [80]. Despite this success, there are considrable concerns regarding the application of adenoviral vectors in the clinic. One major obstacle is the pre-existing immune reactivity against adenoviral vectors [81]. Recent study demonstrated that monomethoxy polyethylene glycol-succinimidyl propionate modified adenoviral vector could attenuate both adaptive immune response and hepatotoxicity, leading to the improved delivery efficacy of shRNA into the liver [82]. In the setting of RNAi delivery, another issue is that adenovirus highly expressed small non-coding RNAs, which can inhibit the RNAi pathway by acting as competitive substrates which saturate the Dicer and RISC machinery [83-84]. This limitation is potentially circumstance in the newest, third-generation, helper-dependent adenoviral vector that all viral encoding sequences are replaced by stuffer DNA and transgene [85]. The proof of concept has been provided by recent studies showing that the third-generation adenoviral vector mediated shRNA expression allows inhibition of target gene expression in the liver, although high level shRNA expression seems to result in activation of the interferon response [86-87].

Adeno-associated viral (AAV) vectors have potential for shRNA delivery. Up to date, summer 2010, 75 gene therapy trials using AAV vectors have been registered at The Journal of Gene Medicine Clinical Trial site (http://www.abedia.com/wiley). These trials were approved for treating various diseases, including cancer, monogenic diseases, neurological diseases, ocular diseases, cardiovascular diseases and infectious diseases (Figure 2). There is a single liver-directed Phase I trial in patients with severe hemophilia B using AAV to deliver the normal human Factor IX (FIX) gene. This study achieved only transient therapeutic gene expression, likely due to the preexisting immunity to the AAV2 serotype [88]. Two additional

trials of liver-directed AAV gene therapy for hemophilia B are currently recruiting participants (ClinicalTrials.gov).

Although AAV serotype 2 (AAV2) is the first AAV that was vectored for gene delivery, pre-existing immunity against AAV2 likely led to the failure of the clinical hemophilia B gene therapy trial [88]. AAV serotype 8, a new member of the AAV family isolated from rhesus monkeys, is less prone to recognition by pre-existing antibodies in humans and also show a 10- to 100-fold increased transduction efficiency in mice liver [89-90]. A further improvement of conventional single-stranded AAV vectors is the construction of self-complementary AAV vectors which can anneal to form stable transcriptional active DNA dimers after uncoating [91].

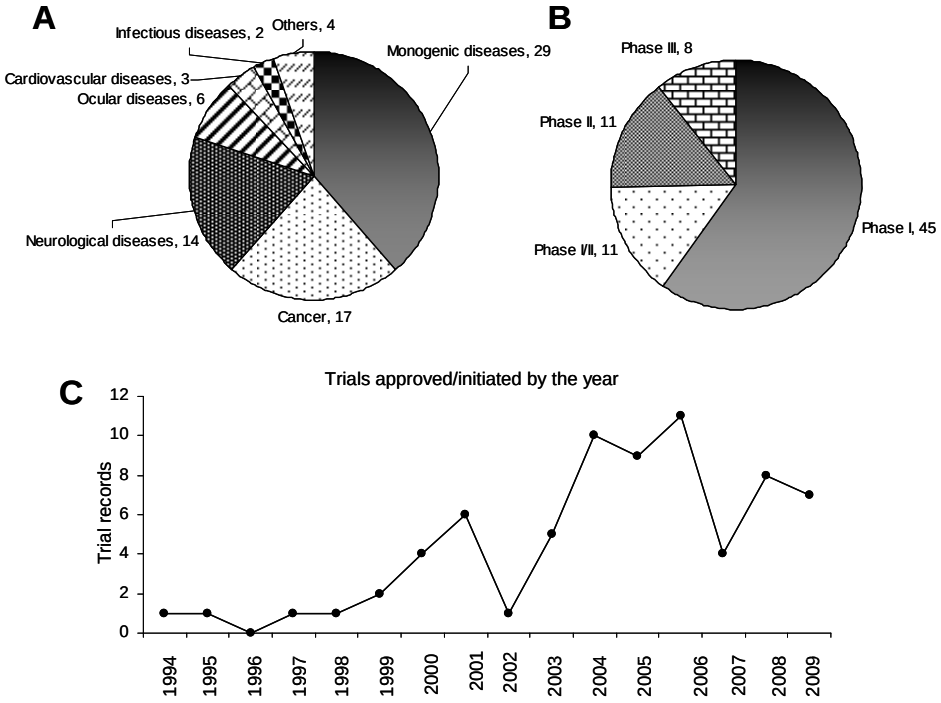

Figure 2. Clinical trials of AAV-mediated gene therapy registered at the Journal of Gene Medicine Clinical Trial site. (**A**) In total, there are 75 registered AAV trials, including 29 for monogenic diseases, 17 for cancers, 6 for ocular diseases, 3 for cardiovascular diseases, 2 for infectious diseases and 4 for other indications. (**B**) 45 trials are at the stage of Phase I, 11 at Phase I/II, 11 at Phase II and 8 at Phase III. (**C**) Distribution of trials according to the year of approved/initiated. Two trials lacking the information of the year approved/initiated and two from 2010 are not listed in the graph.

Lentiviral vectors are derived from HIV-1 and are distinguished from the other vectors in the fact that they can integrate into host genome and therefore are capable of mediating long-term expression of shRNA [92-94]. However, production limitation and potential safety issues related to genomic integration and recombination limits their widespread clinical

application. Currently, new generation vectors have been developed such as the third generation VSV-G pseudotyped HIV-1 vectors, HIV-2 vectors and simian immunodeficiency virus vectors [95-97] to overcome these problems and further modification of the envelope protein with a HBV peptide could improve targeting of the liver [98]. Moreover, the safety and specificity of RNAi delivery can be further enhanced by transcriptional regulation. Different from the first-generation shRNA, the second-generation mimics the structure of pri-miRNA, which therefore can be expressed by polymerase II promoters [99]. Polymerase III promoters, in particular U6, often overly expresses shRNA, shown to induce acute cytotoxicity [100]. Using liver-specific pol II promoter driving shRNA expression has been shown improved tolerability [101].

3.3 Delivery of viral vector-expressed shRNA during isolated liver perfusion

With end-stage liver disease due to chronic HCV infection being the leading indication for liver transplantation worldwide, management and treatment of recurrent disease remains a major clinical challenge. RNAi, in particular delivered by viral vector, can be ideal to modify the new graft and to protect HCV recurrence. During the process of liver transplantation, the donor undergoes multiple perfusions to clear the liver of blood, preserve it, and prepare it for transplant. Ex vivo perfusion of the liver could provide a good opportunity for liver targeted delivery of gene therapy.

Early studies have demonstrated that ex vivo perfusion of cold-preserved rat liver grafts with replication-defective adenoviral vectors resulted in uptake and expression of transgenes [102-103]. Similar, vector delivery during graft perfusion showed efficacy in protecting renal or cardiac graft from acute rejection [104-105]. In order to use RNAi to protect liver grafts from re-infection by HCV, integrating self-inactivating lentiviral vectors would be most suitable, because of their capability of stable and long-time expression of shRNA. Interestingly, a commonly used graft preservation solution, University of Wisconsin solution, improved the lentiviral transduction of hepatocytes under both normothermic and hypothermic conditions. Hydroxyethyl starch was found to be responsible for the increase in transduction [106]. In the context of liver transplantation, this opens a distinct window of opportunity to increase the ex vivo transfer of vectors, even under cold conditions.

4. The role of microRNAs in HCV infection

4.1 Regulation of HCV replication by microRNAs

Increasing evidence has suggested the involvement of microRNAs (miRNA) in viral infection [107]. A liver specific miRNA, miR-122, is the first identified host miRNA linked to HCV replication [16]. Two putative binding sites located in the 5' NCR are considered for miR-122 targeting, and binding to both sites was found to be necessary for viral replication. The two sites are adjacent and are separated by a short spacer, which is largely conserved between HCV genotypes [108]. These targets are notably very different from the normal miRNA targeting of mRNA. Generally, miRNA targets the 3' NCR, leading to suppression or degradation of mRNA [109]. Interestingly, insertion of the HCV miR-122 binding sites into the 3' NCR of a reporter mRNA leads to downregulation of mRNA expression, indicating that the location of the miR-122 binding site is crucial in gene regulation [108].

In addition to the direct interaction, miR-122 can indirectly facilitate HCV replication by regulation of heme oxygenase-1 (HO-1) expression, a key cytoprotective enzyme capable of suppressing HCV replication [110-111]. More recently, miR-122 has been shown to stimulate HCV translation by enhancing the association of ribosomes with the viral RNA at an early initiation stage [112]. Moreover, interferon-beta has been shown to rapidly modulate the expression of numerous cellular miRNAs including miR-122, and that eight of these miRNAs have sequence-predicted targets within the HCV genome. These findings may support the notion that mammalian species can use cellular miRNAs to combat viral infections through the interferon system [113]. A recent clinical study showed that miR-122 expression was markedly decreased in HCV patients who did not develop a sustained virological response during IFN- therapy, confirming the link between the IFN response and the miRNA pathway [114]. Recently, an additional miRNA, miR-199a*, was identified as a potential inhibitor of HCV replication [115].

4.2 Therapeutic targeting of miRNAs

MiRNAs represent a novel class of cellular molecules involved in HCV life cycle. The discovery of the positive regulatory function of miR-122 in HCV replication and the existing technology

of manipulating miRNA function have provided the basis of developing miRNA-targeted therapeutic strategies for HCV infection. Inhibition of miR-122 function can be achieved through the use of conventional antisense technology. This antisense approach can act at multiple levels to affect miR-122, such as by binding to the mature, pre- or pri-miR-122 and block their function. However, the low efficacy of the conventional antisense oligonucleotides (ASO) often requires additional modification to improve their biological activity.

The first report on the successful use of 2'-O-methyl (OMe) ASO was to knockdown let-7 function in Drosophila [116]. Subsequently, ASO was applied to inhibit miR-122 in mice, which was termed “antagomiRs” [117]. Transfection of miR-122 antagomir reduced HCV RNA up to 84% in two independent subgenomic models, respectively [110]. Another approach, MOE (2'-O-methoxyethyl phosphorothioate) modification, has also shown to effectively inhibit miR-122 activity in the liver [118].

Locked nucleic acid (LNA) modification represents a more advanced approach. LNA is a class of ASO with the ribose ring ‘locked’ by a methylene bridge connecting the 2’-O atom with the 4’-C atom. By “locking” the molecule with the methylene bridge, LNA ASO is constrained in the ideal conformation for Watson-Crick binding, making the pairing with a complementary nucleotide strand more rapid and increasing the stability of the resulting duplex [119]. A simple systemic delivery of an unconjugated, PBS-formulated LNA-antimiR has shown to effectively antagonize miR-122 in non-human primates, resulting in uptake of the LNA-antimiR in the cytoplasm of primate hepatocytes and formation of stable heteroduplexes between the LNA-antimiR and miR-122. As miR-122 was shown to be a key regulator of cholesterol and fatty-acid metabolism in the adult liver [118], this treatment led to a long-lasting and reversible decrease in total plasma cholesterol without any evidence for LNA-associated toxicities or histopathological changes in these animals [120]. Similar results were reported in mice by the same group [121].

Additionally, miR-122 silencing can be achieved by LNA/2′ -*O*-methyl mixmer, peptide nucleic acids (PNA), PNA–peptide conjugates, or a chimeric 2'Fluoro/2'OMe modified ASO. An antisense PNA conjugated to a cell-penetrating peptide can inhibit miR-122 without the need for transfection or electroporation of the human or rat cell lines, highlighting the potential therapeutic applications [122]. The chimeric 2'Fluoro/2'OMe anti-miR-122 ASO

displays improved efficacy and a 5-10 fold improvement in potency compared to LNA ASO, indicating its potential for further development as anti-HCV therapeutic agent [123]. In contrast, miR-199a* was shown to inhibit viral replication, therefore overexpression could be a rational therapeutic strategy [115].

5. Potential limitations of RNAi therapy

5.1 Resistance and mutational escape

Similar to existing antiviral monotherapies, RNAi monotherapy may fail to completely control infection and will allow the evolution of resistant quasispecies. Therefore, a combined strategy is likely required to successfully control the HCV infection. The strict specificity of RNAi has as disadvantage that a single nucleotide mutation in the targeted HCV sequence can abrogate recognition and thus silencing. A first straightforward solution to this problem is to simultaneously target multiple viral sequences [32]. Also for conventional antiviral compounds it became clear from the treatment of HIV/AIDS that only a combinational strategy as used in the highly active anti-viral therapy, HAART, is effective in preventing the development of viral resistance. In vitro treatment of HCV with a library of endoribonuclease-prepared siRNAs, which simultaneously target multiple sites of the viral genome, was shown to effectively inhibit the replication of subgenomic and genomic HCV replicons and avert escape [124]. Alternatively, multiple-targeted siRNAs can be generated from raw or vector-derived long double stranded RNA molecules by Dicer [125-126].

A second strategy is the simultaneous targeting of both viral and host sequences. Cellular proteasome α-subunit 7 (PSMA7) and Hu antigen R (HuR) have been found to interact with HCV [127-128]. Combination of PSMA7- and HuR-directed siRNAs with HCV-directed siRNAs revealed additive antiviral effects in HCV replicons [129]. Similarly, both entry and replication can be simultaneously targeted using shRNAs directed against two regions of the HCV RNA and one region of the host cell receptor, CD81. The triple shRNA cassettes driven by three H1 promoters were effective in concurrently reducing HCV replication and CD81 mediated E2 binding, with comparable efficacy to the single shRNA vectors [93]. Invention of the second-generation shRNA that mimics the structure of pri-miRNA provided the possibility of expressing multiple shRNA in a single cassette. Multimerization of a single miR-155-based

cassette up to at least eight copies of anti-luciferase shRNAs can be used to increase the inhibition of a single target mRNA [130]. HIV-1 replication can be efficiently inhibited by simultaneous expression of four antiviral siRNAs in the single polycistron mir-17-92 context [131]. Certainly, such an approach could well fit in the design of multi-shRNAs strategies for HCV treatment.

IFN-α possesses indirect antiviral activity by stimulating genes that can lead to a non-virus-specific antiviral response. Based on their complementary antiviral mechanisms, combining RNAi with IFN-α may prevent therapeutic resistance and exhibit additive antiviral activity. Promisingly, we found that lentiviral mediated RNAi and IFN- α act independently on HCV replication showing combined antiviral activity with no evidence of cross-interference. Treatment with IFN-α had no effect on either lentiviral vector transduction or gene silencing efficacy [132]. In addition, upregulation of certain host genes, such as the ubiquitin specific protease 18 (USP18), have been determined to be associated with patients who do not respond to interferon therapy. It was subsequently demonstrated that silencing of USP18 enhanced the anti-HCV activity of interferon, which provided another approach of combining RNAi with IFN-α [133]. Additional combination of RNAi with ribavirin, ribozymes, ASO or small molecule inhibitors would be reasonable, but needs to be further evaluated.

5.2 Viral suppression of RNAi

It has been shown that RNAi is involved in the inhibition of viral infection and silencing of transposable elements in plants, insects, fungi and nematodes [134-138]. Although it remains controversial whether RNAi also acts as an antiviral defence mechanisms in mammalian species [139], the interplay of viral infection with the RNAi pathway is more established [140].

Given the fact that the replication of the HCV genome involves dsRNA and the IRES region contains double stranded hairpin structures, RNAi may exert anti-HCV action. It has been shown that either incubation of HCV IRES with Dicer enzyme or transfection of HCV sequence into cells can result in generation of IRES-related siRNA, indicating that both exogenous and endogenous Dicer can target HCV IRES. Furthermore, NS5B-related siRNA can be detected in a HCV subgenomic replicon, suggesting that a dsRNA intermediate may also be targeted by the RNAi pathway [141].

Like many other viruses, HCV can overcome the antiviral effects of RNAi through several mechanisms. It was found that siRNA-mediated gene silencing of the reporter gene GFP was inhibited when the entire HCV polyprotein was expressed. The structural protein E2 was found to be responsible for this inhibition by bounding to Argonaute-2 (Ago-2), a component of the RISC complex [142]. HCV core protein was also found to inhibit shRNA but not siRNA mediated gene silencing, suggesting functional inhibition of Dicer enzyme without affecting the activity of RISC [141, 143]. It was further recognized that the N-terminal 62 amino acids of the core were required for RNAi suppression [143].

6. Expert opinion

An overwhelming number of studies so far have extensively demonstrated the power of RNAi in understanding HCV biology and has revealed its potency as therapeutic approach. Identification of host factors involved in the various stages of the viral life cycle by RNAi screening has provided important new knowledge regarding the virus-host interactions and revealed many potential therapeutic targets. In particular, the discovery of cellular miR-122 as a positive modulator of HCV replication has attracted broad interest to develop ASO-based therapeutic strategies. However, targeting miRNA and other host factors has to be considered with caution, as long as their exact physiological function is not fully understood. The observed active interplay of HCV with the RNAi pathway may re-ignite a smouldering debate on whether RNAi in human is contributing to the innate immune defence against viral infections.

Although HCV RNA genome is a prime target for RNAi, it has become clear that the therapeutic application is far from straightforward. Single RNAi target-based strategies likely fall short due to the high mutation rate, genetic diversity, complicated genomic structure and RNAi inhibition by the virus. Therefore, multiple viral targets, combined targeting of viral and host factors, or even combination with conventional treatments, may be necessary to achieve the best therapeutic effects. Together with the rapid improvement of gene delivery technology, RNAi based therapy against HCV infection has a very promising future. However, before clinical applications can be considered, it is essential to bridge the gap between experimental in vitro research and clinical application of RNAi therapy in patients by

evaluate the long-term in vivo efficacy of RNAi in better, immune competent, small animal models for HCV.

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Chapter 6.2

A dynamic perspective of RNAi library development

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Trends in Biotechnology, 2012, in press

Abstract

Shortly after the dissertation of the mechanism of RNA interference (RNAi), various RNAi libraries for invertebrates, plants or mammalians that enable loss-of-function genetic screens on a genome-wide scale have been developed. Joint academic and industrial effort has led to the commercial launch of many of these libraries and this field is expected to continuously evolve at incredible speed. This article comparatively reviews the principles and applications of different RNAi libraries: from earlier synthetic to recent lentiviral RNAi libraries. The unique properties and limitations of each library will be important references for instigators to choose particular library for their specific application.

RNAi library: conception and development

The definition of the central dogma of molecular biology in 1970, which is that "DNA makes RNA makes protein", has led to the realization that antisense base pairing might constitute an efficient strategy to interfere with gene expression for mechanistic studies or therapeutic purposes [1]. Initially, development of this approach for practical purposes was haphazard, fraught with setbacks and false starts. The field progressed slowly with most advances being made in plant biology, and especially in petunia. In 1993, the discovery of microRNA (miRNA), a class of small noncoding RNAs that act as master regulators in eukaryotic cells [2], accelerated development in the RNA research field. In 1998, the first antisense drug (Fomivirsen, injected to treat cytomegalovirus retinitis) was approved by the food and drug administration [3] t the same time that the mechanism behind RNA interference (RNAi) was dissected in Caenorhabditis elegans (C. elegans) [4]. The discovery of RNAi changed the face of gene regulation. Moreover, its application for studying gene function and for therapeutic development evolved at incredible speed (Figure 1) [5]. The dissection of the RNAi mechanism in C. elegans was recognized with the Nobel Prize in 2006, although most researchers recognize the earlier work in petunia as just as important [6].

An important step in RNA research was the generation of RNAi libraries in 2000 [7-8] that allowed performing loss-of-function screens on a genome-wide scale, superior to the candidate gene approach employed before. RNAi libraries represented a major step forward for gene function investigations and led to the identification of new genes related to various biological functions as well as new therapeutic targets for many types of diseases. Sequence-specific inhibition of gene expression at the posttranscriptional level, RNAi in invertebrates [4, 9] and plants [10] can be induced by introducing long double-stranded RNA (dsRNA, usually hundreds of nucleotides in length) that subsequently is processed into functional small interfering RNA (siRNA, usually 21-25 nucleotides in length) by the host gene silencing/miRNA machinery. RNAi in mammals is triggered by siRNA [11]: siRNA can be introduced into cells as chemically synthetic siRNA or delivered by vectors, commonly viral vectors, that express a short hairpin RNA (shRNA) precursor [12]. Based on their different processing mechanisms, various types of RNAi libraries have been developed for invertebrates, plants and mammals. In this article, we comprehensively review the development of RNAi libraries: from early synthetic to recent lentiviral RNAi libraries (Table

1). In addition, we propose a combinatorial approach of different RNAi libraries as an enhanced tool.

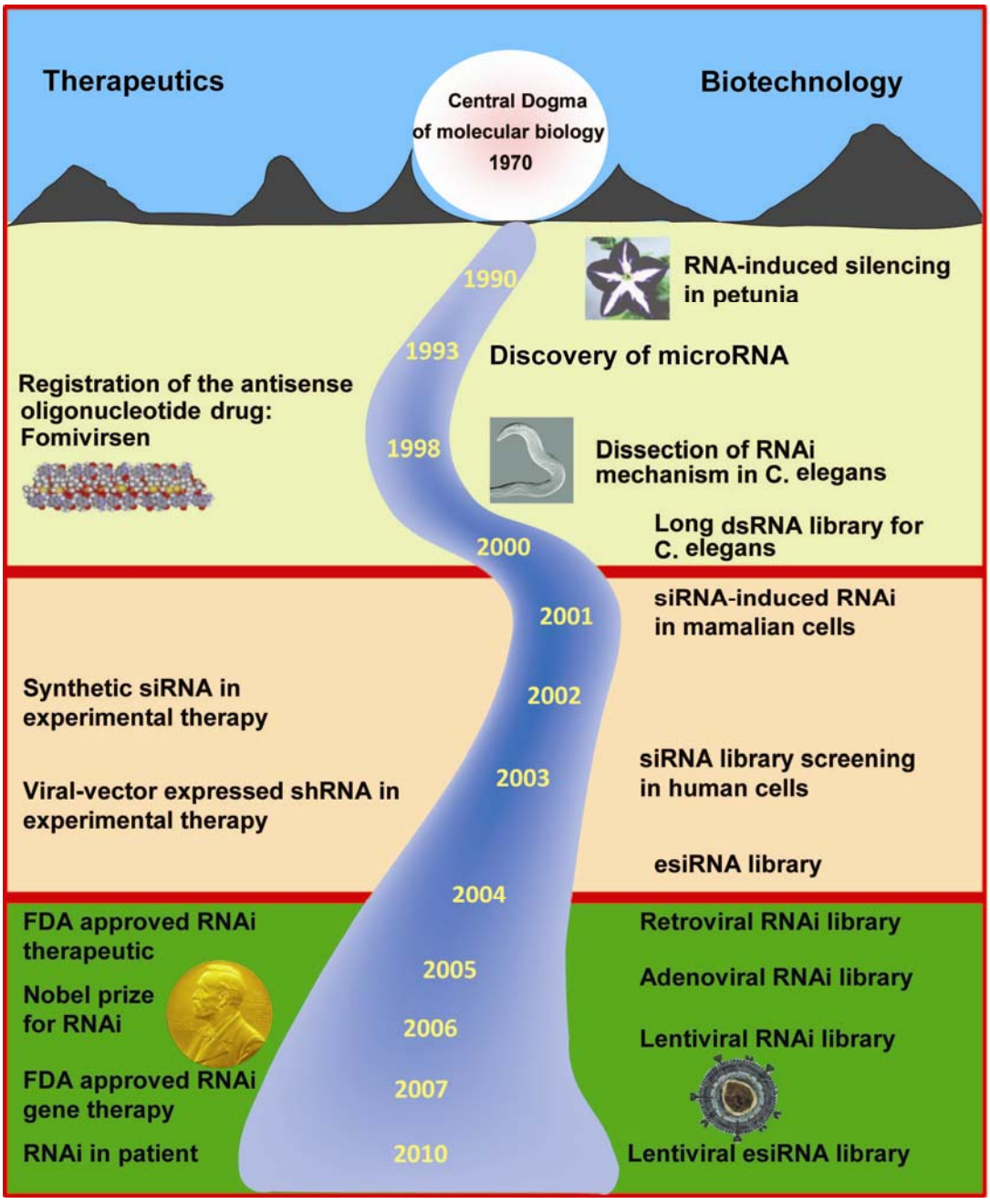


Figure 1. Timeline of RNAi and RNAi library development.

Long dsRNA libraries for invertebrates and plants

High throughput RNAi screening was pioneered in C. elegans [7-8]. In 2000, a bacterially expressed long dsRNA library (2,445 independent clones) targeting nearly 90% of predicted genes on C. elegans chromosome I, was first presented [7]. This is a re-usable library that permits unlimited RNAi screens simply by feeding bacterial clones to the worms, though it is still a rudimentary form of RNAi library. At the same time, a dsRNA library targeting 96% of the approximately 2,300 predicted open reading frames on chromosome III of C. elegans was developed [8]. The library was constructed in a step process, first involving PCR amplification of the individual open reading frame to generate DNA templates and then by *in vitro* transcription to produce dsRNAs. These dsRNA were then micro-injected into the worms for functional analysis, allowing large-scale screens. Subsequently, these libraries have been extended to cover 86% [13] and 98% [14] of the predicted genes of the C. elegans genome. A similar approach was also applied to construct a genome-wide long dsRNA library in Drosophila theoretically targeting 91% of the predicted genes of this important model organism [15]. Long dsRNA sub-genomic libraries (containing a few hundred targets) were also developed for trypanosome [16], a protozoan pathogen, and for plants including barley [17], Arabidopsis [18] and maize [19]. A few hundred transgenic RNAi lines targeting a set of chromatin-associated genes have been produced for Arabidopsis and maize, which are commercially available at ChromDB (www.chromdb.org).

Library	Type	General feature	Supplier/Institute	Refs
Long dsRNA				
Ahringer	Bacterial expressed	16,757 clones target 86% of 19,427 prediected *C. elegens* genes	University of Cambridge, UK	13
Sonnichsen	In vitro transcription	20,326 dsRNAs target 98% of predected *C. elegens* genes	Cenix Bioscience GmbH, Germany	14
Perrimon	In vitro transcription	19,470 dsRNAs target 91% of predicted Drosophila genes	Harvard Medical School, USA	15
siRNA				
Dharmacon siRNA	Chemical synthesis	Targeting 18,236 human genes and 19,040 mouse genes. A mixture of 4 siRNAs targets one gene	Thermo Scientific	
Silencer siRNA	Chemical synthesis	37,755 siRNAs target 12,585 human genes; 33,402 siRNAs target 11,134 mouse genes	Ambion	
MISSION esiRNA	Endoribonuclease prepared	Targeting >16,000 human and >8,600 mouse genes. a mixture of siRNAs targets each gene	Sigma-Aldrich www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/mission-esirna.html	38
Vector delivered shRNA				
Arabidopsis amiRNA constructs	Plasmid	shRNA mimics miRNA; amiRNA design tool for over 100 plants; vectors or clones targeting much of the Arabidopsis genome	amRNAi design tool http://wmd.weigelworld.org Vector and clones available at Arabidopsis Biological Resource Center www.arabidopsis.org	23-24
SilenceSelect library	Adenoviral	>11,500 shRNAs target ~5,000 human druggable genes	SilenceSelect www.silenceselect.com	46
NKI library	Retroviral	23,742 shRNAs target 7914 human genes; Puromycin selection	The Netherlands Cancer Institute	56
Hannon library V1	Retroviral	28,659 shRNAs target 9,610 human genes; 9,119 shRNAs target 5,563 mouse genes; Puromycin selection	Cold Spring Harbor Laboratory, USA	57
Hannon library V2	Retroviral	79,805 shRNAs target 30,728 human and 67,676 shRNAs target 28,801 mouse genes. The shRNA structure mimics miR-30 backbone; Puromycin selection	Cold Spring Harbor Laboratory, USA	58
MISSION TRC library	Lentiviral	The TRC1 contains ~159,000 shRNAs targeting ~16,000 human and ~15,950 mouse genes; Additional ~150, 000 TRC2 constructs; Puromycin selection	Sigma-Aldrich The Broad Institute www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna.html	64

GeneNet library	Lentiviral	200,000 shRNAs target 47,400 human genes; 150,000 shRNAs target 39,000 mouse genes; Pooled genome-wide or pathway focused vectors Puromycin selection	System Biosciences http://www.systembio.com/rnai-libraries	
GIPZ library	Lentiviral	62,000 shRNAs target human genome; 62,000 shRNAs target mouse genome; miR-30 backbone; GFP marker; Puromycin selection	Thermo Scientific; Cold Spring Harbor Laboratory, USA; Harvard University, USA https://www.openbiosystems.com/RNAi/shRNAmirLibraries/GIPZLentiviralshRNAmir/	
TRIPZ inducible library	Lentiviral	~159,000 shRNAs targeting ~16,000 human and ~15,950 mouse genes; miR-30 backbone; Doxycycline inducible; RFP marker; Puromycin selection	Thermo Scientific; Cold Spring Harbor Laboratory, USA; Harvard University, USA https://www.openbiosystems.com/RNAi/shRNAmirLibraries/TRIPZlentiviralinducibleshR/	

Table 1. A list of RNAi libraries available for high throughput screening

Short hairpin RNA (shRNA)-based RNAi: applicable for invertebrates, plants and mammals

Another means of RNAi delivery is the vector (plasmid)-driven transgenic expression of shRNA. This shRNA can be processed by the cellular RNAi/miRNA biogenesis machinery into functional siRNA. The conventional shRNA constructs (equivalent to pre-miRNA structure) are often expressed by RNA polymerase (Pol) III promoters, such as H1 or U6 promoters. The second generation of shRNA (also termed artificial miRNA, amiRNA) was developed by mimicking the pre-miRNA structure and replacing the mature miRNA sequence with the target of interest. It can be transcribed either by Pol II or Pol III promoters [20]. Figure 2 illustrates the design of shRNA and amiRNA constructs and their implication in RNAi library development. In Drosophila, the conventional shRNA approach has been widely used to generate transgenic RNAi lines and this approach works well in all somatic tissues of this organism, but for unknown reasons is ineffective in the female germ line [21]. Nevertheless, amiRNAs modelled on Drosophila miR-1 silence gene expression during oogenesis. This approach is being used to make a genome-wide RNAi transgenic resource, which should greatly aid the study of Drosophila oogenesis [22]. Currently, a range of transgenic RNAi Drosophila stocks either generated by the conventional shRNA or the amiRNA approaches is available through the Transgenic RNAi Project (TRiP) at Harvard Medical School. In Arabidopsis, high specific gene silencing using amiRNA-based RNAi is available through expression driven by constitutive, inducible or tissue-specific Pol II promoters [23-24]. For automating amiRNA design, the WMD (Web MicroRNA Designer) platform has been

developed (Table 1). In mammals, the invention of the shRNA expression cassette has formed the basis for developing viral vector-delivered RNAi.

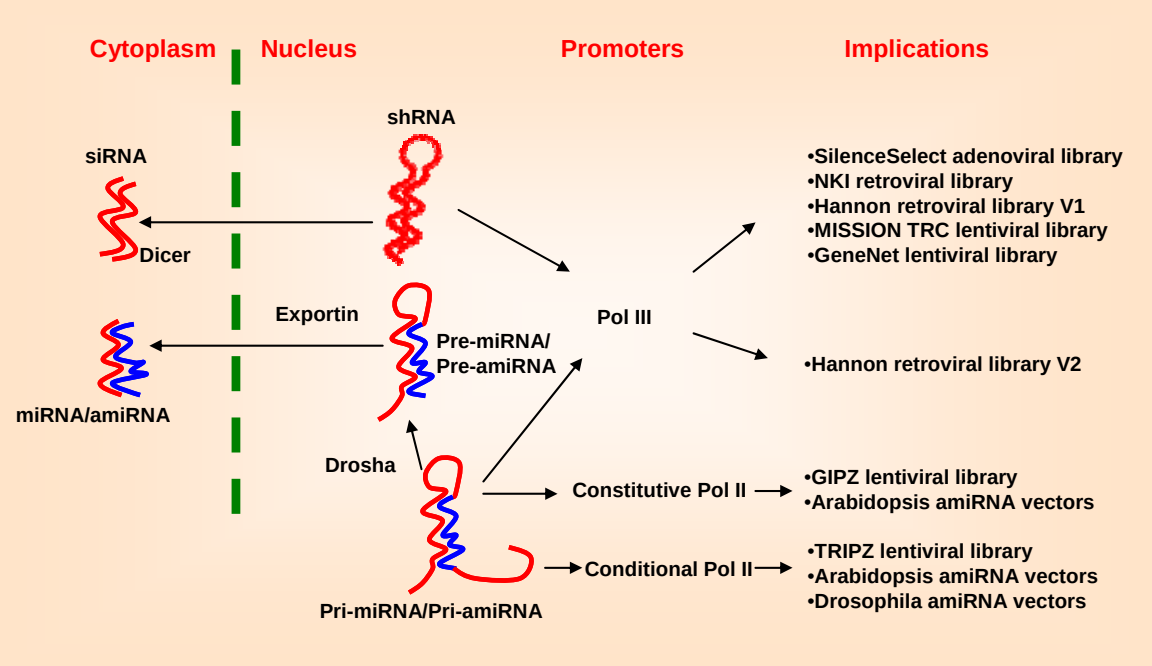


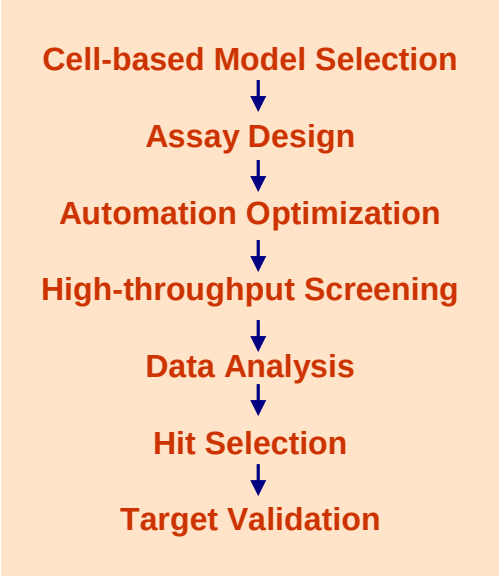
Figure 2. Principles and implications of shRNA constructs in RNAi library development. The first generation of shRNA construct is similar to the structure of pre-miRNA that is often expressed by RNA polymerase (Pol) III promoters, such as H1 or U6 promoters. The second generation of shRNA (also termed as artificial miRNA, amiRNA) was developed by modelling on the pri-miRNA structure and replacing the mature miRNA sequence with the target of interest. It can be transcribed either by Pol II or Pol III promoters. Therefore, amiRNA can also be conditionally expressed by using tissue specific or inducible Pol II promoters. shRNAs are transcribed in the nucleus and exported and processed into functional siRNA/amiRNA by the cellular RNAi/miRNA biogenesis machinery. Both generations of shRNAs have been widely implied for developing viral vector-based RNAi libraries or constructs for generating RNAi transgenic Drosophila stocks or Arabidopsis lines.

siRNA libraries and the bloom of high throughput screening in mammalian cells

In mammalian cells, long dsRNA often triggers an innate immune, such as interferon response, resulting in a non-sequence-specific down-regulation of protein expression [25]. The innate immune system utilizes sensory molecules (protein kinase R, retinoic acid-inducible gene I and melanoma differentiation-associated gene 5) to trigger the interferon response to non-self dsRNAs that are longer than 30 bp [26]. Therefore, siRNA (usually 21 bp) has become the primary form of inducing RNAi in mammals [11].

RNAi screening in mammals began with the identification of modulators of TRAIL-induced apoptosis in HeLa cells using synthetic siRNAs targeting 510 human genes [27]. Combinatorial chemistry, used to generate multiple synthetic siRNAs, facilitated a flurry of studies using synthetic siRNA-based high throughput genetic screens (from sub-genome to genome-wide) and has now become routine for use in mammalian cell culture. The general workflow is described in Figure 3. Numerous lists of genes linked to various functions in cancer, stem cell biology and virus infection, have been generated and facilitate our understanding of these biological and pathological processes [28-34].

Figure 3. The general workflow of siRNA library screening



Despite the bloom of RNAi screening using chemically synthesized siRNA, considerable cost associated with chemical synthesis and transfection reagents limits the number of academic laboratories that can afford genome-wide siRNA screens. To circumvent this issue, several groups have attempted to develop more cost-efficient approaches, termed enzymatically prepared siRNA (esiRNA) libraries [35-38]. The clear advantage of esiRNA libraries over chemically synthetic siRNA libraries is that esiRNAs originate from a DNA template (a cDNA library or a library of plasmids carrying the DNA of interest) and are processed into a heterogeneous mixture of siRNAs that all target the same mRNA sequence, leading to higher efficacy. esiRNA libraries could be potentially created for organisms with as yet un-sequenced genomes.

The construction of an esiRNA library requires the generation of long dsRNAs by PCR-amplification of a DNA template and subsequent *in vitro* transcription by T7 RNA polymerase. The resulting dsRNA is digested with either RNase III or a recombinant Dicer enzyme to generate a pool of siRNAs, which range in length from 18–25 bp after purification. A similar strategy was also applied to generate shRNA libraries: amplified DNA templates were directly digested by DNase into multiple short dsDNAs that were subsequently cloned into plasmid for expression of siRNAs [39-42].

As described above, the generation of an esiRNA library requires enormous effort. Thus, it is generally not cost effective for academic laboratories to construct individual esiRNA

libraries. Fortunately, the genome-scale MISSION esiRNA libraries (Table 1) have recently been released by Sigma-Aldrich [38]. Although some experimental data has shown a more than 10 fold higher target specificity of esiRNAs as compared to individual siRNAs, subsequent follow up work will reveal more about the pros and cons of esiRNA libraries [37].

A common issue for both chemically synthetic and enzymatically prepared siRNA is that the transfection protocol is accompanied by substantial non-specific or toxic effects. In addition, many mammalian cells are inaccessible to chemical (e.g. lipofectamin) or electrochemical means of transfection. Furthermore, neither siRNA nor esiRNA libraries are suitable for screens of primary untransformed cells because of poor transfection efficiency, either *in vitro*, *ex vivo* or *in vivo*. Viral vectors represent advanced tools that potentially can overcome these limitations [43].

Viral vector-delivered RNAi libraries for mammals

Adenoviral libraries

Adenovirus is a large non-enveloped dsDNA virus encoding more than 40 viral proteins from the 36 kb genome. Adenoviruses are efficient for gene transfer, are relatively safe and scale up easily. They have been widely used in clinical gene therapy trials [44] and even approved for delivery of p53 gene for anti-cancer treatment in particular cancers in China [45]. Replication deficient adenoviral vectors [44] are currently favored to deliver protein coding genes or siRNAs. The SilenceSelect™ library is based on an adenoviral vector with shRNAs that can be used to silence different human drugable targets (Table 1) [46]. This library has not been widely used so far, but a study comprehensively identifying the cellular factors that regulate hepatitis C virus replication shows its potential [47].

