KNOCKING OUT HCV RNA REPLICATION BY RNA INTERFERENCE

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Background: Hepatitis C Virus (HCV) is a serious problem of chronic liver disease. It may lead to development of liver cirrhosis and hepatocellular carcinoma. Therapy used to treat patients with chronic HCV infection includes treatment with interferon either on its own or in combination with ribavirin. Some infection cases do not respond to treatment and an alternative treatment is mandatory. **Method:** RNA interference (RNAi) is an antiviral mechanism which is present in plants that induces double-stranded RNA degradation. Oligonucleotides corresponding to different parts of the HCV NS3 coding region were ligated into pSUPER, and expressed as small interfering RNAs (siRNAs) after transfection. **Results:** Fluorescent Activated Cell Staining (FACS), northern blotting, Western blotting, and Real Time-PCR demonstrated that one of these siRNAs was able to ablate HCV RNA by RNAi. **Conclusion:** RNAi may represent a new approach for the treatment of HCV infection.
Keywords: Hepatitis C virus, HCV, RNA interference, RNAi

INTRODUCTION

Hepatitis C Virus (HCV) was identified as the causative agent for most post-transfusion and sporadic non-A, non-B hepatitis cases in 1989.[1] According to recent estimates, about 170 million individuals worldwide are infected and 3 to 4 million people are newly infected each year.[2] One important characteristic of HCV is its strong propensity to persist in the infected individual which leads to severe liver damage, ranging from chronic hepatitis to liver cirrhosis and hepatocellular carcinoma.[3] It is a serious infection, affecting 1 to 2% of the population in most developed countries. Around 90% of HCV infections become chronic, up to 20% of these develop into liver cirrhosis, and 1 to 5% of the cases lead to hepatocellular carcinoma.[4] Although treatment of this infection using interferon-α and ribavirin has been effective in a minority of cases (about 40%), more than 60% of patients show no response to treatment.

HCV is a single stranded RNA with positive polarity. The HCV genome is approximately 9.6 Kb in length, shorter than that of Flaviviruses and consists of a 5` NCR, a single open reading frame (ORF) encoding a polyprotein of between 3010 and 3033 amino acids, which contains both the structural proteins and non-structural proteins and a 3` NCR.[5] The 5` NCR is highly conserved and has an internal ribosomal entry site (IRES) which directs cap independent translation of the HCV ORF. The HCV ORF encodes a single polyprotein that is 3,008–3,037 aa in length and is post-translationally modified to produce at least ten different proteins: core, envelope proteins E1 and E2, p7, and nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The NS3 protein is approximately 70 kDa in size and has been shown to possess several important features for HCV replication and host cell interactions. This protein has protease activity in the N-terminal one third of the protein which is necessary for the cleavage of certain regions of the HCV polyprotein[6], helicase and NTPase activities in the C-terminal two thirds.[7,8]

cRNA generated by *in vitro* transcription of cloned viral DNA and transfection into cells, is stable and although the transcripts may not be replicating, the RNA may be detected within cells for prolonged periods.[9] From these findings culture systems, based on the replication of HCV RNA, have been developed. This involves the stable replication of subgenomic selectable HCV RNAs, known as replicons. These replicons were originally generated from a HCV consensus genome isolated from a chronically infected individual. The structural region of the HCV polyprotein up to NS3 was deleted, but the 5` UTR and the 3` UTR were both still in place. A neomycin phosphotransferase selectable marker gene as well as the IRES of the EMC virus were also inserted between the 5` UTR and the NS3 gene. These replicons are bicistronic: translation of the first cistron (neo) is directed by the HCV IRES and the second cistron (NS3-5B) by the EMCV IRES.[9]

