

# INHIBITION OF HEPATITIS C VIRUS (HCV) CORE PROTEIN-INDUCED CELL GROWTH BY NON-STRUCTURAL PROTEIN 4A (NS4A) IS MEDIATED BY MITOCHONDRIAL DYSREGULATION.

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## ABSTRACT

Hepatitis C virus (HCV) is a significant health problem facing the world. More than 170 million people are infected with HCV worldwide. HCV encodes a large polyprotein precursor that is processed into at least 10 distinct products including structural (core, E1 and E2) and non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B). Besides its importance in virus replication, NS4A functions as a cofactor for NS3 and contributes to viral pathogenesis by influencing cellular functions. Here, we investigated the effect of NS4A protein on the growth rate induced by core protein in liver cells. Using our established tetracycline inducible system, we demonstrated the ability of NS4A protein to inhibit core protein-induced cell growth in Hepatoma cell line, HepG2. Induction of both core and NS4A proteins in HepG2-core/NS4A transfectants inhibited core-induced growth advantage in HepG2-core transfectants and blocked NS4A protein-induced cell growth inhibition in HepG2-NS4A transfectants. Using both immune fluorescence staining and Western blot analysis, we confirmed the localization of NS4A protein to the mitochondria in HepG2-NS4A transfectants expressing NS4A protein. Data obtained from flow cytometry analysis, using JC-1 demonstrated the loss of mitochondrial membrane potential ( $\Delta\Psi_m$ ) by the expression of NS4A protein in HepG2-NS4A transfectants, but not by the expression of core protein in HepG2-core transfectants. Whereas, the induction of the expression of both core and NS4A proteins in HepG2-core/NS4A transfectants blocked NS4A-induced loss of  $\Delta\Psi_m$  in HepG2 cells. Taken together, our data suggest an important role for mitochondria in the modulation HCV NS4A-induced inhibition of HCV core-mediated cell growth.

KEY WORDS: HCV, Core, NS4A, Cell Growth.

## INTRODUCTION

Hepatitis C virus (HCV) is a significant health problem facing the world. More than 170 million people are infected with HCV worldwide (1). HCV infection causes acute hepatitis, which is naturally cleared in 20-30% of patients (2). However, in 70-80% of cases persists causing chronic hepatitis, which mostly leads to a spectrum of diseases including steatosis, fibrosis, cirrhosis and hepatocellular carcinoma. Up to date there is no effective vaccine available for the virus, and the current therapies for many HCV-infected patients show little efficacy (3). HCV genome has a long open reading frame, flanked with 5' and 3' untranslated region, which encodes a polyprotein precursor of about 3,010 to 3,033 amino acids (aa) residues (4). This polyprotein is cleaved by both host and viral proteases to generate four structural proteins (C, E1, E2 and P7) and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B)(4). Core protein is derived from the first 191 amino acids of the N-terminal of the precursor polyprotein (4). Besides its function as a component of viral nucleocapsids, core protein has been shown to act on transactivation of numerous cellular and viral promoters (5-8), many of which are involved in the regulation of cellular proliferation. In addition, core targets a wide spectrum of cellular factors and different signalling pathways (9,10). Furthermore, transgenic mice with constitutive expression of core show liver steatosis and eventually develop HCC (11). NS4A is a 54-residue amphipathic peptide with a hydrophobic N-terminus and a hydrophilic C-terminus (12). Its known functions include acting as cofactor for NS3 protease, possibly by assisting in the membrane localization of NS3 and other viral replicase components, and stimulating phosphorylation of NS5A (13). In addition, the NS4A has been reported to inhibit cell proliferation (14,15) and to enhance mitochondria-mediated apoptosis (16). In this study, we demonstrated for the first time the ability of NS4A protein to inhibit core protein-induced cell growth by a mechanism including mitochondrial dysregulation.