The relatively large genome of adenoviruses and the associated increased potential for non-specific interaction between vector and host proteins are factors that limit application of this type of virus for library-based screening. In addition, adenovirus highly expresses non-coding RNAs, viral associated RNAI and RNAII, which can inhibit RNAi pathways by acting as competitive substrates that saturate the Dicer and the RNA-induced silencing complex, RISC machinery, and thus interfere with RNAi silencing [48-49]. These considerations prompted the development of third generation adenoviral vectors, where all viral encoding sequences were replaced by noncoding DNA and cargo sequences and thus at least theoretically have circumvented this issue [50]. This system has not yet been implemented in a large scale RNAi

library setting because of technical issues in vector engineering and production. Unfortunately, adenoviral approaches use episomal delivery, which results in only transient transgene expression. Nevertheless, the relative ease of large-scale virus production will ensure that adenoviral RNAi libraries remain a choice for particular *in vivo* application.

Adeno-associated viral vector-based libraries

Adeno-associated viral (AAV) vectors have been developed from the wild type AAV that consists of a small single-stranded DNA genome of about 4,700 bp. In addition to the favorable economics of vector production, AAVs infect both non-dividing and dividing cells. AAV mediated transgene expression is stable despite the vector's largely episomal nature, and lack of apparent pathogenicity. As a result, AAV vectors are the prime candidates for clinical application as well as for RNA interference approaches and exon skipping [51-52]. As a single-stranded DNA vector, synthesis of the complementary DNA to generate a dsDNA template for transcription has rate-limited its gene transfer efficacy. The self-complementary (sc) AAV vector has been developed to bypass this rate-limiting conversion step, dramatically improving the speed and efficiency of transduction [53]. A downside to the use of scAAV is that the transgene size should not exceed 2,000 bp in order to fit in the viral capsule, but this is not a consideration in the context of delivery of RNAi [12, 54]. Thus, although no RNAi library has been built based on AAV so far, we do believe it represents a promising vehicle, in particular for *in vivo* screening.

Retroviral libraries

Retroviral vectors, such as the one derived from the Moloney murine leukemia virus [55], have the ability to integrate into the host genome and therefore support long-term transgene expression even in quickly dividing cell types. Thus, retroviral vectors have been favored for RNAi delivery. Table 1 lists various libraries, their sizes and the number of genes they target. The application of these highly evolved retroviral RNAi libraries has indeed pushed the field forward [56-63]. However, retroviral vectors only infect dividing cells, resulting in low transduction efficacy or even complete resistance to infection in slowly dividing and post-mitotic cells such as neuronal cells.

Lentiviral libraries

Efficient transduction of both dividing and non-dividing cells is a distinct and defining competitive advantage of lentiviral over retroviral vectors. Lentiviral vectors were engineered from the human immunodeficiency virus type 1 (HIV-1) genome pseudotyped with VSV-G envelop proteins and have become popular for RNAi delivery as well as library development because of their wide-range tropism, their propensity to integration into the host genome and their effective transduction of most cell types. The first lentiviral RNAi library was developed by The RNAi Consortium (TRC) at the Broad Institute of MIT and Harvard, termed the TRC library [64]. Several other companies, including System Biosciences, Santa Cruz and Thermo Scientific, also devote substantial efforts to the development of new lentiviral RNAi libraries (Table 1). There are two lentiviral RNAi libraries recently launched by Thermo Scientific in cooperation with Cold Spring Harbor Laboratory and Harvard University that are especially interesting. One is engineered for constitutive expression and one is Tet-On-controlled for inducible expression of shRNAs, both employing a design based on miR-30 shRNA. A recent approach combines the advantages of esiRNA and lentiviral delivery: a shRNA library containing ≈150 shRNAs per gene enzymatically generated from human cDNA was cloned into an inducible lentiviral vector. The method could be further improved by expressing multiple shRNAs per lentiviral vector [65].

Although various enormous high throughput screens have been published that purportedly use retroviral or lentiviral RNAi libraries, most of these studies actually use a pooled approach as illustrated in Figure 4a [60, 66-67], since the cost of large scale gene-by-gene screen is practically unaffordable. Upon transduction of a pooled library, the first step is usually a general section of transduced cells by either chemical resistance or fluorescent-based cell sorting (these vectors often express a puromycin resistance gene or fluorescent protein marker). The next step is a specific selection and clonal expansion according to the particular experimental design, for instance screening of chemo-resistance in tumor cells [60]. The final step of identifying positive hits could be achieved by either sequencing [66] or microarray hybridization of integrated shRNAs [56].

Ex vivo application of retroviral or lentiviral RNAi libraries is relatively straightforward, but the technical challenges surrounding large-scale vector production remains a bottleneck for *in vivo* applications. One affordable and thus commonly used approach is the *ex vivo* transduction of stem cells or cancer cells followed by xenografting animals (Figure 4b) [61-62,

[68]. Another approach is generating tissue-specific or systemic RNAi transgenic mice by *ex vivo* transduction of embryonic stem cells (Figure 4c) [69-71]. However, the unmet need for high throughput gene-by-gene screen and direct *in vivo* application will continuously drive the field to optimize the current libraries or develop new ones.

Perspectives

Minimizing non-specific effects

The use of siRNA is fraught with potential off-target effects, due to the convergence of the processing machinery for delivered siRNA with the endogenous miRNA biosynthesis pathway. Like miRNA, particular siRNA may suppress the expression or translation of a subset of genes in a sequence-dependent manner via partial base-pairing, in addition to the cleavage of the perfect base-paired target gene. The off-target effects on mRNA expression can be detected by genome-wide expression profiling, whereas the effects on translation are often impossible to assess [72]. Bioinformatics provides some tools that help optimize shRNA design in this respect [56]. In theory, esiRNAs have a high risk of inducing off-target effects because a mixture of siRNAs is generated for each target gene without pre-selection. Although, some argue that because each single siRNA in a pool has different off-targets but the same on-target effects, siRNA pools can dilute out off-target effects [37, 72-73]. The second position in the guide strand for the siRNA is essential to recognize off-targets whereas an otherwise perfectly-matched on-target is much less affected by mismatch at this position. Therefore, chemical modification by adding a 2'-O-methyl substantially reduces off-target effects [74]. Likewise a single nucleotide bulge placed at position 2 of the antisense strand substantially alleviates off-target effects [75]. Incorporation of unlocked nucleic acid modification into position 7 is also able to reduce off-target effects, without affecting RNAi potency [76].

In addition to sequence-specific off-target effects, the convergence of siRNA and miRNA machinery could potentially affect the biogenesis of cellular miRNAs. Because of the broad regulatory function of miRNAs on gene expression, disturbance of miRNA biogenesis may fundamentally change cell physiology. Vector expressed shRNA requires export from the nucleus to the cytoplasm and processing into functional siRNA. Thus, it is likely to have a bigger impact on miRNA machinery than raw synthetic siRNA. AAV-mediated over-expression of shRNA has been shown to evoke liver toxicity in mice, ultimately even causing

death [77]. Here, cellular RNAi factors including exportin-5 and four Argonaute proteins were likely saturated [78]. Saturation of the miRNA pathway was also observed using some lentiviral RNAi libraries as a consequence of over-expression of shRNA, although no significant cell toxicity was observed [79]. The lethal toxicity that was observed [77] could have been caused by the combination of AAV vector and over-expressed shRNA. Saturation of miRNA machinery was also observed with transfection of raw siRNA [80]. Given the broad functions of miRNAs in regulating gene expression and other biological processes such as virus infection [79, 81], this type of sequence-independent off-target effect is an obvious issue in almost all types of screen. For siRNA libraries, transfection reagents are another factor in the induction of non-specific effects. Thermo Scientific has been at the forefront of attempts to develop transfection reagent free approaches for siRNA delivery [82]. For viral vector-delivered shRNA, transduction of the vector could also potentially induce non-specificity. Any type of screening will require validation by an alternative technology. A further consideration when choosing raw siRNA or vector expressed shRNA is the competence of particular cell types to process shRNA. Ovarian cancer cells, for example, have largely lost the ability to process shRNA and miRNA, as a result of reduced expression of Dicer and Drosha [83]. Therefore, it is crucial to include appropriate experimental controls to minimize risks of misinterpretation of RNAi screening data due to non-specific effects [84]. For siRNAs or shRNAs, one or multiple scrambled sequences are often recommended to be included as negative controls. For viral vector delivered RNAi, it is also crucial to balance the vector input between the control and experimental conditions. For esiRNAs, Renilla Luciferase, Firefly Luciferase or eGFP-derived esiRNAs are recommended as negative controls by Sigma-Aldrich. However, these are questioned as fair controls because of the heterogeneity of the pooled siRNAs mixture. The field urgently awaits clear and valid control standards.

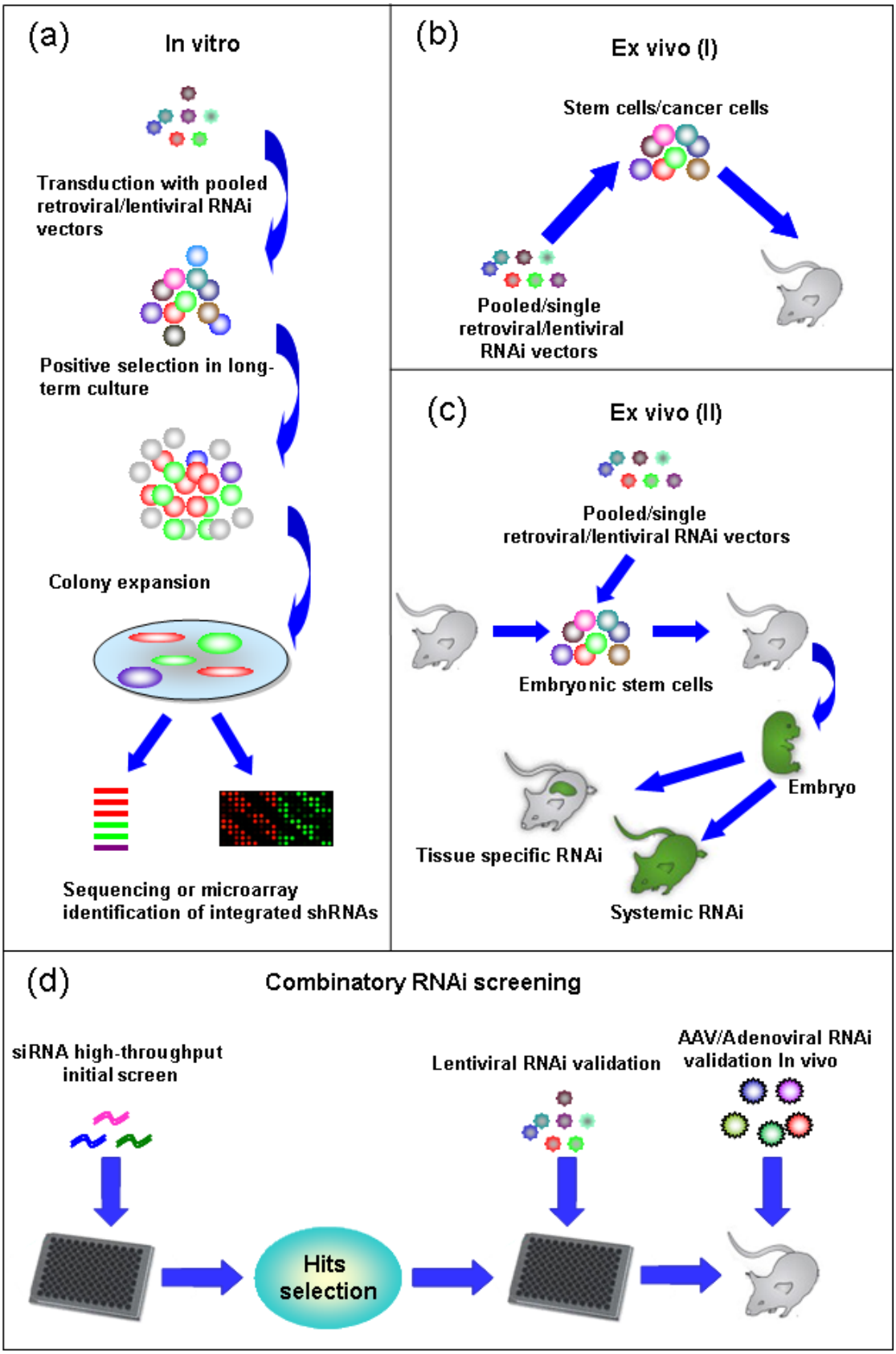
Confirming gene silencing

It is difficult in any high-throughput screen to validate the gene silencing efficacy of each siRNA or shRNA individually. Differences in the efficacy, however, may account for the variation between different screens for the same phenotype. For instance, identified hits in the screens of host factors for HIV or HCV infection, had very little overlap and many well-established host factors were not confirmed (i.e. false negatives) in those screens [34, 79]. A fully-validated library would certainly move the field forward, and efforts such as the

comprehensive validation of the TRC library currently attempted by Sigma-Aldrich are well under way. Interestingly, a ‘‘Sensor assay’’ that enables high-throughput identification of effective shRNAs in a massively parallel format has recently been developed. This unbiased assay has identified potent shRNAs by constructing and evaluating 20,000 RNAi reporters that cover every possible target site in nine mammalian transcripts [85]. Ultimately these efforts will result in higher reproducibility between different screens.

Optimizing hit selection

Another potential cause of significant variability among different RNAi screens is the criteria for hit selection. Various analyses, including z score, z* score, strictly standardized mean difference (SSMD), SSMD* and t statistic, have been well-used to determine RNAi screen criteria [86]. However, issues remain. For example, it is unclear how to deal with values just below a threshold. This probably depends to a large extent on the relative importance of reducing validation work (which requires less false positives) as opposed to the missed opportunities created by false negatives [87]. Bibliohistoric approaches can provide a second pass filter, but require substantial human resources. Integrating RNAi screening datasets with other genomic information, particularly those represented in the form of networks has been shown to facilitate the identification of false-positives and false-negatives [88]. We urge the field to set forth accepted standards that would ensure the quality and accuracy of information in the large datasets generated from RNAi genome-wide screens.


(a)
In vitro
Transduction with pooled retroviral/lentiviral RNAi vectors
Positive selection in long-term culture
Colony expansion
Sequencing or microarray identification of integrated shRNAs
(b)
Ex vivo (I)
Stem cells/cancer cells
Pooled/single retroviral/lentiviral RNAi vectors
(c)
Ex vivo (II)
Pooled/single retroviral/lentiviral RNAi vectors
Embryonic stem cells
Embryo
Tissue specific RNAi
Systemic RNAi
(d)
Combinatory RNAi screening
siRNA high-throughput initial screen
Hits selection
Lentiviral RNAi validation
AAV/Adenoviral RNAi validation In vivo

Combined use of different libraries

Improving RNAi libraries may ultimately require a combinatory strategy for optimal screening (Figure 4d). For example, first, a genome-wide screen could be performed using siRNA library in cell culture. Then, the selected hits could be validated by lentiviral RNAi *in vitro* and further validated *in vivo* by AAV or adenoviral RNAi.

Conclusions

The power of unbiased library approaches (Figure 5) has motivated investigators to develop RNAi arrays. New RNAi libraries with confirmed gene knockdown efficacy, fewer off-target effects and both *in vitro* and *in vivo* high throughput screening capabilities need to be developed and will further foster progress in the field. More sophisticated hit selection methodologies are urgently called for and probably require the collective efforts of industry and academy. Nevertheless, the very substantial fruits that genome-wide RNAi screens have delivered justify the energy devoted to them.

Figure 4. *In vitro* and *ex vivo* application of lentiviral RNAi library, and a proposed combinatorial use of different RNAi libraries. (a) Lentiviral RNAi libraries are often used as a pool of sub- or genome-scales of vectors. For *in vitro* screening, cells are first transduced with vectors and then are selected by either chemical resistance or cell sorting based on reporter gene expressing, depending on the types of vectors used. Upon clonal expansion according to particular experimental design, positive hits could be identified by either sequencing or microarray hybridization of integrated shRNAs. (b) For *ex vivo* application, stem cells or cancer cells can be transduced with single of pooled vectors. Phenotypes are screened in animal by xenograftment assay. (c) Another *ex vivo* approach is generation of tissue-specific or systemic RNAi transgenic mice by vector transduction of embryonic stem cells. (d) siRNA library can be used as an initial screening in cell culture (one or multiple cell culture models). Selected hits can be validated by lentiviral RNAi *in vitro*. AAV or adenoviral RNAi can be used for *in vivo* further validation.

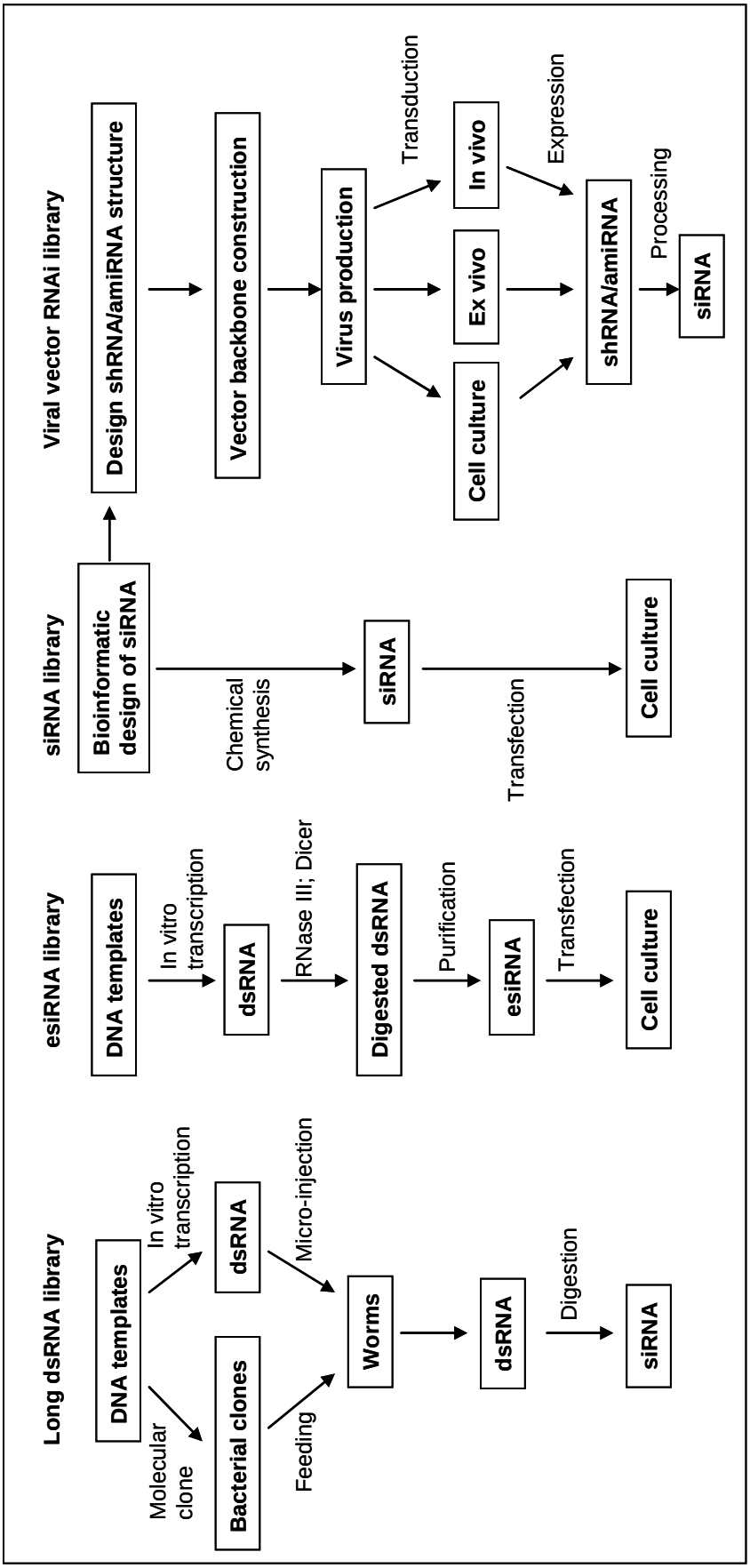


Figure 5. Comparative summary of algorithms for the construction and application of long dsRNA, esiRNA, siRNA and viral vector delivered RNAi libraries.

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Chapter 7

Disturbance of the microRNA pathway by commonly used lentiviral shRNA libraries limits the application for screening host factors involved in hepatitis C virus infection

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FEBS Lett, 2011, 6;585(7):1025-30

Abstract

RNA interference (RNAi) is widely used as a screening tool for the identification of host genes involved in viral infection. Due to the limitation of raw small interfering RNA (siRNA), we tested two commonly used short hairpin RNA (shRNA) lentiviral libraries to identify host factors involved in hepatitis C virus (HCV) infection. It was found that these shRNA library vectors caused non-specific disturbance of HCV replication that was not due to toxicity or interferon response, but related to the high shRNA levels disturbing the endogenous microRNA biogenesis. The high shRNA levels achieved with these vectors reduced the levels of mature microRNAs, including miR-122 known to promote HCV replication. Our findings extend the caution of potential off-target effects of lentiviral shRNA libraries which appear unsuitable to screen microRNA regulated phenotypes, such as HCV replication.

1. Introduction

The development of RNAi libraries, which allowed performing genome wide loss-of-function screens, has been a major step forward for the study of gene function and resulted in identification of new genes related to various biological functions and discovery of new therapeutic targets for many types of diseases [1]. The RNAi Consortium (TRC) library is the first and the currently most widely used lentiviral RNAi library, which uses multiple distinct short hairpin RNAs (shRNA) to knockdown most of the known human and mouse genes [2-3]. Broad application of this library has already been reported in numerous publications on the identification of human disease-related genes [4-6]. The TRC library appears to have advantages over conventional synthetic small interference RNA (siRNA) libraries. Transfection of synthetic siRNA only evokes transient gene silencing often accompanied with non-specific or toxic effects, and many types of mammalian cells are ineffective or resistant to the transfection [7]. Lentiviral vector delivery of RNAi possesses great advantages over raw siRNA applications owing to their wide-range tropism, genomic integration property and effective *in vitro* delivery.

Given the need to better understand the biology of hepatitis C virus (HCV) infection and the search for new therapeutic targets, several recent studies have attempted to screen host factors using siRNA libraries [8-11]. However, mainly due to the limitation of synthetic siRNA, host factors identified had very little overlap and many well-established host factors were not identified (i.e. false negatives) in those screens. Our previous studies showed that lentiviral vectors expressing shRNAs could sequence specifically knockdown HCV genomic RNA or host factors, whereas vectors expressing no or irrelevant shRNAs had no significant effect on HCV replication [12-13].

In the present study, we have tested different lentiviral shRNA expression systems for screening of HCV host factors and compared their on-target and off-target effects. Here we report sequence non-specific effects of two commonly used lentiviral shRNA libraries on HCV replication. Further analysis showed that this off-target effect is caused by a disturbance of the microRNA biosynthesis in the transduced cells associated with the high level of shRNA expression by these vectors. Solutions to minimize the off-target effects are discussed.

2. Materials & Methods

2.1. Cell culture

Cell monolayers of the human embryonic kidney epithelial cell line 293T and the human hepatoma cell line Huh7 were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen–Gibco, Breda, The Netherlands) complemented with 10% v/v fetal calf serum (Hyclone, Logan, Utah, USA), 100 IU/ml penicillin, 100 mg/ml streptomycin, and 2mM L-glutamine (Invitrogen–Gibco) (cDMEM). Huh7 or Huh6 cells containing a subgenomic HCV replicon were maintained with 250 μg/ml G418 (Sigma, Zwijndrecht, The Netherlands). Effects on HCV replication were determined based on luciferase activity or quantitative real time PCR.

2.2. Purchase or poduction of lentiviral vectors

TRC lentiviral vectors (Sigma-Aldrich) were obtained from Erasmus Center for Biomics. Santa Cruz lentiviral RNAi vectors were ordered from Santa Cruz Biotechnology, Inc., California, USA. Other vectors were preserved in van-der-Laan or Berkhout's lab.

2.3. In vitro vector transductions, GFP and CD81 analysis

Concentrated virus stocks were titrated using 293T cells 24h after infection, with transduction efficiency based on the number of GFP-positive cells as determined by flowcytometry (FACSCalibur; BD BioSciences, Mountain View, CA, USA) after 72 hours. CD81 expression was determined using flowcytometry by staining with phycoerythrin (PE) conjugated mouse anti-human CD81 monoclonal antibody (BD Pharmingen, San Diego, USA). Mouse IgG1 was used as isotype-matched control antibody (BD Pharmingen).

2.4. Selection of cells transduced with low dose TRC or home made shCD81 lentiviral vectors

Huh7 cells were transduced with low dose TRC or home made shCD81 lentiviral vectors, in order to generate stable cell lines with comparable low copy number of vector integration. Since TRC vector contains puromycin resistance gene, transduced cells were selected by culturing in 1 ug/ml puromycin to clear the non-transduced cells. Whereas our home made

shCD81 vector contains GFP reporter gene, transduced GFP positive cells were sorted by FACS Aria sorter (BD BioSciences).

2.5. Production of RNAi conditioned medium (CM)

Huh7 cells were cultured with normal culture medium. When cultures reached 60-70% confluence, cells were un-transduced or transduced by vector for 6 hrs, washed three times with PBS and subcultured in normal medium for more than six days. Conditional medium (CM) was collected from the second refreshment of the culture medium. To avoid transfer of free LV-sh vectors or cells, the CM was centrifuged and filtered (0.25 μM pores).

2.6. Luciferase assay

100 mM luciferin potassium salt (Sigma) was added to Huh-7 ET cells and incubated for 30 min at 37ºC. Luciferase activity was quantified using a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

2.7. Quantification of gene expression by real time RT-PCR

Cells were lysed using Trizol (Invitrogen–Gibco), RNA was precipitated using 75% EtOH and captured with a Micro RNeasy silica column (Qiagen, Venlo, The Netherlands). RNA was quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from 1 μg total RNA using the iScript cDNA Synthesis Kit from Bio-Rad (Bio-Rad Laboratories, Stanford, CA, USA). The cDNAs of HCV, FKBP8, CyB, CD81 and GAPDH were quantified using real-time PCR (MJ Research Opticon, Hercules, CA, USA) performed with SYBR Green PCR Master Mix (Bioline USA Inc., Tauton, MA) according to manufacturer's instructions.

2.8. Quantification of miRNA

The kits for total RNA isolation, cDNA preparation and real-time PCR detection of miRNA were purchased from Exiqon, Denmark or Applied Biosystems, USA, and the experiments were performed according to manufacture’s guidelines. RNA was quantified using a Nanodrop ND-1000 (Wilmington, DE, USA).

2.9. Quantification of shRNA

The TRC shCD81 and our home made shCD81 vector express shRNA targeting the same region of human CD81 gene (TRC shCD81: CAAGGATGTGAAGCAGTTCTA; home made shCD81: GGATGTGAAGCAGTTCTAT). Therefore, it allows designing a customized kit for quantification of both shCD81. To avoid amplification of CD81 mRNA, we specially amplified the antisense sequence of shCD81 (UAGAACUGCUUCACAUCC) using a custom TaqMan-based real-time PCR assay ordered from Applied Biosystems.

In order to perform a fair comparison of shCD81 expression between two vector systems, Huh7 cells were transduced with low dose TRC or home-made shCD81 lentiviral vectors to generate stable cell lines with comparable low copy number of vector integration. Cells transduced with TRC vector were selected by culturing in puromycin, whereas cells transduced with home-made shCD81 vector were sorted based on GFP positivity.

2.10. Quantification of lentiviral vector integration by real time RT-PCR

Genomic DNA of TRC or home made shCD81 tranduced cells was isolated using QIAamp DNA Blood Mini Kit (Qigan, Venlo, The Netherlands). DNA was quantified using Nanodrop ND-1000 (Wilmington, DE, USA). The following primers were used to amplify the vector sequence: forward primer, 5-CTGGAAGGGCTAATTCACTC-3; reverse primer, 5-GGTTTCCCTTTCGCTTTCAG-3 [14]. 250 ng, 1.25 µg or 2.5 µg of genomic DNA with SYBR Green PCR Master Mix (Bioline USA Inc.) and primer were used per RT-PCR reaction to determine the relative copy number of integrated lentiviral vector.

2.11. Statistical analysis

Statistical analysis was performed by using either matched pair nonparametric test (Wilcoxon signed-rank test) or the non-paired, nonparametric test (Mann–Whitney test) usingGraphPad Prism software. P-values less than 0.05 were considered as statistically significant.

3. Results & Discussion

The TRC lentiviral shRNA library (Sigma-Aldrich, St. Louis, MO, USA) [3] is based on the third-generation self-inactivating lentiviral vector contains a shRNA cassette driven by the human polymerase III promoter U6 and the puromycin resistance gene (puromycin n-acetyl transferase) driven by the PGK promoter element. Using these vectors, long-term gene silencing can be achieved at low Multiplicity Of Infection (MOI) transduction by puromycin selection of stable vector-integrated cells. We first screened in the established HCV subgenomic replicon model, Huh7-ET, which contains HCV non-structural sequences and a luciferase reporter gene [15]. Viral replication was monitored by measuring luciferase activity. As expected, a pool of five different shRNA vectors targeting a known HCV host factor, FKBP8 [16], resulted in knockdown of FKBP8 gene expression and inhibition of HCV replication (Fig. 1A). However, a control vector containing shRNA targeting green fluorescent protein (shGFP), which is not expressed in these HCV replicon cells, unexpectedly resulted in inhibition of HCV replication without affecting FKBP8 gene expression (Fig. 1A). Surprisingly, any set of shRNA vectors we tested from the TRC library (fourteen target genes without known relation to HCV replication), evoked a significant inhibition of HCV replication compared to untreated replicon cells or cells treated with the control vector (LV-con) without shRNA cassette (Fig. 1B). The tetraspanin CD81 is known to be involved in HCV viral entry but not viral replication. Direct comparison of our home-made shCD81 vector [12-13] and the TRC shCD81 vector containing the exact same CD81 target sequence showed both vectors had similar efficacy in reducing CD81 gene expression, however, only the TRC vector affected HCV replication (Fig. 1C). The off-target and the gene-specific silencing effects of the TRC shCD81 vector had similar dose-dependency for a range of MOI (Fig. 1D). The off-target effect was even seen after four weeks of puromycin selection of cells with low vector integration rates (Fig. 1E). This was tested in another hepatoma cell line, Huh6 replicon, a model that is more suitable for long-term maintaining HCV replication in culture [17]. These results suggest that optimization of the dose of the shRNA vector is not able to circumvent the off-target effect. We further tested another independent lentiviral shRNA library commercially available from Santa Cruz Biotechnology (http://www.scbt.com/gene_silencers.html.) For each target gene, pools of three to five

individual vectors are provided, each containing different shRNA species driven by the same H1, human polymerase III promoter. As shown in Figure 1F, all the vectors induced specific silencing of target genes by 70-80% but also evoked off-target inhibition of HCV replication.

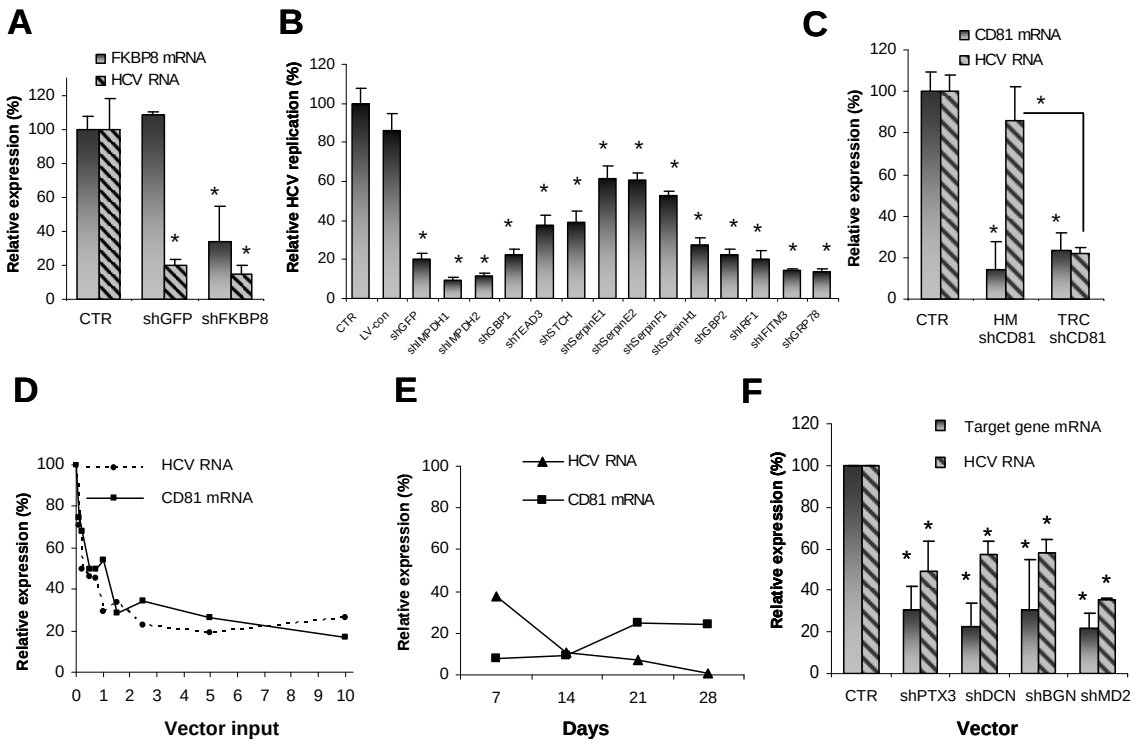

Figure 1. Sequence non-specific disturbance of HCV replication by widely used lentiviral shRNA libraries. (A) HCV replication was dramatically reduced in Huh7-ET replicon cells after transducing the TRC shFKBP8 vector. However, a control vector containing shRNA targeting GFP also inhibited HCV replication without affecting FKBP8 gene expression. (B) Set of fourteen TRC shRNA vectors (pool of 4-5 vectors for each target gene) tested in the subsequent screening showed clear non-specific effects on HCV replication. (C) TRC shCD81 and our home made (HM) shCD81 vector, which target the same sequence of CD81 mRNA, significantly reduced CD81 expression at comparable levels. However, the TRC vector but not our original vector showed a clear off-target effect on HCV replication. (D) The TRC shCD81 vector down regulated CD81 expression in a dose-dependent fashion as determined by qRT-PCR, but is closely accompanied with off-target effects on HCV replication. (E) The non-specific effect on HCV replication was also observed in Huh6 replicon cells transduced with low dose TRC shCD81 vector and long-term culture after puromycin selection and it was comparable with the specific knockdown of the CD81 target gene. Data of HCV and CD81 RNA expression was calculated as the percentage of cells transduced with a control vector, LV-con. (F) Also vectors obtained from Santa Cruz lentiviral shRNA library showed significant off-target effects on HCV replication. * P<0.01.

It is known that expression of double-stranded RNA can induce the production of type I interferons via activation of PKR. In order to exclude the possible induction of interferon as well as other paracrine antiviral cytokines/compounds by the TRC shRNA vectors, we collected culture medium from transduced Huh7 cells and determined the antiviral activity

in HCV replicon cells. As shown in Figure 2A, conditioned medium of transduced cells had no effect on HCV replication suggesting that no significant amounts of interferon or other antiviral cytokines/compounds are produced.

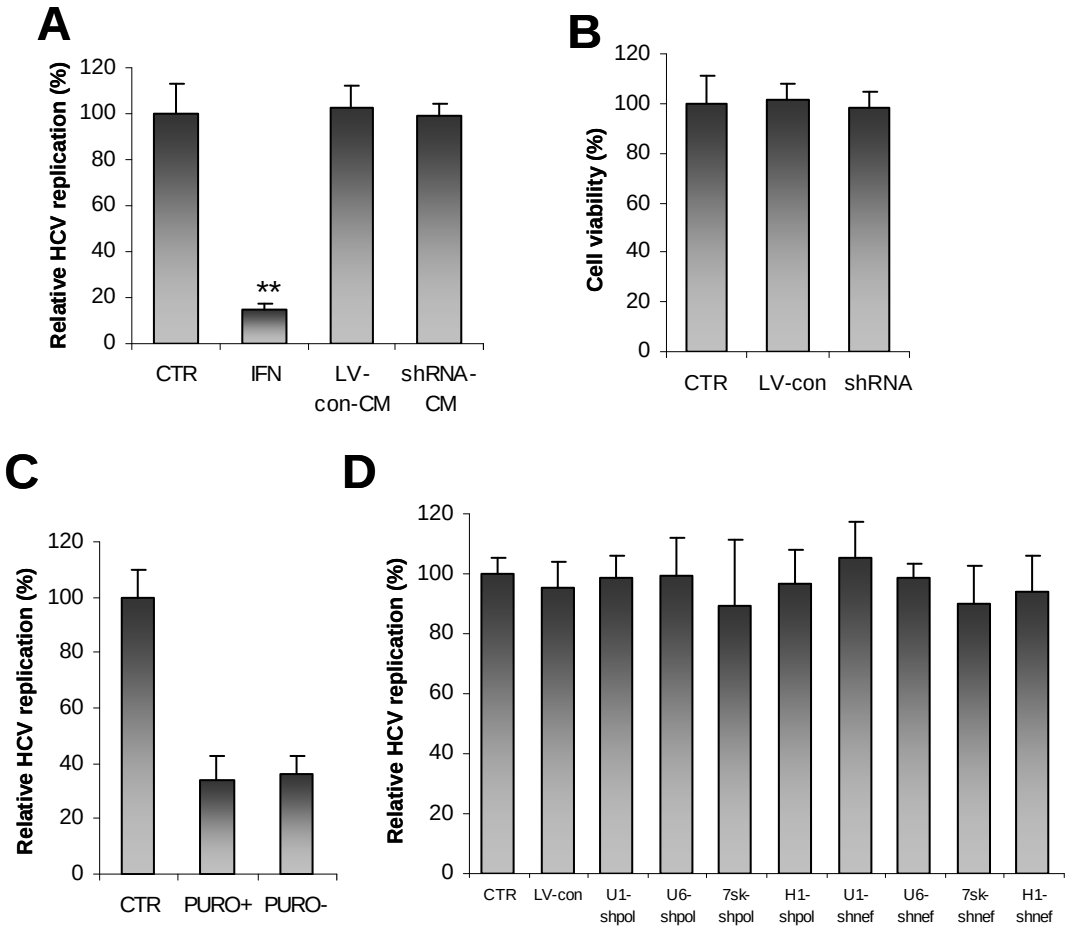

Figure 2. The off-target effect of lentiviral shRNA libraries is independent of interferon production, cytotoxicity, the puromycin resistance gene or the type of promoter used. (A) 0.3 IU/ml of interferon-alpha (IFN) significantly inhibits HCV replication by 85% ± 2 (mean ± SD, n=5, **P<0.01), whereas conditioned medium (CM) derived from Huh7 cells transduced with TRC LV-con or TRC LV-shRNA vectors did not show inhibition of viral replication in Huh7-ET replicon cells, indicating no production of significant amounts of interferon or other antiviral cytokines/compounds after transduction of lentiviral vectors. Data presented in the shRNA-CM group is the mean of five experiments with different LV-shRNA vectors. (B) Transduction of either control or shRNA vectors did not affect cell viability determined by MTT cell metabolism assay. (C) Deletion of the puromycin resistance gene from TRC vector did not circumvent the off-target effect. (D) Two sets of shRNAs, directed against HIV targets which are not expressed in the HCV replicon cells, driven by four different promoters (the polymerase III promoters U6, H1, 7SK and the polymerase II promoter U1) did not induce any non-specific effect on HCV replication, indicating that promoters by themselves are not responsible for the difference in off-target effects. Shown is mean ± SD of three independent experiments.