RNA Interference (RNAi) is a process in which double stranded RNA (dsRNA) can inhibit gene expression in a sequence dependent fashion. The discovery that dsRNA can act as a gene silencing trigger was only made after the direct exposure of the nematode worm *Caenorhabditis elegans (C. elegans)* to purified double-stranded RNA (dsRNA), with sequence identity to the *unc22* gene, abrogated *unc22* expression.[10] Silencing of a functional gene by the exogenous introduction of complementary dsRNA was termed RNA interference (RNAi), and has since been shown to be the underlying mechanism of both Post Transcriptional Gene Silencing (PTGS) and gene quelling.[11] The HCV genome is a single-stranded RNA which functions as both a messenger RNA and replication template. This makes HCV an attractive target for the study of RNA interference. A number

of groups have demonstrated that siRNAs interfere with HCV gene expression and replication. These studies used RNAi to investigate HCV replication in hepatocytes and to define host genes that are significant for viral replication.[12] However, the potential for RNAi as an antiviral therapy is still not clear, as there are many potential difficulties for *in vivo* RNAi. siRNA molecules, designed to target a HCV which were delivered to a HCV replicon cell line reduced HCV protein expression and RNA synthesis to levels that were 90% less than cells treated with non-specific siRNAs.[13] The antiviral effect of siRNAs was also shown to be independent of IFN. These results are encouraging for the development of RNAi as a tool and as a new approach for the treatment of persistent HCV infections.[14]

The goal of this study was to assess the efficacy of RNAi against HCV RNAs.

MATERIAL AND METHODS

Tissue Culture and Generation of Stable HCV Subgenomic Replicon Cell Line
The S1179I HCV subgenomic replicon (Rep 4) (genotype 1b) was obtained from Charles Rice (The Rockefeller University, New York) and passaged as described.[15,16] Huh-7 cells were transfected with HCV subgenomic replicon RNA and maintained as described.[15,16] An Huh-7 cell line that stably replicated HCV RNA replication (called S1179I herein) was used in all experiments.

Generation and Transfection of siRNA
Five different regions of the HCV NS3 (1b) coding sequence were chosen as potential targets for siRNAs. These sequences were chosen using the criteria described by Brummelkamp[17] and DNA oligonucleotides which encoded the corresponding siRNAs were synthesized for subsequent ligation downstream of a polIII promoter in pSUPER. For each siRNA, a 19 nucleotide sequence was derived from the target RNA, separated by a short spacer of 9 nucleotides from the reverse complement of the same 19 nucleotide sequence. The resulting transcript is predicted to fold back on itself to form a 19-base pair step-loop structure. For the sense sequence, a cleavage site for restriction enzyme *Bgl* II was added to the 5' region and a *Hind* III cleavage site was added to the 5' of the anti-sense sequences. The sense and anti-sense sequences of the DNA oligonucleotides that encoded siRNAs were as follows in Table-1.

Cells (Huh7, 293T, replicon cell lines, Rep 3 and Rep 4) were grown in 6 well (35 mm) cell culture plates with or without cover slips until they were approximately 70% confluent. Two µg of pSUPER-siRNA constructs were transfected and left for 3 days.

Table-1: Sense and anti-sense sequences of the DNA oligonucleotides that encoded siRNAs

HCV NS3i1:	GAT CCC CTA TGT GAT GAG TGC CAC TCT TCA AGA GAG AGT GGC ACT CAT CAC ATA TTG GAA A.
HCV NS3iC1:	AGC TTT TCC AAA AAT ATG TGA TGA GTG CCA CTC TCT CTT GAA GAG TGG CAC TCA TCA CAT AGG G.
HCV NS3 i2:	GAT CCC CCT GAC TCG ACC ACT ATC CTT CAA GAG AAG GAT AGT GGT CGA GTC AGT TTT TTG GAA A.
HCV NS3iC2:	AGC TTT TCC AAA AAC TGA CTC GAC CAC TAT CCT TCT CTT GAA GGA TAG TGG TCG AGT CAG GGG G.
HCV NS3 i3:	GAT CCC CTG CTG TAG CAT ATT ACC GGT TCA AGA GAC CGG TAA TAT GCT ACA GCA TTT TTG GAA A.
HCV NS3iC3:	AGC TTT TCC AAA AAT GCT GTA GCA TAT TAC CGG TCT CTT GAA CCG TAA TAT GCT ACA GCA GGG G.
HCV NS3i5:	GAT CCC CTG GCG TGT GTT GGA CTG TCT TCA AGA GAG ACA GTC CAA CAC ACG CCA TTT TTG GAA A.
HCV NS3iC5:	AGC TTT TCC AAA AAT GGC GTG TGT TGG ACT GTC TCT CTT GAA GAC AGT CCA ACA CAC GCC AGG G.