## MATERIAL AND METHODS

### Cell lines

Human hepatoma cell line HepG2 (ATCC, Rockville, MD, USA). RetroPack™ pT67 cells (Clontech Inc). Cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum.

### Construction of HCV core plasmid

The construction of the HCV core and HCV NS4A plas-

mids encoding for the complete HCV core- and HCV NS4A proteins respectively was performed as described (10,14,17). Briefly, the viral RNA was extracted from 100 µl of serum using QIAamp viral RNA extraction kit (Qiagen). The complete HCV cDNA was synthesized with Genscript (Genecraft). The HCV ds cDNA region (nt 280-8306) was amplified from the complete HCV cDNA by conventional PCR using the following primer pairs: 5'-GAC CGT GCA CCA TGA GCA CG-3' (sense) and 5'-CTG CCT ACC GAG CAG GCA GCA-3' (anti-sense). The PCR products were modified to generate blunt ends and then cloned into the EcoR V site of pcDNA3.1 (+) (Invitrogen) to generate pcDNA3.1-HCV. The HCV core protein-encoding region (nt 280-825) was amplified from the pcDNA3.1-HCV plasmid comprising the complete HCV cDNA of the genotype 4A using the following primer pairs having a start codon (bold) and a Hind III restriction site (underlined): 5'-CCC AAG CTT GGG GAC CGT GCA CCA TGA GCA CG-3' (sense) and 5'-CCC AAG CTT GGG TCG GCG AAG CGG GGA CAG TC-3' (antisense). Whereas, the HCV NS4A protein- encoding region (nt 5244-5406) was amplified from the pcDNA3.1-HCV plasmid comprising the complete HCV cDNA of the genotype 4A using the following primer pairs having a start codon (bold) and a ClaI restriction site (underlined): 5'-CCA TCG ATA TGG TGA CAA GTA CGT GGG TCT TG -3' (sense) and 5'-CCA TCG ATC TCC TCC ATT TCG TCC AAC TG -3' (antisense). The cDNA sequences of both HCV core and NS4A proteins were first sequenced and then inserted into the Hind III and ClaI sites of pRevTRE vector, respectively.

### Generation of viruses

The generation of viruses was performed as described (10,14,17). Briefly, the packaging cell line RetroPack™ pT67 (Clontech) was grown in DMEM with 10% FCS, 2 mM L-glutamine (all from Sigma-Aldrich) at 37°C and in 5% CO<sub>2</sub>. The cells were transfected with the appropriate retroviral construct e.g. pRev Tet-Off, pRevTRE-core, or pRevTRE-NS4A by nucleofector™ Kit (Amaxa biosystems). Forty-eight hours post-transfection, the supernatant was collected, filtered through a 0,45-µm-syringe filter, and spun at 50.000xg for 1,5h. Pelleted virus was resuspended in a 0,1 or 0,05 the original volume of medium at 4°C for 4h.

### Infection of target cells

The development of HepG2- Tet-Off as well as the double stable Tet-off clones (HepG2-Core, HepG2-NS4A, and HepG2-core/NS4A) allowing controlled expression of HCV core under the control of tetracycline was performed as described (10, 14, 17). G418 and Hygromycin

resistant clones, termed, HepG2-core, HepG2-core/NS4A and HepG2-NS4A transfectants were screened for expression of HCV core, and NS4A proteins by RT-PCR. Positive clones, with high induction efficiency, were expanded and rescreened by RT-PCR and immunoblotting using anti-HCV core or anti-NS4A antibodies for the expression of HCV core protein as described (10, 14,17).