The number of viable cells was also not changed after transduction as measured by MTT conversion assay (Fig. 2B). One clear difference between our vectors and the vectors from the TRC and Santa Cruz libraries is the fact that these latter ones contain the puromycin resistance gene. To investigate whether the puromycin resistance gene cause the inhibition of HCV replication, a modified version of the TRC shGFP vector was made by removing the coding sequence of the puromycin resistance gene. As shown in Figure 2C, this modified lentiviral vector had a similar effect on HCV replication as the wild type shGFP vector. An alternative explanation for the different off-target effects of shRNA vectors is the use of a particular promoter element, as it was reported that shRNA expression levels could significantly differ between, for instance, the U6 and H1 promoters [18]. To investigate whether different promoters cause distinct off-target effects, two sets of shRNAs vectors were tested with four different promoters (the polymerase III promoters U6, H1, 7SK and the polymerase II, U1)[19]. The shRNAs were directed against the *Pol* and *Nef* HIV sequences which are not present in the HCV replicon cells. As shown in Figure 2D, none of vectors induced any inhibition of HCV replication, suggesting that a difference in promoter does not, by it self, explain the difference in off-target effects.

Previous study has reported that adeno-associated virus delivered shRNA in certain context could over-saturate the cellular microRNA pathways [20]. Numerous studies have consistently shown that HCV replication is tightly regulated by the endogenous microRNA, miR-122 [21-22], and treatment of chronically infected chimpanzees with a locked nucleic acid (LNA)-modified oligonucleotide complementary to miR-122 leads to long-lasting suppression of HCV viremia [23]. Therefore, we investigated the effects of library shRNA vectors on the miRNA biogenesis. As shown in Figure 3A, we found that in transduced cells levels of mature miR-122 (detected by mature miRNA specific RT-PCR) were significantly reduced by 68% ± 10 (mean ± SD, n=7, $P<0.01$), which was accompanied by a more than 3-fold increased expression of cyclin G1 (CCNG1), known to be negatively regulated by miR-122 [24] (Fig. 3B). Similar results were obtained with cells having low vector integration rates selected by puromycin treatment, showing long-term suppression of miR-122 coinciding with a persistent reduction of HCV replication (Fig. 3C). Disturbance of miRNA biogenesis was not restricted to miR-122 but also seen for five other hepatic abundant miRNAs (Fig. 3D).

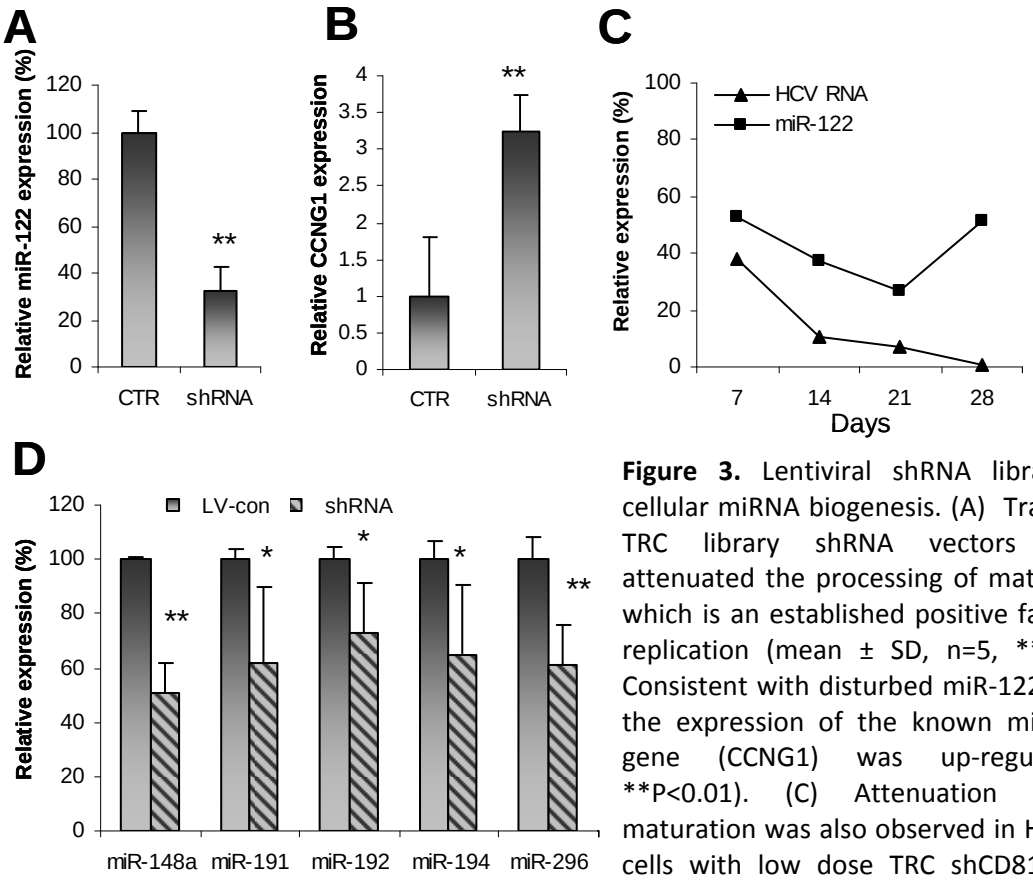

Figure 3. Lentiviral shRNA libraries disturb cellular miRNA biogenesis. (A) Transduction of TRC library shRNA vectors significantly attenuated the processing of mature miR-122, which is an established positive factor for HCV replication (mean ± SD, n=5, **P<0.01). (B) Consistent with disturbed miR-122 maturation, the expression of the known miR-122 target gene (CCNG1) was up-regulated (n=5, **P<0.01). (C) Attenuation of miR-122 maturation was also observed in Huh6 replicon cells with low dose TRC shCD81 vector and long-term culture after puromycin selection. Data of HCV RNA and miR-122 expression was calculated as the percentage of the control cells transduced with the control vector, LV-con. Shown is one representative experiment of three. (D) Significant reduction of mature miRNA was further confirmed in a set of other hepatocytes abundant miRNAs, including miR-148a, miR-191, miR-192, miR-194 and miR-296. *P<0.05, ** P<0.01.

To further investigate the cause of miRNA biogenesis disturbances, we directly compared shRNA expression levels in cells transduced with the TRC shCD81 vector or our home-made shCD81 vectors using a customized quantitative RT-PCR assay. The shRNA of both vectors target the same region of CD81 mRNA, allowing direct comparison of expression levels. Both vectors resulted in a similar number of integrated vectors per cell (1.0 vrs 1.8 copy numbers per cells, P=0.51). As shown in Figure 4A, both vectors resulted in a comparable knockdown of CD81 cell surface expression. However, the TRC shCD81 vector resulted in an over 10-fold higher expression of shRNA then our own shCD81 vector (Fig. 4B). Consistently, significant lower expression of miR-122 was observed in the TRC transduced cells (Fig. 4C). Apparently, the commercial lentiviral shRNA vectors have been optimized for efficient shRNA expression intended to maximize the knockdown efficiency of the target genes. Additional experiments were performed to confirm the relation between shCD81 and miR-122 level. As shown in

Figure 4D, escalating doses of the vector input resulted in an increased level of shCD81 and, reversely, a decreased level of miR-122. This is consistent with the earlier observation that increasing dose of TRC shRNA vectors results in increasing inhibition of HCV replication (Fig. 1D). Combined, these results indicate that over-expression of shRNA affects the miRNA levels, likely by causing saturation of the cellular components of the miRNA biosynthesis pathway including exportin-5 and Argonaute proteins [25].

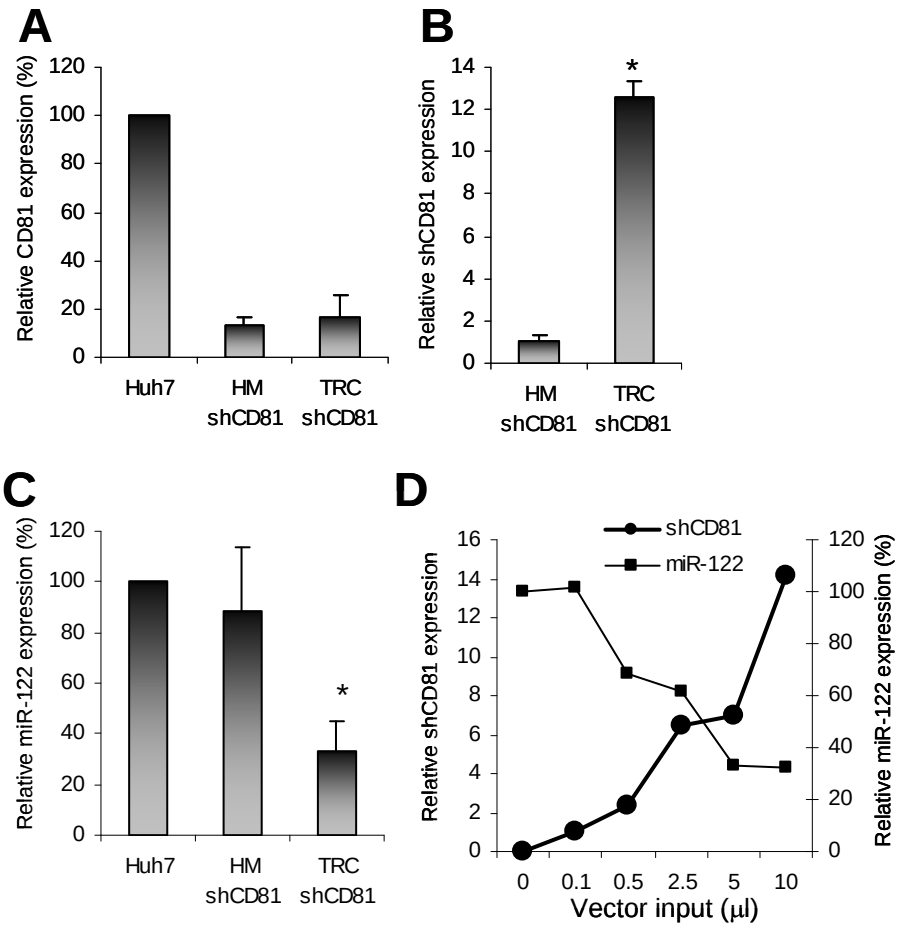

Figure 4. Over-expression of shRNA by the TRC library vector causes disturbance of mature miRNA levels. Huh7 cells transduced with low dose TRC or home-made (HM) shCD81 lentiviral vectors were selected by puromycin resistance or cell sorting, respectively. (A) Comparable CD81 knockdown by TRC or home-made shCD81 vectors was observed (mean ± SD, n=4). (B) The shCD81 expression level was significantly higher in TRC vector transduced cells (12.6 fold ± 0.8 SD, n=4, *P<0.01) as compared to home-made vector transduced cells. (C) Accordingly, miR-122 expression was significantly down-regulated in TRC shCD81 cells and not the HM shCD81 cells (n=4, * P<0.01). (D) Increasing doses of the vector input of TRC shCD81 vector resulted in increased level of shCD81 and reversely decreased the levels of miR-122. Shown is one representative experiment of two.

In conclusion, we found that two widely used lentiviral shRNA libraries have unexpected effects on HCV replication, due to the disturbance of endogenous miRNA production by high

the levels of shRNAs. The model system we have used maybe particular sensitive to regulation by miRNA, but these findings raise concerns about potential off-target effects seen in lentiviral RNAi screens due to effects on miRNA regulated phenotypes. Therefore, based on our findings, caution should be taken in interpretation and validation of positive hits obtained in lentiviral RNAi screening, and appropriate control vectors should be tested to exclude effects on miRNA processing. A solution to this problem is proposed to balance the optimization of shRNA expression levels in order to obtain effective RNAi and the minimization of disturbance of miRNA levels.

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Chapter 8

Hepatic cell-to-cell transmission of small silencing RNA can extend the therapeutic reach of RNAi

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Gut, 2011, Epub

Abstract

RNA interference (RNAi), a sequence-specific gene silencing technology triggered by small interfering RNA (siRNA), represents promising new avenues for treatment of various liver diseases, including hepatitis C virus (HCV) infection. In plants and invertebrate, RNAi provides an important mechanism of cellular defense against viral pathogens and is dependent on the spread of siRNA to neighboring cells. In this study, we investigated whether vector-delivered RNAi can transfer between hepatic cells in vitro and in mice and whether this exchange could extend the therapeutic effect of RNAi against HCV infection. Transmission of RNAi was investigated in culture by assessing silencing of HCV replication and expression of viral entry receptor, CD81, using a human hepatic cell line and primary B lymphocytes transduced with siRNA-expressing vectors. In vivo transmission between hepatic cells was investigated in NOD/SCID mice. Involvement of exosomes was demonstrated by purification, uptake and mass spectrometric analysis. We found that human and mouse liver cells as well as primary human B cells have the ability to exchange small RNAs, including cellular endogenous microRNA and delivered siRNA targeting HCV or CD81. The transmission of RNAi was largely cell-contact independent and partially mediated by exosomes. Evidence of RNAi transmission in vivo was observed in NOD/SCID mice engrafted with human hepatoma cells producing CD81 siRNA, causing suppression of CD81 expression in mouse hepatocytes. In conclusion, both human and mouse hepatic cells exchange small silencing RNAs, partially mediated by shuttling of exosomes. Transmission of siRNA potentially extends the therapeutic reach of RNAi-based therapies against HCV as well as other liver diseases.

Introduction

The translation of molecular biology research has recently fuelled a rapid progress in the drug development for hepatitis C virus (HCV) infection. The directly acting antivirals, including a range of protease and polymerase inhibitors, are at various stages of clinical development.[1] These compounds have potent antiviral activity but also dramatically potentiate the efficacy of the current standard of care, based on pegylated interferon-alpha (IFN-α) combined with ribavirin.[2-3] However, given the large infected population (approx. 170 million carriers), accumulated non-responders, poor tolerability to interferon or the directly acting antivirals, and special populations (e.g. human immunodeficiency virus (HIV) co-infected patients and transplanted patients), novel antivirals remain urgently required, which ideally should act on distinct mechanisms and be applicable in the current non-responders and special populations with less side effect.

RNA interference (RNAi) is a sequence-specific inhibition of gene expression at posttranscriptional level. It is triggered by small interfering RNA (siRNA), which can be introduced into cells as synthetic siRNA or synthesized from a transgene in the cells as the short-hairpin RNA (shRNA) precursor.[4] By using the cellular gene silencing/microRNA (miRNA) biogenesis machinery, these delivered siRNA induces degradation of mRNA by targeting the complementary sequences.[5] This technology has now emerged as a new avenue to combat viral infections and recent developments in the field of gene therapy have increased the feasibility of clinical applications with dozens of RNAi clinical trails currently underway (www.ClinicalTrials.gov). Both the viral genome and host cellular factors involved in the viral life cycle, such as viral receptor CD81, can be targeted by RNAi and convey protection against infection.[6-7] In the context of treating chronic HCV or preventing recurrence in HCV-positive transplant, a single dose administration with long-lasting therapeutic effects would be ideal. Therefore, integrating lentiviral vector expressing shRNA represents a suitable strategy.[8]

In plants and invertebrates, RNAi naturally provides an important defense mechanism against pathogens. Pathogen-derived siRNA, formed by processing of double stranded RNA (replication) intermediates during infection, spread to neighboring cells and even propagate throughout the entire organism.[9-11] This transmission of RNAi was shown to be of critical

importance for plant and insect resistance against infections.[9, 11-12] RNAi transmission is also able to direct epigenetic modification in recipient cells in plants and conveys protection against pathogenic challenges.[13-14] Mammalian cells, like mouse or human mast cell lines,[15] the African green monkey kidney fibroblast-like cell line,[16] and human glioma, embryonic kidney, Epstein–Barr virus positive nasopharyngeal carcinoma and B lymphocyte cell lines,[16-19] were shown to be able to transfer cellular or viral encoded miRNAs in culture via secreted exosomes in cell-contact independent manner. In contrast, transmission of endogenous miRNA, viral miRNA or delivered small RNA between B and T cell lines in culture occurs in a cell contact-dependent.[20]

In this study, we investigated transmission of vector-derived RNAi in culture of human hepatic cells and primary human B cells and in mice liver. We found that human and mouse liver cells and primary human B cells have the ability to exchange small RNAs, including small silencing RNA as well as miRNA. We further demonstrated that transmission of gene silencing is cell-cell contact independent and, like reported for miRNA, can be partially mediated by exchange of secreted exosomes. The property of hepatic cells to exchange small silencing RNAs can significantly extends the therapeutic reach of RNAi-based therapy against HCV infection and other liver diseases.

Materials & Methods

Cell culture

Cell monolayers of the human embryonic kidney epithelial cell line 293T and human hepatoma cell line Huh7, Huh6 and HepG2 were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen–Gibco, Breda, The Netherlands) supplemented with 10% v/v fetal calf serum (Hyclone, Logan, Utah, USA), 100 IU/ml penicillin, 100 g/ml streptomycin, and 2 mM L-glutamine (Invitrogen–Gibco). Huh7 cells containing a subgenomic HCV bicistronic replicon (I389/NS3-3V/LucUbiNeo-ET, Huh7-ET) were maintained with 250 μg/ml G418 (Sigma, Zwijndrecht, The Netherlands).

Primary human B cells were expanded from liver transplant donor splenocytes using a mouse fibroblast cell line stably transfected with human CD40L. The detailed protocol was described in our previous study.[21]

Luciferase assay

Effects on HCV replication were determined based on luciferase activity. 100 mM luciferin potassium salt (Sigma) was added to Huh-7 ET cells and incubate for 30 min at 37°C. Luciferase activity was quantified using a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

miR-122 reporter assay

pMiR-Luc reporter vector expressing firefly luciferase gene incorporated with a unique miR-122 target site at its 3'UTR was purchased from Signosis, Inc. (Sunnyvale, CA, USA). 293T cells were transfected with the plasmid and treated with concentrated Huh7-CM or control medium for 24h. Luciferase activity was measured as described above.

Lentiviral vectors, conditioned medium (CM) and RNAi transfer experiments

Lentiviral vectors LV-shCD81 and LV-shNS5b, were constructed and produced as previously reported.[8] LV-shNS5b contains expression cassettes of shRNA targets the viral NS5b region (GACACUGAGACACCAAUUGAC 6367-6388). LV-shCD81 targets human and mouse CD81 mRNA (GGAUGUGAAGCAGUUCUAU). Lentiviral vector expressing miR-122 (LV-miR-122) was constructed by cloning of the precursor sequence of mature miR-122 amplified by PCR from human genomic DNA. A third-generation lentiviral packaging system pND-CAG/GFP/WPRE was used to produce high-titer VSV-G-pseudotyped lentiviral vectors in 293T cells. Vector supernatants were removed 36 and 48 hr post transfection, passed through a 0.45 m filter and concentrated 1000-fold by ultracentrifugation. Concentrated virus stocks were titrated using 293T cells 24h after infection, with transduction efficiency based on the number of GFP-positive cells as determined by flowcytometry (FACSCalibur; BD BioSciences, Mountain View, CA, USA) after 72 hours. Vector concentration was determined in 293T cells based on the number of GFP-positive cells as determined by flowcytometry. CD81 expression was determined using flowcytometry by staining with phycoerythrin (PE) conjugated mouse anti-human CD81 monoclonal antibody (BD Pharmingen, San Diego, USA). Mouse IgG1 was used

as isotype-matched control antibody (BD Pharmingen). Effects of RNAi on CD81 expression was determent by flowcytometry.

Huh7 cells were cultured with normal culture medium. When cultures reaching 60-70% confluence, cells were un-transduced or transduced by LV-shCD81, LV-shNS5b or LV-shCon for 6 hrs, washed three times with PBS and subcultured in normal medium for more than 8 days. Conditioned medium (CM) was collected after the second refreshment of the culture medium. To generate CM specifically containing miR-122, 293T cells were transduced with LV-miR-122 or control lentiviral vector (LV-CTR). After overnight transduction, 293T cells were washed three times and cultured for up to 8 days. The CM from 293T was prepared using fresh culture medium and collected after 48 hrs. All CM were centrifuged at 4 000 rpm for 30 min to remove cell contaminants. Concentrated CM (approx. 25- to 100-fold) was prepared using ultrafiltration units with a 3-kDa cutoff membrane (Millipore, Bedford, MA, USA). Huh7 cells were treated with conditioned medium for 48 hrs at 1:1 dilution.

Cell co-culture experiments

To generate stable shRNA integrated cell lines, naïve Huh7 cells were transduced with the lentiviral vectors and were expanded in culture for at least 8 days before using in experiments. Co-culture experiments were performed for 48 hrs in 96-well culture plates, with 20 000 Huh7-ET HCV replicon cells per well mixed with 20 000, 10 000 or 2 000 control LV-shRNA or LV-shNS5b transduced Huh7 cells. HCV replication was determined by luciferase activity. Co-culture with control (parental) Huh7 cells had no effect on HCV replication/luciferase activity and did not effect Huh7-ET cell proliferation at any condition, as measured by CFSE dilution assays.

Similarly, primary human B cells were also transduced with LV-shRNA or LV-shNS5b vector to generate stable shRNA donor cells. Co-culture experiments were performed by mixing with Huh7-ET cells.

RNA transfer experiments in mice

Immunodeficient NOD/SCID mice (Charles River Laboratories, Wilmington, MA, USA) aged 3-4 weeks were used. The use of animals was proved by the institutional animal ethics committee at Erasmus Medical Center Rotterdam. Mice were engrafted with 0.5×10^6 Huh7-shCD81 (four mice) or Huh7-shCon (seven mice) cells injected intrasplenic. Cell

transplantations and surgical procedures were performed under 1.5% isoflurane inhalation anaesthesia and a prophylactic antibiotic was given. Two and half weeks after engraftment, mice were sacrificed and liver tissue obtained for analysis. To demonstrate cell-free transfer of small RNA, NOD/SCID mice were intravenous injected with 200 µL of 100-fold concentrated shCD81-CM or shCon-CM every two days for three times (four animals per group). After 6 days, mice livers were procured, dissociated by collagenase digestion[22] and analyzed for CD81 expression by flowcytometry.

Exosome purification and electron microscopy imaging

Exosomes were prepared from the supernatant of Huh7 cells by differential centrifugation. Briefly, supernatant was centrifuged at 3 000 g for 20 min to eliminate cells and at 10 000 g for 30 min to remove cell debris. Exosomes were pelleted by ultracentrifugation (Beckman SW28) at 64,047 g for 110 min followed by a sucrose gradient isolation at 100 000 g (Beckman SWTi60). For uptake experiments, 0.1% Rhodamine C18 solution was added to the sucrose before centrifugation. For electron microscopy, exosomes were visualized by negatively staining using uranyl acetate.

Exosome uptake and RNAi transfer

For visualization of exosome uptake, Huh7 cells were seeded on glass cover slips. Rhodamine labeled exosomes were added to live cells on coverslips in heated-chamber (37°C) and uptake was measured real-time using confocal microscopy (Zeiss LSM510META). To determine the kinetics of exosome uptake, images were taken every minute for 45 minutes. Paraformaldehyde (PFA)-fixed cells served as control to exclude passive transfer of Rhodamine by exosome cell-fushion. In order to specify the subcellular localization of exosomes, nuclear staining using the Hoechst dye were performed. In these experiments, only two time points were measured, 1 and 30 min after adding exosomes. This is to avoid cytotoxicity of Hoechst induction by the laser and decay of the nuclear staining.

RNAi transfer by purified exosomes was tested by treating Huh7-ET cells with shNS5 containing exosomes for 48h and viral replication was measured based on luciferase activity. Similarly, Huh7 cells were treated with shCD81-containing exosomes for 48h and CD81 cell surface expression was quantified by flowcytometry.

Mass spectrometric analysis

Two batches of purified exosomes were subjected to mass spectrometry at the Erasmus MC Proteomics Center. Briefly, 1D SDS-PAGE gel lanes were cut into 2-mm slices using an automatic gel slicer and subjected to in-gel reduction with dithiothreitol, alkylation with iodoacetamide and digestion with trypsin (Promega, sequencing grade), essentially as described by Wilm et al.[23] Nanoflow LC-MS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ-Orbitrap mass spectrometer (Thermo) operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm × 100 µm, packed in-house) at a flow rate of 8 µl/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm × 50 µm, packed in-house) using a linear gradient from 0 to 80% B (A = 0.1 % formic acid; B = 80% (v/v) acetonitrile, 0.1 % formic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode. Peak lists were automatically created from raw data files using the Mascot Distiller software (version 2.3; MatrixScience). The Mascot search algorithm (version 2.2, MatrixScience) was used for searching against a customized database containing all IPI_human protein sequences (release 2010_09). The peptide tolerance was typically set to 10 ppm and the fragment ion tolerance was set to 0.8 Da. A maximum number of two missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 65. Individual peptide MS/MS spectra with Mascot scores below 40 were checked manually and either interpreted as valid identifications or discarded. Typical contaminants, also present in immunopurifications using beads coated with pre-immune serum or antibodies directed against irrelevant proteins were omitted from the table.

RNA isolation and real-time RT-PCR analysis

Total RNA was extracted using the miRNeasy mini kit according to manufacturer's instructions (Qiagen, Hilden, Germany). Mouse liver tissues were mechanically disrupted and lysed using Trizol (Invitrogen–Gibco). RNA was quantified using a Nanodrop ND-1000

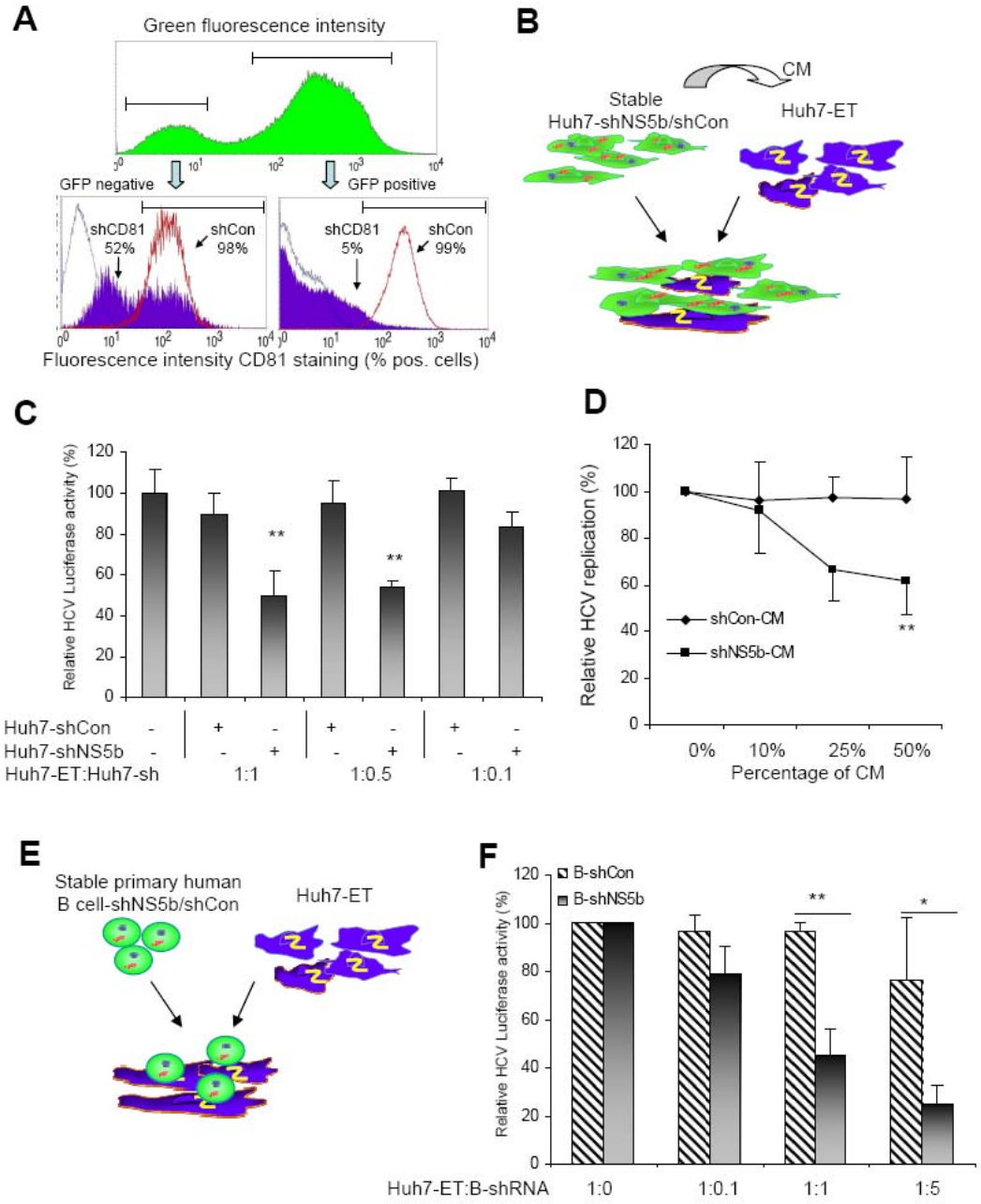
(Wilmington, DE, USA). cDNA was prepared from 1 µg total RNA using a iScript cDNA Synthesis Kit from Bio-Rad (Bio-Rad Laboratories, Stanford, CA, USA). The cDNA of mouse CD81, TBP, CyB, and GAPDH were quantified using real-time PCR (MJ Research Opticon, Hercules, CA, USA) performed with SybrGreen (Sigma-Aldrich) according to manufacturer's instructions. CD81 mRNA levels were normalized to the average level of the three independent reference genes using the ddCT method. TaqMan-based real-time PCR kit for detection of miR-122 was purchased from Applied Biosystems and analysis was performed according to manufacture's guideline. A customized kit for quantification of small silencing RNA was designed by amplification of the antisense sequence of shCD81 (UAGAACUGCUUCACAUCC) using TaqMan-based real-time PCR technique ordered from Applied Biosystems. The assay supposes to preferentially amplify the mature miR-122 or siCD81, but possibly also detect the precursors.

Fluorescent immunohistochemistry

Mouse liver tissue was dissected and cryoprotected in 30% sucrose for generation of frozen sections. Serial 6 µm cryosections were air-dried for 48 h at room temperature followed by a washing step with PBS. Sections were fixed with 50% acetone in PBS for 10 min on ice and blocked in PBS containing 4% fat free milk for 1 h at room temperature. Subsequently, sections were incubated with Alexa Fluor647 labeled anti-mouse CD81 antibody (AbD Serotec, Oxford, UK) at the dilution of 1:100 for 30 min. After three washes, nuclear staining was achieved by incubating with DAPI (Sigma-Aldrich) at the dilution of 1:50 for 5 min. Multiple areas from the mouse liver tissue surrounding nodules of engrafted Huh7 cells were analyzed by confocal microscopy. The Huh7 nodules were distinguished from liver parenchyma based on GFP-positivity and tumor morphology.

Statistical analysis

Statistical analysis was performed by using either matched-pair nonparametric test (Wilcoxon signed-rank test) or the non-paired, nonparametric test (Mann–Whitney test) using GraphPad Prism software. P-values less than 0.05 were considered as statistically significant.



Results

Transmission of lentiviral vector-delivered RNAi targeting HCV receptor or viral genome

We have constructed a lentiviral vector, LV-shNS5b, which contains both the green fluorescent protein (GFP) reporter gene and a shRNA targeting the HCV NS5b region, which encodes the viral RNA-dependent RNA polymerase. We used a subgenomic HCV replication

model, based on a Huh7 hepatoma cell line containing the non-structural sequence of HCV genome with a luciferase reporter gene (Huh7-ET), mimicing viral replication without virus particle production.[24] As reported, LV-shNS5b resulted in a maximum inhibition of HCV replication of 98% ± 0.5 (mean ± standard deviation, n = 8, P<0.001) at highest transduction efficiency.[8, 25] However at suboptimal transduction efficiency, the percentage inhibition of viral replication, as measured by luciferase activity, significantly exceeded the percentage of transduced cells, as measured by GFP expression. For instance, with a transduction efficiency of 45% GFP the observed inhibition of HCV replication was 58%, suggesting possible extension of RNAi to non-transduced cells. Similar results were observed with the LV-shCD81, a vector containing GFP and shRNA targeting the HCV receptor CD81. LV-shCD81 profoundly reduced CD81 cell surface expression in transduced Huh7 cells (mean inhibition 92.9% ± 5.9 SD, n=8, P<0.001), but also significantly reduced CD81 expression in, the non-transduced, GFP-negative cells (30.1% ± 12.9 inhibition, P<0.001) (Fig. 1A). CD81 reduction was not related to loss of cell viability as dead/permeable cells were excluded from analysis.

Figure 1. Evidence for intercellular functional transmission of small silencing RNAs. (A) Silencing of CD81 expression by LV-shCD81 extended to, GFP-negative, non-transduced cells. Huh7 cells were transduced by LV-GFP containing either a CD81 targeting shRNA (LV-shCD81) or a scrambled control shRNA (LV-shCon). Shown in the upper panel is representative histogram of GFP fluorescence intensity. Lower panels show flowcytometric analysis of CD81 staining in gated GFP negative (left panel) and GFP positive (right panel) cells transduced with LV-shCon (Red line) or LV-shCD81 (Purple area). Blue lines show isotype-matched control staining. The percentages of CD81 positive cells are indicated. (B) HCV replicon cells (Huh7-ET) were directly co-cultured with cells stably expressing shNS5b (Huh7-shNS5b) or control shRNA (Huh7-shCon) or treated with conditioned culture medium (CM) of these cells. (C) Significant inhibition of HCV replication was observed at a 1:1 and 1:0.5 ratio of Huh7-ET with Huh7-shNS5b as compared to co-cultures with Huh7-shCon or untreated cells. Shown is the mean ± SD of six independent experiments. ** P<0.01 (D) Huh7-ET replicon cells treated with shNS5b-CM (at final concentration 50%), but not shCon-CM, showed a significantly reduced HCV replication of 39% ± 12 (n=9, **P<0.01) compared to untreated controls. (E) Huh7-ET cells were co-cultured with primary human B cells stably expressing shNS5b or shCon. (F) Significant reduction of viral replication was observed when co-cultured with B cells expressing shNS5b at 1:1 ratio (n=4, **P<0.01). Such an effect was also confirmed at 1:5 ratio (n=3, *P<0.05), although high density of B cells appears to cause some non-specific effects.

To ensure that the gene silencing effect on GFP negative cells was not due to insensitivity of GFP detection or silencing of transgenic expression, additional co-culture experiments were performed (Fig. 1B). A significant inhibition of HCV replication was observed when Huh7-ET HCV replicon cells were co-cultured with naïve Huh7 cells stably expressing shNS5b at a 1:1 ratio (51% ± 12 SD, n=6, P<0.01) as compared to Huh7-shCon co-cultures and untreated Huh7 cells (Fig. 1C). A similar effect was observed at a lower ratio of 1:0.5 of Huh7-ET and Huh7-shNS5b cells, but lost significance when co-culturing at very low ratios (Fig. 1C). To confirm in primary human cells, B cells were generated from splenocytes and stably transduced with shRNA vectors (Fig. 1E). Similarly, a robust inhibition of viral replication was observed in replicon cells co-cultured with B cells stably expressing shNS5b at 1:1 (n=4, P<0.01) or 1:5 ratio (n=3, P<0.05) as compared to B cell-shCon co-cultures (Fig. 1F).

RNAi transmission has been reported as acting via cell contact-dependent [20] or independent[15] fashions depending on the models used, although the exact mechanisms remain largely elusive. Using immunofluorescence microscopy we observed that LV-shCD81-dependent knockdown of CD81 expression in GFP negative cells was not restricted to cells in direct contact with GFP positive cells, but rather a general pattern of CD81 reduction was seen (data not shown). To further investigate whether RNAi can be transmitted in the absence of direct cell-cell contact, conditioned medium (CM) was prepared from stably transduced Huh7 cells expressing shCon, shCD81 or shNS5b (Fig. 1B). As shown in Figure 1D**,** exposure of Huh7-ET cells to shNS5b-CM (at final concentration of 50%) specifically reduced HCV replication by 39% ± 12 SD (n=9, P<0.01), without transfer of GFP positivity. Treatment with shCD81-CM also significantly reduced CD81 expression in Huh7 cells (23.5% ± 5.1 inhibition, n=7, P<0.01). These results suggest that transmission of RNAi is cell-contact independent but rather seem to involve the uptake of released silencing RNA components.

Functional transmission of liver abundant miRNA

We further investigated whether such a cell-contact independent manner of small RNA transmission also exist for endogenous miRNA. Huh7 cells highly express miR-122, a liver abundant miRNA that has been reported to be a crucial positive regulator of HCV replication and translation.[26] We found that cell-free conditioned medium of Huh7 cells (Huh7-CM)

contained high levels of miR-122 (data not shown). Concentration of Huh7-CM (Huh7-C-CM) using ultrafiltration resulted in a 10-fold increase of miR-122 levels. The miR-122 level of Huh7 cells is more than 200-fold higher than another hepatoma cell line HepG2 and over 50,000-fold higher than the embryonic kidney epithelial cell line 293T (data not shown). Treatment of HepG2 cells with Huh7-CM or Huh7-C-CM significantly increased intracellular miR-122 levels by 3- to 4-fold ($p<0.01$), indicating uptake of miR-122 from the medium. An even more pronounced miRNA uptake was observed in 293T cells, leading to about a 20- or 1750-fold elevation of cellular miR-122 levels after exposure to Huh7-CM and Huh7-C-CM, respectively (Fig. 2A). The miRNA transfer was also observed in freshly isolated human peripheral blood mononuclear cells incubation with Huh7-CM, resulted in approx. 100-fold increase in cellular miR-122 levels (Fig. 2B). To more specifically demonstrate the transfer of miRNA and to exclude possible induction of miRNA gene expression by other factors present in conditioned medium, we generated a lentiviral vector specifically expressing the precursor of miR-122 (LV-miR-122). Conditioned medium were produced from LV-miR-122 or control vector (LV-shCon) transduced 293T cells. 293T cells naturally expressed very low levels of miR-122 and transduction with LV-miR-122 (~5% transduction efficiency) resulted in approx. 10-fold increase of cellular miR-122 levels. As shown in Figure 2C, miR-122-CM but not shCon-CM specifically increased the cellular miR-122 levels in 293T cells by approx. 5-fold. Similarly, incubation with miR-122-CM increased the cellular miR-122 levels of the T cell line, SupT1 cells, by approx. 15-fold (Fig. 2D). To evaluate the functional consequence of miRNA transmission, a reporter plasmid expressing luciferase gene coupled with miR-122 complementary sequence was used to transfect 293T cells. Treatment of concentrated Huh7-CM significantly reduced miR-122 associated luciferase activity compared with either untreated or control medium treated group ($P<0.01$) (Fig. 2E), suggesting functional regulation of target reporter gene expression by transferred miRNA.