Fluorescent-activated cell staining (FACS) analysis of siRNA transfected mammalian cells
The siRNA transfected cells were washed with RPMI and then re-suspended in RPMI and pelleted by centrifugation at 1200 rpm for 5 minutes. This washing procedure was repeated once more. 300µl of fixative (4% paraformaldehyde in PBS) was added to each cell pellet on ice for a minimum of 10 minutes. To block non-specific binding of antibodies the cells were incubated with a 1:25 dilution of swine normal serum (Dako Ltd, Denmark) for 30 minutes. 50 l of saponin A (0.1% saponin, 0.01 M hepes in RPMI) was added to each tube and left for 30 minutes at room temperature. Fifty µl of primary anti-NS3 antibody (NS3-7) was added and left for 30 minutes at room temperature. One ml of saponin A was added to each tube, which were then spun at 1200 rpm, and supernatant was removed. Fifty µl of FITC conjugated swine anti-rabbit antisera was added to each tube and left for 30 minutes at room temperature. The tubes were spun at 1200 rpm and the supernatant was removed. The tubes were washed with 1 ml of saponin B (0.1 M Hepes, 0.1% saponin, RPMI), spun at 1200 rpm, and supernatant was discarded. Again, the tubes were washed with 1 ml of RPMI alone, spun at 1200 rpm, and the supernatant was discarded. Finally, cell fluorescence signals were determined immediately after staining using a FACS caliber flow cytometer (Becton Dickinson). FITC was identified by using a 530 band pass filter. The analysis was performed using CELLQUEST software (Becton Dickinson). A primary gate was set to exclude dead cells or debris. The background level was estimated by omitting the primary antibody.

Imunofluorescent *in situ* staining
Glass cover slips were sterilised (dipped in alcohol and flamed) and placed in the bottom of the wells of Sterilin 6 well plates. HCV RNA replicon cells were seeded in half of the wells while the rest of the wells were seeded with Huh7 cells. The cells left to grow up to 80% confluence.

Staining of cells to detect HCV NS3
Three (3) ml of methanol: acetone mix was added to the wells which contained growing cells onto cover slips within a 6 well plate for a minimum of 1 hour at -20 °C. The mix was then removed from the wells and the cells were re-hydrated by washing with PBS. To block non-specific binding of antibodies the slides were incubated with 1:25 dilution of swine normal serum (Dako) for 30 minutes. The swine serum was discarded and the excess was wiped away. Each of the cover slips were removed from the corresponding well and placed, cell coated face down, onto 50 µl of rabbit polyclonal anti-NS3 antibody (NS3-7)[18] diluted 1 in 50 in sterile PBS at room temperature on a 2×2 cm square of parafilm for 30 minutes. Unbound antibody was washed off by washing three times in PBS and the cover slips were then placed face down onto 50 µl of FITC conjugated swine anti-rabbit antibody (DAKO), diluted 1 in 40 in sterile PBS for 30 minutes. The slide was washed again three times in sterile PBS, once in water, and placed face down on mounting medium (Vectashield) on a clean microscope slide. The cells were viewed at 400× magnification with an inverted microscope.

Western blot
Western blot analysis was performed on total lysates from negative and positive controls and siRNA-transfected S1179I cells as described.[19] The gels were transferred to Hybond-N membranes (Amersham Pharmacia), blocked, and incubated with antibodies to NS3 followed by incubation with a horseradish peroxidase-conjugated mouse anti-rabbit antibody. The blots were developed with (Hyperfilm ECL, Amersham Biosciences).