#### **Immune fluorescence staining of HCV core transfected cells**

HepG2-core, HepG2-NS4A, and HepG2-core/NS4A transfectants were plated in glass bottom culture dishes (MatTek Corporation, USA) at a density of  $1 \times 10^4$  cells and allowed to grow for 24h under normal condition in the presence of tetracycline (4 $\mu$ g/ml). After the withdrawal of tetracycline for 48h, the cells were subjected to immunofluorescence staining as described (18). Briefly, cells were fixed for 20 min in ice-cold PBS containing 4% formaldehyde. Blocking and all following procedures were performed in PBS supplemented with 4% BSA and 0,05% saponin. Anti- HCV NS4A (Research Diagnostics, Inc) and anti-Tom20 (SC-11415; Santa Cruz, USA) antibodies were applied at 4°C overnight at the appropriate concentrations. Then, cells were washed three times for 20 min and incubated with the secondary antibody (1:500). Finally, samples were washed extensively and mounted in fluorescent mounting medium (Dako Corporation, Carpinteria, CA) with or without 10 ng/ml 4', 6-diamidino-2-phenylindole. Pictures were taken on an Axiovert 135 Microscope (Zeiss, Germany) with an Aplanachromat x63 oil immersion lens using OpenLab software (Improvision, Tübingen, Germany).

#### **Immunoblot**

Immunoblot analysis was performed according to the standard procedures. The following antibodies were used at the indicated dilution: anti-HCV core protein antibody and anti-HCV NS4A protein (Research Diagnostic, Inc, USA), 1:1,000; anti-Tom20 (SC-11415), 1:500; anti- actin (SC-1615), 1:5,000 (Santa Cruz Biotechnology, Inc, USA).

#### **MTT assay**

The cell number was determined by MTT assay using cell proliferation kit (Roche, Mannheim, Germany) as described (10,14,18,19). HepG2-core, HepG2-NS4A and HepG2-core/NS4A transfectants as ( $1 \times 10^3$ /well) were plated into a microtiter plate (Nunc) and cultured in medium with (+Tc) or without (-Tc) 4  $\mu$ g/ml tetracycline. The MTT assays were performed at regular time intervals up to 7 days.

#### **Preparation of mitochondria and endoplasmic reticulum (ER) from cultured cells**

HepG2-core, HepG2-NS4A and HepG2-core/NS4A transfectants were allowed to grow in the presence and

in the absence of tetracycline (4mg/ml), 48h later, the cells were washed with PBS, and then scraped off with 5 ml of PBS. Collected cells were precipitated by centrifugation at 600 g for 5 min, and washed with HE buffer (10 mM Hepes-KOH, pH 7,5; and 1 mM EDTA) containing 10% (wt/vol) sucrose. The cells were resuspended in 1 ml of the same buffer containing 20  $\mu$ g/ml 2-macroglobulin, homogenized by five-times aspiration through a 27-gauge needle, and then centrifuged at 600 g for 5 min to obtain a post-nuclear supernatant. The supernatant was layered over a discontinuous gradient of 40 and 60% sucrose in HE buffer (3 and 1 ml, respectively). After centrifugation at 100,000 g for 3 h, 0,5-ml aliquots were collected from the top of the tube. 100  $\mu$ l of each fraction was precipitated with 10% TCA and subjected to SDS-PAGE and immunoblotting using antibodies against Tom20 (mitochondria) or NS4A proteins.

#### **Detection of the loss of mitochondrial membrane potential ( $\Delta\Psi_m$ ) using JC-1**

The measurement of mitochondrial membrane potential was performed as described (18). Briefly, HepG2-core, HepG2-NS4A and HepG2-core/NS4A transfectants were allowed to grow in the presence and in the absence of tetracycline (4mg/ml), 48h later, the cells were trypsinized, counted, and washed twice in ice-cold PBS and resuspended in PBS. The cells were stained with 10  $\mu$ M JC-1 for 30 min at room temperature in the dark. The intensities of green fluorescence at 520–530 nm (PMT 2) and of red fluorescence at more than 550 nm (PMT 3) of 50.000 individual cells were analyzed by using a flow cytometer. The intensity voltages of the photomultipliers of detector 2 (PMT 2) and detector 3 (PMT 3) were set at 470 V. The value of  $\Delta\Psi_m$  in response to the test compound was expressed as a ratio of PMT 3 to PMT 2.