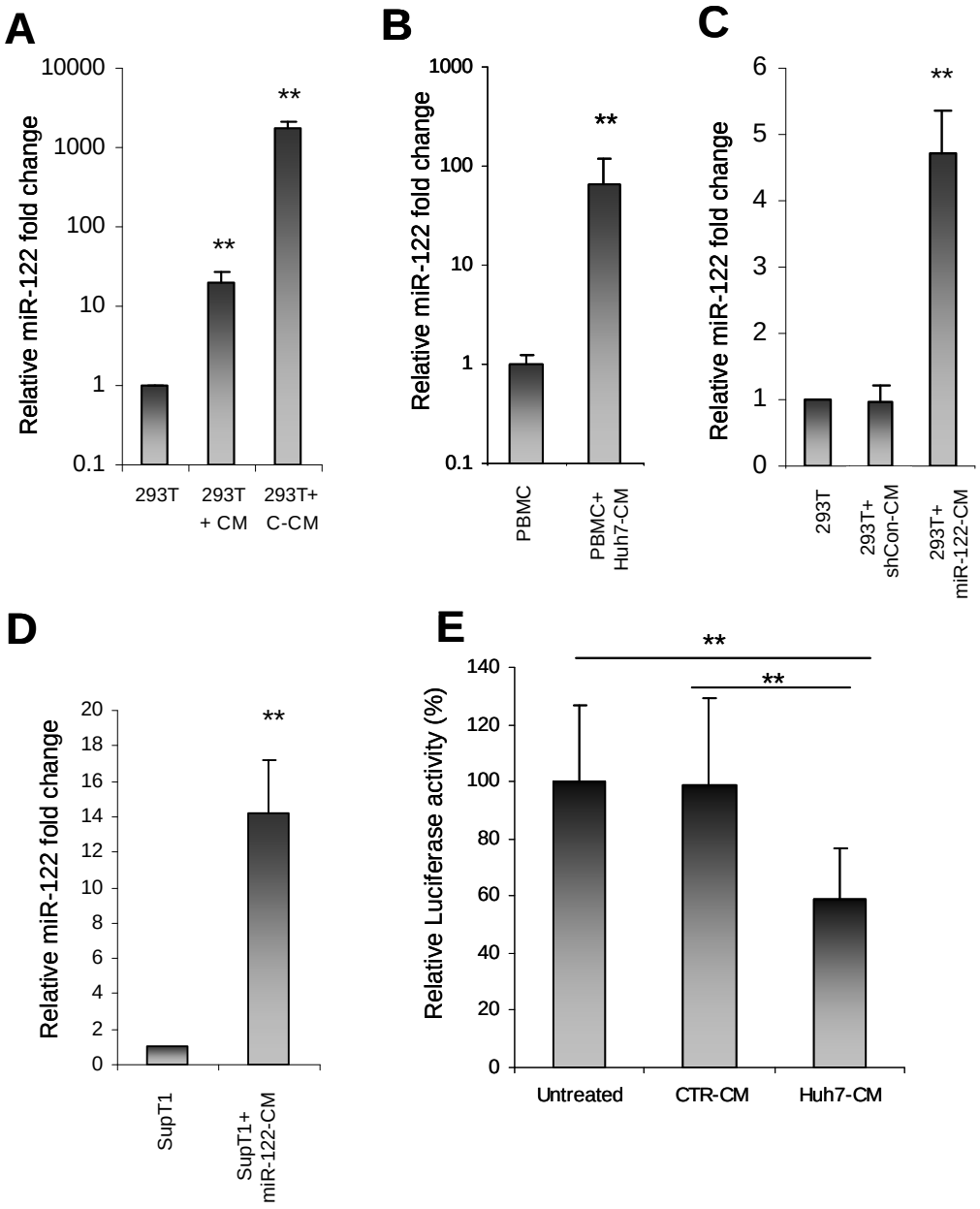
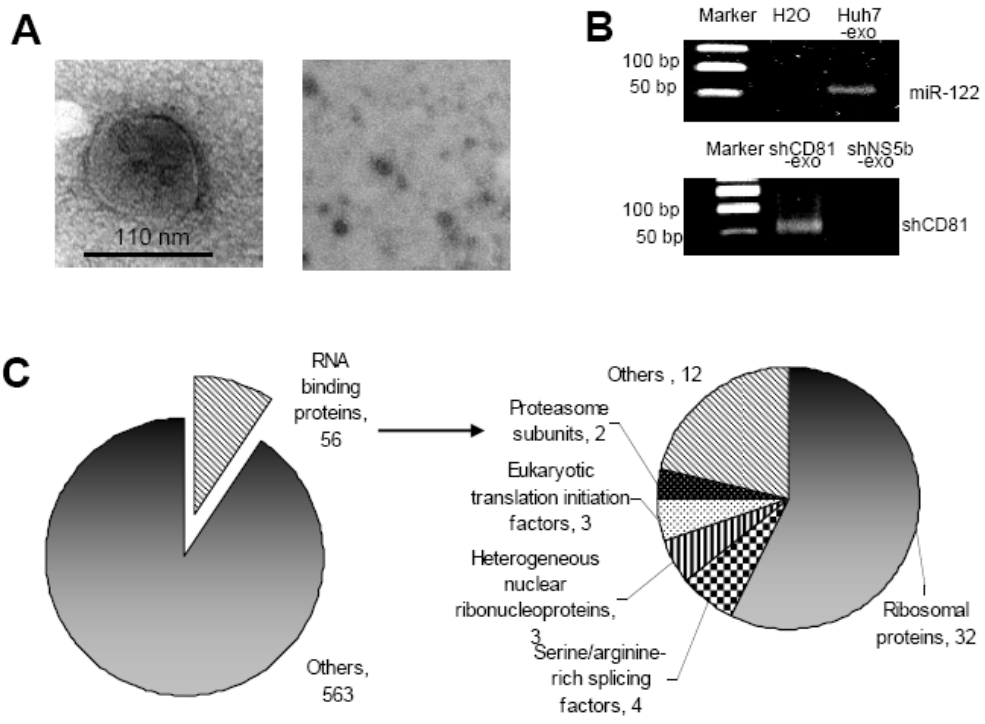


Figure 2. Evidence for intercellular functional transmission of liver abundant miRNA. (A) Uptake of miR-122 by 293T cells after exposure to Huh7-CM or Huh7-C-CM. (B) PBMCs from healthy controls, showing about 100-fold increase in cellular miR-122 level after 6 hours of incubation with Huh7-CM. (C) To confirm miRNA transfer and rule out the induction of miRNA gene expression by other factors present in CM, we generated CM of 293T cells either transduced LV-miR-122 or LV-shCon. Treatment of naïve 293T cells with miR-122-CM but not shCon-CM increased the cellular miR-122 level by approx. 5-fold. (D) Incubation with miR-122-CM resulted in about 15-fold increase of cellular miR-122 levels in the T cell line, SupT1 cells. Data shown above is the mean ± SD of three or four independent experiments. (E) Treatment of concentrated Huh7-CM resulted in significant reduction of miR-122 related luciferase activity in 293T cells transfected with miR-122 reporter plasmid, compared with control medium treated or untreated group. Shown is mean ± SD of three independent experiments (n=11 replicates in total). **P<0.01.

Secreted exosomes contain small RNAs and RNA binding proteins

Previous studies have shown that cellular miRNA can be released from cells by secretion of microvesicles/exosomes.[15, 17] To further investigate whether exosomes are involved in the transfer of small silencing RNA, we purified secreted exosomes from Huh7-CM or CM of stably transduced Huh7 cells expressing shCon, shCD81 or shNS5b using density gradient ultracentrifugation. Figure 3A shows an electronmicrograph of a purified exosome. RT-PCR analysis of shCD81-CM exosomes showed the presence of both miRNA (miR-122) and shCD81 (Fig. 3B). Huh7-CM derived exosomes were analyzed by mass spectrometry to characterize the protein content. From two independent preparations of exosomes, over 600 common proteins were detected, including the established exosome markers Tsg101, CD63, CD9, Alix, Flotillin and RAB5.[27] Importantly, 56 distinct RNA binding proteins were present, including ribosomal proteins, serine/arginine-rich splicing factors, heterogeneous nuclear ribonucleoproteins, eukaryotic translation initiation factors and proteasome subunits (Fig. 3C). The presence of RNA binding proteins is consistent with a previous study, showing exosomes derived of primary rat hepatocytes are highly enriched for nucleotide binding proteins.[28] Relevant to the content of miRNA and siRNA, we identified four proteins in exosomes which are known to be important for the miRNA pathway and which are potential binding partners of the small silencing RNA cargo in hepatic exosomes (Fig. 3D). In particular interesting is the nucleolar phosphoprotein B23, NPM1, which has been recently shown to specifically protect the degradation of miRNAs.[29] RAN, the Ras-related nuclear protein, is known for its involvement in nucleo-cytoplasmic transport. Interestingly, recent studies have shown that Exportin-5 mediated nuclear export of pre-miRNA or shRNA acts in a Ran–GTP dependent manner.[30-32] Further studies will be required to identify the exact molecular machinery which is involved in the sorting and packaging of small silencing RNA into exosomes.



D

Gene symbol	Protein name	emPAI value	Functions
RAN	Ras-related nuclear protein	2.70	Nucleo-cytoplasmic transport/miRNA biogenesis
NPM1	Nucleolar phosphoprotein B23	1.12	Protection of miRNA degradation
TUT1	U6 snRNA-specific-terminal uridylyltransferase 1	0.76	U6 snRNA terminal uridylyltransferase
SSB	Sjogren syndrome antigen B	0.08	RNA III polymerase transcription

Figure 3. Exosomes contain small RNAs and RNA binding proteins. Secreted exosomes were purified from conditioned medium (CM) from Huh7 cells using density gradient ultracentrifugation. (A) Electronmicrograph imaging shows presence of exosomes in purified fraction. (B) RT-PCR analysis of purified exosomes from shCD81-CM showed the presence of both miRNA and shCD81. Markers indicate the anticipated amplicon size for miR-122 and shCD81 No-template (H2O) and purified exosomes from shNS5b-CM served as negative controls. This assay supposes to preferentially amplify the mature miR-122 or siCD81, but possibly also detect the precursors. (C) Mass spectrometry was performed to analyze the protein content of two independent batches of Huh7-CM derived exosomes. Using a Mascot cutoff for specificity (Mascot >40), in total over 600 common proteins were identified including many exosome-specific proteins. There are 56 proteins are known RNA binding proteins, including 32 ribosomal proteins. (D) Of the RNA binding proteins, four are known to be involved in the miRNA pathway and are potentially involved in the selection, sorting and packaging of small silencing RNA in hepatic exosomes. Shown are protein name, main function and relative abundance in exosomes indicated by the amPAI value (mean of two samples).

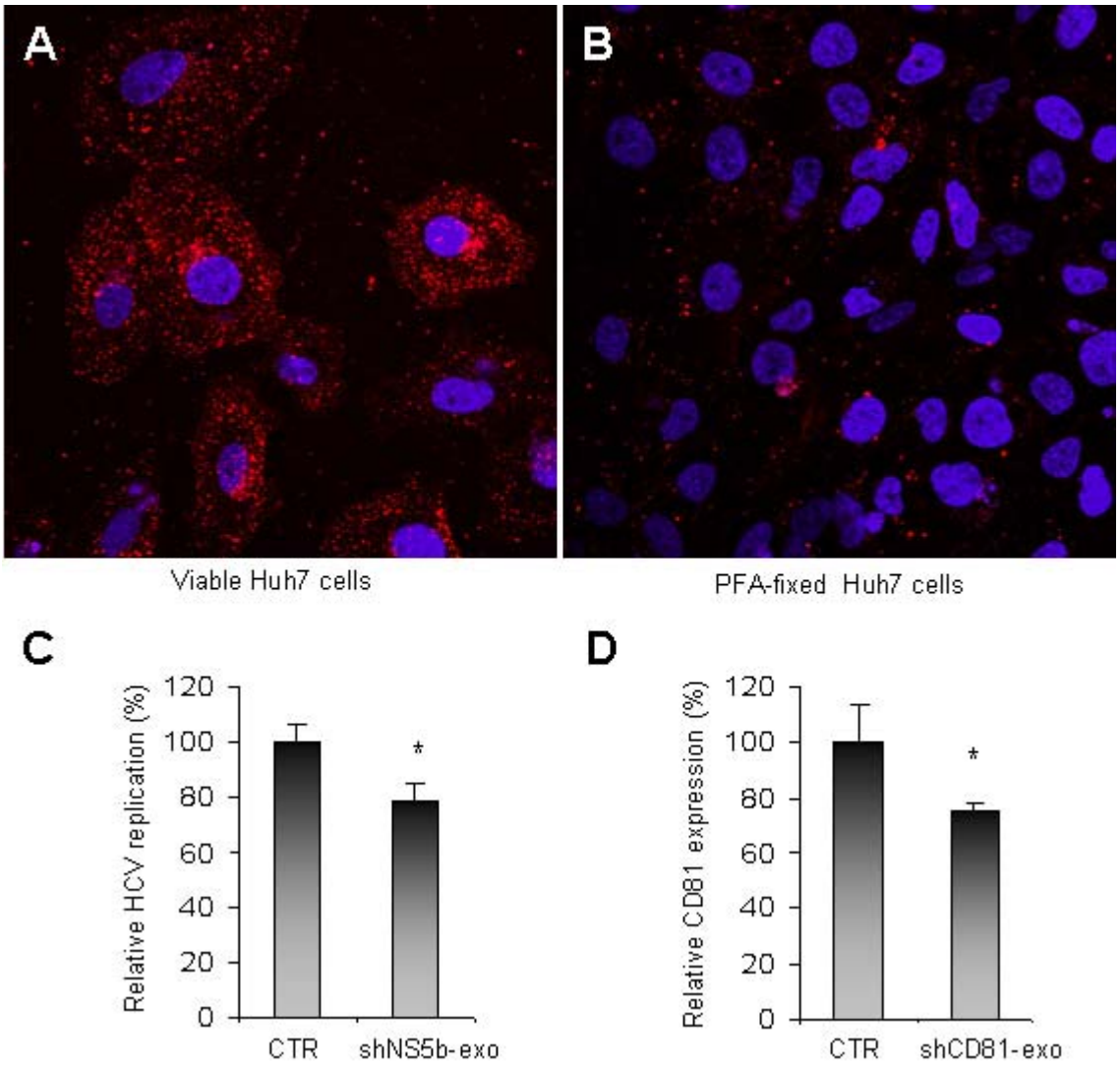


Figure 4. Exosome-mediate functional transmission of small silencing RNAs. (A) Dynamic visualization of rhodamine-labeled exosome uptake by live Huh7 cells shows intracellular accumulation in viable cells but not (B) in PFA-fixed cells. Red staining represents exosomes and blue staining marks the nucleus. Shown is one of three independent experiments. 800 x magnification. (C) Treatment of Huh7-ET replicon cells with purified exosomes derived from shNS5b-CM significantly reduced viral replication by 21.6% ± 6.4. (D) Treatment of normal Huh7 cells with purified exosomes derived from shCD81-CM resulted in a significant down regulation of CD81 cell surface expression by 24.5 % ± 3.1. Shown is the mean inhibition ± SD of four independent experiments. *P<0.05.

Transmission of gene silencing is partially mediated by exosomes

To investigate the involvement of exosomes in small RNA transfer, real-time live cell imaging was performed with Huh7 cells exposed to fluorescent-labeled exosomes using confocal microscopy. Real-time analysis showed that exosome uptake is rapid and occurs within 45 minute (data not shown). As shown in Figure 4A, ingested exosomes predominantly accumulate in the cytoplasm or other intracellular compartments but not in the nucleus.

Exosome uptake was observed in most of the living cells (>80%), but hardly uptake was observed in PFA-fixed cells (Fig. 4B), confirming that uptake is an active process. Treatment of HCV replicon cells with purified exosomes derived from shNS5b-CM resulted in a significant reduction of viral replication (mean inhibition 21.6% ± 6.4 SD, n=4, P<0.01) (Fig. 4C). Similarly, treatment of Huh7 cells with exosomes derived from shCD81-CM resulted in a significant down regulation of CD81 cell surface expression (24.5 % ± 3.1 reduction, n=4, P<0.05) (Fig. 4D). These findings confirm that secreted exosomes contain small RNAs, including miRNA and small silencing RNA, can mediate transmission of functional gene silencing. In addition, recent studies have suggested the co-existence of exosome dependent and independent pathways of small RNA release and transfer.[20, 32, 33]

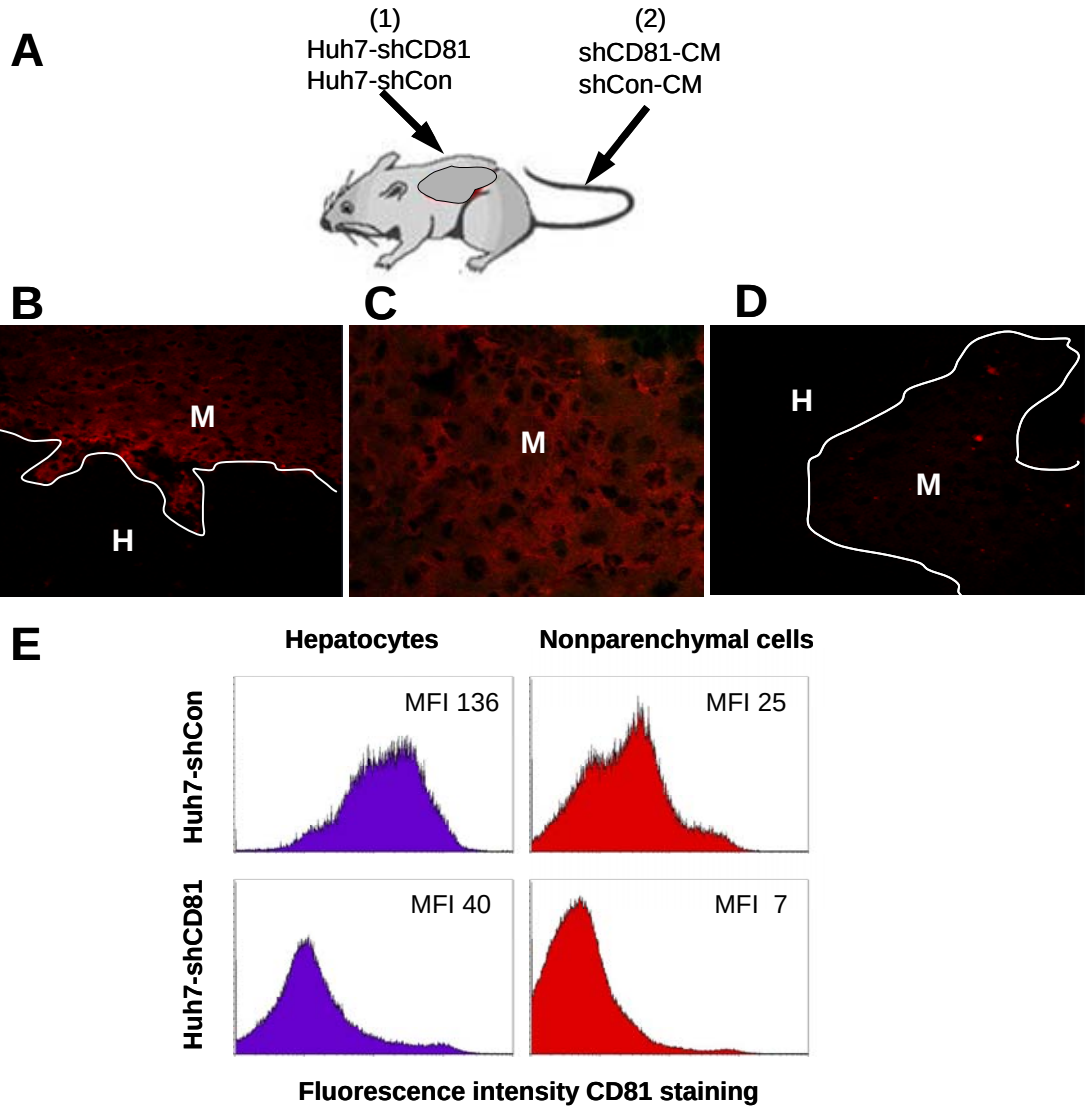
Transmission of gene silencing in mouse liver

To explore the evidence for small RNA exchange *in vivo*, we engrafted Huh7-shCD81 cells, stably expressing shRNA targeting mouse CD81, or Huh7-shCon cells, containing irrelevant shRNA, in the liver of immunodeficient NOD/SCID mice by intrasplenic injection (Fig. 5A). Human hepatomas in the mouse liver tissue were visualized based on green fluorescent protein (GFP) positivity. Mouse liver tissue surrounding nodules of Huh7-shCon cells showed comparable CD81 expression (Fig. 5B) as untreated mice (Fig. 5C). Contrary, liver tissue adjacent to Huh7-shCD81 nodules showed a marked reduction in CD81 expression (Fig. 5D). Flowcytometric quantification of mouse-specific CD81 expression on dissociated liver cells showed an average reduction of 71.3% on both hepatocytes and non-parenchymal cells in Huh7-shCD81 versus Huh7-shCon engrafted mice (P=0.002, Fig. 5E). This finding suggests transfer of RNAi from the human cells to the primary mouse cells *in vivo*. In order to determine whether RNAi transfer *in vivo* is cell-contact dependent, NOD/SCID mice were intravenously treated with shCD81-C-CM or shCon-C-CM (Fig. 5A). At day six, a significant reduction of CD81 mRNA level was observed in mouse livers by shCD81-CM treatment (mean reduction of 31.6% ± 15.6, n=4) as compared to the shCon-CM controls (n=4, P<0.05) (Fig. 5F). Consistent with the gene expression levels, an approximate 20% reduction of CD81 cell surface expression was observed in both hepatocytes and non-parenchymal cell populations by flowcytometry (Fig. 5G). The gene silencing by shCD81-CM was comparable to that of liposome or nanoparticle delivery of siRNA observed in a transgenic mouse model of HCV or in human tumors.[35-36] Despite earlier reports of hepatotoxicity by adeno-

associated vector mediated RNAi,[37] we observed no evidence of liver injury by histology or serum transaminases as a result of shCD81-CM treatment.

Discussion

From the discovery of RNAi in 1998[38] to the approval of RNAi therapeutics or RNAi-based gene therapy by FDA, the face of its application has been dramatically changing. Much attention has been received for developing antiviral RNAi against such as HIV[39], HBV[40] or HCV[6] infection. If RNAi therapies are to be utilized as an effective treatment or prevention of HCV infection, long-term, stable siRNA expression needs to be achieved. Raw synthetic siRNA or plasmid-encoded shRNA transfections elicit only short-term silencing, whereas viral vectors that encode for shRNA can potentially induce long-term and continuous gene-silencing.[6] Adeno-associated viral (AAV) vectors are currently considered the prime candidate for clinical gene therapy applications, including for the treatment of various liver diseases. Biotech companies, such as Tacere therapeutics, have pioneered the development of an AAV-based anti-HCV RNAi regimen, termed "TT-033" (http://www.tacerebio.com). However, AAV-mediated expression of shRNA was shown to evoke liver toxicity in mice ultimately causing death.[37] It was suggested that the saturation of endogenous miRNA processing machinery by overexpressed shRNA is the potential cause,[41] but the exact mechanism remains unclear. Lentiviral vector represents another promising candidate for clinical RNAi delivery. Although certain lentiviral RNAi systems, such as some commercial RNAi libraries, express high levels of shRNA and cause disturbance of cellular miRNA machinery, no significant cell toxicity was observed.[42] The lethal toxicity observed by Grimm et al.[37] could be caused by the combination of AAV vector and overexpressed shRNA. Of note, the lentiviral RNAi vectors used in this study express moderate levels of shRNA without clear effect on miRNA pathway.[42] To overcome the potential toxicity and off-target issues, liver-specific promoters[43] or miRNA-based RNAi constructs[44] have been used to generate safer vectors.


A
(1)
Huh7-shCD81
Huh7-shCon
(2)
shCD81-CM
shCon-CM
B
M
H
C
M
D
H
M
E
Hepatocytes
Nonparenchymal cells
Huh7-shCon
MFI 136
MFI 25
Huh7-shCD81
MFI 40
MFI 7
Fluorescence intensity CD81 staining
F
Relative CD81 mRNA (%)
120
110
100
90
80
70
60
50
*
shCon
shCD81
G
Relative CD81 surface expression (%)
Hepatocytes
Nonparechymal cells
120
110
100
90
80
70
60
50
*
shCon
shCD81

Although studies have demonstrated the feasibility of combating HIV infection via ex vivo delivery of lentivral RNAi[45], it remains challenge to produce sufficient vectors targeting the entire liver organ. Virtually for any type of vectors, it is not possible to achieve 100% transduction efficacy in patients. The phenomenon we described in the current study that gene silencing could transfer to neighboring non-transduced cells could indeed potentially overcome the issue of suboptimal vector transduction to certain extend. Whether it would be sufficient to silence the virus in the non-transduced cells solely via the RNAi transmission route remains questionable. Like HIV[46], HCV is prone to develop resistant mutants, if the antiviral potency is suboptimal. Vector simultaneous delivery of multiple shRNAs targeting different regions of the virus or combination of targeting host factors could be one solution to prevent mutagenesis[47], since the non-transduced cells could receive multiple antiviral shRNAs even though the levels are not so abundant. Like other new antivirals,[2-3] combining interferon is likely required for RNAi-based therapy to achieve ultimate success in chronically infected HCV patients.[48]

Figure 5. In vivo evidence for transmission of RNAi in mice. (A) Schematic representation of in vivo experiments with immunodeficient mice. (1) NOD/SCID were either engrafted with control Huh7 cells expressing irrelevant shRNA targeting NS5b (Huh7-shCon) or Huh7 cells expressing shRNA targeting murine CD81 mRNA (Huh7-shCD81) in the liver. (2) Alternatively, NOD/SCID we injected intravenously with 200 µl of 100-fold concentrated cell-free conditioned medium (CM) from Huh7-shCD81 or Huh7-shCon cells, three times with 48 hours intervals. All groups had four animals. Confocal immunofluorescence staining using an anti-mouse CD81-specific antibody showed normal CD81 expression (Red fluorescence) in the mouse liver tissue (M) surrounding nodules of Huh7-shCon cells (H) (B), comparable to expression in untreated mice (C). (D) Contrary, CD81 expression in mouse liver tissue (M) surrounding Huh7-shCD81 cells (H) was markedly reduced. (E) Flowcytometric quantification of dissociated liver cells showed a significant reduction of CD81 expression in mouse hepatocytes and non-parenchymal cells (average reduction of 71.3%, P=0.002) in mice engrafted with Huh7-shCD81 (bottom panels) as compared to mice engrafted with Huh7-shCon (top panels). GFP positive human cells were gated out and a mouse specific anti-CD81 antibody was used to specifically determine mouse CD81 expression. Number indicated the average geometric mean fluorescence intensity (F) Analysis of liver mRNA showed a significant knockdown of CD81 expression in mice treated with shCD81-CM as compared to shCon-CM treatment. (G) Knockdown of CD81 surface expression was confirmed by flowcytometry in both hepatocyte and non-parenchymal cell populations (Approx. 20%). * $P<0.05$.

The mechanism of RNAi transmission in plants and invertebrates has been proposed via direct cell-to-cell contact or systemic spreading, although the exact mechanism remains largely elusive. Rechavi et al. has reported transmission of small RNA between B and T cell lines in culture occurring in a cell contact-dependent,[20] whereas many others[15-19] described the secretory transmission pathway involving exosomes in different mammalian cell culture systems. In this study, we also observed the release and uptake of small RNA-packed exosomes by hepato-like cells. We further performed mass spectrometric analysis to characterize the protein content of these exosomes. Along with the previous studies charactering exosmes derived from monocytes[49-50] or hepatocytes[28] there appears to be some cell type specificity. For instance, AGO2, a protein involved in the RNAi machinery, is detectable in monocytes[49-50] but not hepatocytes-derived[28] exosomes. The differential enrichment of nucleotide and nucleic acid binding proteins was observed between Huh7 and primary hepatocytes-derived exosomes[28]. Interestingly, we found several proteins present in our exosomes that potentially contribute to the functional transmission of small RNAs. RAN, the known nucleo-cytoplasmic transport, was demonstrated to be involved in Exportin-5 mediated nuclear export of pre-miRNA or shRNA.[30-32] The co-presence of NPM1 that can specifically protect the degradation of miRNAs[29] and TUTase that can potentially edit miRNAs or shRNAs[51] suggests that the process of degradation and modification of small RNAs can be potentially regulated within the exosmes. Conceivably, both the protein and RNA content deters the function of transferred exosomes.

In this study, a comprehensive *in vitro* and *in vivo* demonstration of RNAi transfer was achieved by using shRNA donor cells, conditioned medium and purified exosomes. We assumed that exosmes only partially mediated the transmission of gene silencing and the other part would be contributed by the secreted small RNAs independent of exosomes. Consistently, recent study showed that substantial amount of extracellular miRNAs are associated with Argonautes but not with exosomes.[33] Further studies will be required to identify the exact molecular machinery which regulates the release and uptake functional small RNAs.

In summary, this study provided *in vitro* and *in vivo* evidence that small RNA could be exchanged between hepatic cells and that this property extended RNAi-mediated gene silencing against HCV receptor or viral genome. Exchange of small RNAs, in our models, was independent of direct cell-to-cell contact and appeared to be mediated by the secretory

pathway partially involving exosomes. Cells stably expressing shRNA, like stem cells, may represent an effective way for the therapeutic delivery of RNAi *in vivo*. These findings might bear relevance for clinical application of RNAi-based therapy in the treatment of chronic hepatitis C as well as metabolic and immunomediated liver diseases.[52]

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Chapter 9

Combined anti-viral activity of interferon-α and RNA interference directed against hepatitis C without affecting vector delivery and gene silencing

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J Mol Med, 2009, 87(7):713-22

Abstract

The current standard interferon-alpha (IFN-α)-based therapy for chronic hepatitis C virus (HCV) infection is only effective in approximately half of the patients, prompting the need for alternative treatments. RNA interference (RNAi) represents novel approach to combat HCV by sequence-specific targeting of viral or host factors involved in infection. Monotherapy of RNAi, however, may lead to therapeutic resistance by mutational escape of the virus. Here we proposed that combining lentiviral vector mediated RNAi and IFN-α could be more effective and avoid therapeutic resistance. In this study, we found that IFN-α treatment did not interfere with RNAi mediated gene silencing. RNAi and IFN-α act independently on HCV replication showing combined antiviral activity when used simultaneously or sequentially. Transduction of mouse hepatocytes in vivo and in vitro was not effected by IFN-α treatment. In conclusion, RNAi and IFN-α can be effectively combined without cross-interference and may represent a promising combinational strategy for the treatment of hepatitis C.

Introduction

The hepatitis C virus (HCV) infection remains a major cause of chronic liver disease with an estimated 170 million carriers worldwide. The current standard therapy, pegylated interferon-alpha (IFN-α) in combining with ribavirin, has achieved substantial success [1, 2]. However, still half of the patients fail to develop a sustained virologic response after this therapy. To improve treatment outcomes, novel monotherapies or alternative combination of IFN-α based therapies are urgently required.

RNA interference (RNAi), the degradation of cognate mRNA by small interfering RNA (siRNA), has emerged as a novel therapeutic entity for viral infections. Since the HCV genome is a single-stranded RNA that functions as both a template for transcription and template for a negative strand replication intermediate, it is a prime candidate for RNAi [3]. Instead of a 5' cap, the IRES, located at the 5' NCR of the viral genome, plays an essential in initiating translation [4]. Because of the most conserved sequence within the viral genome, IRES seems an ideal target for RNAi and indeed several studies have demonstrated inhibition of HCV replication by targeting this region [5-7]. HCV replication is mediated by NS5B, an RNA-dependent RNA-polymerase that lacks proofreading abilities. As a result of extremely high mutation rate (10^3 per nucleotide per generation) and replication rate (10^{12} virions per day) in patients, HCV quasispecies are generated [8]. NS5b region has been shown to be very effective for RNAi induced suppression of HCV replication [9]. Host cellular factors involved in the viral entry, such as CD81, Claudin-1, Occludin, or SR-B1 could also be candidate targets for RNAi antiviral therapy [10-12]. Knockdown of CD81 by RNAi significantly prevented the binding of Huh7 cells to E2 protein [13]. A definitive proof of the therapeutic value of CD81 in vivo was provided by a recent study showing that treatment with anti-CD81 antibodies completely protected human liver-uPA-SCID mice from a subsequent challenge with HCV consensus strains of different genotypes [14].

The success of RNAi in therapeutic application also depends on an efficient delivery system, which can support long-term siRNA production and continuous gene silencing. Integrating self-inactivating lentiviral vector (LV) can achieve this criterion by encoding small hairpin RNA (shRNA), a precursor of siRNA that is cleaved into biologically active siRNA by host cell enzyme, Dicer[15, 16].

Similar to existing antiviral monotherapies, a new monotherapy based on a single RNAi target will likely fail due to the development of resistance. HCV can potentially overcome the antiviral effects of RNAi through several mechanisms, including genomic diversity, mutational escape and attenuation of RNAi machinery [17-20]. RNA viruses like HIV and HCV are highly adaptive in this context and mutational escape from therapy has been documented against single RNAi treatments [18, 21]. Thus, a combined strategy would be necessary for eliminating HCV infection. Previous studies reported by our group and others, have shown that simultaneously targeting both viral and host cell elements by RNAi could increase the potency of antiviral therapies[13, 21-23]. IFN-α possesses indirect antiviral activity by stimulating genes that can lead to a non-virus-specific antiviral response, whereas RNAi can directly interfere with viral entry and replication through targeting viral RNA genome or mRNA of cellular factors. Based on their complementary antiviral mechanisms, we propose that combining RNAi with IFN-α may prevent therapeutic resistance and exhibit enhanced antiviral activity. Moreover, the additional combination of ribavirin to RNAi and IFN-α may further improve the therapeutic effects in the treatment of chronic hepatitis C.

To date, very little is known about the interaction of exogenous IFN-α with lentiviral vector delivery and the anti-viral action of RNAi. Reports have suggested that the administration of lentiviral vectors to mice triggers a rapid and transient type I IFN response (i.e. IFN-α and IFN-β). In animals that lack the capacity to produce type I IFN, dramatic increases in transduction to hepatocytes were seen, indicating that endogenous IFNs may interfere[24].

In the current study, we investigated the effect of combining lentiviral mediated RNAi with IFN-α in an in vitro HCV replication model. The results indicate that IFN treatment does not affect the transduction efficiency of hepatocytes in vitro and in vivo. Moreover, IFN did not interfere with RNAi mediated knockdown of host cell target genes, including CD81, and showed combined antiviral activity with shRNAs directed against the HCV genome. The combined efficacy of RNAi and IFN- may provide new opportunities for highly effective antiviral therapy for hepatitis C.

Materials & Methods

Cell culture

Cell monolayers of the human embryonic kidney epithelial cell line HEK293 and human hepatoma cell line Huh7 and Huh6 were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen–Gibco, Breda, The Netherlands) and complemented with 10% v/v fetal calf serum (Hyclone, Logan, Utah), 100 IU/ml penicillin, 100 mg/ml streptomycin, and 2mM L-glutamine (Invitrogen–Gibco) (cDMEM). Huh7 cells containing a subgenomic HCV bicistronic replicon (I389/NS3-3V/LucUbiNeo-ET, Huh7-ET) were maintained with 250 μg/ml G418 (Sigma, Zwijndrecht, The Netherlands).[25]

Construction and production of lentiviral vectors (LV)

The vectors, LV expressing Green Fluorescent Protein (LV-GFP), LV-shCD81, LV-shIRES and LV-shNS5b containing shRNA cassettes were constructed and produced as previously reported [13]. LV-shIRES targets HCV IRES region (AGGUCUCGUAGACCGUGCA 321-340). LV-shNS5b targets the viral NS5b region (GACACUGAGACACCAAUUGAC 6367-6388). LV-shCD81 targets human CD81 mRNA (GGAUGUGAAGCAGUUCUAU 594-614). Briefly, a third-generation lentiviral packaging system pND-CAG/GFP/WPRE containing shRNA expression cassette targeting HCV IRES, NS5b region or viral receptor CD81 was used to produce high-titer VSV-G-pseudotyped lentiviral vectors in HEK293 cells. Vector supernatants were removed 36 and 48 hr post transfection, passed through a 0.45 M filter, and concentrated 1000-fold by ultracentrifugation. Plasmid SHC005 containing both shGFP cassette and puromycin selectable marker was obtained from Erasmus Center for Biomics (Rotterdam, The Netherlands). LV-shGFP was produced in the third-generation lentiviral packaging system as mentioned above.

In vitro LV transductions, GFP and CD81 analysis

Concentrated LV stocks were titrated using HEK293 cells 24h after infection, with transduction efficiency based on the number of GFP-positive cells as determined by flowcytometry (FACSCalibur; BD BioSciences, Mountain View, CA, USA). Transduction efficiency of LV-GFP in the presence of IFN-α was tested using this method in Huh7 cells 3

days post-infection. Transduction efficiency of LV-shIRES and LV-shNS5b in Huh7-ET cells was also tested by this method. Pegylated IFN-α 2a (dissolved in water) was provided by Roche Ltd (Basel, Switzerland). CD81 expression was determined using flowcytometry by staining with phycoerythrin (PE) conjugated mouse anti-human CD81 monoclonal antibody (BD Pharmingen, San Diego, USA). Mouse IgG1 was used as isotype-matched control antibody (BD Pharmingen).