Northern Blot Analysis
Total RNA was extracted from transfected cells and 5 g of total RNA was loaded onto the gel. NS3 probe was purified by using MicroSpin G-50 columns (Amersham Pharmacia). Blots were visualized and quantitated as described.[20]

Real Time-PCR of siRNA
Real-time PCR (SYBR® Green) was performed as described.[21] To quantitate HCV transcript levels, dilutions of plasmids containing the HCV NS3 or the beta-actin gene were always run in parallel with cDNA from the S1179I cells for use as standard curves (dilutions ranged from 10^8 to 10^0 copies of each plasmid). The PCR primers for HCV NS3 amplification in real-time PCR were 5' ATGGCGCCTATTACGGCCTA (forward) and 5' ACACACGCCATTGACGCAGG (reverse). Primers used for beta- actin amplification in real-time PCR were 5' GAACTCCAGCAGGACCATGTG (forward) and 5' CACTTCATGATGGAATTGA (reverse).

RESULTS

**Inhibition of NS3 expression by HCV siRNA constructs**
Huh7 cells did not stain with NS3-7 antibody and were represented by a purple peak after FACS (Figure-1 A). Rep 4 cells which stained with NS3-7 antibody showed a peak (green) of fluorescence, corresponding to NS3 expressing cells, and this is shown as being separate from the position of Huh7 cells which did not express NS3 (purple peak) (Figure-1 B). Rep 4 cells which were transfected with pSUPHBV also had a fluorescence peak corresponding to NS3 expressing cells (Figure-1 C). In most of the cases where siRNA constructs against NS3 were used, the Rep 4 cells could still be stained (Figure-1 D, E, F, G, J, K, L, and M). However, the FACS results showed that pSUPNS3-3 successfully inhibited the expression of NS3 in Rep 4 cells, as the FACS peak of these cells was completely coincident with that of the untransfected Huh7 cells (purple) (Figure-1 H & I). This experiment was repeated several times with the same results. Initially 10 µg of each siRNA construct was used per well, however it was found in subsequent experiments that 2 µg per well of pSUPNS3-3 could abolish the NS3 expression.