## **RESULTS**

#### **Tetracycline-regulated expression of HCV core and NS4A proteins in HepG2 cells**

The expression of both HCV proteins core and NS4A proteins were detected in HepG2-core, HepG2-NS4A and HepG2-core/NS4A transfectants by Western blot analysis after the withdrawal of tetracycline from the culture medium for 48h (Figure 1). The induction expression of HCV core and-NS4A proteins was found to be time-dependent, and could be quantitatively regulated by the variation of tetracycline concentration in the culture medium (data not shown).

#### **Inhibition of core protein-induced cell growth by NS4A protein**

To investigate whether core protein-induced cell

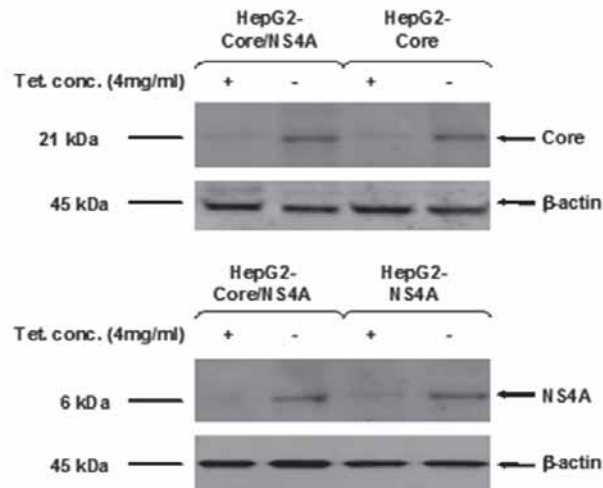


FIGURE 1. Expression of both HCV core and-NS4A proteins in HepG2 cells. Detection of HCV core and HCV NS4A proteins in HepG2- core, HepG2-NS4A, and HepG2- core/NS4A transfectants cultured in the presence (+Tc) or in the absence (-Tc) of 4 µg/ml tetracycline for 48h. 50 µg protein was separated by 12 % SDS-PAGE and analysed by immunoblotting using anti- core and anti- NS4A antibodies The same blots were reprobred with an anti-actin antibody to compare loading and transfer.

growth should be influenced by the expression of NS4A protein, the transfectants HepG2-core, HepG2-NS4A and HepG2-core/NS4A were allowed to grow in the presence and in the absence of tetracycline (4µg/ml) up to 7 days and the cell growth rate was determined using cell viability assay (MTT). Data obtained from MTT assay (Figure 2) confirmed the ability of core protein to induce cell growth advantage in HepG2 cells, and demonstrated the inhibition of the same cells by the expression of NS4A. Interestingly, the expression of both core and NS4A in HepG2-core/NS4A transfectants suppressed core protein-induced growth rate in HepG2-core transfectants and blocked NS4A-induced cell growth inhibition of HepG2-NS4A transfectants (Figure 2). Taken together, these data suggest

an important role for NS4A protein in the inhibition of core protein-induced growth rate of HepG2 cells. **Subcellular localization of NS4A protein to mitochondria triggers the loss of mitochondrial membrane potential in HepG2 cells**

To investigate the role of mitochondria in the modulation NS4A-induced cell growth inhibition of HepG2 cells, the HepG2 transfectants HepG2-core, HepG2-NS4A and HepG2-core/NS4A were allowed to grow in the presence and in the absence of tetracycline (4µg/ml). Forty eight hours later, the cells were subjected either to immune fluorescence staining, Western blot analysis or to the measurement of mitochondrial membrane potential ( $\Delta\Psi_m$ ) using JC-1. To confirm the subcellular localization of NS4A protein, the HepG2-NS4A tran-