In vivo LV transductions in mice

11-week-old female NOD/LtSz-scid/scid (NOD/SCID) mice were injected intravenously with suboptimal dose of LV-GFP (5 x 10^6 transducing units in 0.25 ml PBS) in the tail vein. Three mice were injected subcutaneously with pegylated IFN-α 2b (Intron A, Schering-Plough, Kenilworth, NJ) at six hours before and 72 hours after intravenous administration of LV-GFP. The dose of peg-IFN-α per injection was 30 g/kg body weight, as previously described [26]. Two mice were injected with vector alone and one animal served as negative control. After seven days, mice were sacrificed and livers were harvested. Mouse liver tissue was digested by collagenase type IV (Sigma, Zwijndrecht, The Netherlands) for 30 min at 37ºC in order to generate a single-cell suspension. Transduction efficiency was determined by the percentage of GFP-positive hepatocytes using flowcytometric analysis (FACSCalibur, BD BioSciences).

Luciferase assay

100 mM luciferin potassium salt (Sigma) was added to Huh7-ET cells and incubate for 30 min at 37ºC. Luciferase activity was quantified using a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

Statistical analysis

Statistical analysis was performed by using either matched pair nonparametric test (Wilcoxon signed-rank test) or the non-paired, nonparametric test (Mann–Whitney test) (GraphPad Prism software). P-values less than 0.05 were considered as statistically significant.

Results

Potent inhibition of HCV replication by lentiviral vector mediated RNAi

A subgenomic replicon cell line (Huh7-ET), containing HCV non-structural sequences and a luciferase reporter gene[25, 27], was used to determine the anti-viral potency of IFN-α and RNAi (Fig. 1A). HCV replication was monitored by measuring luciferase activity. Robust reduction of luciferase activity (97 ± 2% inhibition, mean ± SD, n = 9) was observed from 2.5 to 100 IU/ml of IFN-α.

LV-shNS5b vector containing both GFP and shRNA targeting HCV NS5b sequence was tested. Huh7-ET treated with increasing dose of LV-shNS5b resulted in higher levels of transduction efficiency and inhibition of HCV replication as monitored by GFP reporter gene expression and luciferase activity, respectively. Maximum inhibition of HCV replication was observed at MOI of 20 and 25 by 98 ± 3% (mean ± SD, n = 6) (Fig. 1B). The results indicate that RNAi mediated inhibition of HCV replication can be as effective as IFN-α in Huh7-ET model.

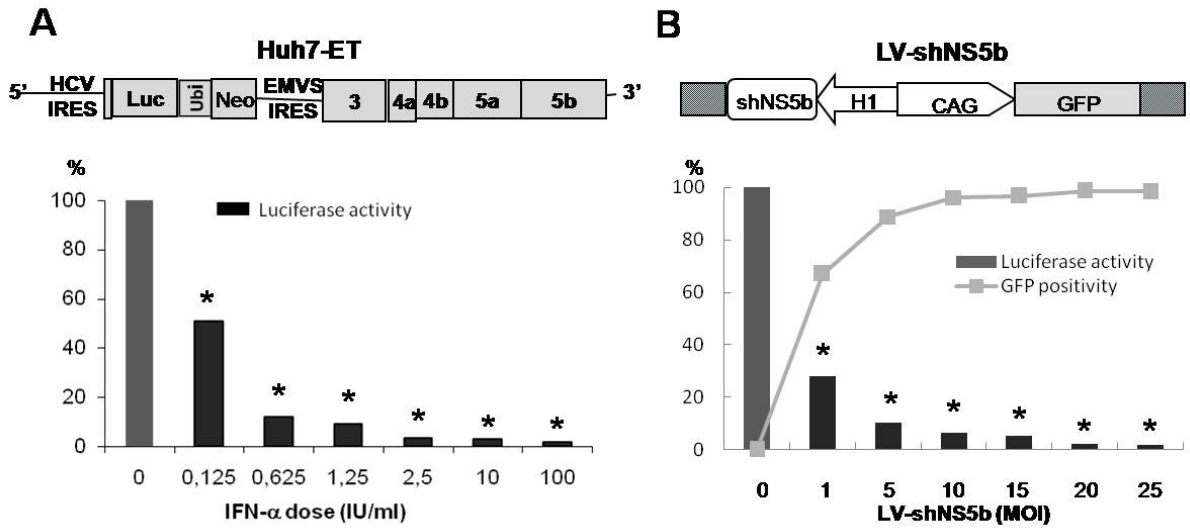

Figure 1. Inhibition of HCV replication by IFN-α or LV-shNS5B. (A) Huh7-ET replicon was used for testing HCV replication by monitoring luciferase activity. IFN-α treatment inhibits viral replication in a dose-dependent manner. Profound reduction of luciferase activity (97 ± 2% inhibition, mean ± SD, n = 9) was observed from 2.5 to 100 IU/ml concentration of IFN-α. (B) LV-shNS5b contains both GFP reporter gene and shRNA targeting HCV were tested. Huh7-ET treated with increasing dose of LV-shNS5b resulted in higher levels of transduction efficiency and inhibition of HCV replication, monitored by GFP positive population and luciferase activity respectively. Maximum inhibition of HCV replication was observed at high dose (20 or 25 MOI) by 98 ± 3% (mean ± SD, n = 6). *P<0.01 (Wilcoxon test) significantly different from untreated conditions.

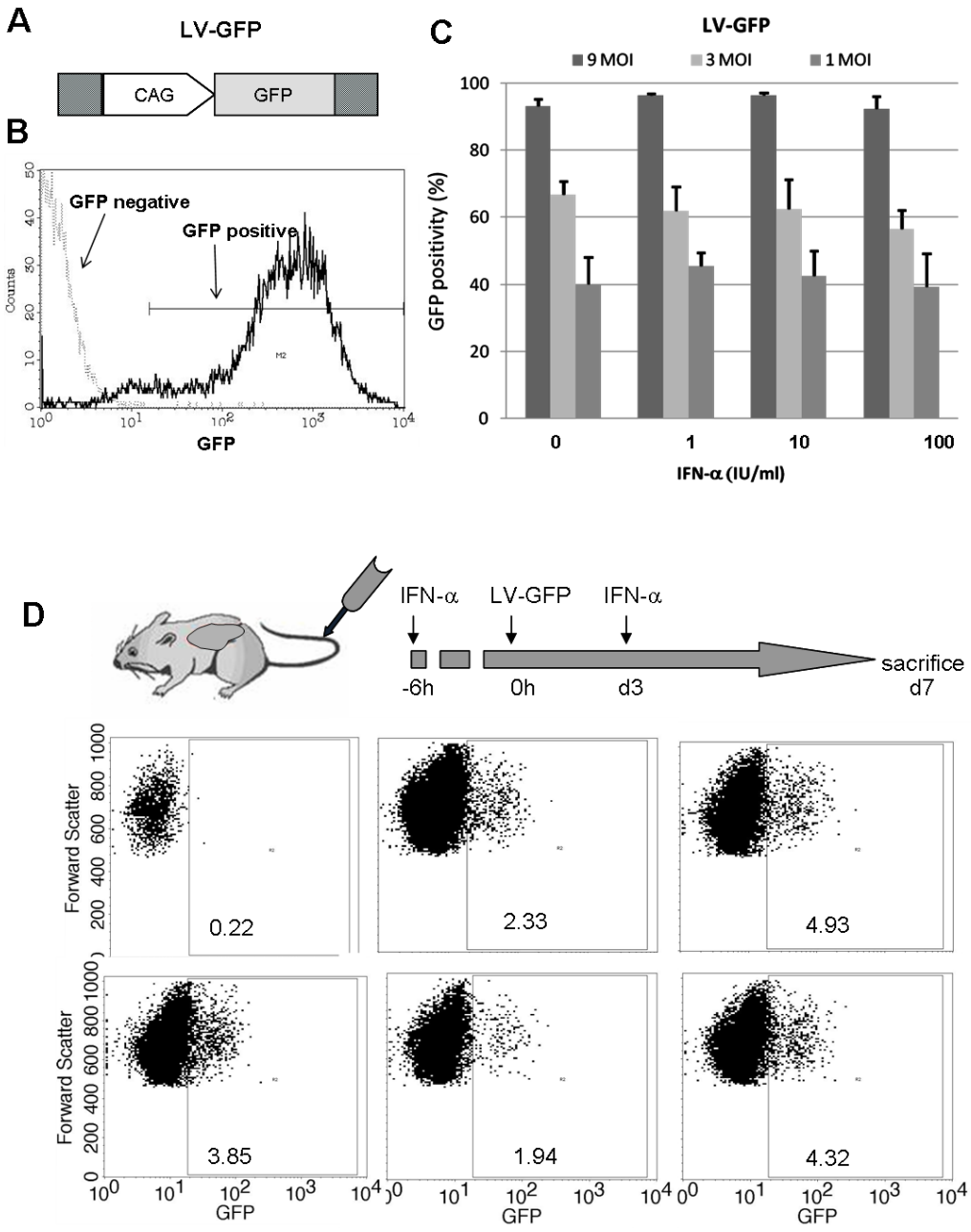

Figure 2. Effect of exogenous IFN-α on lentiviral vector transductions in vitro and in mice. (A) LV-GFP vector expressing GFP under control of CAG promoter was used for transduction of Huh7 cells. (B) Representative histogram of GFP expression determined by flowcytometry of control cells and transduced cells three day after culture. (C) No significant (P>0.05) differences of transduction efficiency, compared with non-treated control group, were observed with 1, 10 or 100 IU/ml of IFN-α for high (9 MOI), intermediate (3 MOI) and low (1 MOI) vector concentrations. Shown is the mean ± SD of four independent experiments. MOI, Multiplicity Of Infection. (D) Mice treated with exogenous IFN-α 6h before and 3 days after administration of LV-GFP (subotimal dose: 5 x 106 transducing units) showed comparable transduction efficiency in the liver (1.7 to 4.1% transduced hepatocytes), compared with the group injected with vector only (2.1 and 4.7% transduced hepatocytes). Percentage of GFP-positive cells was indicated in the FACS picture for each individual mouse.

Exogenous IFN-α has no negative impact on lentiviral transduction in vitro and in vivo

To address whether exogenous IFN-α has a negative impact on lentiviral transduction, a vector transduction assay was performed by infecting Huh7 cells with LV-GFP vector (Fig. 2A) in the absence or presence of 1, 10 or 100 IU/ml of IFN-α. At day 3, transduction efficiency was assessed by flowcytometry for GFP positive cells (Fig. 2B). When compared with non-treated control group (93 ± 2%, mean ± SD, n = 4), 1, 10 or 100 IU/ml concentration of IFN-α had no significant effect on vector transduction at MOI of 9 (97 ± 0.4%, 97 ± 0.9% and 93 ± 4%, respectively; P >0.05). Similar results were observed at medium (3 MOI) and low (1 MOI) vector concentrations (Fig. 2C). Also pretreatment of cells with IFN-α for 24 h did not influence vector transduction (data not shown).

To further investigate the effects of exogenous IFN-α on lentiviral transductions in vivo, mice were treated with peg-IFN-α six hours before and 72 hours after to intravenous administration of LV-GFP. As shown in Fig. 2D, there was no significant effect of peg-IFN-α treatment on vector transduction in mice livers (1.7 to 4.1% transduced hepatocytes) compared to untreated transduction group injected with vector only (2.1 and 4.7% transduced hepatocytes).

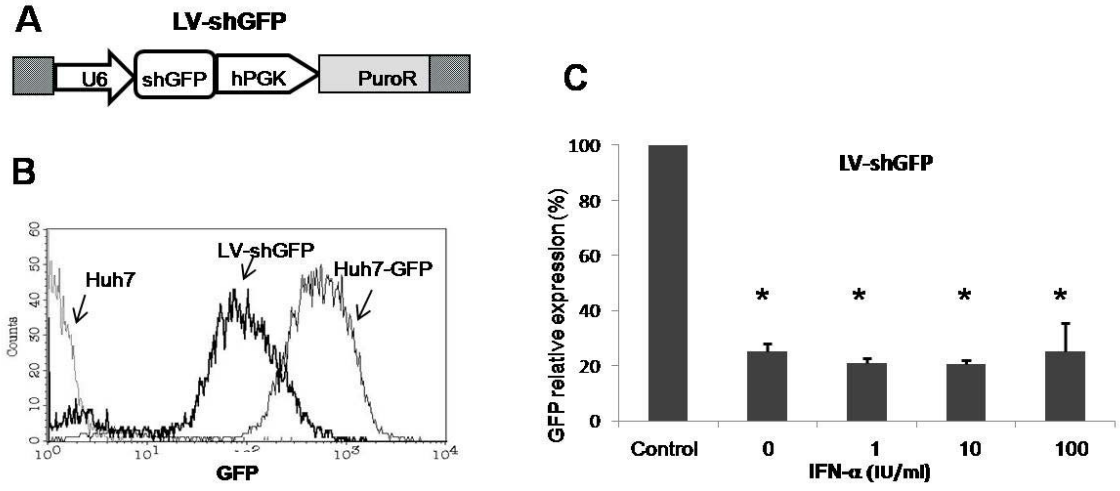

Figure 3. IFN-α does not interfere with RNAi-mediated knockdown of GFP. (A) LV-shGFP vector containing a shGFP cassette driven by U6 promoter were used. (B) Control (Huh7) and stable GFP expressing cell line (Huh7-GFP) were treated with LV-shGFP. GFP expression was measured by flowcytometry three days post- transductions, clearly showing inhibition of GFP expression by LV-shGFP in Huh7-GFP cells. (C) Shown is the relative GFP expression based on the mean fluorescence intensity. LV-shGFP significantly inhibited GFP expression by 74.7 ± 2.6% (*P*<0.01) and treatment of 1, 10 or 100 IU/ml of IFN-α did not interfere with the knockdown by LV-shGFP. Shown is the mean ± SD of three independent experiments (**P*<0.01).

IFN-α does not interfere with RNAi mediated host gene silencing

In the setting of combining RNAi with IFN-α, a critical concern would be the influence of exogenous IFN-α on RNAi gene silencing efficiency. To evaluate this issue, a lentiviral vector expressing shRNA to target report gene GFP (LV-shGFP; Fig. 3A) was produced and gene-silencing efficacy was determine in the absence or presence of IFN- . First, a stable GFP expressed cell line (Huh7-GFP) was created by infecting naive Huh7 cells with LV-GFP and expanded for several passages. Huh7-GFP cells were treated with LV-shGFP with or without IFN-α. LV-shGFP significantly inhibited GFP mean fluorescence intensity in Huh7-GFP cells (Fig. 3B), by 74.7 ± 2.6% (mean ± SD, n=3, P < 0.01). Treatment of 1, 10 or 100 IU/ml IFN-α did not significantly interfere with LV-shGFP mediated GFP knockdown (79.1 ± 1.9%, 79.5 ± 1.4% and 74.9 ± 10%, respectively, P>0.05) (Fig. 3C).

To further confirm these findings, LV-shCD81 was used to target HCV entry receptor CD81. The LV-shCD81 vector contains a GFP reporter gene to track transduction efficiencies (Fig. 4A). CD81 knockdown was determined by flowcytometry based on the GFP positive population (Fig. 4B, C). LV-shCD81, compared with LV-GFP control vector, significantly reduced CD81 expression by 90.8 ± 8.1% (mean ± SD, n=3, P<0.01). Importantly, knockdown of CD81 in Huh7 cells dramatically reduced infection by the JFH1-derived infectious HCV particles (Fig. 4D). As shown in Fig. 4E, LV-shCD81 retained robust gene silencing efficacy in the IFN-α (1, 10 or 100 IU/ml) treatment groups (83.9 ± 12.9%, 87.2 ± 9.6% and 84.3 ± 11.6%, respectively). Similar results were obtained using other hepatoma cell line, Huh6 (data not shown). Taken together, these results indicate that exogenous IFN-α does not interfere with RNAi mediated gene silencing.

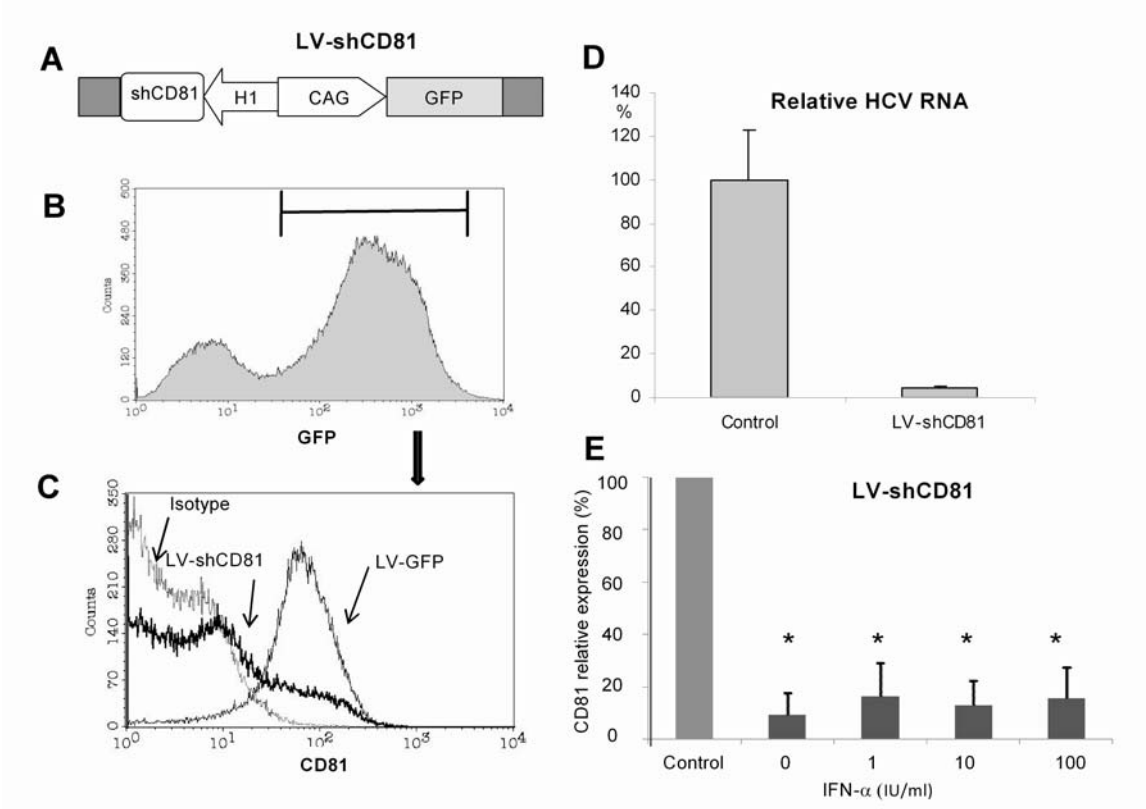

Figure 4. IFN-α does not interfere with RNAi-mediated silencing of CD81. (A) LV-shCD81 vector containing a GFP reporter gene and shRNA targeting CD81 was used to transduce Huh7 cells. LV-GFP vector without shRNA cassette was used as control. (B) Three days after transfection, GFP positive transduced cells were gated to determine CD81 expression. (C) Representative CD81 expression histogram of GFP positive cells is shown. Isotype-matched control antibody staining of LV-shCD81 cells was included as negative control. (D) Knockdown of CD81 in Huh7 cells profoundly reduced HCV infection. Three days after LV-shCD81 transduction, Huh7 cells were exposed to JFH1-derived infectious HCV particles for 6 hours. Three days after infection, the LV-shCD81 treated cells showed a clear reduction in intracellular viral RNA levels as determined by quantitative RT-PCR. (E) Relative CD81 expression was calculated based on mean fluorescence intensity. LV-shCD81 significantly reduced CD81 expression by 90.8 ± 8.1% (*P*<0.01), compared to LV-GFP cells. LV-shCD81 retained robust gene silencing efficacy at different concentrations of IFN-α. Shown is the mean ± SD of three independent experiments (**P*<0.01).

Simultaneous and sequential combined anti-viral activity of IFN-α and RNAi

The finding that IFN-α did not affect transduction efficacy and gene silencing prompted the question whether IFN and RNAi have combined anti-viral effects on HCV replication. As shown in Fig. 1B, LV-shNS5b has potent anti-viral activity. Combination of low dose IFN-α (<1 IU/ml) with low vector dose (MOI ≤ 5) resulted in a significant enhanced anti-viral effect (Fig. 5A). For example, combination of 1 MOI vector with 0.9 IU/ml IFN-α showed 92.1 ± 8.1% (mean ± SD, n = 6, *P*<0.01) inhibition versus 72.2 ± 9.4% with vector alone or 71.5 ± 4.1% with IFN-α alone. Real-time quantative PCR confirmed the results in this group of treatments

(data not shown). At high dose vector (MOI ≥ 10) inhibition of HCV replication was nearly complete and, therefore, no significant combinational effect with IFN could be observed.

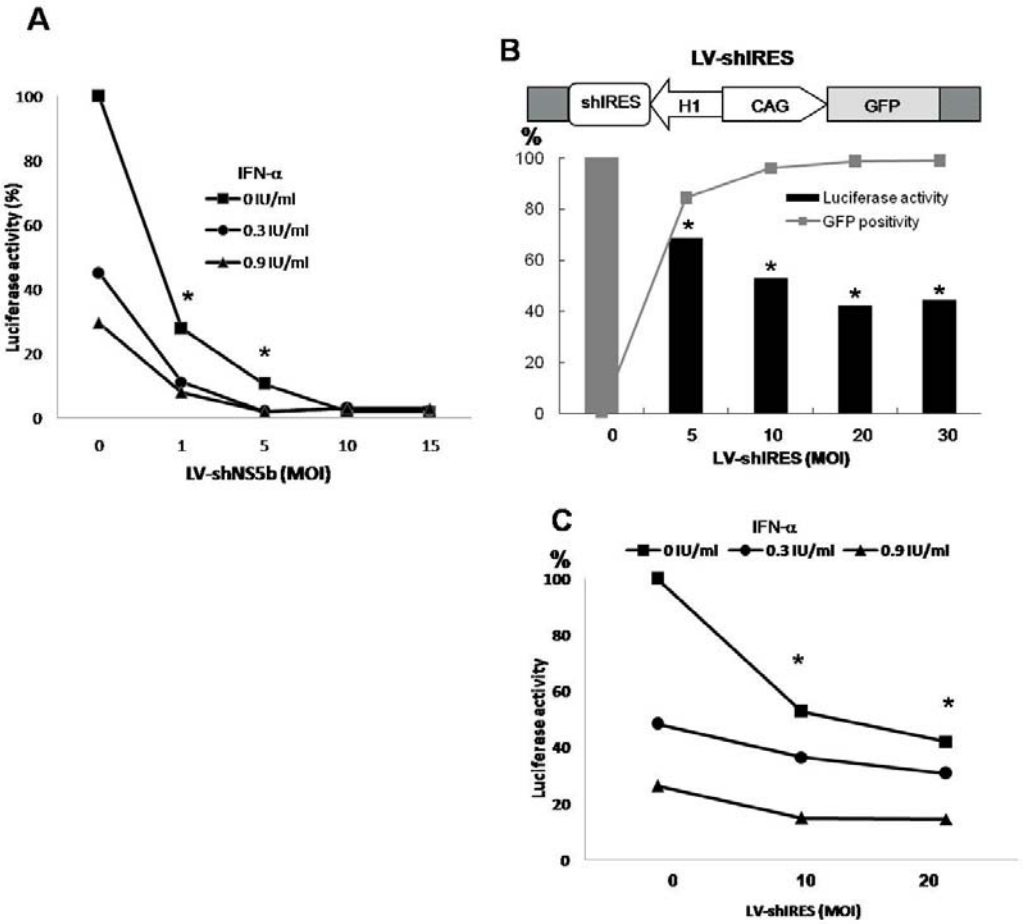

Figure 5. Enhanced inhibition of HCV replication by simultaneous treatment with IFN-α and RNAi. (A) The structure and action of LV-shNS5b on HCV replication have been shown in Fig. 1B. The combination of low dose IFN-α (< 1 IU/ml) with low dose vector (≤10 MOI) resulted in enhanced anti-viral effects. For example, combination of 1 MOI vector with 0.9 IU/ml IFN-α showed 92.1% ± 8.1 inhibition, versus 72.2% ± 9.4 with vector alone or 71.5 ± 4.1% with IFN-α alone (mean ± SD, *P*<0.01). Also with 5 MOI a significant combinational effect was observed (*P* <0.05), but at higher MOIs inhibition of HCV replication was nearly complete and no significant additive effect of IFN-α was observed. (B) LV-shIRES vector containing GFP reporter gene and shRNA targeting HCV IRES was used to transduce Huh7-ET cells. Increasing dose of LV-shIRES resulted in higher levels of transduction efficiency and inhibition of HCV replication, shown by GFP positivity and luciferase activity, respectively. (C) Combining low dose IFN-α with LV-shIRES resulted in enhanced inhibition of HCV replication at each combined condition. Shown is the mean ± SD of six independent experiments (*P<0.05).

In order to more clearly show the combined antiviral effect of IFN-α and RNAi, a less potent vector containing both GFP and shRNA targeting HCV IRES, was used (Fig. 5B). Huh7-

ET treated with increasing dose of LV-shIRES vector resulted in higher levels of transduction efficiency and inhibition of HCV replication, monitored by GFP positive population and luciferase activity, respectively. There was a clear threshold for the level of HCV silencing observed at vector dose of MOI 20 and higher (Fig. 5B). Similar to the results with LV-shNS5B vector, a combination of low dose IFN-α with LV-shIRES resulted in enhanced inhibition of HCV replication at each combined condition. For example, the combination of LV-shIRES (MOI 20) with 0.9 IU/ml IFN-α enhanced inhibition of HCV replication to 86.8% ± 3.4 compared to vector alone (56.4%, n = 6, $P<0.001$) or IFN-α alone (72.5%, $P<0.05$) (Fig. 5C). Real-time quantative PCR confirmed the results in this group of treatments (data not shown).

To further investigate this combined antiviral activity, Huh7-ET cells were sequentially treated with IFN- and RNAi. Cells were treated with suboptimal dose of IFN-α (0.5 IU/ml) for 24 hours followed by a secondary treatment with either LV-shNS5b or again with IFN-α. As shown in figure 6A, cells were more sensitive to subsequent treatment with RNAi than to re-treatment with IFN-α. Maximal inhibition of HCV replication with IFN-α was 81.3% ± 1.3 (n = 7) versus 98.2% ± 0.2 inhibition (n = 6) with LV-shNS5b ($P<0.001$). This indicates that HCV treated with IFN-α remains sensitive to RNAi, more so then to additional IFN-α. Conversely, treatment of LV-shIRES led to partial inhibition of viral replication and was more sensitive to secondary treatment with IFN-α than re-treatment of the same vector (Fig. 6B). At 48h after the initial treatment of LV-shIRES, additional treatment of IFN-α (0.9 IU/ml) resulted in 96.0% ± 0.6 inhibition of viral replication versus 75.1% ± 3.7 inhibition after re-treatment with LV-shIRES (n = 6, $P<0.001$).

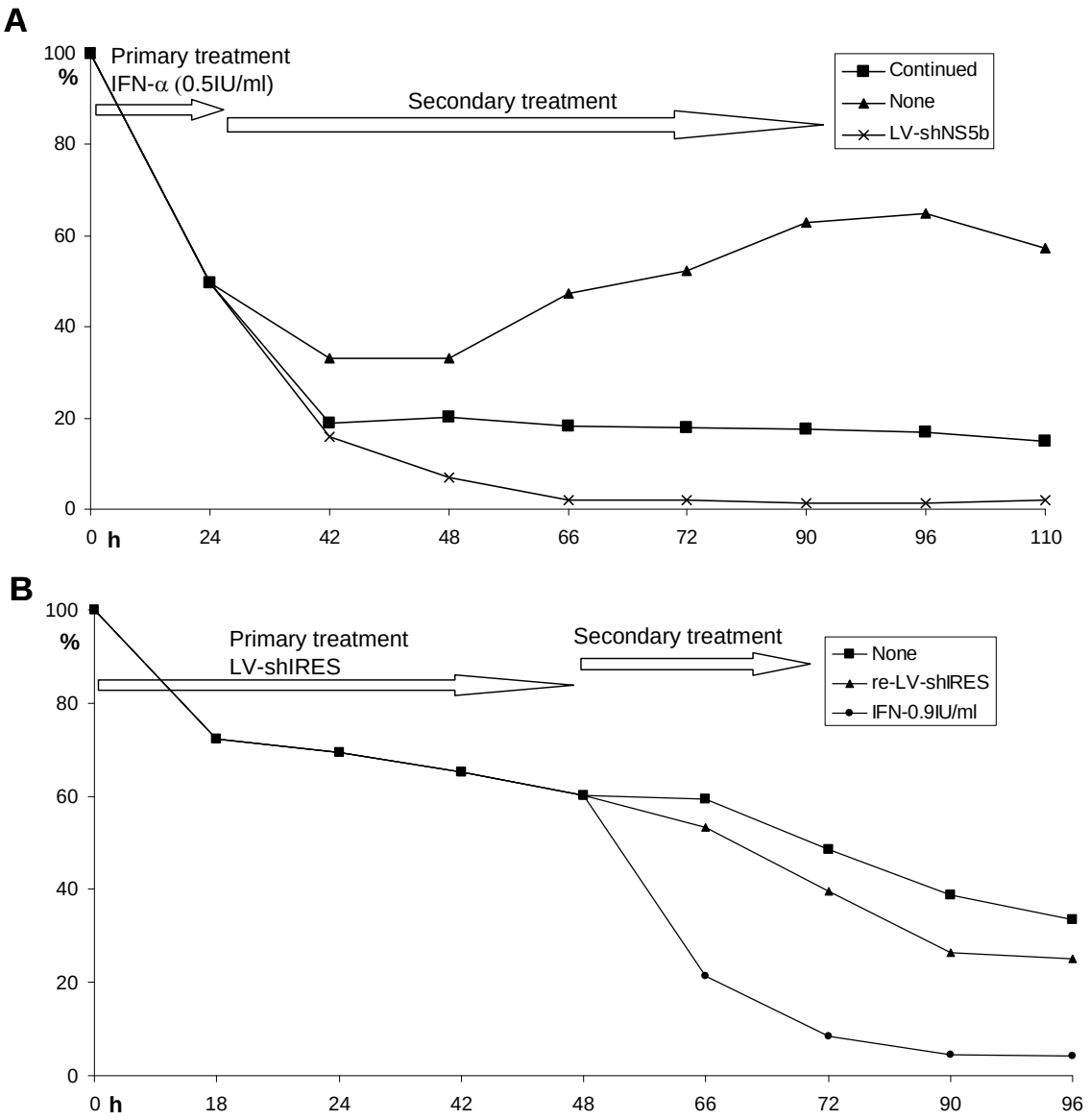

Figure 6. Subsequential treatment of IFN-α and RNAi reciprocally enhances inhibition of HCV replication. (A) Huh7-ET cells were treated with low dose (0.5 IU/ml) of IFN-α 24 hrs after which medium was replaced and cells were treated a second time for an additional 86hrs. Secondary treatment with IFN-α resulted in a maximum inhibition of 81.3% ± 1.3 (n = 7, *P*<0.001) from t= 42hr onward. However, subsequently switching IFN-α to LV-shNS5b resulted in a significantly greater inhibition of viral replication (98.2% ± 0.2 inhibition, n = 6, *P*<0.001). In Huh7-ET cells without secondary treatment HCV replication was partially restored to approx. two-third of baseline levels at two days after switching. (B) Conversely, Huh7-ET cells primarily treated with LV-shIRES were more sensitive to a secondary treatment with IFN-α than re-treatment with the same LV-shIRES vector. Secondary treatment with 0.9 IU/ml IFN-α resulted in profound inhibition of viral replication (96% ± 0.6 inhibition at t=96h, n = 6, *P*<0.001) as compared to secondary treatment with vector (75.1 % ± 3.7 inhibition, n = 6). In Huh7-ET cells without additional treatment HCV replication was (66.4 % ± 5.4 inhibition, n = 6). Overall these findings indicate that cells treated with IFN-α are more sensitive to subsequential treatment with RNAi than re-treatment with IFN-α. Equally, cells treated with RNAi are more sensitive to subsequential treatment with IFN-α than re-treatment with RNAi.

Taken together, these results indicate that RNAi and IFN-α act independently on HCV replication and that combination of these agents resulted in an enhanced anti-viral effect. Furthermore, IFN-α and RNAi appear complementary suggesting that viral resistance to treatment of IFN-α remains sensitive to treatment with RNAi and, reversely, that viral resistance to RNAi remains sensitive to inhibition by IFN-α.

Discussion

Peg-IFN-α in combination with ribavirin is currently the standard therapy for chronic HCV leading to a sustained viral response in approximately half of patients with the common genotypes [28]. In order to improve treatment outcomes and to provide opportunities to treat previous non-responders, it is urgent to exploit alternative therapies. RNAi represents as a novel anti-viral strategy that could be effective for HCV. To be applicable in therapeutic setting, it is critical that RNAi induces long-term and stable gene silencing. Since integrating lentiviral vectors have shown great advantages in transgenic delivery [29], third-generation LV are currently the best option for stable shRNA delivery. We found that LV and RNAi can be applied in combination with IFN-α without effecting transduction to hepatocytes or interfering with RNAi-mediated gene silencing.

The action of RNAi technology is clearly different from conventional anti-viral compounds. Particularly when delivered by a HIV derived lentiviral vector, the first concern would be whether the vector itself could have complicated interaction with innate immune system, especially IFN response. The induction of an endogenous IFN-response by shRNA expressing vectors is dependent on the cell type and the sequence. Studies have demonstrated that human dendritic cells can mount an IFN response after exposure to wild-type HIV or lentiviral vectors [30-32]. To our knowledge, our current study is the first report on the effect of exogenous IFN-α on lentiviral transduction and RNAi. A recent study has shown that lentiviral vector triggers a type I interferon (IFN-α/β) response that restricts hepatocyte gene transfer and promotes vector clearance in mice [24]. These mouse experiments contradict our experiment in NOD/SCID mice where no negative effect of exogenous peg-IFN-α treatment was observed on transduction of hepatocytes (Fig. 2D). Also previous studies by others [33, 34] and our group [13] showed no in vitro evidence of

endogenous type I interferon responses after transduction with lentiviral vectors. The fact that exogenous IFN-α does not have an apparent impact on LV transductions, makes a combined approach feasible.

In the setting of combining RNAi with IFN-α, the most critical issue would be the influence of exogenous IFN-α on gene silencing efficiency. This study has clearly shown that IFN-α does not have significant negative effects on RNAi mediated silence of both GFP report gene and native host gene CD81. Down regulation of viral-binding co-receptor CD81 expression was previously shown to reduce HCV E2 envelope binding [35]. Now we (Fig. 4D) and others [36] have shown RNAi mediated reduction of CD81 also inhibit infection of replication competent HCV virus, JFH1. Therefore, it is conceivable that lentiviral mediated RNAi has the potential not only to inhibit viral replication, but also to prevent infection. Most importantly, a simultaneous combination of IFN-α with RNAi directly targeting viral genome (IRES or NS5b region) significantly enhanced their individual anti-viral effects. Furthermore, IFN-α and RNAi clearly acted complementary shown by the fact that cells treated with IFN-α were more sensitive to subsequent treatment with RNAi than to re-treatment with IFN-α. Conversely, treatment of LV-shIRES led to partial inhibition of viral replication and was more sensitive to secondary treatment with IFN-α than re-treatment of the same vector (Fig. 6B). These findings could suggest that HCV that develops resistance to treatment with IFN-α would still be sensitive to RNAi therapy and, reversely, that potential viral resistance developed against RNAi would still be susceptible to inhibition by IFN-α.

Owing to the high risk of escape and resistance development, combination of RNAi with IFN-α may be necessary to completely cure HCV infection by attacking the virus in two distinct ways. In particular, this approach possesses unique advantages in preventing and treating HCV recurrence after liver transplantation. Lentiviral mediated RNAi could be used to modify a donor graft and prevent or slow HCV recurrence after transplantation; meanwhile, low dose IFN-α can be used to systemically treat HCV enhancing the therapeutic effects of both. Additional combinations with ribavirin are conceivable in order to further enhance therapeutic effects. Our preliminary results show that ribavirin has no negative effects on the action of RNAi (data not shown).

In conclusion, in this study we found that exogenous IFN-α had no significant negative influence on lentiviral transduction. In the presence of IFN-α, RNAi-mediated gene knockdown was unaffected and moreover, a combination of viral genome targeted RNAi

with IFN-α achieved enhanced anti-viral effects. Therefore, this novel combination strategy may offer the potential to eliminate HCV infection in chronically infected patients.

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Chapter 10

Hepatocyte-derived MicroRNAs as Serum Biomarker of Hepatic Injury and Rejection after Liver Transplantation

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Liver Transplantation, 2011, Epub

Abstract

Recent animal and human studies highlight the potential of hepatocyte-derived microRNAs (HDmiRs) in serum as early, stable, sensitive, and specific biomarkers of liver injury. Their usefulness in human liver transplantation, however, has not been addressed. Aim of this study is to investigate serum HDmiRs as markers for hepatic injury and rejection in liver transplantation. Serum samples of healthy controls and liver transplant recipients (n = 107), and peri-transplant liver allograft biopsies (n = 45) were analyzed by RT-PCR quantification of HDmiRs, miR-122, miR-148a and miR-194. The expression of miR-122 and miR-148a in liver tissue was significantly reduced with prolonged graft warm ischemia times. Conversely, serum levels of these HDmiRs were elevated in patients with liver injury and positively correlated with transaminase levels. HDmiRs appears to be very sensitive, as patients with normal transaminase values (below 50 IU/L) had 6 to 17-fold higher HDmiRs levels as healthy controls ($P < 0.005$). During an episode of acute rejection, serum HDmiRs were elevated up to 20-fold and appear to rise earlier than transaminase levels. HDmiRs proved stable during repeated freezing and thawing of serum. In conclusion, this study shows that liver injury is associated with release of HDmiRs into the circulation. HDmiRs represent promising candidates as early, stable and sensitive biomarkers for rejection and hepatic injury after liver transplantation.

Introduction

MicroRNAs (miRNAs), a class of small non-coding RNAs, are important regulators of gene expression and they control many cellular processes by post-transcriptional suppression of gene expression (1, 2). Altered tissue expression levels of miRNAs have lately been linked to various pathologic conditions in humans, including malignant, infectious, metabolic, autoimmune, and cardiovascular diseases (3-9). These findings have lead to increased interest in miRNAs as potential diagnostic markers as well as targets for therapeutic interventions.

Hepatocytes express a distinct set of miRNAs of which miR-122 is most abundant (10). MiR-122 was found to be an important regulator of cholesterol metabolism (11), iron homeostasis (12) and a crucial host factor for hepatitis C virus infection and replication (13, 14). In addition to these important cellular functions, recent studies in rodents have demonstrated that miR-122, as well as other hepatocyte-abundant miRNAs, are released from cells during drug-induced liver injury (15, 16). These hepatocyte-derived miRNAs (HDmiRs) were detectable in serum or plasma and levels increased dependent on the dose and duration of drug exposure. HDmiRs were found to correlate with serum transaminases, aspartate transaminase (AST) and alanine transaminase (ALT), as well as liver histology. Importantly, the rise in serum miRNA in these animals appeared earlier than the rise in transaminases. In addition to the diagnostic potential of miRNA, experimental animal studies have shown that miRNAs are a feasible target for therapeutic intervention to minimize and even reverse severe tissue injury caused by ischemic insults (17). In humans, it has recently been shown that the HDmiR miR-122 can also be detected in serum and was found to be elevated in patients with hepatocyte injury caused by viral, alcoholic or chemical-related hepatotoxicity (18, 19). Also in these patients, serum and plasma miR-122 showed a close correlation with transaminases and liver histology. However, this has not been evaluated in the setting of liver transplantation.