Effects of HCV siRNA constructs on the expression of NS3 in Rep 4 cells: Imunofluorescent *in situ* staining
The immunofluorescence results showed that there was no inhibition of NS3 expression with pSUPNS3-1, 2, 4, and 5 as previously shown by FACS analysis, but there was a large reduction in the level of NS3 expression with pSUPNS3-3. The pSUPHBV also showed no reduction in NS3 expression. The positive control (Rep 4 cells alone) showed positive staining with anti-NS3 antibody and no staining was seen in the negative control (Huh7 cells) (Figure-2).
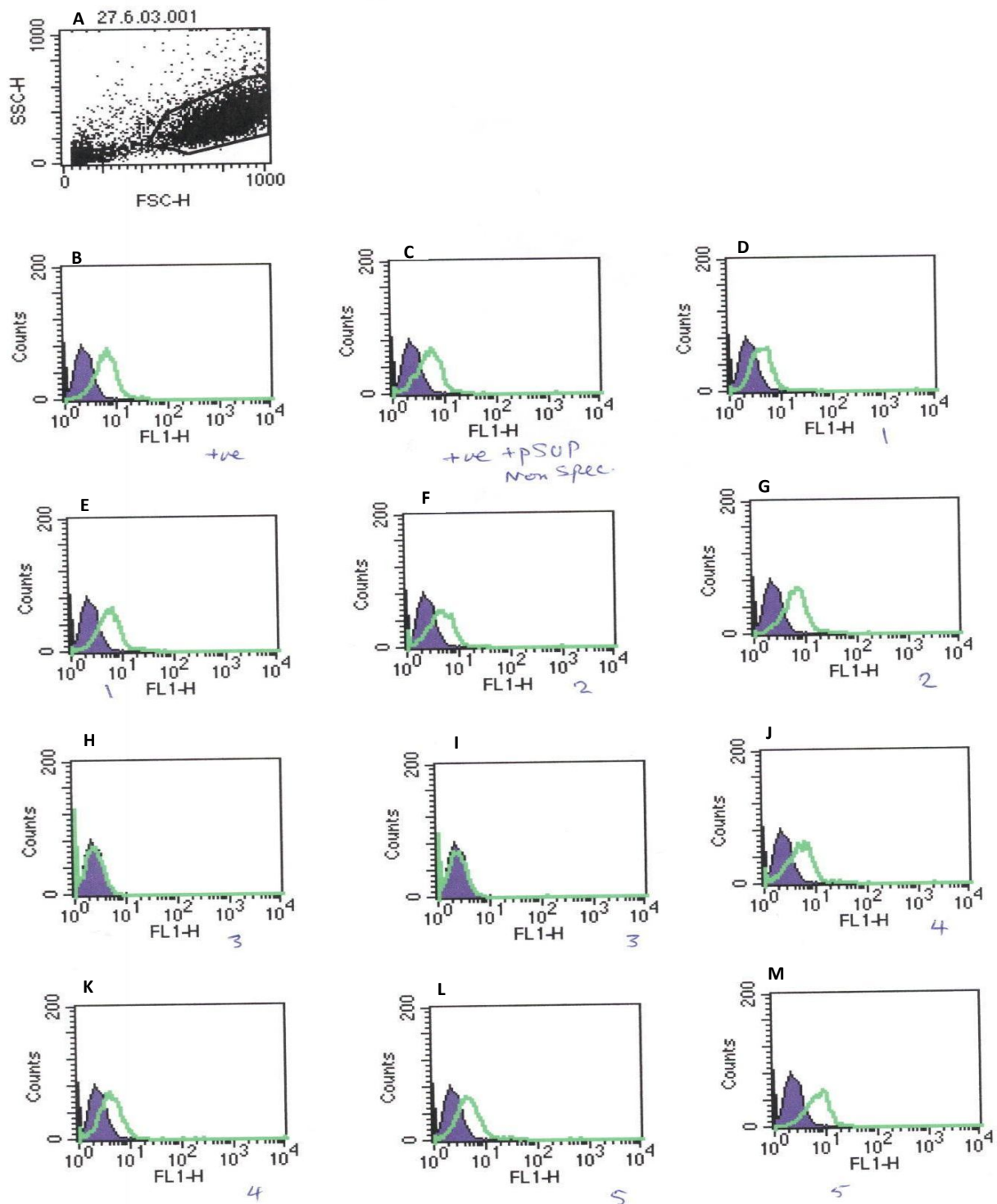
Figure-1: The effects of HCV siRNA constructs on NS3 expression detected by FACS. A: Non-stained Huh7 cells