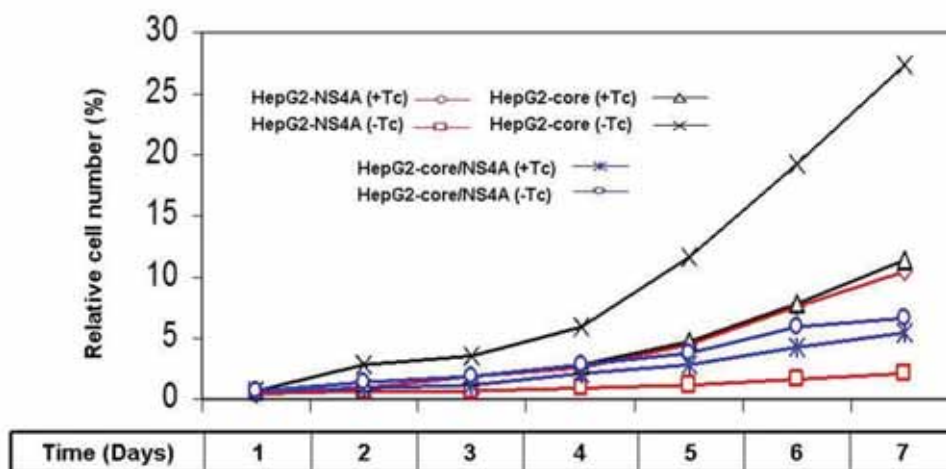
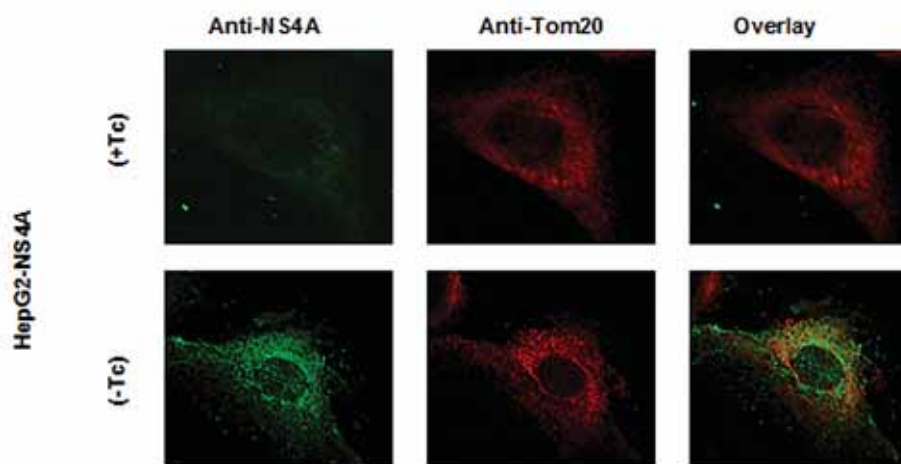
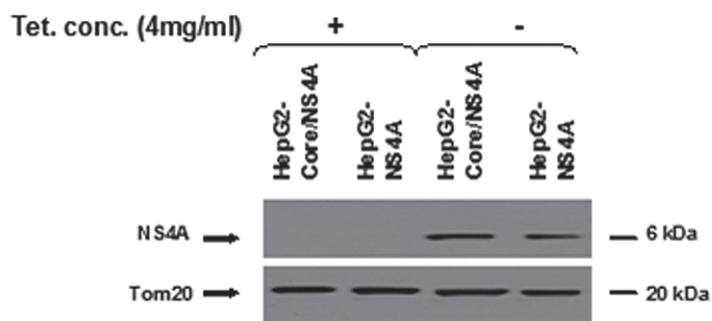