Liver transplantation has developed from a risky experimental procedure to a life-saving and effective treatment of end-stage liver failure. However, despite this success, transplant recipients can suffer from serious side effects of long-term immune suppression and remain at risk of de novo malignancies (20) or lose their allograft due to rejection, recurrent disease

or biliary complications (21, 22). The potential benefit of tapering immunosuppressive medication in patients to reduce toxicity is countered by the potential risk of losing the graft by immune mediated rejection. Therefore, there is an urgent need for better biomarkers that could provide earlier and more sensitive signs of rejection or liver graft dysfunction in a non-invasive fashion. Given their cell-type specific distribution, their biological stability and sensitivity of detection, HDmiRs could represent promising candidates for this. Indeed, several recent studies in the setting of kidney transplantation have highlighted the potential of mRNA and miRNA as biomarkers for assessing renal allograft status (23-26). Current protein-based markers for liver injury, AST and ALT, are also expressed outside the liver in muscle tissue and they can cause false elevations during muscle injury (27). Therefore, assessment of liver allograft status often still requires tissue biopsies for more definite proof of hepatic injury. Particularly after liver transplantation, taking trough-cut biopsies is a relative perilous procedure associated with pain, bleeding and infections (28-31). Alternatively, more sensitive, specific and non-invasive methods for monitoring graft injury are needed to minimize the need for liver biopsies and allow safer weaning-off of immunosuppressive medication.

The aim of the current study was to investigate the utility of serum HDmiRs as markers for hepatic injury and acute rejection after liver transplantation. We found that the expression of miR-122 and miR-148a in liver tissue was significantly diminished with prolonged graft warm ischemia times and, conversely, was elevated in serum during ischemia and reperfusion injury and acute rejection. HDmiRs were found to represent promising candidates as biomarkers for assessing allograft status after liver transplantation.

Patients & Methods

Patient samples

All liver transplantations were performed at Erasmus Medical Center, Rotterdam, The Netherlands. Liver graft biopsies (n = 45) were obtained during transplantation 60 minutes after portal reperfusion and directly snap frozen for storage. Serum samples were taken from 12 healthy controls and 43 recipients at different times after liver transplantation and included 13 patients with histologically proven acute rejection. All blood samples were

collected using a standardized protocol and serum was processed within 2 hours and quickly stored in -80˚C. Serum samples with signs of red blood cell lysis were not used. Patient demographics and clinical variables were extracted from a prospectively filled database and summarized in Table 1. The intrinsic stability of HDmiRs in serum was determined by subjecting four individual serum samples from liver transplant recipients to five freezing and thawing cycles (-80°C / +20°C). The Medical Ethical Council of the Erasmus MC approved the use of human samples and all patients provided informed consent for the use of materials for medical research.

		Pre-transplant	Post-transplant	
	Healthy controls	**End-stage liver disease**	**Non-rejectors**	**Rejectors**
Serum samples (n)	12	10	34	47
Mean AST (UI/L)	-	89 ± 17	785 ± 221	151 ± 45
Mean ALT (UI/L)	-	95 ± 33	732 ± 166	219 ± 34
Patients (n)	12	10	20	10
Age (Years)	42 ± 3	51 ± 3	50 ± 2	40 ± 8
Gender (M:F)	7 : 5	7 : 3	14 : 6	6 : 4
Underlying disease				
Viral	-	7	10	4
Cholestatic	-	1	3	2
Alcoholic	-	1	2	2
Other	-	1	5	2

Table 1. Characteristics of patients and healthy controls

Serum levels of AST and ALT <50 UI/L were considered normal. Acute cellular rejection was defined by the presence of all three following criteria: a transient rise in AST and ALT levels above the upper limit of normal, a rejection activity index (RAI) of 6 or more in the consequent needle biopsy at histological examination and a decrease in transaminase levels upon treatment with methylprednisolone (32).

RNA isolation

Total RNA was extracted from approximately 10 mg of liver tissue using the miRNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). A modified protocol was used to isolate total RNA from serum. For this, 1.5 ml of Qiazol Lysis Reagent was added to 200 µl of serum and extensively mixed by vortexing. Chloroform (300 µl) was added and after centrifugation (15 minutes, 16.000 RCF), 800 µl of an aqueous RNA-containing layer was obtained, which was further processed according to the manufacturer's protocol (Qiagen). RNA extracted from liver tissue was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and normalized to a concentration of 50 ng/7.5 µl. RNA extracted from serum could not be quantified due to its low concentration and was normalized only for initial serum input.

Reverse transcription and real-time polymerase chain reaction (RT-PCR)

The TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) was used to prepare cDNA, for multiple miRNAs in one reaction, using a modified protocol. Every multiplex cDNA reaction consisted of 0.4 µl dNTP mix, 1.35 µl Multiscribe RT enzyme, 2.0 µl 10x RT Buffer, 0.25 µl RNase Inhibitor, 1.0 µl of each RT primer, and 7.5 µl of diluted template RNA. The total reaction volume was adjusted to 20 µl with nuclease free water. Based on literature, 15 miRNAs were initially tested, namely miR-30a, miR-30c, miR-30e, miR122, miR-133a, miR-148a, miR-191, miR-192, miR-194, miR-198, miR-200c, miR-222, miR-296, miR-710 and miR-711 (15, 33-36). Three highly expressed hepatocyte-rich miRNAs, miR-122, miR-148a and miR-194, were selected and further used. For analysis of liver biopsies, additional cDNA was prepared for a small nuclear RNA, RNU43, which served as reference gene for normalization of RNA input. For serum samples two additional non-liver-abundant miRNAs, miR-133a (muscle-abundant) and miR-191 (blood-abundant), served as controls. All cDNA reactions were performed according to the manufacturer's instructions. PCR reactions were carried out in duplicate and according to the manufacturer's instructions. Each reaction consisted of 10 µl TaqMan Universal PCR Master Mix, 0.5 µl microRNA-specific PCR primer (Applied Biosystems) and 5.0 µl of the previously diluted (1:10 dilution) cDNA. The final volume of every PCR reaction was adjusted to 20 µl with nuclease-free water.

Statistical analyses

Statistics for correlation were generated using Spearman's Rank Correlation test. Comparative statistics between groups were tested using the Mann-Whitney U and the Wilcoxon matched pairs test by GraphPad Prism software (GraphPad Software Inc., San Diego, USA). P-values < 0.05 were considered significant.

Results

Reduced hepatic miRNA levels in liver grafts with long warm ischemic times

To investigate changes in intrahepatic miRNA expression in response to ischemia-reperfusion injury, 45 biopsies taken from liver grafts one hour after reperfusion were analyzed. Average cold ischemia time was 484 ± 25 minutes (mean ± SEM) and the mean warm ischemia time was 35 ± 2 minutes. As shown in Figure 1A, there was a significant positive correlation between the levels of hepatocyte-abundant miRNAs. Levels of miR-122 strongly correlated with miR-148a and miR-194 ($R \geq 0.85$, $P < 0.001$), but were approximately 20-fold higher than those of miR-148a and miR-194. As shown in Figure 1B, the levels of miR-122 and miR-148a, but not miR-194, in these liver graft biopsies showed a significant reverse correlation with the length of warm ischemia time ($R = -0.307$, $P = 0.038$ and $R = -0.404$, $P = 0.005$ respectively). No significant correlation of miRNA levels and cold ischemia times was observed (data not shown). These findings suggest that graft injury associated with longer warm ischemia times reduced levels of specific hepatocyte-abundant miRNAs, possibly by the release of miRNAs from injured cells.

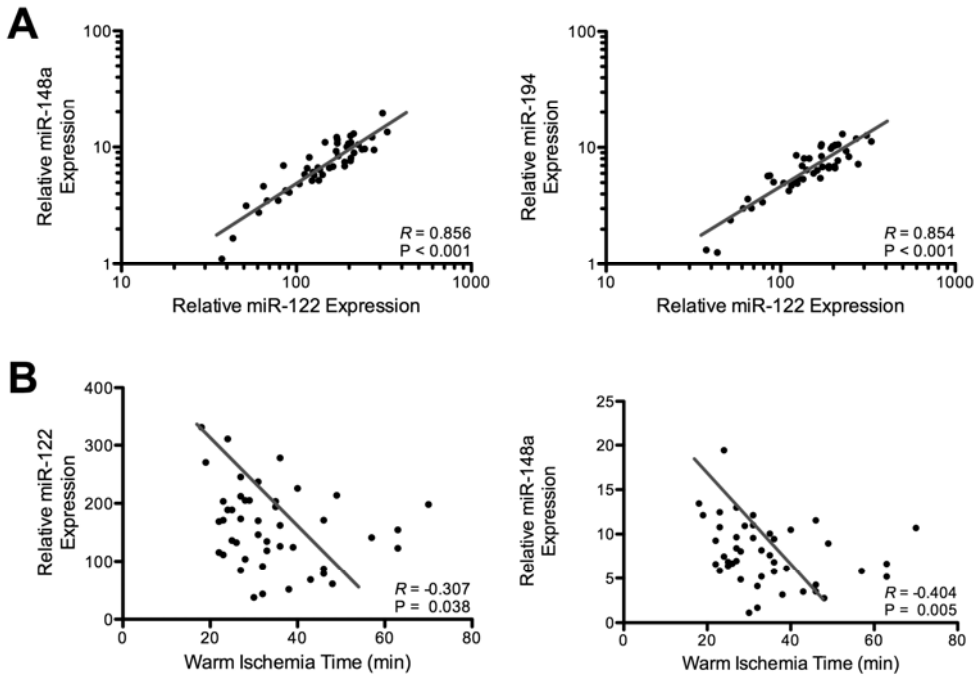

Figure 1. Decreased levels of hepatic miRNAs in liver grafts with extended warm ischemia times. Liver graft tissue biopsies (n = 45) were analyzed for the hepatocyte-abundant miRNAs, miR-122, miR-148a and miR-194, by quantitative RT-PCR. MiRNA levels were normalized to small nuclear RNA RNU43, which served as a reference gene. (A) Relative expression levels of miR-122 correlated significantly with miR-148a and miR-194 in the liver grafts ($R \geq 0.85$, $P < 0.001$). MiR-122 levels were approximately 20-fold higher than miR-148a and miR-194. (B) Decreased levels of miR-122 and miR-148a in liver graft biopsies correlated significantly with length of the warm ischemia time to which the graft had been exposed during liver transplantation ($P < 0.05$).

Serum HDmiRs are associated with peri-transplant ischemic liver injury

Serum samples from healthy individuals and liver allograft recipients within 2 weeks of transplantation were analyzed for the presence of HDmiRs. All three HDmiRs, miR-122, miR-148a and miR-194, and both control miRNAs, 133a and 191, were detectable in the serum from healthy individuals and patients. As shown in Figure 2, the levels of HDmiRs were significantly elevated in patients after liver transplantation as compared to healthy controls. In serum samples with high transaminase levels (AST or ALT > 50 UI/L), the levels of miR-122 were respectively 124-fold and 102-fold elevated with respect to average levels in healthy ($P < 0.0001$). When compared to healthy controls, levels of miR-148a and miR-194 were respectively 30-fold and 40-fold higher in the high transaminase groups ($P < 0.0001$). Levels of all HDmiRs were significantly higher in the high AST and ALT groups compared to the low AST and ALT groups ($P < 0.005$, Fig. 2) with the exception of miR-194 in the high ALT group,

which was only 2-fold elevated and not statistically significant. Levels of the control miRNAs, miR-133a and miR-191, were not significantly different between any of the groups (Fig. 2). The HDmiRs appeared to be sensitive, as patients with normal transaminase values had significantly elevated levels of miR-122, miR-148a and miR-194 compared to healthy controls (respectively 11, 7, and 9-fold higher in the low AST group and respectively 8, 6 and 17-fold higher in the low ALT group, $P < 0.005$). As shown in Figure 3, a positive correlation was observed between serum HDmiRs levels and transaminases in patients. The correlation with AST and ALT resulted in a coefficient *R* of respectively 0.80 and 0.77 for miR-122, while for miR-148a the coefficient *R* was 0.60 for both AST and ALT ($P < 0.0001$). No significant correlations were found for miR-194 ($R < 0.30$, $P > 0.05$).

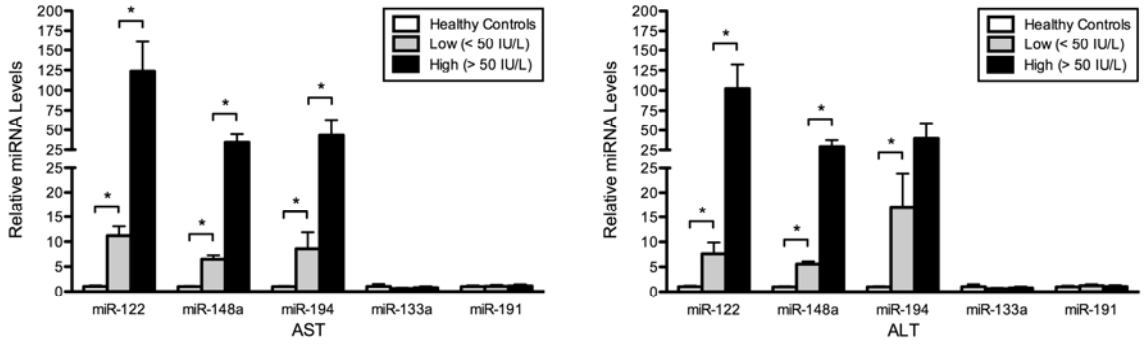

Figure 2. Hepatocyte-derived miRNAs (HDmiRs) are elevated in serum during peri-transplant ischemic liver injury. HDmiRs miR-122, miR-148a and miR-194, were quantified using RT-PCR in 92 serum samples obtained from liver transplant recipients (n = 40) and healthy controls (n = 12). Compared to healthy controls, levels of miR-122, miR-148a and miR-194 were significantly elevated in serum samples of patients with low AST and ALT levels by 11-, 7-, 9-, and 8-, 6- and 17-times, respectively. Levels were further elevated in serum of patients with transaminase levels above the clinical diagnostic threshold of 50 UI/L. For the high AST group miR-122, miR-148a and miR-194 were 11-, 5- and 5-fold higher and for the high ALT group 13-, 5- and 2-fold higher compared to the low AST and ALT groups. Levels of control miRNAs, miR-133a and miR-191, were not significantly elevated in any of the serum samples compared to healthy controls. * $P < 0.005$

Additional experiments to test the stability of HDmiRs in serum showed that levels of miR-122, miR-148a and miR-194 in serum were not significantly affected after five cycles of freezing (-80˚C) and thawing to room temperature (mean 120% ± 11 SEM, 100% ± 6 and 99% ± 19 of untreated baseline values, respectively).

Elevated serum HDmiRs during acute rejection

Serum HDmiRs were analyzed in liver transplant recipients experiencing an episode of acute rejection. As shown in Figure 4A, serum HDmiR miR-122, was significantly elevated during rejection. An average 9-fold increase was observed at the time of rejection compared to levels 6 months after resolving rejection ($P < 0.005$). For five patients a longitudinal series of serum samples before, during and after acute rejection was analyzed. One representative patient is shown in Figure 4B. Serum levels of miR-122 and miR-148a showed kinetics similar to those of AST and ALT and increased up to 20-fold during acute rejection. Levels of the control miRNAs, miR-133a and miR-191, did not increase during acute rejection (Fig. 4B). Although miR-122 showed similar kinetics, it appeared to rise and drop one or two days earlier than transaminase levels (Fig.4B). As shown in Figure 4C, in pooled date of five patients a similar trend was observed. At the moment of diagnosis and start of treatment of the acute rejection (0 hr) miR-122 was already elevated to its maximum level. Levels of miR-122 dropped quickly after the start of intravenous methylprednisolone treatment, while levels of AST and ALT continued to rise even after the start of treatment and took longer to normalize.

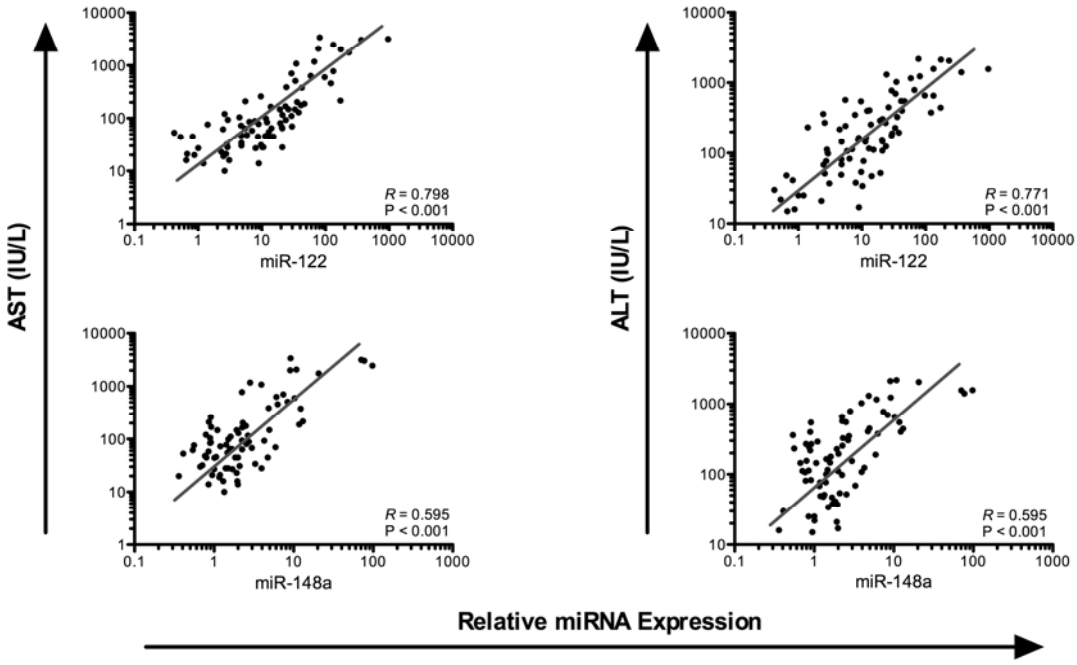

Figure 3. Levels of serum HDmiRs in liver transplant recipients correlate with AST and ALT. HDmiRs, miR-122 and miR-148a, were quantified using RT-PCR in eighty serum samples obtained from liver transplant recipients. Serum levels of miR-122 and miR-148a correlated significantly with levels of AST and ALT in the same samples.

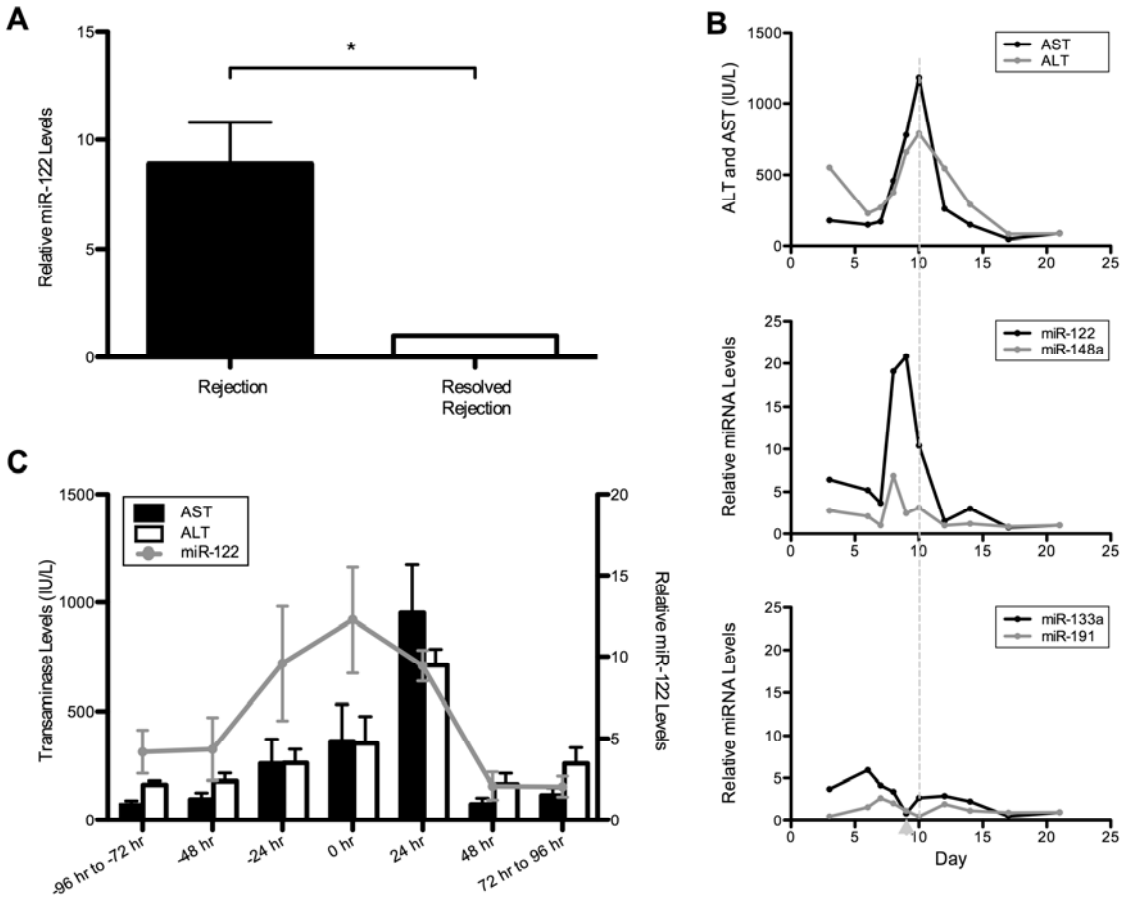

Figure 4. Changes in serum HDmiRs during acute rejection. Serum samples from 13 liver transplant recipients experiencing one or more episodes of biopsy-proven acute rejection were analyzed. **(A)** Levels of serum miR-122 were significantly elevated during acute rejection by approximately 9-fold compared to levels in the same recipients 6 months after rejection was resolved ($n = 13$, $P < 0.005$). **(B)** From five of these patients a longitudinal series of serum samples, taken at daily intervals, was analyzed. Representative results from one patient are shown. Serum levels of miR-122 and miR-148a increased up to 20-fold during acute rejection (middle panel) and showed similar kinetics to those of AST and ALT (top panel). The peak of HDmiRs appears to precede the peak of transaminases (indicated with dashed line) and quickly normalized after starting treatment with intravenous methylprednisolone (arrow on axis). Levels of control miRNAs, miR-133a and miR-191, did not show an increase during acute rejection (lower panel). **(C)** Levels of serum transaminases and miR-122 of the 5 patients at the histologic diagnosis and start of methylprednisolone treatment (t = 0 hr) and up to 96 hrs before and after are shown. Levels of miR-122 reached a maximum level at the start of treatment and quickly decreased after treatment whereas transaminase levels still continued to rise 24 hrs later.

Discussion

Small non-coding RNAs, in particular miRNAs, have emerged as important genetic regulators of cellular processes, including tissue injury and repair responses (17). Recent studies in small animal models as well as humans have demonstrated that HDmiRs are highly stable and sensitive serum biomarkers of liver injury (15, 16, 18, 19). In both humans and rodents, HDmiRs appeared to increase earlier and more rapidly in serum than AST and ALT. In particular miR-122 was significantly elevated even in subjects with transaminases below the threshold of 50 IU/l (15, 16, 18, 19). In the current study we provide evidence that the concept of miRNAs as biomarkers of hepatic injury is also feasible in the setting of liver transplantation. Serum levels of HDmiRs were elevated in patients with liver injury after liver transplantation (Fig. 2) and during acute rejection (Fig. 4). Conversely, hepatic miRNA levels in liver graft biopsies exhibited diminished expression with prolonged warm ischemic times (Fig. 1). During acute rejection, serum HDmiRs showed similar kinetics, however, miRNA levels increased and decreased earlier than transaminases (Fig. 4B and C). As in previous studies (15, 18), miRNAs showed higher sensitivity than transaminases and miRNA stability was confirmed as proposed by earlier studies (6, 9, 37-40).

HDmiRs could provide a solution for the urgent need for better non-invasive biomarkers that could serve as earlier and more sensitive signs of rejection or liver graft dysfunction. Better markers would greatly help the management of liver transplant recipients and could allow the safer reduction of immunosuppressive medication to achieve a better balance between effects (prevention of graft rejection) and side effects (toxicity, infection and malignancy). Long-term complications of immunosuppressive drugs, such as nephrotoxicity and *de novo* cancer, are becoming a bigger problem due to the long survival of liver transplant recipients (20). Currently, the potential benefit of tapering immunosuppressive medication in patients is countered by the potential risk of losing the graft by immune mediated rejection. Serum ALT and AST are often insufficient for the early and definitive diagnosis of acute rejection, necessitating the use of liver biopsies. Particularly in the setting of liver transplantation, liver biopsies pose a significant risk for complications such as pain, bleeding and infections (28-31). Feasibility of the concept of minimally invasive diagnosis of

acute rejection, based on the detection of messenger RNA, has been demonstrated for kidney transplants (24, 25).

Currently, little is known about the mechanism and biology of release of hepatocyte-abundant miRNAs in response to liver injury. Ideally an unbiased genome-wide approach would be preferred to study release, but it is very challenging to perform gene-array analysis on serum samples because of the low yields of RNA and the relative high amounts required. In our initial analyses we tested 15 different types of hepatocyte and cholangiocyte abundant and control miRNAs selected from other studies (15, 33-36). These included miR-30a, miR-30c, miR-30e, miR122, miR-133a, miR-148a, miR-191, miR-192, miR-194, miR-198, miR-200c, miR-222, miR-296, miR-710 and miR-711, but only the three HDmiRs were found to be significantly elevated during acute rejection. Likely, many other miRNAs expressed in hepatocytes and other liver cells are released during hepatic injury, but only the most abundant and liver-specific miRNAs will be detectable in serum. Nevertheless, the hepatocyte-abundant miRNA miR-194, with expression levels in liver tissue significantly correlating with miR-122 (Fig. 1A), did not correlate with transaminase levels (data not shown). This suggests that there may be sequence specificity or selectivity regarding the release of miRNAs, rather than just a general leakage of all miRNAs from the injured cell. This hypothesis is supported by the observation that cellular miRNAs can be released from cells by secretion of microvesicles including exosomes and that only distinct sets of miRNAs are selectively packaged into microvesicles (40, 41).

This specificity in release and the distinct repertoires of miRNAs expressed by various cell types in the liver may allow in the future distinguishing between different causes and types of liver injury, like cholangiocyte injury in bile ducts and endothelial cell injury in veins and arteries. Preliminary data from our research group indeed suggests that tissue levels of specific miRNAs expressed by biliary epithelial cells could be used to quantify biliary injury and can predict the development of long-term biliary complications and graft loss after liver transplantation (42). In addition, miRNA-based diagnostics could facilitate allograft selection, particularly of marginal donors, and potentially enlarge the pool of grafts. For example, several experimental studies demonstrated a role of hepatic miRNAs, including miR-122, in regulation of cell proliferation during liver regeneration after partial hepatectomy (43-48). Although the exact biology is not clear, it is conceivable that the decrease in miR-122 expression during graft storage may be related to hepatic cell cycle progression in response

to ischemic injury. It is tempting to speculate that manipulation of miRNAs using anti-sense, anti-miRNA technology (11) could allow therapeutic manipulation for rescue of marginal grafts or allow the use of smaller size split grafts by minimizing injury and stimulating cell proliferation (17).

In summary, we demonstrate that circulating HDmiRs, miR-122, miR-148a and miR-194, are stable and detectable during hepatic injury in patients after liver transplantation. The levels of two of these HDmiRs closely correlate with AST and ALT during post-transplant liver injury and acute rejection. These data support the potential of miRNA-based diagnostic tools for various types of liver injury in liver transplant recipients.

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Chapter 11

Exosome-Mediated transmission of Hepatitis C Virus

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Submitted

Part III.

Stem Cell-based Strategies

Chapter 12

Mobilization of hepatic mesenchymal stem cells from adult human liver grafts

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Liver Transplantation, 2011, 17(5):596-609

Abstract

Extensive studies have demonstrated the potential applications of bone marrow–derived mesenchymal stem cells (BM-MSC) as regenerative or immunosuppressive treatments in the setting of organ transplantation. The aims of the present study were to explore the presence and mobilization of mesenchymal stem cells (MSC) in adult human liver grafts and to compare their functional capacities to those of BM-MSC. The culturing of liver graft preservation fluids (perfusates) or end-stage liver disease tissues resulted in the expansion of MSC. Liver-derived mesenchymal stem cells (L-MSC) were equivalent to BM-MSC in adipogenic and osteogenic differentiation and in wingless-type-stimulated proliferative responses. Moreover, the genome-wide gene expression was very similar, with a 2-fold or greater difference found in only 82 of the 32,321 genes (0.25%). L-MSC differentiation into a hepatocyte lineage was demonstrated in immunodeficient mice and in vitro by the ability to support a hepatitis C virus infection. Furthermore, a subset of engrafted MSC survived over the long term in vivo and maintained stem cell characteristics. Like BM-MSC, L-MSC were found to be immunosuppressive; this was shown by significant inhibition of T cell proliferation. In conclusion, the adult human liver contains an MSC population with a regenerative and immunoregulatory capacity that can potentially contribute to tissue repair and immunomodulation after liver transplantation.

Introduction

The adult liver harbors a population of facultative progenitors (oval cells in rodents and hepatic progenitor cells or hepatoblasts in humans) that respond to specific injuries and can differentiate into hepatocytes and biliary cholangiocytes.[1,2] These liver progenitor cells are quiescent in the healthy liver but are activated when certain liver diseases impair the regenerative capacity of mature hepatocytes, cholangiocytes, or both.[3] However, oval cells/hepatic progenitors do not constitute a homogeneous population, and their precise origin and the signals governing their activation are not entirely clear.[4,5] Previous studies have indicated the presence of a stem cell niche at the proximal biliary tree (the canals of Hering) that contains hepatic stem cells serving as precursors to hepatic progenitor cells.[6,7] Recent studies have further characterized these hepatic stem cells, which are abundant in human fetal and adult livers and have been proposed to be precursors of hepatic progenitors.[8] This population is located in ductal plates in fetal and neonatal livers and in the canals of Hering in pediatric and adult livers.[9]

The mesenchymal stem cell (MSC) is one of a few cell types on the brink of being used clinically in different areas of therapeutic application, including organ transplantation.[10] The bone marrow (BM) compartment harbors resident MSC with multilineage differentiation potential and anti-inflammatory and immunomodulatory properties, which have been proposed to play a role in the response to liver injury.[11,12] Encouragingly, various studies have demonstrated the therapeutic potential of bone marrow–derived mesenchymal stem cells (BM-MSC) in different liver disease models, such as liver resection, fulminant hepatic failure, and, in particular, liver transplantation.[13-15] Besides their hepatic differentiation potential, MSC produce trophic factors that have been shown to provide paracrine support for hepatocyte proliferation, angiogenesis, tissue repair, and immunomodulation.[16-18]

In contrast to the experimental significance of BM-MSC in liver injury responses and in contrast to the reports on the presence of MSC in fetal human livers and on the presence of mesenchymallike stem cells in adult rat livers,[19-22] sufficient studies describing MSC in the adult human liver are lacking. In this study, we first investigated the presence of MSC in adult human liver tissue and their mobilization during graft cold storage at the time of liver transplantation. Secondly, phenotypic and functional analyses were performed to evaluate

the biological characteristics and therapeutic potential of isolated liver-resident MSC. Gene array analysis revealed a high degree of similarity between gene expression profiles of BM-MSC and liverderived mesenchymal stem cells (L-MSC). Furthermore, Wnt responsiveness and hepatic differentiation in vitro and in mice confirm that L-MSC represent a bona fide stem cell/progenitor population in the adult human liver.

Materials & Methods

Isolation and culture of MSC from liver tissue and liver perfusate solution

End-stage liver disease tissue samples were obtained from the explanted livers of liver transplant recipients. Liver graft preservation fluid (perfusates) was collected from human liver grafts at the time of transplantation as described previously.[23] Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 IU/mL penicillin, and 100 lg/mL streptomycin. The use of liver tissues and perfusates was approved by the medical ethics committee of Erasmus MC.

Flow cytometry

MSC were stained for 30 min at 4°C with directly-labeled mouse monoclonal antibodies directed against CD90, CD105 (R&D systems, Abingdon, UK), CD34 (Miltenyi Biotec, Bergish Gladbach, Germany), CD45 (Beckman Coulter, Inc. Fullerton, CA, USA), HLA-DR (BD Pharmingen, San Diego, CA, USA), and CD166 (BD Pharmingen). Flow cytometric analysis was performed using FACS Calibur and CellQuest Pro software (FACS Calibur, BD Biosciences, San Jose, CA).

Adipogenic and osteogenic differentiation

For adipogenic differentiation, MSC were cultured in DMEM supplemented with 10% FCS, 1 μM dexamethasone, 500 μM isobutyl-methylxanthine, 5 μg/ml insulin and 60 μM indomethacin (Sigma-Aldrich) for 3 weeks. Oil Red O staining (Sigma-Aldrich) was used for detection of adipocytes. For osteogenic differentiation, cells were cultured in DMEM with 10% FCS supplemented with 0.2 mM ascorbic acid, 100 nM dexamethasone, and 10 mM β-

glycerol phosphate (Sigma-Aldrich) for 3 weeks. Alizarin Red S staining (Sigma-Aldrich) was performed to detect deposited calciumphosphates.

Cell proliferation assays

Liver MSC (5 x 10^3) were plated in 96-well plates and treated with Wnt3a-conditioned medium (Wnt3a-CM) and control L-cell conditioned medium (L-CM), as described previously [15]. At the indicated times, the number of metabolically active cells was quantified by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 0.5 mg/ml) assay.

Liver MSC (5 x 10^4) were stained with 0.2 μM carboxy-fluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Leiden, The Netherlands) for 5 min at 37°C and plated in 6-well plates. At different time points, cells were harvested and stained with 7AAD (BD Pharmingen), and measured by flow cytometry. Generation analysis was performed with ModFit LT v3.0 software (Verity Software House, Topsham, USA) and was gated to exclude 7AAD-positive dead cells. The proliferation index, which is the sum of the cells in all generations divided by the computed number of original parent cells, was used to indicate the extent of cell proliferation.

Gene expression profiling by microarray

Total RNA of three independent liver MSC cultures at passage 2-5 (one culture derived from liver tissue biopsy and two derived from liver perfusates) and three BM MSC cultures at passage 2-5 (from different donors) and of three hepatoma cell line (Huh7) cultures was used for genome-wide microarray analysis with the HuGene 1.0 ST.v1 Affymetrix Genechip (Affymetrix, Santa Clara, USA) according to the manufacturer's procedures. Transcript level expression measures were generated using RMA as implemented in the Affymetrix Gene Expression Console and probesets annotations were retrieved from Nettafx using the same software. Probesets differentially expressed among conditions were identified using the class comparison tool implemented in BRB-ArrayTools (National Institutes of Health, USA). Principal component analysis was performed using Partek (Partek Incorporated, Missouri, USA). Hierarchical clustering was performed in Spotfire (Spotfire, Inc. Somerville, MA, USA). Pathway analysis was performed using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, USA).

In vitro hepatocyte differentiation

In vitro hepatic differentiation was performed in three steps as reported previously [16], but with modification at the final step. For the final maturation step, cultures were incubated with infectious JFH1-derived HCV particles.[25] Hepatogenic differentiation was determined by quantitative RT-PCR detection of albumin and hepatitis C virus (HCV) IRES RNA.

Real time RT-PCR

RNA was precipitated using a Micro RNeasy Kit (Qiagen, Venlo, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from 1 μg total RNA using a iScript cDNA Synthesis Kit from Bio-Rad (Bio-Rad Laboratories, Stanford, CA, USA). The cDNA of human and mouse albumin, CD90, CD105, HCV IRES, CyB, and GAPDH were quantified using real-time PCR (MJ Research Opticon, Hercules, CA, USA) performed with SybrGreen (Sigma-Aldrich) according to manufacturer's instructions. CyB or GAPDH were used as reference genes to normalize gene expression.

Periodic acid schiff (PAS) staining

Hepatic differentiated and undifferentiated MSC were fixed in 4% paraformaldehyde for 20 min and then intracellular glycogen was stained using PAS staining. Briefly, fixed cells were oxidized in 0.5% periodic acid solution for 5 minutes. After rinse in distilled water, place in Schiff reagent for 15 minutes. Then wash in lukewarm tap water for 5 minutes and Counterstain in Mayer's hematoxylin for 1 minute.

Cell transplantation

Immunodeficient NOD/SCID mice (Erasmus MC institutional breeding) age 6-8 weeks were intraperitoneally injected with 100 μL/20g body weight olive oil containing 10 μL carbon tetrachloride (CCl4). After 24 hrs, 1×10^6 liver MSC (n=3) or BM MSC (n=2) suspended in 0.2 ml PBS were injected in the spleen. Untreated (n=2) or non-engrafted CCl4-treated NOD/SCID mice (n=1) served as negative control. Four weeks after engraftment, the mice were sacrificed and their livers were removed. Secondly, luciferase labeled liver MSC (5×10^5 cells) were subcutaneously injected into NOD/SCID mice (n=2) and luciferase activity was measured at the indicated time points by IVIS camera.

Fluorescent immunohistochemistry

Mouse liver tissue was dissected and cryoprotected in 30% sucrose for generation of frozen sections. Sections were incubated with fluorecent labeled antibody at the dilution of 1:100 for 30 min. After three washes, nuclear staining was achieved by incubating with DAPI (Sigma-Aldrich) at the dilution of 1:50 for 5 min. 20–30 images of each condition were captured by confocal microscopy.

T cell proliferation/suppression assay

Effect of liver MSC on proliferation of T cells was determined by MLR assay. Briefly, PBMC (4 x 10^5) in the presence or absence of MSC were seeded in 96-well round-bottom plates. Irradiated allogeneic PBMC (2 x 10^5) or phytohemagglutinin (PHA; Murex Biotech, UK) was used for stimulation. After 5 days, proliferation was assessed by determination of the incorporation of 0.5 µCi (0.0185 MBq) [^{3}H] thymidine (Radiochemical Center, Amersham, United Kingdom) for 18 hours.

Statistical analysis

Statistical analysis was performed by using either the paired nonparametric test, Wilcoxon signed-rank test, or the unpaired nonparametric test, Mann–Whitney test, by GraphPad InStat software (GraphPad Software Inc., San Diego, USA). P-values lower than 0.05 were considered statistically significant.