The selected population of gated cells is within the area bounded by the dark lines. This population of unstained cells was then, represented by purple peak in the subsequent samples. B: Rep 4 cells stained with anti-NS3 antibody (green) compared to untransfected cells (purple). C: Rep 4 cells transfected with pSUPHBV. D, E (pSUPNS3-1), F, G (pSUPNS3-2), J, K (pSUPNS3-4), L, and M (pSUPNS3-5): showed no inhibition of NS3 expression in Rep 4. H and I: Rep 4 cells transfected with pSUPNS3-3 showed complete overlap of the green and purple peaks, indicating that NS3 expression had been ablated
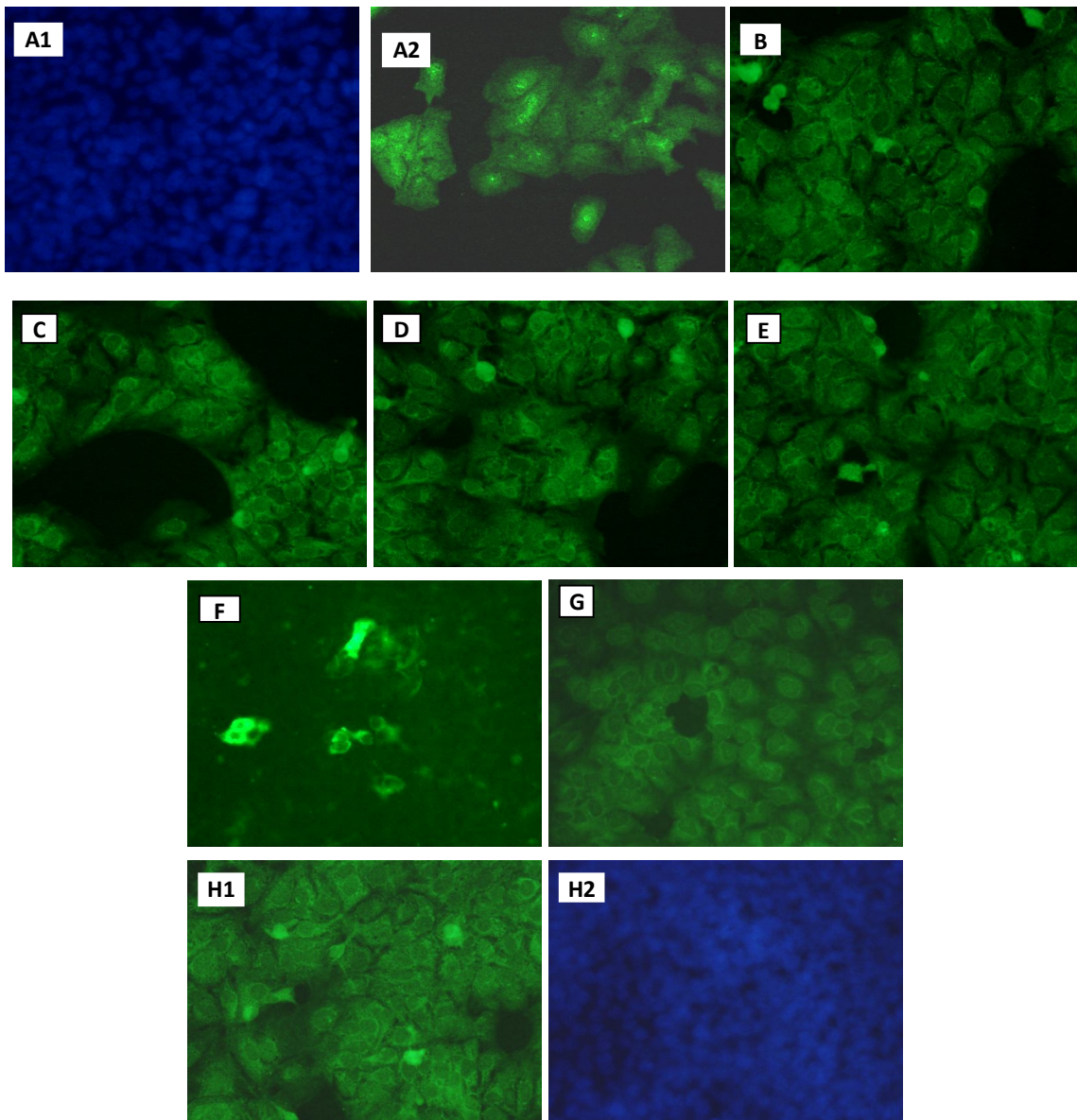
Figure-2: Effects of HCV siRNAs constructs on the expression of NS3 in Rep 4 cells: Immunofluorescence staining using NS3-7 antibody