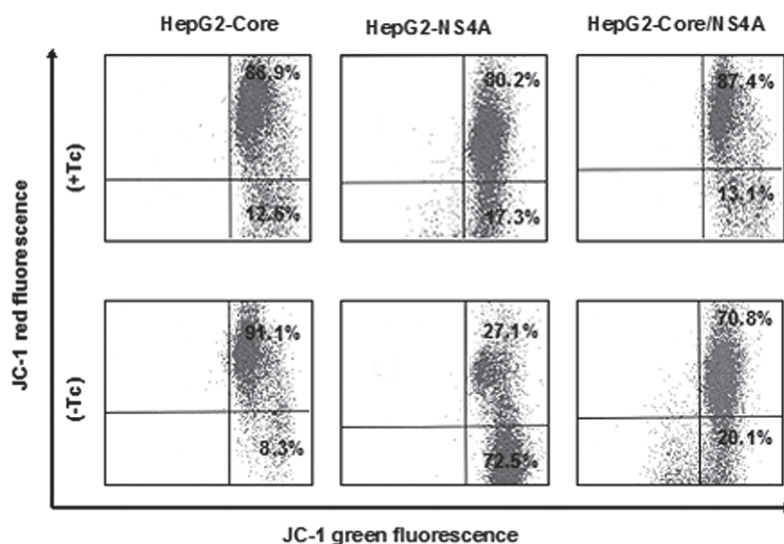
FIGURE 2. Effect of core, NS4A and Core/NS4A proteins on cell growth. HepG2-core, HepG2-NS4A and HepG2-core/NS4A transfectants were cultured in the presence and in the absence of tetracycline for the indicated time points. The proliferation rate was assessed by MTT assay. The results are means  $\pm$  SE of three independent experiments.



A)



B)



C)

FIGURE 3. Subcellular localization of HCV NS4A protein and loss of mitochondrial membrane potential. A) Subcellular localization of NS4A protein to mitochondria using confocal laser scan microscopy. 48h after the withdrawal of tetracycline from the culture medium, the NS4A protein was stained with anti- NS4A antibody (green); mitochondria were stained with anti-Tom20 antibody (Red). The overlay of mitochondria (Tom20) and NS4A indicated NS4A localization to the mitochondria (yellow). B) Subcellular localization of NS4A protein to mitochondria using mitochondrial fractions. C) Loss of mitochondrial membrane potential in HepG2 transfectants. HepG2 transfectants, HepG2-core, HepG2-NS4A and HepG2-core/NS4A cultured in the presence (+Tc) and in the absence (-Tc) of (4µg/ml) tetracycline were stained with JC-1 and analyzed by flow cytometry. HepG2 transfectants with intact mitochondria displayed high red and high green fluorescence and appeared in the upper right quadrant of each scatter plot. In contrast, cells that showed a lower mitochondrial membrane potential displayed high green fluorescence and low red fluorescence and appeared in the lower right quadrant. Data are representative of three independent experiments.



sients cultured in the presence and in the absence of tetracycline (4µg/) were stained with anti-Tom20 antibody (mitochondria) and co-stained with anti- NS4A. Data obtained from confocal laser scanning microscopy confirmed the localization of NS4A protein to mitochondria (Figure 3A). In addition, we confirmed further, the localization of NS4A to the mitochondria by Western blot analysis using mitochondrial fractions prepared from HepG2-NS4A and HepG2-NS4A/core transfectants cultured in the presence (+Tc) and in the absence (-Tc) of tetracycline for 48h. Data obtained from Western blot analysis using anti-NS4A or anti-Tom20 antibodies (Figure 3B) demonstrated the expression of Tom20 in all mitochondrial fractions obtained from either HepG2-NS4A or HepG2-Core/NS4A transfectants cultured in presence and in absence of tetracycline. In contrast, the detection of NS4A protein was noted only in the mitochondrial fractions obtained from either HepG2-NS4A or HepG2-core/NS4A transfectants cultured in the absence of tetracycline. These data confirm the localization of HCV NS4A protein to the mitochondria and suggest further an important role for the mitochondria in the modulation of NS4A-induced cell growth inhibition. Based on the subcellular localization of NS4A protein to mitochondria, we hypothesized that NS4A may cause the loss of mitochondrial membrane potential and subsequently lead to mitochondrial dysfunction. The HepG2-core, HepG2-NS4A and HepG2-core/NS4A transfectants were allowed to grow in the presence or in the absence of tetracycline (4µg/ml). Forty eight hours later, the cells were subjected to flow cytometry analysis for the measurement of mitochondrial membrane potential ( $\Delta\Psi_m$ ) using JC-1. Data obtained from flow cytometry analysis (Figure 3C) demonstrated the loss of mitochondrial membrane potential in response to the expression of NS4A protein in HepG2-NS4A transfectants. In contrast, the induction of both NS4A and core proteins in HepG2-core/NS4A transfectants was found to block NS4A-induced loss of mitochondrial membrane potential in HepG2 cells (Figure 3C). Whereas, in HepG2-core transfectants no alteration was noted in response to the expression of core protein. Taken together, these results suggest an essential role for the mitochondrial pathway in the modulation of NS4A-induced cell growth inhibition of HepG2 cells and subsequently the inhibition of core protein-induced cell growth.