Results

Mobilization of hepatic MSC from adult human liver grafts

Graft perfusion, procurement and cold-storage are associated with ischemia and tissue injury. Previously, we found that the washout of the graft preservation solution (perfusate) collected at time of liver transplantation contain high numbers of mononuclear cells which detach from the liver, including lymphocytes, NK-cells, antigen-presenting cells [23, 27] and hematopoietic stem cells.[28] Flowcytometric analysis of perfusate mononuclear cells revealed the presence of a small but consistent fraction of cells double-positive for MSC surface markers CD90 and CD105 (mean 0.09% ± 0.07 SD, n = 8) and CD90 and CD166 (0.02% ± 0.02; Fig. 1A). Prospectively, fresh perfusates from 15 consecutive liver transplantations were

collected and mononuclear cells were isolated and cultured for the presence of MSC. Fibroblast-like cells were observed in the initial cultures of all perfusates (Fig. 1B). In a majority of cultures the number of these cells rapidly increased (Fig. 1C). These cells could be expanded and passaged for several months under normal non-hypoxic culture conditions, clearly distinct from a previously described albumin^{+}CD105^{-} population of hepatic stem cells.[29] Flowcytometric analysis of expanded cells at passage 4 to 9 revealed a surface marker profile typical for MSC (Fig. 1D). A high percentage of cells stained positive for CD90 (mean 59% ± 18 SD, n=11), CD105 (55% ± 14) and CD166 (44% ± 16) and were mostly negative for the hematopoietic stem cell marker CD34 (0.8% ± 0.7), the leukocyte lineage markers CD45 (0.7% ± 0.7) and HLA-DR (1.9% ± 1). Functional analysis showed that the expanded liver-derived cells have multi-lineage potential with a capacity for adipogenic (Fig. 1E) and osteogenic (Fig. 1F) differentiation, similar to BM MSC.

To confirm the presence of MSC in adult human liver, tissue samples of explant liver from a variety of patients with end-stage liver disease were dissociated and the unfractionated cell suspensions were cultured. After 4 to 10 days, in a majority of cultures, clusters of cells with fibroblast-like morphology were observed. Like MSC from perfusate, these cells were highly positive for CD90, CD105 and CD166, negative for the markers CD34, CD45 and HLA-DR, and had equivalent adipogenic and osteogenic differentiation capacity (not shown). Liver MSC could also be expanded from disease-free liver graft tissue obtained from post-mortem organ donors (data not shown). Cultures of peripheral blood mononuclear cells (PBMC) from end-stage liver disease patients, brain-dead multi-organ donors or healthy controls did not obtain any MSC. Therefore it is unlikely that liver MSC in perfusates are directly mobilized from the BM compartment and derived from residual donor blood.

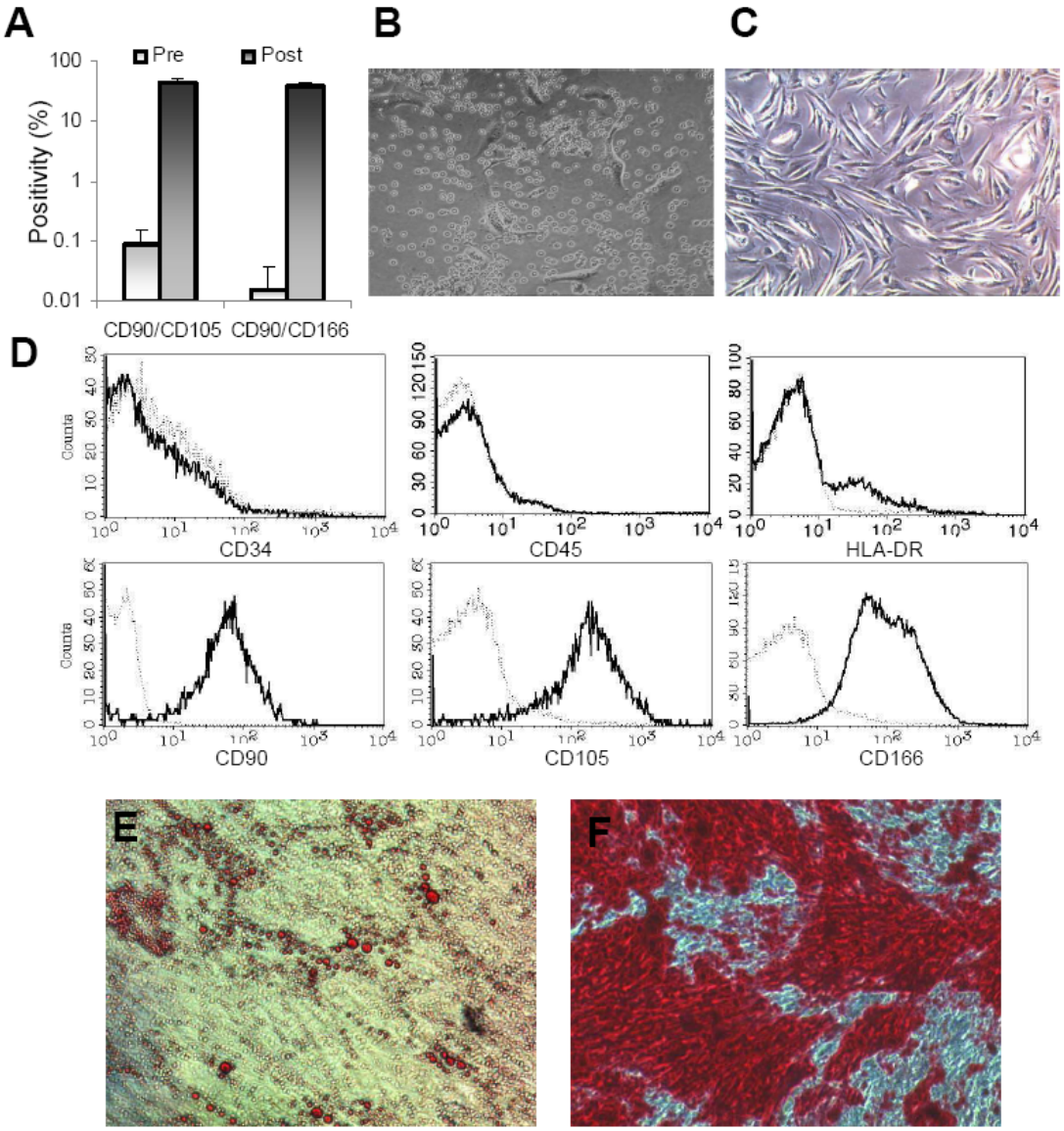

Figure 1. Characterization of MSC mobilized from human liver at time of graft cold storage. (A) Low percentage of $CD90^{+}CD105^{+}$ (mean 0.09% ± 0.07 SD, n = 8) and $CD90^{+}CD166^{+}$ (mean 0.02% ± 0.02 SD, n = 8) double positive cells were present in liver perfusates before culture (Pre), but rapidly expanded upon culture to 44% ± 8 and 37% ± 6 respectively at passage 4 to 9 (Post) (n = 6, $P < 0.001$). (B) In the majority of cultures cells with fibroblast-like morphology appear within ten days. (C) Fibroblast-like cells rapidly proliferated and could be sub-cultured and expanded for 10-20 passages. (D) Flow cytometric analysis of surface markers showed that expanded cells exhibit a typical MSC-like phenotype, positive for CD90 (mean 59% ± 18 SD, n=11), CD105 (55% ± 14) and CD166 (44% ± 16) and were mostly negative for the hematopoietic stem cell marker CD34 (0.8% ± 0.7), the leukocyte lineage markers CD45 (0.7% ± 0.7) and HLA-DR (1.9% ± 1). Black line in histograms represents the specific staining and the grey line shows the background staining of isotype-matched control antibody. (E) Adipogenic differentiation of liver-derived MSC was detected by Oil Red O staining for lipid droplet (red). (F) Osteogenic differentiation of these cells was evaluated by detection of deposited calciumphosphates using Alizarin Red S staining (red). Shown are representative stainings of four independent cultures (Magnification 100×). Similar adipogenic and osteogenic differentiation was observed with BM MSC and MSC obtained from liver biopsies (data not shown).

Wnt signaling promotes liver MSC proliferation

Wnt signaling has been shown to modulate the growth of human BM MSC[30] and plays an important role in liver homeostasis and pathology.[31] As shown in Figure 2A, liver MSC (both from tissue and perfusate) stimulated with Wnt3a exhibited a significant increase in viable (metabolically active) cell numbers compared to control-treated MSC as measured by MTT assay (mean increase of 44% ± 17 SD at day 6, $P < 0.001$). In order to confirm that this increase was related to enhanced cell proliferation, and not due to enhanced cell survival, a CFSE-fluorescence based proliferation assay was used. As shown in Figure 2B, Wnt3a-CM treatment accelerated CFSE-dilution of labeled liver MSC indicative for enhanced cell proliferation. At day 6 of culture, the percentage of cells having undergone eight rounds of cell division (9th generation) was 10% for L-CM versus 50% for Wnt3a-CM treated cells. Similar proliferative responses were seen with BM MSC (not shown).

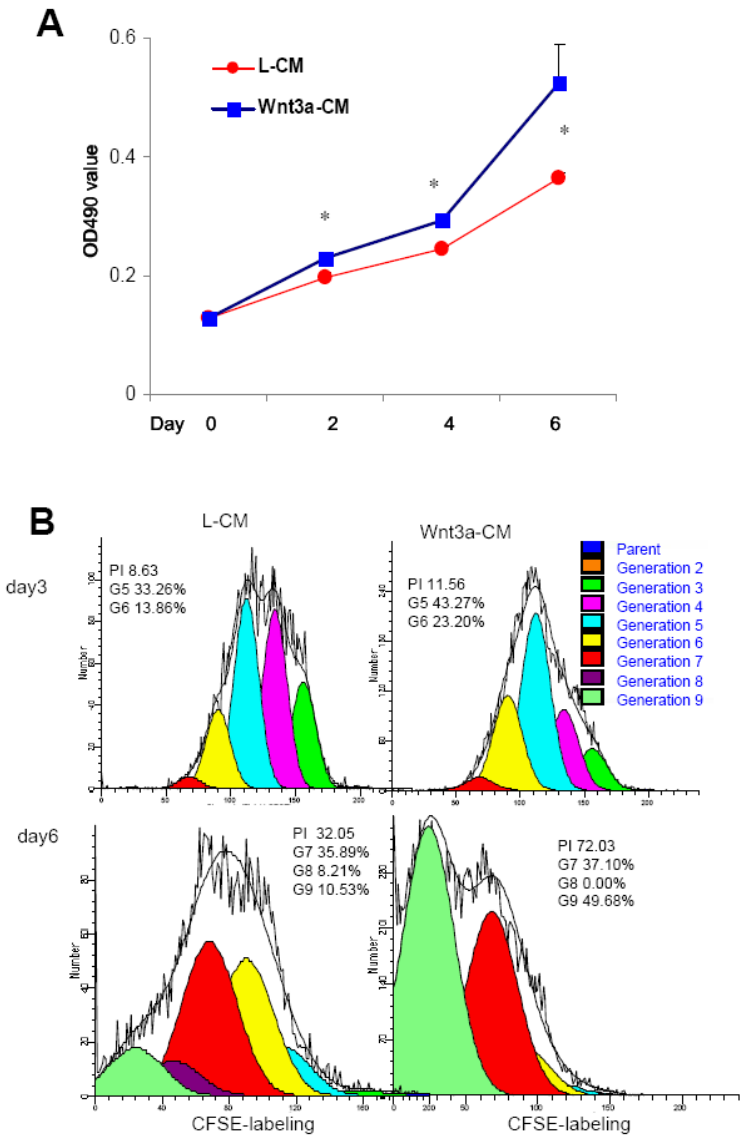

Figure 2. Wnt3a promotes liver MSC proliferation. (A) MTT assay showed that stimulation of MSC with Wnt3a conditioned medium (CM) significantly increased the number of cell as compared to control L-CM treatment, at day 2 (16% ± 6 increase), day 4 (20% ± 3) and day 6 (44% ± 17). Shown is the mean ± SD of six independent experiments (*P < 0.001). (B) Increased Wnt3a-induced cell proliferation was confirmed by CFSE-dilution of labeled liver MSC. A marked increase in the percentage of cells of 5th and 6th generation of proliferation at day 3 (47% L-CM vs 66% Wnt3a-CM) and 8th and 9th generation at day 6 (19% L-CM versus 50% Wnt3a-CM) was observed, which was also reflected in the marked increase of the proliferation index seen in the Wnt3a-stimulated cells at both time points (11.6 and 72.0 for Wnt3a-CM versus 8.6 and 32.1 for L-CM).

Gene expression profiles of liver and BM MSC

In order to gain further insight into the molecular phenotype of liver MSC, we performed genome-wide expression profiling on early passage (passage 2-5) MSC cultures. The expression profile of these liver MSC was compared to that of BM MSC (also three cultures at passage 2-5 from different donors). Cultures of Huh7 hepatoma cells (n=3) serving as a generic control of a replicating cell population of hepatic origin. The principal component analysis of their genome-wide expression profiles, groups the three cell types into three separate clusters on a three-dimensional scatter plot (Fig. 3A). Notably, liver-derived Huh7 cells cluster far apart from both liver and BM MSC, regardless of their hepatic or extra-hepatic origin. Accordingly, when compared to liver MSC, more than 20% of Huh7 genes were differentially expressed. Direct comparison of gene expression of liver tissue obtained from grafts at time of transplantation showed that liver-derived MSC highly express CK19 and HGF, whereas expression of CK18, c-Met, and Lgr5 was lower and albumin mRNA was undetected (Fig. 3B). Comparitive analysis of liver and BM MSC showed comparable expression levels of most of the known MSC-associated genes (Fig. 3C).[32] However, under identical analysis settings, there were less than 1% of genes differentiately expressed between liver and BM MSC (311 of 32,321 genes at $P < 0.001$) (Fig. 3D). Overall, only 45 genes were over 2-fold higher expressed in liver MSC and 37 genes more than 2-fold higher in BM MSC (See Supplementary Table S3). Among the differentially expressed genes in MSC were MMP1, ACTG2 and IL33 more than twenty-fold higher in liver MSC and MFAP5, IGFBP3 and RBP4 over ten-fold higher in BM MSC ($P < 0.001$). However, no significant differences in biological or functional pathways of gene expression were observed between liver and BM MSC using Ingenuity pathway analyses (data not shown). To distinguish liver MSC from hepatic stellate cells, gene array analysis was performed for ten known stellate cells-associated genes and two recently reported markers, CD133 and Oct4.[33-34] Differential gene expression of these twelve markers was comparable between liver MSC and BM MSC (data not shown). Together, these data indicate that in terms of transcriptome composition liver MSC are highly similar to BM MSC and, like BM MSC, appear distinct from hepatic stellate cells.

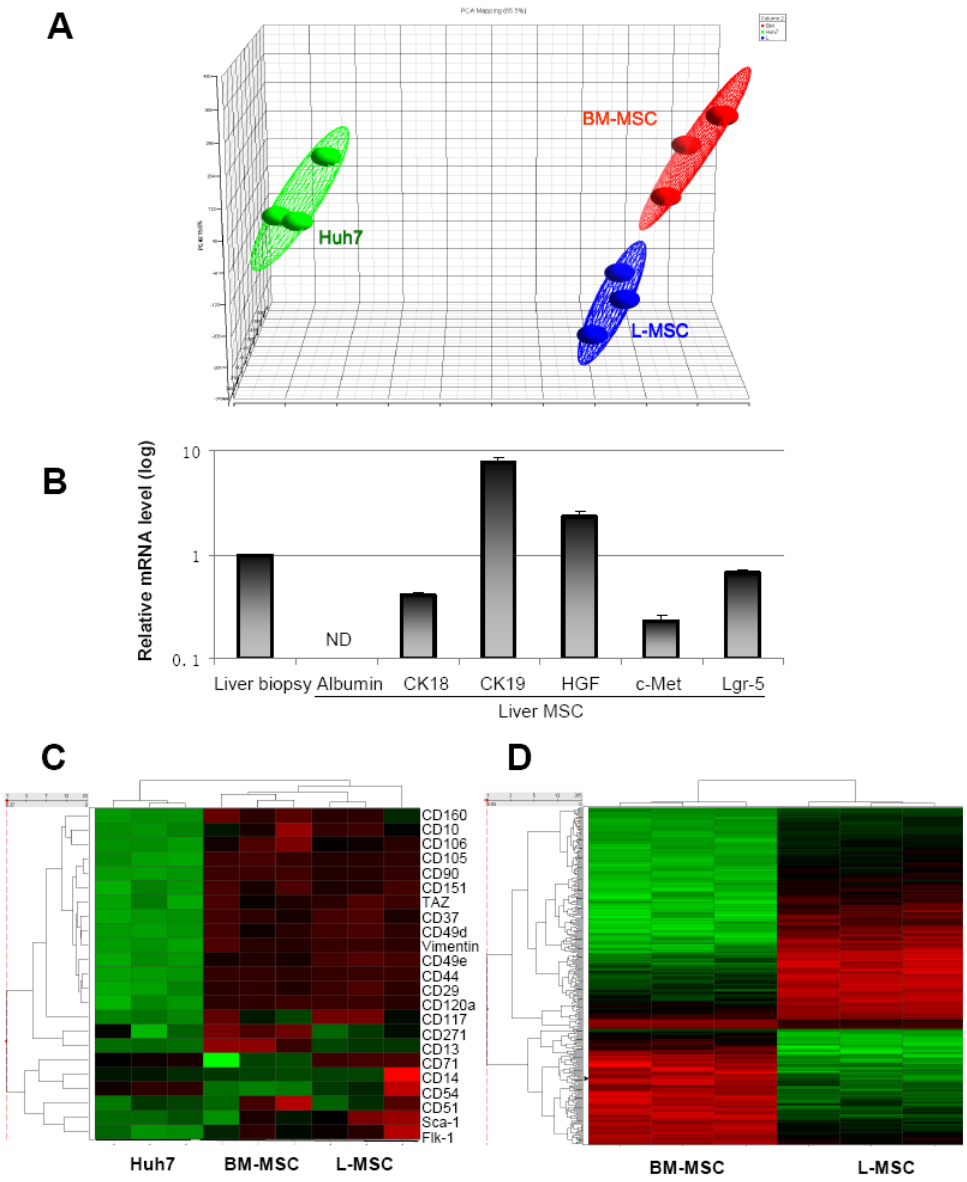

Figure 3. Gene expression profiling of liver MSC, BM MSC and Huh7 hepatoma cells. (A) Principal component analysis of genome-wide expression profiles was used to visualize correlation relationships among samples. The three independent liver MSC (L-MSC) preparations cluster into a separate grouping, apart from both BM MSC and Huh7 cells. The short distance between the liver MSC and BM MSC cluster in the 3D correlation space suggests that the two MSC populations are characterized by a similar pattern of gene expression, at variance with Huh7. (B) Real-time RT-PCR analysis of liver MSC gene expression levels relative to GAPDH and normalized to levels in donor graft liver tissue. MSC highly express CK19 and HGF whereas expression of CK18, c-Met, Lgr5 and albumin are lower or undetectable (ND). Shown is the mean ± SD of one representative experiment in triplicates. (C) Gene array analysis of liver and BM MSC shows comparable gene expression of known MSC markers, whereas Huh7 have generally low expression of these genes. (D) Although the expression profiles of the two cell types appear very similar, liver MSC and BM MSC can be distinguished on the consistent differential expression of a small proportion of their transcriptome (less than 1%, $P < 0.001$).

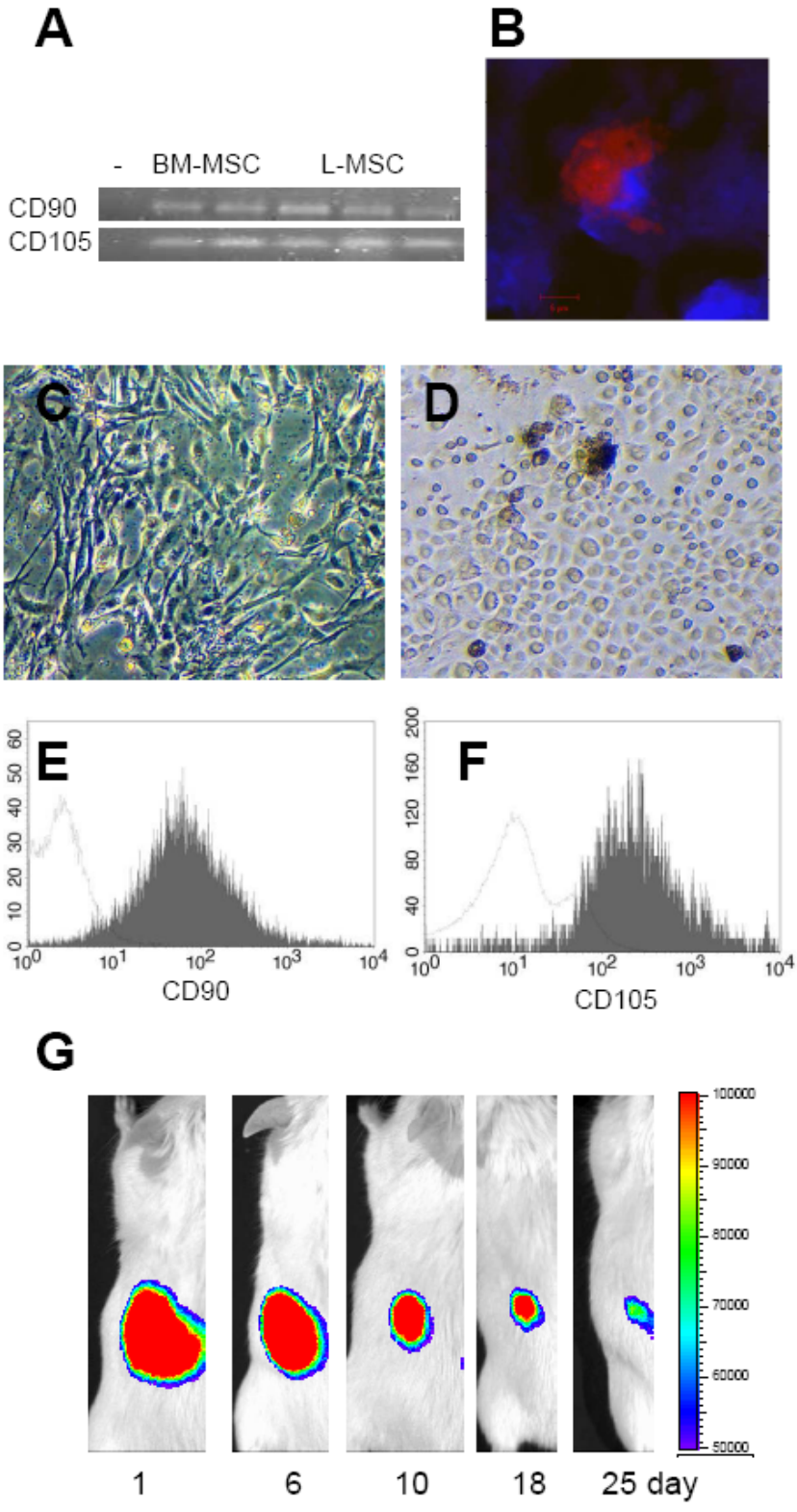

Figure 4. Human liver MSC retain stem cell characteristics after engraftment in mice. Liver or BM MSC engrafted in the liver of NOD/SCID mice subjected to CCl4-induced liver injury. Four weeks after administration, mouse liver tissue was analyzed for the presence of human MSC. (A) RT-PCR analysis showed expression of the human-specific MSC markers CD90 and CD105 in liver tissue from transplanted mice but not in sham-treated controls (-). (B) Immunofluorescent staining of mouse liver tissue showed human-specific CD90 positive cells (red) present in the MSC-engrafted mice, but not control mice (not shown). DAPI nuclear staining shown in blue (Bar represents 5 micrometer). (C) In a majority of cultures of dissociated mouse liver cells, MSC-like cells with typical fibroblast-like morphology rapidly expanded. (D) No such cells were observed in cultures of control mouse livers. Magnification C and D was 400x. Flowcytometric analysis of engrafted mice confirmed that a high percentage of cells in culture were positive for human MSC surface marker CD90 (mean 60.8% ± 18 SD) (E) and CD105 (61.6% ± 21) (F). Gray lines indicated isotype-matched control staining. (G) Survival of subcutaneously engrafted liver MSC in NOD/SCID mice. MSC expressed the lucifease reporter gene and luciferase signal was measured at different time points after engraftment. Luciferase signal gradually declined over time but was clearly detectable for at least 25 days, confirming long-term survival of viable MSC in vivo.

Long-term survival of liver MSC in vivo

To evaluate the fate of MSC in vivo, liver or BM MSC were engrafted in the liver of NOD/SCID mice subjected to CCl4-induced liver toxicity. As shown in Figure 4A, RT-PCR analysis at four weeks showed the expression of the human MSC markers CD90 and CD105 in the liver of MSC-engrafted mice but not in sham-treated controls. Fluorescent immunohistochemstry confirmed the presence of human CD90 positive cells in the mouse liver tissue (Fig. 4B), although these cells did not express cell proliferation marker PCNA. Dissociated mouse liver tissue was cultured for five days. Human MSC with typical fibroblast-like morphology rapidly expanded in culture (Fig. 4C). No such cells were observed in control mice not engrafted with human MSC (Fig. 4D). Flow cytometric analysis revealed that the majority of the explanted liver cells expressed human CD90 (60.8% ± 18.2) (Fig. 4E) and CD105 (61.6% ± 20.8, mean ± SD, n=3) (Fig. 4F). This was further confirmed by RT-PCR analysis showing human specific CD90 and CD105 gene expression in cultured cells. No CD90 and CD105 mRNA was detected in cultures of sham-treated controls (data not shown). To further evaluated the in vivo survival, luciferase labeled liver MSC were subcutaneously engrafted into NOD/SCID. As shown in Figure 4G, luciferase signal was clearly visible up to 25 days after engraftment confirming longer term MSC survival in vivo, though the signal gradually declined over time.

Hepatic differentiation of liver MSC in vitro and in vivo

The hepatocyte-lineage differentiation potential of liver MSC was determined in vitro using an established hepatogenic culture procedure. After 30 days of culture, morphologic changes of most cells were observed in all cultures (n=6). In contrast to fibroblast-like morphology of undifferentiated MSC (Fig. 5A), hepatogenic differentiation induced a polygonal shaped morphology at 15 days (Fig. 5B) and 25 days of culture (Fig. 5C). Fluorescent immunohistochemistry staining showed expression of human albumin protein after MSC differentiation (Fig. 5D), but not undifferentiated MSC (Fig. 5E). Similarly, glycogen-storage was detected in differentiated MSC (Fig. 5F) but not undifferentiated MSC (Fig. 5G). Quantitative RT-PCR analyses confirmed specific expression of albumin mRNA in differentiated BM and liver MSC (Fig. 5H).

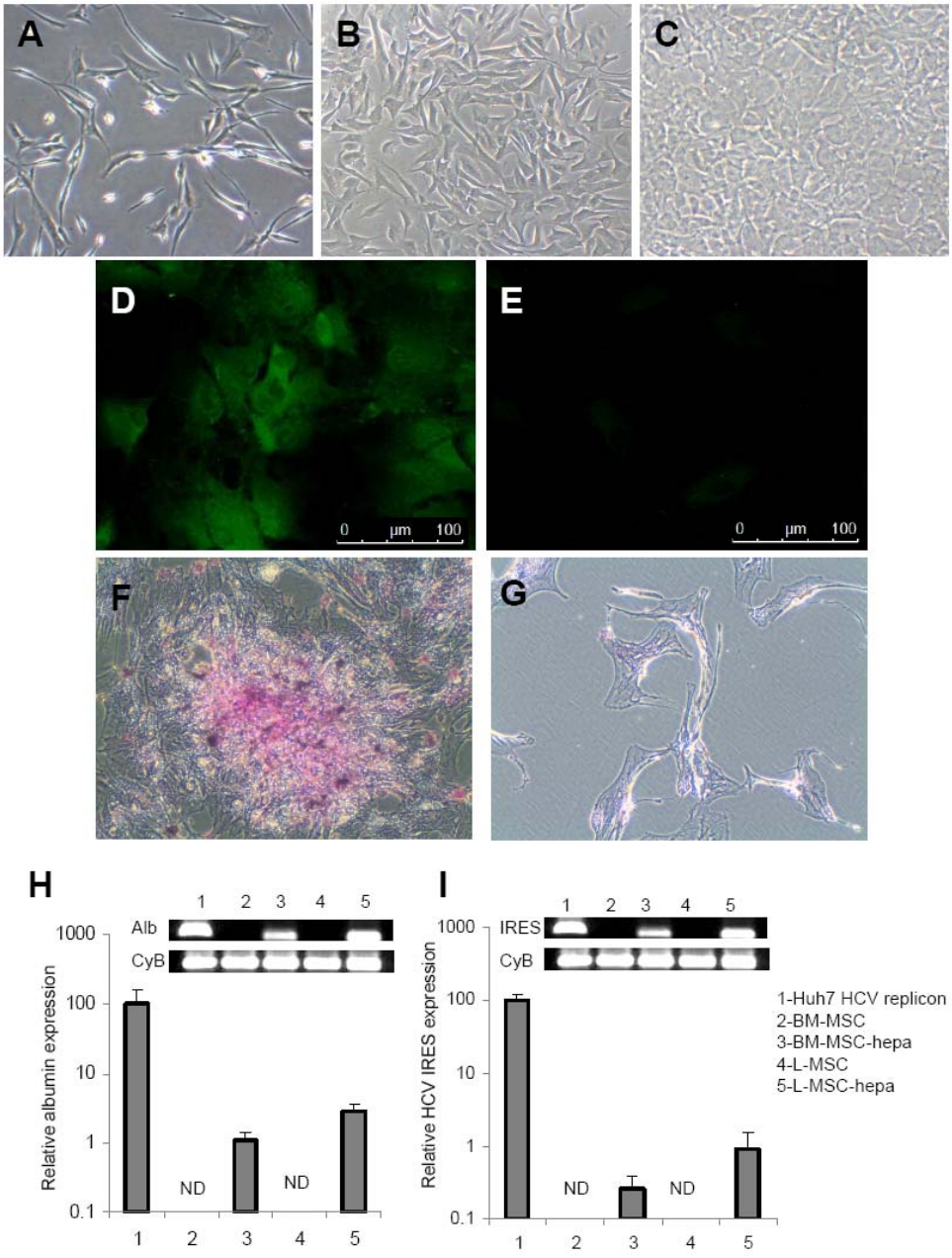

Figure 5. Liver MSC differentiate into hepatocyte-like cells permissive to HCV infection. In contrast to fibroblast-like spreading of undifferentiated liver MSC (A), hepatogenic differentiation of liver MSC induced a polygonal shaped morphology with a granular cytoplasm as shown 15 days (B) and 25 days (C) of differentiation (400× magnification). Fluorescent immunocytochemistry showed human albumin staining (green) in hepatic differentiated MSC at day 30 (D), but not in undifferentiated MSC (E). Glycogen (stained pink) storage was seen in hepatic differentiated MSC (F) but not in undifferentiated MSC (G) (100× magnification). Gene expression analysis of cultured MSC showed clear expression of hepatocyte-specific albumin gene after hepatogenic differentiation (MSC-hepa) by quantitative real-time RT-PCR (H). Albumin mRNA was undetectable (ND) in undifferentiated BM or liver MSC and Huh7 hepatoma cell line served as positive control. To further characterize the MSC-derived hepatocyte-like cells, differentiated and undifferentiated cells were incubated with conditioned medium containing infectious HCV particles (HCVcc). Real-time RT-PCR analysis for the HCV IRES sequence showed that differentiated MSC can be infected by HCV while undifferentiated BM and liver MSC are not permissive to infection (I). Huh7 replicon cells (Huh7-ET) with high level HCV replication served as a positive control.

To further investigate the functionality of MSC-derived hepatocyte-like cells, we challenged differentiated and undifferentiated liver MSC with infectious HCV particles (JFH-1-derived HCVcc), a virus with hepatic tropism produced by Huh7.5 cells. RT-PCR analyses of HCV specific IRES sequence clearly showed that, unlike undifferentiated MSC, MSC-derived hepatocyte-like cells are permissive for HCV infection (Fig. 5I). Levels of HCV RNA was about 100-fold lower than high replicating HCV replicon cells.

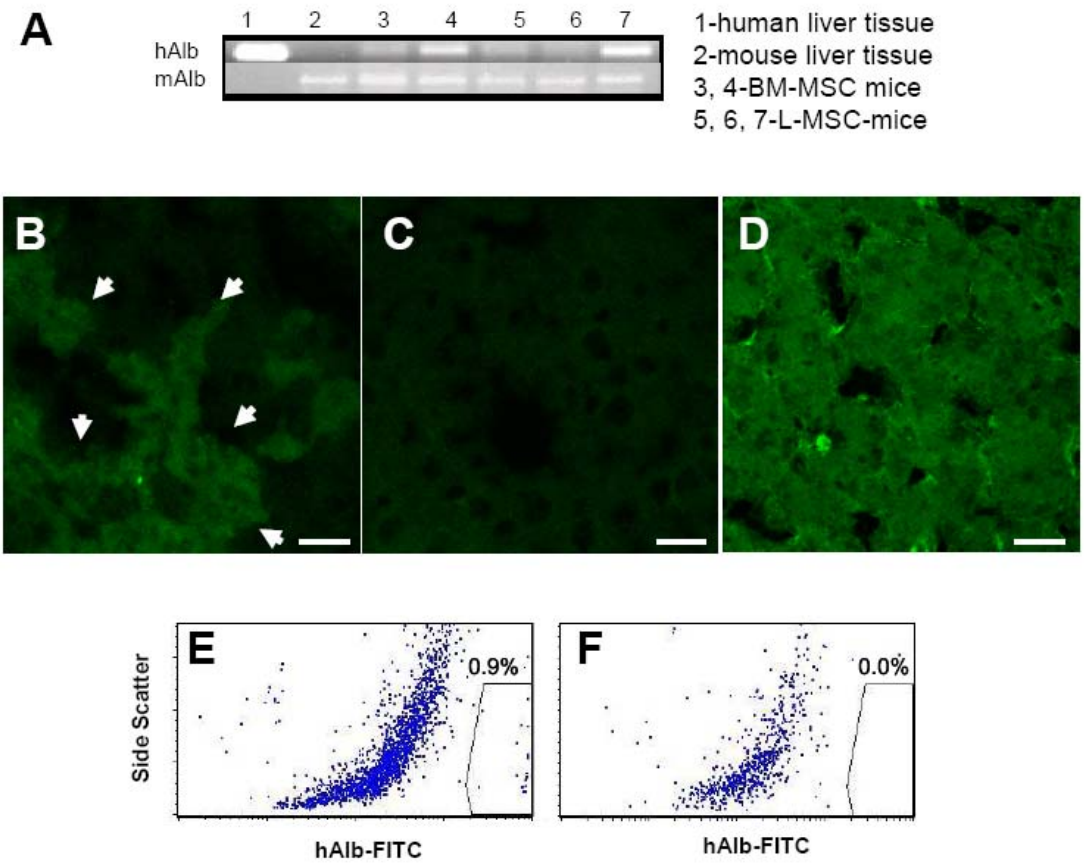

Figure 6. MSC differentiation into hepatocyte-like cells in vivo. L-MSC or BM-MSC were engrafted into the livers of NOD/SCID mice subjected to CCL4-induced liver toxicity. Four weeks after the MSC administration, the mouse livers were harvested and analyzed for evidence of hepatic differentiation. (A) RT-PCR analysis for human- and mouse-specific albumin RNA. Human albumin expression was observed in all MSC-engrafted livers (lanes 3-5) and was not observed in the control mouse liver (lane 2). Human liver RNA served as a positive control (lane 1), and mouse albumin was detected in all mouse livers (lanes 2-7) but not in the human liver. (B) Immunohistochemical staining of the mouse livers confirmed the presence of human albumin–positive cell clusters (indicated by arrows) in mice engrafted with BM-MSC or L-MSC (n=7). (C) Control mouse and (D) human liver tissue served as negative and positive controls, respectively, for the albumin staining (Bar=20 μm). The concentration of human-specific, albumin-positive cells, as quantified by flow cytometry, was (E) 1.09% 6 0.39% in the dissociated mouse livers (n=5) and (F) ND (<0.01%) in the sham-treated control mice (n=3).

Hepatogenic differentiation potential of liver MSC was further evaluated in vivo, in NOD/SCID mice with CCl4-induced liver injury. Four weeks after MSC engraftment, mouse livers were harvested and RT-PCR analyses displayed detectable levels of human-specific albumin gene expression (Fig. 6A). Immunohistochemical staining of mouse liver tissue showed the presence of human albumin positive cell clusters in all MSC engrafted mice (Fig. 6B), although the frequency of these clusters were generally low. No human albumin positivity was observed in untreated of shame CCL4 treated control mice (Fig. 6C). Flow cytometric analyses of dissociated mouse livers confirmed the presence of human hepatocyte-like cells (Fig. 6E), with a mean percentage of albumin positive cells of 1.09 ± 0.39 SD (n=5). No positive cells were detected in livers from control mice (Fig. 6F). Overall, these results indicate that liver MSC, or a subset of, have hepatogenic potential in vitro and in vivo and that MSC-derived hepatocyte-like cells are infectable with HCV.

MSC effectively suppress T cell proliferation

It is well established that MSC populations from various tissues, including BM, spleen, heart and fat, have immune regulatory and suppressive properties including the inhibition of T cell proliferation.[16, 35] The effect of adult human liver MSC on proliferation of mitogen- and alloantigen stimulated T cells was investigated in vitro by measuring [3H] thymidine incorporation. PBMC or purified $CD4^+CD25^-$ T cells stimulated with mitogenic PHA or irradiated allogeneic PBMC cells were co-cultured with MSC at different ratios. As shown in Figure 7A, a significant inhibition of alloantigen-stimulated proliferation was observed at MSC: PBMC ratios of 1: 2 (97% inhibition, $P < 0.001$), 1: 4 (92%, $P < 0.001$), 1: 8 (76%, $P < 0.001$) and 1: 16 (53%, $P < 0.05$; $n = 6$). Comparable inhibition of proliferation was observed with PHA-stimulated PBMC (Figure 7B). Similar results were obtained with BM MSC and when purified $CD4^+CD25^-$ T cells were used as responder cells (not shown).These findings indicate that, like other MSC populations, liver MSC are potent inhibitors of T cell proliferation and may contribute to allo-immune regulation after liver transplantation.