A1 and A2: Huh7 cells (negative control). A1: DAPI staining. A2: Immunofluorescence staining. B: Rep 4 cells (positive control). C: Rep 4 transfected with pSUPHBV. D: Rep 4 transfected with pSUPNS3-1. E: Rep 4 transfected with pSUPNS3-2. F: Rep 4 transfected with pSUPNS3-4. G: Rep 4 transfected with pSUPNS3-5. H1 & H2: Rep 4 transfected with pSUPNS3-3. H1: DAPI staining. H2: immunofluorescence staining

Effects of HCV siRNA construct on the expression of NS3 in Rep 4 cells: Western blot

The results showed bands of approximately 70 kDa corresponding to NS3 with the Rep 4 cells and the Rep 4 cells transfected with pSUPER-HBV but no NS3 band was seen with the Rep 4 cells transfected with pSUPNS3-3 or with Huh7 cells (negative control) (Figure-3). This indicates strongly that the siRNAs expressed by pSUPNS3-3 inhibit the translation of the proteins encoded by the HCV replicon RNA due to the degradation of the RNA.

Real Time-PCR of siRNA

It is evident from the results that pSUPNS3-3 significantly reduced the NS3 copy number in transfected Rep 4 cells compared to the untransfected Rep 4 cells. This reduction in the NS3 copy number was about 80% (Figure-4).

The results confirmed the ablation of HCV RNA in Rep 4 cells treated with pSUPNS3-3.
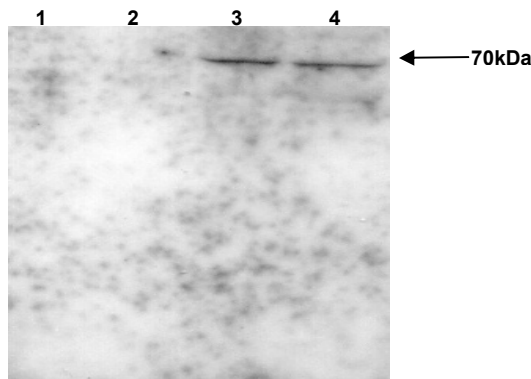
Figure-3: Western blotting of cells treated with HCV siRNA

Lane 1: Huh7 cells. Lane 2: Rep 4 cells transfected with pSUPNS3-3. Lane 3: untransfected Rep 4 cells. Lane 4: Rep 4 cells transfected with pSUPHBV.
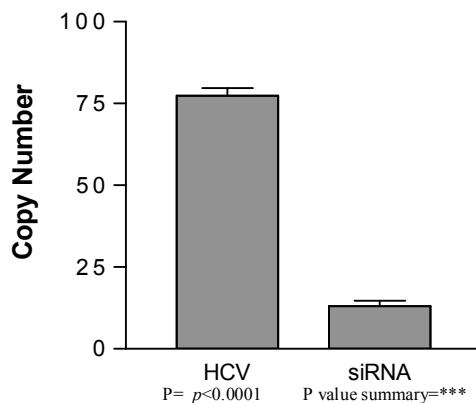
Figure-4: Graph showing the effect of pSUPNS3-3 on Rep 4 cells

The copy number of Rep 4 cells was compared with Rep 4 cells transfected with pSUPNS3-3. The results were represented by using PRISM.

DISCUSSION

In this study, it was shown that HCV RNA can also be silenced by RNAi, providing proof of principle for future RNAi-based therapy of chronic HCV infection. HCV is an ideal target for the inhibition of viral replication by RNAi since the virus genome is a single stranded RNA molecule. The entire NS3 region of HCV-1b was screened for potential target sites for RNAi.

A FACS based RNAi assay was used which was based on the ability of Huh7 cells that expressed NS3, after transfection with a plasmid that expresses NS3, to be stained with an anti-NS3 antibody. Around 80% of the transfected Huh7 cells expressed NS3. The FACS results showed that there was more than 80% inhibition of NS3 expression in the cells transfected with pSUPNS3-3, whereas the pSUPHBV plasmid which contained an siRNA sequence which

was designed to target hepatitis B virus (negative control), showed no inhibition of NS3 expression. However, the other HCV siRNA constructs pSUPNS3-1, pSUPNS3-2, pSUPNS3-4, and pSUPNS3-5 showed little inhibition of NS3 expression although their siRNA sequences were also designed to target the HCV NS3 region. However, experimental results from other studies have shown that not all siRNA which follow the design criteria previously described are able to knock out the target sequence.[22]