## DISCUSSION

In this study, we demonstrated the ability of NS4A protein to inhibit core protein-induced cell

growth in HepG2 cells and specified the role of mitochondria in the modulation of this event. Many viruses, including HCV, possess viral proteins that either promote or inhibit cell death. Among HCV proteins, core (10,20), NS3 (14,21) and NS5A (22,23) have been reported to enhance cell growth rate or to possess antiapoptotic functions. Also, there are reports showing that core (24-27), E1 (28,29), NS3 (30), NS4A (14,31), NS5A and NS5B (32) either cell growth inhibition or possess proapoptotic function. NS4A is known to localize in the endoplasmic reticulum (ER) (33,34) and to accumulate on the mitochondria, and subsequently leads to the loss of mitochondrial membrane potential (31). In this study, we confirmed also the localization of NS4A protein to the mitochondria and demonstrated further the loss of mitochondrial membrane potential. However, the loss of mitochondrial membrane potential together with the inhibition of NS4A protein-induced growth rate in HepG2-NS4A transfectants suggests an important role for the mitochondria in modulation of NS4A-induced cell growth inhibition of HepG2 cells. Interestingly, the induction of both core and NS4A protein expression in HepG2-core/NS4A transfectants was found to block both NS4A-induced loss of mitochondrial membrane potential observed in HepG2-NS4A transfectants and cell growth advantage induced by the expression of core protein. Our results showed that NS4A-induced cell growth inhibition and loss of mitochondrial membrane potential were eliminated by the expression of core protein. However, it should be emphasized, that the mitochondrial morphology and intracellular localization were altered by the expression of both core and NS4A proteins in HepG2-core/NS4A transfectants. These results imply the possibility that both core and NS4A proteins, after being transported to mitochondria, exert a significant effect on the function of mitochondria without affecting mitochondrial membrane potential. Previously, the detection of core protein on rough ER surrounding the mitochondria was reported in both core protein-transfected liver cell lines and in core protein-transgenic mice (35) as well as in direct association with mitochondria (36-38). Also, this association has been observed in core protein-transgenic mice (37,39) and was linked to both mitochondrial injury and oxidative stress. Our data collectively in agreement with a recent study (36) demonstrate that core protein localizes to the surfaces of mitochondria via its mitochondrial targeting motif and thereby reduces the binding of NS4A to the mitochondria and subsequently blocks NS4A-induced mitochondrial damage. On

the other hand, the accumulation of NS4A protein on the mitochondria leads to the induction of mitochondrial damage that finally influences negatively

the cell functions and subsequently inhibit core protein induced cell growth advantage in HepG2 cells.

## CONCLUSION

In conclusion, our present data imply the possibility that NS4A protein is responsible at least, in part, for the inhibition of Core protein-induced proliferation of hepatocytes in HCV infected patients.

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