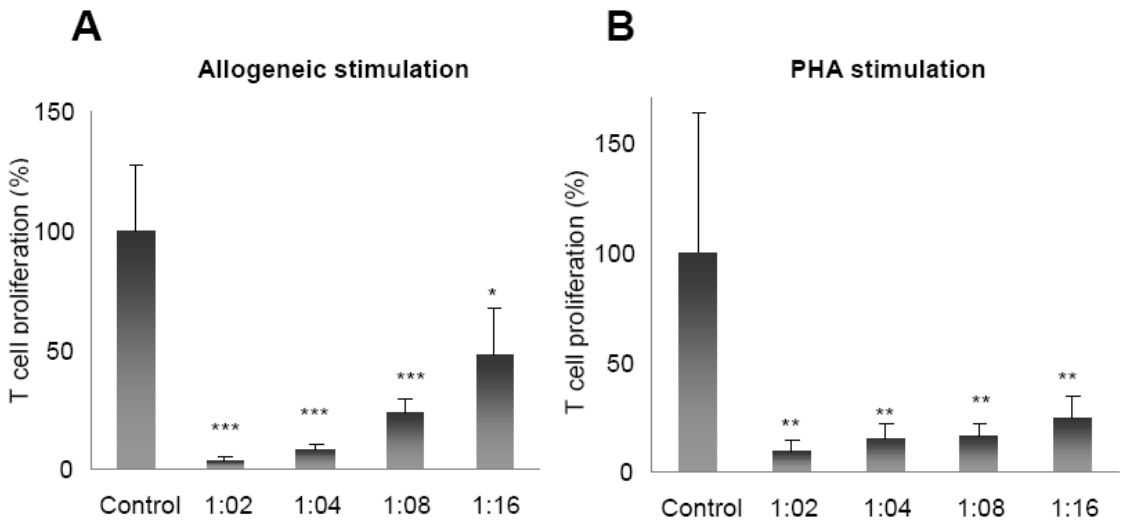

Figure 7. Suppression of T cell proliferation by liver MSC. (A) T cells were stimulated with irradiated allogenic PBMC and co-cultured with allogeneic MSC in 1:2, 4, 8, and 16 ratios (MSC: PBMC) for 5 days. (B) T cells were stimulated with PHA and co-cultured with MSC for 5 days at different ratios. Significant inhibition of cell proliferation was observed at different ratios. Similar results were observed with BM MSC and when purified CD4+CD25- T cells were used as responders (data not shown). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Shown is the mean $\pm$ SD of six experiments.

Discussion

Currently, the therapeutic potential of stem cells, such as MSC, are being explored at incredible pace for the treatment of liver disease as well as many other diseases [4, 14]. In vitro hepatic differentiation has been described for MSC derived from different sources, including BM, fat, lung, cord blood and amniotic fluid.[25, 36-38] In the current study we provide evidence of the presence of MSC in the adult human liver itself. Gene expression profiling showed a high degree of similarity between liver and BM MSC (Fig. 3). Overall, only 82 genes (0.25% of the transcriptome) were more than 2-fold different in expression between liver MSC and BM MSC. Their high similarity, including the expression profiling of most of these MSC markers (Fig. 3C), supports that the cells type we isolated is a *bona fide* MSC population. In addition, the distinct genomic signature provides indication of their distinct origin, which in turn provides potential markers to distinguish these two MSC populations.

In order to determine hepatic differentiation of liver MSC in vitro, we combined the conventional methods with a novel approach of infecting the differentiated cells with HCV (Fig. 5). We found that hepatic differentiated occurred in a subpopulation of liver MSC that

not only changed morphology and expressed albumin, but also supported HCV infection. Since HCV infection is largely restricted to mature hepatocytes, the results strongly indicated that at least part of the MSC-derived hepatocytes are fully differentiated and functionally support viral entry and replication.

Consistently, hepatic differentiation of MSC has been reported in vivo as well. Several studies mentioned the occurrence of MSC oriented hepatic differentiation in models of liver injury and regeneration.[14, 25, 37, 39] Though hepatic differentiation generally occurs at a relatively low frequency (also seen in Fig. 6) and concerns are raised regarding the fate of stem cells after transplantation in immune competent hosts,[40] these findings still brought new hope for MSC-based cell therapies for regenerative liver diseases. Of note, is that the hepatic differentiation of MSC appears to be less robust that seen with a different, more committed, population of hepatic stem cells identified from human fetal and postnatal livers,[8] but seems to be improved upon in vitro hepatic differentiation of MSC before engraftment.[41] If MSC are used to treat liver diseases, both hepatic differentiation and in vivo survival of the stem cells are crucial. In this study, we found that liver MSC not only can differentiate towards hepatocytes but can also be retrieved four weeks after transplantation into NOD/SCID mice (Fig. 4), indicating their long-term survival property in vivo.

It is of note that MSC can migrate to injured tissue and contribute to tissue repair and wound healing. This mobilization is likely regulated by specific danger signals and chemotactic factors.[42-43] MSC have a profoundly greater capacity to survive under conditions of ischemia, because in the absence of oxygen MSC can survive using anaerobic ATP production.[44] Our previous studies have shown that several types of liver-derived hematopoietic cells are mobilized during perfusion of the graft and are continuously released into the recipient after liver transplantation.[23, 27] Continuous migration of donor leukocytes into recipient's circulation leading to chimerism occurs more often in liver transplantation compared to other organ transplantation and has been associated with graft acceptance.[45] We hypothesize that ex vivo vascular ischemic perfusion of liver grafts may stimulate MSC mobilization at the time of transplantation. Indeed, substantial numbers of liver MSC could be isolated from liver perfusates by culturing the cell fraction, demonstrating migration of graft MSC during cold-storage and perfusion (Fig. 1). Thus, liver preservation fluid can be considered a novel source for MSC, which is of particular importance, because normal healthy human liver tissue is usually not available. Given the fact that liver MSC

possess potent immunomodulatory properties (Fig. 7), we speculate that like many liver leukocytes, graft-derived MSC may also migrate to the recipient after liver transplantation and subsequently play a role in immune-regulation, tissue repair and regeneration.

In conclusion, this study provides evidence for the presence of MSC in human adult liver. These cells have similar characteristics as BM MSC regarding phenotype and function. The migration of graft MSC at the time of liver transplantation provides an alternative source of liver MSC, which also suggest that continuous release of graft MSC and systemic contribution may occur after transplantation in a recipient. We believe that our observation has paved way for further study on the role of these cells in physiological as well as under particular pathological conditions.

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Chapter 13

Mesenchymal stem cells inhibit hepatitis C virus infection by paracrine triggering of host innate immunity involving IFITM3

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Patent WO2010/053350, filing date Nov 7th 2008;

Manuscript in preparation

Chapter 14

Recognition of spontaneous malignant transformation of human mesenchymal stem cells

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Submitted

Chapter 15

Summary and discussion

From the identification of the virus in 1989 to the development of interferon-based standard of care, the face of managing chronic hepatitis C has indeed been dramatically changing. Overall, approx. half of patients can achieve sustained virologic response (SVR) with the current standard peg-IFN-α in combination of ribavirin therapy, whereas the SVR rates is only about 40% in patients with genotype 1 infection, the most common genotype in Europe and North America[1]. With the launching of directly acting antivirals (DAAs), triple combination of DAAs, peg-IFN-α and ribavirin is expected to increase SVR rates in genotype 1 patients up to approximately 60%, or more[2]. However, given the large infected population, accumulated non-responders, poor tolerability to interferon or DAAs, and special populations, liver transplantation remains and will continue to be an important treatment option for HCV–induced end-stage liver disease. Furthermore, novel antivirals remain urgently required to be developed, which ideally should act on distinct mechanisms and be applicable in the current non-responders and special populations with less side effects.

Interaction of immunosuppressants with antiviral interferon signaling

The universal occurrence of re-infection post-transplantation is associated with reduced graft and patient survival in HCV-positive liver transplants[3]. Using of particular immunosuppressive medication has attributed to the aggravated infection course and poor response to antiviral therapy in post-transplant patients. However, clear evidence of distinct effects of different immunosuppressants on HCV infection or interferon therapy so far come only from research of cell culture models (**Chapter 2**). A recent study[4] by Hirano and coworkers reported that tacrolimus (Tac) but not cyclosporine A (CsA) could interfere with interferon signaling and thereby reducing the antiviral activity of IFN-α in a subgenomic HCV replicon model promoted us to more extensively evaluate this issue in two state-of-the-art

HCV culture models. In contrast, we found no evidence that either CsA or Tac interferes with IFN-α induced gene expression or IFN-α mediated antiviral activity against HCV infection (**Chapter 3**). Therefore, no mechanistic argument is found to break the clinical controversy about choice of calcineurin inhibitor during post-transplantation antiviral therapy.

Previous work[5] from our group showing MPA, an immunosuppressant approved for use in 1994, can effectively suppress HCV replication and in synergy with IFN-α, inspired us to continue explore its mechanism-of-action. In **chapter 4**, we confirmed the potent antiviral effects of MPA at clinically relevant concentrations in the JFH1-derived infectious HCV model. In mice bearing subgenomic HCV replicon cells, systemic MPA treatment led to efficient inhibition of viral replication and confirms its *in vivo* anti-HCV evidence. Though MPA is known to act trough inhibition of IMPDH2 enzyme which accounts for the anti-T cell proliferation effects, we found that IMPDH activity had only minor contribution to the anti-HCV effect of MPA in hepatocytes. In stead, MPA induced the expression of multiple antiviral Interferon-Induced Genes (ISGs), including IRF1, by augmenting the transcriptional activity of so-called Interferon Stimulated Response Element (ISRE). Using RNAi-based loss-of-function approach, IRF1 was shown to be directly involved in the anti-HCV action of MPA. This mechanism was found to be similar to ribavirin (**Chapter 5**), another type of IMPDH inhibitor used in the standard care of chronic HCV for synergizing the antiviral interferon response. These two chapters provided new mechanistic insights into the antiviral mechanism of MPA and ribavirin and their molecular basis of synergy with interferon.

RNA interference and micoRNA in the biology and therapy of HCV

RNA interference (RNAi)-based gene silencing technologies have fuelled a rapid progress in the basic understanding of the HCV biology and revealed numerous new viral and host cell factors as potential targets for therapy (**Chapter 6.1**). The development of RNAi libraries, which allowed performing genome wide loss-of-function screens, has been a major step forward for the study of gene function and resulted in identification of new genes related to various biological functions and discovery of new therapeutic targets for many types of diseases (**Chapter 6.2**). Lentiviral vector-based RNAi libraries appear to have advantages over conventional synthetic siRNA libraries, owing to their wide-range tropism, genomic integration property and effective in vitro delivery. However, certain commercial lentiviral RNAi libraries overexpressed small hairpin RNA (shRNA) that subsequently saturate the

cellular microRNA (miRNA) biosyntheses machinery. Since HCV replication is largely dependent on miR-122, we found that these libraries failed to screen host factors involving HCV replication due to the disturbance of miR-122 maturation (**Chapter 7**).

In the context of treating chronic HCV or preventing recurrence in HCV-positive transplant, a single dose administration with long-lasting therapeutic effects would be ideal. Therefore, integrating lentiviral vector expressing shRNA represents a suitable strategy. A phenomenon described in **Chapter 8** that RNAi-mediated gene silencing can spread to non-transduced cells has indeed extended the therapeutic reach of RNAi. Virtually for any type of vector, it is not possible to achieve 100% transduction efficacy in patients. Therefore, RNAi transmission to neighboring cells could potentially overcome the issue of suboptimal vector transduction to certain extend. Like other new antivirals[6-7], combining interferon is likely required for RNAi-based therapy to achieve ultimate success in chronically infected HCV patients. **Chapter 9** has demonstrated that interferon did not interfere with lentivral transduction and RNAi gene silencing efficacy with combined antiviral activity when combined with anti-HCV lentiviral RNAi.

MiRNAs have been extensively investigated for their biological roles in regulating gene expression but also considered as therapeutic targets as well as biomarkers for prognosis and diagnosis of various diseases. In **chapter 10**, we have demonstrated that circulating miR-122, the liver abundant miRNA, represents potential biomarkers of hepatic injury in liver transplantation patients. Along with the finding in Chapter 8 that secreted miRNAs are packed in exosomes, **Chapter 11** provided evidence that exosomes can mediate the transmission of HCV virus, as a novel transmission route that can escape from the attack by neutralizing antibodies.

Mesenchymal stem cells and liver diseases

Mesenchymal stem cells (MSC) were originally identified as heterogeneous population of stem cells in the bone marrow (BM), but more recent studies demonstrated that MSC can be readily isolated from many other adult and fetal tissues. Owing to their multi-potency together with anti-inflammatory and immune-modulatory properties, their biological roles and therapeutic potentials have been explored at incredible speed. We have identified a resident MSC population in adult human liver that are phenotypically and functionally similar to BM-derived MSC (**Chapter 12**). Liver MSC were releases during preservation of the graft at

the time of liver transplantation and may continuously migrate into recipient and modulate graft acceptance after transplantation. In addition, MSC produce a broad spectrum of soluble factors, including growth factors, extracellular matrix and enzymes. Interestingly, we found MSC can potently inhibit HCV infection, which is triggered by the soluble factors. The MSC secretome stimulated the host innate immune system involving the broad antiviral interferon-inducible transmembrane protein (IFITM) family member, IFITM3 (**Chapter 13**). Since the presence of resident MSC in the adult liver, this finding clear bears both biological and clinical implications. In terms of clinical application of MSC, it often requires ex vivo expansion of MSC to reach sufficient numbers for applying in patients. However, we observed spontaneous malignant transformation of MSC after extensive in vitro culture (**Chapter 14**). We further provided a method with a panel of biomarkers that can potentially screen transformation events. This approach would be particular useful in the clinical application of stem cell therapy and alleviate the concern of transplanting malignant cells into patients.

Final remarks

- Laboratory investigation of the effects of immunosuppressants on HCV infection and interferon antiviral therapy will not resolve the clinical controversy regarding the choice of particular immunosuppressive medication in HCV-positive liver transplants. However, it does provide important insight into the virus life-cycle and how these drugs can act on this. This knowledge is of value for future drug development as well as promotes the initiation of relevant clinical studies.

- RNAi represents futuristic but promising option for treatment of HCV infection. Likely, it will not be applicable for all the patients but do give hope to special populations, including previous non-responders or patients with poor tolerability to the standard therapy, HIV co-infected patients or transplant recipients.

- Discovery of the antiviral property of mesenchymal stem cells has indeed brought up another novel avenue of combating HCV infection. Together with their hepatic differentiation potential, anti-inflammatory and immune-modulatory properties and nourishing capability, therapeutic application of MSC in HCV patients may achieve “one stone hits multiple birds” effects. Given the presence of hepatic MSC, their biological roles and the interaction with other cell types could be particular interesting in healthy and diseased liver.

Samenvatting en discussie

Vanaf de ontdekking van het Hepatitis C Virus (HCV) tot aan de ontwikkeling van de huidige antivirale therapie met interferon is er veel veranderd met betrekking tot de behandeling van chronische HCV. Bij ongeveer de helft van alle patiënten verdwijnt het virus volledig na behandeling met de standaard combinatietherapie van peg-IFN-α en ribavirine. Bij patiënten die geïnfecteerd zijn met genotype I HCV, het meest voorkomende genotype in Europa en Noord-Amerika, gebeurt dat in slechts 40% van de gevallen[1]. Met de introductie van nieuwe antivirale middelen zoals protease remmers en andere "direct acting antivirals" (DAAs), die gegeven worden in combinatie met met peg-IFN-α en ribavirine, verwacht men echter dat meer dan 60% van de patiënten met type I HCV wel zal reageren op deze therapie[2]. Toch zullen nu en in de toekomst levertransplantaties nodig blijven voor de behandeling van vergevorderde leverziekten als gevolg van HCV infectie. De reden hiervoor is het grote aantal geïnfecteerde individuen, het toenemende aantal patiënten dat niet op de huidige therapie reageert of andere moeilijk te behandelen patiënten, en de niet geringe bijwerkingen van interferon en DAAs. Daarnaast blijft de ontwikkeling van nieuwe antivirale middelen, die bij voorkeur een ander werkingsmechanisme hebben dan de huidige middelen, en die toegepast kunnen worden bij patiënten die niet reageren op de huidige standaardtherapie, noodzakelijk.

Interactie van immuunsuppressiva met het antivirale werkingsmechanisme van interferon

Bij HCV-positieve levertransplantaties wordt het donororgaan vaak opnieuw geïnfecteerd met HCV, wat negatieve gevolgen heeft voor het behoud van het donororgaan en het overlevingspercentage van de patiënten[3]. De toenemende ernst van de infectie en de verminderde respons op antivirale therapie na transplantatie wordt in verband gebracht met het gebruik van bepaalde immuunsuppressiva. Met behulp van onderzoek met celkweek modellen is bewezen dat verschillende immuunsuppressiva effecten hebben op HCV infectie en de werking van interferon (**Hoofdstuk 2**). Recent onderzoek door Hirano en medewerkers[4] heeft uitgewezen dat Tacrolimus (Tac), in tegenstelling tot Cyclosporine A (CsA), kan interfereren met de signaaltransductie van interferon. In een HCV replicatiemodel resulteerde dit in de vermindering van de antivirale werking van IFN-α. Naar aanleiding van

deze resultaten hebben wij dit mechanisme onderzocht in twee geavanceerde HCV kweek systemen. In tegenstelling tot Hirano vonden wij, dat zowel Tac als CsA geen invloed hebben op IFN-α geïnduceerde genexpressie en de antivirale activiteit van IFN-α in deze kweeksystemen (**Hoofdstuk 3**). Op de vraag welke calcineurineremmer de voorkeur zou moeten hebben tijdens antivirale therapie na transplantatie kan op grond van onze bevindingen nog steeds geen duidelijk antwoord worden gegeven.

Bij voorgaand onderzoek van onze groep is aangetoond dat MPA, een immuunsuppressivum dat vanaf 1994 goedgekeurd is voor de behandeling van patiënten, op een effectieve manier de replicatie van HCV kan onderdrukken en de antivirale werking van IFN-α kan versterken[5]. Op grond van deze resultaten hebben wij het werkingsmechanisme van MPA nader onderzocht. In **hoofdstuk 4** konden wij de antivirale werking van MPA bij klinisch relevante concentraties bevestigen, zowel in het subgenomisch model voor HCV infectie, als in het JFH-1 model voor infectieus HCV. *In vivo* bewijs voor de antivirale werking van MPA werd geleverd door muizen te implanteren met cellen die het subgenomisch HCV replicon bevatten en deze te behandelen met MPA. Door de behandeling met MPA werd de replicatie van het virus op een effectieve manier geremd. Hoewel het bekend is dat de werking van MPA verloopt via de remming van het enzym IMPDH2, is uit ons onderzoek gebleken dat deze remming slechts een gedeeltelijke bijdrage levert aan de inhibitie van HCV door MPA. In plaats daarvan werd de expressie van verschillende antivirale, interferon geïnduceerde genen (ISGs) waaronder IRF1, door MPA geïnduceerd door middel van de verhoogde activering van een interferon gestimuleerd respons element (ISRE). Door de expressie van IRF1 uit te schakelen met RNAi kon worden aangetoond dat IRF1 direct betrokken is bij de HCV remmende werking van MPA. Ook de werking van ribavirine, een ander type IMPDH remmer die gebruikt wordt in de standaard behandeling van chronische HCV en die het effect van interferon versterkt, bleek gebaseerd te zijn op het zelfde werkingsmechanisme (**Hoofdstuk 5**). De hoofdstukken 4 en 5 van dit proefschrift hebben nieuwe inzichten opgeleverd met betrekking tot het antivirale mechanisme van MPA en ribavirine, en de moleculaire basis voor hun synergie met interferon.

RNA Interferentie en microRNA in de biologie en therapie van HCV

Het uitschakelen van genen met behulp van op RNA interferentie (RNAi) gebaseerde technologie heeft het begrip van de biologie van HCV aanzienlijk vergroot. Bovendien

werden met behulp van RNAi vele nieuwe virulentie factoren ontdekt, zowel in het virus als in de gastheercel, die een potentieel doelwit zijn voor therapie (**Hoofdstuk 6.1**). De ontwikkeling van RNAi-banken waarmee door het gehele genoom genen kunnen worden uitgeschakeld heeft geresulteerd in de ontdekking van nieuwe genen die betrokken zijn bij verschillende biologische functies en de ontdekking van nieuwe therapeutische doelwitten voor een groot aantal aandoeningen (**Hoofdstuk 6.2**). RNAi banken die gebaseerd zijn op lentivirale vectoren hebben het voordeel ten opzichte van synthetische siRNA banken dat ze een brede toepasbaarheid hebben, in het genoom integreren en effectief *in vitro* toegediend kunnen worden. Sommige commercieel verkrijgbare lentivirale RNAi banken geven echter een zodanige overexpressie van shRNA, dat het cellulaire systeem dat verantwoordelijk is voor de productie van microRNA (miRNA) verzadigd raakte. HCV replicatie is voor een groot deel afhankelijk van miR-122. Wanneer wij gebruik maakten van lentivirale RNAi banken om gastheer factoren op te sporen die betrokken zijn bij HCV replicatie, bleek de expressie van functioneel miR-122 ernstig verminderd, waardoor een algemene remming van HCV replicatie op trad. Door dit aspecifieke effect op virus replicatie is deze onderzoeksmethode minder geschikt gebleken voor het onderzoeken van HCV gastheer factoren (**Hoofdstuk 7**).

De toepassing van RNAi technologie biedt mogelijkheden voor de behandeling van chronische HCV en de preventie van herinfectie van de getransplanteerde lever. Een lentivirale vector die shRNA tot expressie brengt kan in principe via een eenmalige dosis toegediend worden, waarbij een langdurig therapeutisch effect bereikt kan worden. De therapeutische mogelijkheden voor RNAi zijn inderdaad uitgebreid sinds uit ons onderzoek is gebleken dat het uitschakelen van genen met behulp van RNAi zich vanuit de getransduceerde cellen kan uitbreiden naar naastgelegen cellen (**Hoofdstuk 8**). Het is tot nu toe niet mogelijk gebleken om 100% transductie-efficientie te verkrijgen in patiënten, onafhankelijk van welke vector hier voor gebruikt wordt. Het feit dat RNAi overgedragen kan worden op naburige cellen, zou dus een gedeeltelijke oplossing kunnen bieden voor de suboptimale vector transductie. Op RNAi gebaseerde therapie zal waarschijnlijk wel moeten worden gecombineerd met een behandeling met interferon om optimaal resultaat te verkrijgen bij patiënten met chronische HCV infectie, net zoals dat het geval is bij andere nieuwe antivirale middelen[6-7]. Uit ons onderzoek is gebleken dat interferon geen negatief effect heeft op de lentivirale transductie en het uitschakelen van genen met RNAi, en dat de

combinatie van anti-HCV RNAi met interferon een versterkt antiviraal effect geeft (**Hoofdstuk 9**).

Behalve dat miRNAs betrokken zijn bij genregulatie, en potentieel doelwit zijn voor therapie, kunnen ze mogelijk ook fungeren als biomarkers bij de prognose en diagnose van verschillende ziekten. In **Hoofdstuk 10** hebben wij aangetoond dat miR-122, een lever abundant miRNA, in bloed en serum kan worden aangetoond bij levertransplantatie patiënten, waar het een gevoelige biomarker is voor leverschade. Op basis van de bevinding in hoofdstuk 8, dat het vrijkomen van miRNA uit cellen deels plaatsvindt via het uitscheiden van kleine blaasjes (exosomen), tonen we in **Hoofdstuk 11** aan dat het uitscheiden van exosomen door hepatocyten ook een belangrijke route is voor het overdragen van HCV infectie. Gebleken is dat de exosoom-route van HCV infectie grotendeels ongevoelig is voor remming door virusspecifieke antistoffen en mogelijk kan bijdragen aan herinfectie van hepatocyten na levertransplantatie.

Mesenchymale stamcellen en leverziekten

Mesenchymale stamcellen (MSC) werden voor het eerst geïdentificeerd als een heterogene populatie in het beenmerg (BM). Recent is echter gebleken dat deze cellen ook geïsoleerd kunnen worden uit vele andere humane en foetale weefsels. Omdat deze multi-potente cellen zowel ontstekingsremmende als immuunmodulatoire eigenschappen hebben is er recent veel onderzoek gedaan naar hun biologische rol en mogelijke therapeutische toepassingen. Wij hebben in de lever een populatie MSC gevonden die fenotypisch en functioneel vergelijkbaar is met BM-MSC (**Hoofdstuk 12**). Lever MSC worden gemobiliseerd uit het weefsel tijdens de preservatie van donorlevers gedurende de levertransplantatie procedure, en komen mogelijk ook terecht in de circulatie van de ontvanger waar zij een rol kunnen spelen bij de acceptatie van het donororgaan. Bovendien produceren MSC een breed spectrum aan factoren, zoals groeifactoren, extracellulaire matrixeiwitten en enzymen. Uit ons onderzoek is gebleken dat MSC, via deze factoren, infectie met HCV mogelijk kunnen verhinderen. De door MSC uitgescheiden factoren stimuleerden de expressie van het antivirale eiwit IFTIM3 (interferon-inducible transmembrane protein 3), dat onderdeel is van het aangeboren immuunsysteem (**Hoofdstuk 13**). De vondst van een populatie MSC in de lever heeft zowel biologische als klinische implicaties. Voordat MSC toegepast kunnen worden in patiënten, moeten deze cellen eerst *ex vivo* vermeerderd

worden. Wij vonden echter dat MSC tijdens langdurige *in vitro* kweek spontaan kunnen transformeren tot maligne cellen (**Hoofdstuk 14**). Wij hebben echter een methode kunnen ontwikkelen, waarbij met behulp van een aantal biomarkers mogelijk getransformeerde cellen kunnen worden opgespoord. Deze methode zal vooral bruikbaar zijn voor de klinische toepassing van stamceltherapie, omdat op deze wijze de transplantatie van maligne cellen kan worden voorkomen.

Algemene conclusie

- De resultaten van het laboratoriumonderzoek naar de effecten van immuunsuppressiva op HCV infectie en antivirale therapie met interferon biedt op dit moment geen uitsluitsel welke immuunsuppressieve medicatie in HCV-positieve levertransplantatie patiënten het beste gebruikt kan worden. Wel heeft het inzicht opgeleverd met betrekking tot het begrip van de werking van deze medicijnen, en levert het een aanzet tot de ontwikkeling van nieuwe medicatie en het opstarten van relevante klinische studies.

- RNAi is een veelbelovende behandelingsoptie voor HCV infectie, die zich echter nog in een experimenteel stadium bevindt. Deze behandeling zal waarschijnlijk niet toegepast kunnen worden bij alle HCV patiënten, maar biedt wel extra behandelingsmogelijkheden voor speciale patiëntengroepen, zoals patiënten die niet reageren op de standaardtherapie of deze slecht verdragen, patiënten die met HCV en HIV zijn geïnfecteerd, of patiënten die een transplantatie hebben ondergaan.

- De ontdekking van de antivirale eigenschappen van mesenchymale stamcellen biedt nieuwe opties voor het bestrijden van HCV infectie. Omdat deze cellen kunnen differentiëren tot levercellen, ontstekingsremmende en regeneratieve eigenschappen hebben en het immuunsysteem kunnen beïnvloeden kan de therapeutische toepassing van MSC in HCV patiënten op verschillende vlakken een positief effect hebben. Het feit dat de lever MSC bevat maakt het interessant om hun biologische functie en de interactie met andere celtypen nader te bestuderen, zowel in de gezonde als in de zieke lever.

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Acknowledgements （致谢）

With the great blessing of ALLAH (God), it has finally reached the end of my PhD career. During the past five years, people whom I have ever met, worked or contacted with, have indeed helped or taught me if not to be good but at least not to be evil. I sincerely hope to thank all of you.

To my promoter, Prof. Harry Janssen, thank you for your great and continuous support! I shall never forget your full support for my first 4 years and the extended 1 year PhD research, and particularly the future opportunity you have provided. I indeed appreciate that I could stand on the platform you have established in the field of viral hepatitis to develop my own research line.

To my promoter, Prof. Hugo Tilanus, for your guidance, kindness, continuous encourage and willing to help. I thank you.

For my co-promoter, supervisor and friend, Dr. van der Laan; dear Luc, with you, I have indeed enjoyed my PhD life. You have taught me not only do's but importantly also many don'ts. You taught me, a good scientist is not only publishing a paper in a top journal. Thank you for your patience and encourage to all the bitter rejections. For the great help with our daily life, I sincerely thank you and your family.

To Prof. Maikel Peppelenbosch; dear highly learned Maikel, thank you for providing the future opportunity of working with you. Because of your extreme willing to help young people, it almost changed my mind "extremely smart and learned people can also be easy to approach".

To Prof. Herold Metselaar, your pioneer on the topic of immunosuppressants on HCV recurrence has indeed paved the way of the first part of my thesis. I am honored to have you as Inner Committee of my defense. Thank you.

To Marion (Hoogendoorn), thank you for all your help, suggestion and assistance during my PhD work and during the preparation of the defense.

To Scot, with your help, I could smoothly start my work in the lab, and with your previous work, my thesis has become enriched. Thank you.

To Petra, many thanks for your enormous help with my lab work, proof-reading, Dutch translation and to be my Paranimf.

To Waqar and Veda, thank you for the contribution of chapters to my thesis. I have enjoyed much of working with you.

To Suomi, a highly skilled “mouse surgeon”, thank you for your help with all the mouse operation. Thank you for your collaboration on MSC project.

To Geert (Kazemier) and Jeroen (de Jonge), thank you for providing the liver transplant materials and thank you for the Friday morning work discussion.

To Jaap, thank you for every Friday morning work discussion. You have indeed “forced” me to learn some immunology, although I am still quite “naïve”.

To Ron (Smits), when I first met you at the Liver day in 2007 and knew you would join our lab, I was very excited, because I want to know more about cancer research. Thank you for your conveying on knowledge of Wnt signaling. Thank you for your suggestions to many of my projects and your help with the EDC mouse system.

For Andrea and Andre, from the joint Friday morning meeting we had during my early PhD period and the Monday lab seminar, I have had opportunity to hear a lot about HBV and HCV immunology from you, although I still do not understand much. I would thank you for your knowledge.

To Hanneke, thank you for always having a warm smile and always ready to help.

To Jan (Francke), thank you for helping with reading my Dutch letters and particularly for to be my Paranimf.

To Raymond and Leonie, without your help, my lab work could not go smoothly. Thank you for the ordering, paper work, organizing and assistance.

To Greta, you are indeed the “mother” of our lab, without you, the lab would not function so well. Thank you for your almost unnoticed work and for your smile.

To Paula, our “’sister” in the lab, I hope I am saying correctly. You always take care of everything. Because of you, every cleaning day is so well-organized. Thank you!

To Wendy, thank you for all the discussion and help with my project and grant application.

To Alexander and Gwenny, thank you for both of you are so collaborative.

To Werner and Bisheng, thank you for always ready to solve my computer problem.

To Jasper, with you, the molecular lab is always well-organized. Thank you for help with finding out all the reagents and for help with all the useful technical suggestions.

To my roommates, dear Angela (Tjon), thank you for organizing the Friday morning meeting, for your suggestions for my grant application and for your always sweet smile; to Emmeloes, thank you for your always ready to help. Even if you do not know the solution, you will try to find a way to help; to Rajesh, from you, I learned many new things. Thank you for your broad knowledge; to Timon, best wishes to your study.

To all the MDL laboratory members, whom have left and whom are present, with all of you, I have indeed enjoyed my time in the lab.

To Prof. Gerard Wagemaker, Fatima, Trui, Shazia, Niek and Merel, thank all of you for the collaboration on hematopoietic and mesenchymal stem cell projects.

To all the co-authors, thank you for the contribution to my thesis and to the journal publications.

Thank Dr. Pascal van der Wegen, Dr. Scot Henry, Dr. Bart Haagmans and Sohrab Khatab (Erasmus MC, Rotterdam) for technical support.

Thank Prof. Takaji Wakita (National Institute of Infectious Diseases, Japan) for providing the full-length JFH1-derived infectious HCV replicon, Prof. Ralf Bartenschlager and Dr. Volker Lohmann (University of Heidelberg, Germany) for generously providing the Huh6 and Huh7 subgenomic HCV replicon cells, Prof. Ben Berkhout (University of Amsterdam) for providing

the SupT1 cell line and lentiviral RNAi vectors, and Dr. Jiing-Kuan Yee (City of Hope National Medical Center, USA) for providing the lentiviral backbone plasmid expressing mutated IMPDH2.

For Prof. Xinyuan Liu and Prof. Cheng Qian (supervisors of my Master research), 感谢刘新垣院士和钱程教授对学生的指导，对学生工作和生活的关心！是你们引导学生步入了生命科学的研究领域。

For Prof. Zhuo Li (teacher of my Bachelor study), 是你的生理课让我第一次对生命科学产生了兴趣。感谢你，李倬老师！

For all my teachers, thank you for what you have taught me! 感谢所有的老师对学生的教导！

For my neighbors, friends and Muslim community in Rotterdam, because of you, we do not feel lonely in Rotterdam. Thank you for inviting us for delicious food, for many times dropping off or picking up us at airport and for taking care of my family when I was abroad.

For my parents and brother:（父母和弟弟），感谢你们自始至终对我的支持，无论在艰难时还是在容易时。无以回报你们对我的付出。我为你们做最好的祈祷！

For my parents in law （岳父母），感谢你们的信任和支持，感谢为我们做的美好祈祷！

Last but not the least, to my wife, Umme Kulsum, daughter Sumayyah and son Muhammad; Dear Umme Kulsum, because of your enormous effort, I could focus on my work. Because of you, I could eat hot meal every lunch at work, whereas most of others were chewing bread. Thank you is indeed not enough for you! Dear Sumayyah and Muhammad, because of you, our home is always filled with happiness. I love all of you!

Curriculum vitae

Qiuwei (Abdullah) Pan was born in December 31, 1981, in Changxing, Zhejiang provice, China. He grew up and attended elementary, middle and high school at his hometown, the southeast part of China.

In 2000, he moved to the northwest of China to start his Bachelor study in veterinary medicine and animal breeding at Northwest University for Nationalities, Lanzhou. At the same year, he converted to Islam, thereby named as Abdullah. Soon after graduation in 2004, he moved back to Hangzhou, the capital of Zhejiang provice, to start his research Master degree at Zhejiang Sci-Tech University. Under supervision of Prof. Xinyuan Liu and Prof. Cheng Qian, he initiated the development of oncolytic adenoviral vector delivering RNA interference (RNAi) for the experimental gene therapy of hepatocellular carcinoma (HCC). However, he soon realized the challenge and limitation of cancer gene therapy for clinical application. In stead, he believes it is a right time to tackle the main cause of HCC: chronic viral hepatitis infection.

In 2007, he moved to the department of Gastroenterology and Hepatology, Erasmus Medical Center Rotterdam, the Netherlands, to carry out his PhD research on hepatitis C virus (HCV) infection. Under supervision of a renowned hepatologist Prof. Harry Janssen, a well-known surgeon Prof. Hugo Tilanus and a sharp-minded transplant researcher Dr. Luc van der Laan, he focused on multiple aspects and aimed to improve the outcome of HCV positive liver transplantation. Specifically, he devoted to projects, including (i) development of lentiviral vector delivered RNAi for prevention and treatment of HCV recurrence; (ii) the effects of different immunosuppressants on HCV infection and interferon treatment; (iii) using mesenchymal stem cells for anti-HCV and regenerative therapy. In addition to a list of publications, his discovery of antiviral activity by mesenchymal stem cells has led to a patent application and potential valorisation. In 2009, he was elected as Rising Star by the International Liver Transplantation Society.

Currently, he is recruiting PhD students and building a novel translational research line at the same lab in close collaboration with Prof. Maikel Peppelenbosch (head of the lab) and Prof. Harry Janssen, aiming at better understanding of hepatitis virus-host interaction and developing novel anti-viral therapies.

PhD Portfolio

Name PhD Student	Qiuwei Pan
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PhD Period	April 2007 - February 2012
Promotors	Prof.dr. H.L.A. Janssen & Prof.dr. H.W. Tilanus
Copromotor	Dr. L.J.W. van der Laan

General Courses

- The international course on laboratory animal science (Art. 9)
- Animal imaging workshop
- Partek data analysis courses (three series)
- Basic data analysis on gene expression arrays
- Valorisation workshop
- Photoshop CS3 workshop
- Grant proposal writing workshop

Oral Presentations at International Conferences

- 2008, ESF-EMBO symposium: antiviral applications of RNA interference, Sant Feliu de Guixols, Spain
- 2008, The annual congress of the International Liver Transplantation Society, Paris, France
- 2008, The 59th annual meeting of the American Association for the Study of Liver Diseases, San Francisco, CA, USA
- 2009, The annual congress of the International Liver Transplantation Society, New York, USA (two presentations: plenary Rising Star Award session and parallel session)
- 2009, The 60th annual meeting of the American Association for the Study of Liver Diseases, Boston, USA
- 2009, The 16th International Symposium of Hepatitis C Virus, Nice, France
- 2010, The annual congress of the Asian Pacific Association for the Study of the Liver, Beijing, China
- 2010, The annual congress of the International Liver Transplantation Society, Hong Kong, China
- 2010, The 61st Annual meeting of the American Association for the Study of Liver Diseases, Boston, USA
- 2011, The annual American Transplant Congress, Philadelphia, USA
- 2011, The 62nd annual meeting of the American Association for the Study of Liver Diseases, San Francisco, CA, USA

Oral Presentations at National Conferences

- 2008, The annual conference of the Dutch Association of Hepatology (plenary Best Abstract Award session)

- 2008, The annul conference of the Netherlands Transplantation Society (two presentations)
- 2009, The annul conference of the Dutch Association of Hepatology (plenary Best Abstract Award session)
- 2009, The annul conference of the Netherlands Transplantation Society (two presentations)
- 2010, the NVH - Dutch Liver Science Retreat

Academic Awards

Top Scored Abstract Awards:

- 2008, Altana voordrachtprijs, The annul conference of the Dutch Association of Hepatology (€450)
- 2009, Altana voordrachtprijs, The annul conference of the Dutch Association of Hepatology (€500)
- 2009, Rising Star Award, the annual meeting of International Liver Transplantation Society ($3.000)

Young Investigator Travel Awards:

- 2008, ESF-EMBO symposium: antiviral applications of RNA interference, Sant Feliu de Guixols, Spain (€250)
- 2009, The 59th annual meeting of The American Association for the Study of Liver Diseases ($500)
- 2010, The annual congress of the International Liver Transplantation Society ($1.000)
- 2011, The 62nd annual meeting of The American Association for the Study of Liver Diseases ($500)

Publications

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Patent

VAN DER LAAN, Luc J.W; **PAN, Qiuwei;** CROP, Meindert Johannes. 2008, INHIBITION OF VIRAL INFECTION AND REPLICATION BY MESENCHYMAL STEM CELLS (MSC) AND MSC-DERIVED PRODUCTS. Patent application No.: PCT/NL2008/050711. Pub. No.: WO/2010/053350. **(Contribution: 40%)**