One possible reason for this is that the target sequences could have secondary structures which can hinder access by siRNAs. Another possible reason, though unlikely in this case, is that point mutations could have occurred in the HCV target sequence preventing the correct binding of the siRNAs. Another factor that could reduce siRNA induced degradation of target RNA sequences is the steady-state level of an RNA species, i.e. the balance between the rate of mRNA synthesis, and the rate of degradation of the mRNA. RNAi acts by targeting specific mRNA molecules for degradation, reducing the amount of mRNA available for translation and making transcription the rate limiting step in protein production.

The *in situ* staining also showed that pSUPNS3-3 could inhibit the expression of NS3 protein in Rep 4 cells while pSUPNS3-1, pSUPNS3-2, pSUPNS3-4, and pSUPNS3-5 had little effect on the NS3 protein expression in agreement with the results of the FACS assay.

Although *in situ* staining and the FACS assay are indirect tests for the degradation of HCV replicon RNA by siRNA, they strongly indicate that the NS3 coding sequence was rapidly degraded. Moreover, the inhibition of NS3 expression by siRNAs was also shown by extracting protein from the siRNA treated cells and carrying out Western blotting. This clearly showed the absence of NS3 protein in Rep 4 cells treated with pSUPNS3-3 while a band of 70 kDa was seen with untreated Rep 4 cells and Rep 4 cells transfected with pSUPHBV. RNAs extracted from the different transfected cells, under very stringent conditions, showed that, in cells in which NS3 expression was knocked out by pSUPNS3-3, there was no PCR band when amplification was carried out using NS3 primers. Though by increasing the quantity of the PCR product from pSUPNS3-3 treated Rep 4 cells by five fold, a faint band could be observed. However, a strong PCR band was present for the positive control.

Northern blotting was also used to show the presence of HCV replicon RNA in total RNA extracts from Rep 4 cells. As expected from RT-PCR results, there was a large reduction in the quantity of HCV replicon RNA in Rep 4 cells treated with pSUPNS3-3 while a much stronger hybridization band was shown in

Rep 4 cells which were not treated with pSUPNS3-3. Quantitative analysis by using Real-Time PCR showed a large reduction in the copy number of HCV replicon RNA in Rep 4 cells transfected with pSUPNS3-3 (80%) compared to untreated Rep 4 cells.

In summary, the data from FACS, RT-PCR, northern blotting, Western blotting, and *in situ* staining have shown that RNA interference against the NS3 coding region of HCV, with the siRNAs expressed by pSUPNS3-3 inhibited the expression of the NS3 protein and caused the degradation of HCV replicon RNA in Rep 4 cells.

These results are encouraging and they provide further evidence for the potential use of RNAi as a highly specific antiviral therapy to treat HCV infections. In addition, the results presented here show that siRNAs which target the NS3 region display similar levels of HCV RNA degradation to those published by the other groups who have investigated RNAi with other regions of the HCV genome, e.g., the 5' UTR[23,24], NS3[23,25], NS5A[13], and NS5B[13,23,25] This indicates many areas in the HCV genome are good targets for RNAi.

It is known that the clearance of virus from the mammalian host usually requires the destruction of infected cells, either by the immune system or by apoptosis induced by cytopathic effects of the virus. So using RNAi as an antiviral approach for treating HCV infections may help to eliminate chronic HCV infections without harming liver cells. Although RNAi is able to substantially reduce or eliminate target HCV RNAs *in vitro*, there are many questions that still have to be addressed. These include: how can siRNAs be efficiently delivered to the infected cells *in vivo*; how long does the effect of RNAi last in the infected cells; can strategies be developed, e.g., simultaneous targeting of several sites that will reduce the problems of RNAi nucleotide mismatches generated by mutations in the siRNA target sequence caused by the very high mutation rate of HCV replication *in vivo*?

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