Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Shiyin Jiao April 10, 2014

The Effects of HCV Core Protein on Mitochondrial Respiration

by

Shiyin Jiao

William Lewis, M.D.
Adviser

Department of Biology

William Lewis, M.D.
Adviser

Ronald L. Calabrese, Ph.D.
Committee Member

Rachelle Spell, Ph.D.
Committee Member

2014

The Effects of HCV Core Protein on Mitochondrial Respiration

By

Shiyin Jiao

William Lewis, M.D.

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2014

Abstract

The Effects of HCV Core Protein on Mitochondrial Respiration

By Shiyin Jiao

Hepatitis C virus (HCV) infection is one of the major causes of chronic hepatitis, which may contribute to cirrhosis, culminating in liver failure and hepatocellular carcinoma. According to the World Health Organization (WHO), more than 170 million people worldwide are estimated to be affected. Many promising antiviral drugs are under investigation, but the mechanisms of pathogenesis have not been completely understood. The most common regimens of treatment have not reached a stably high viral response, whereas newly approved drugs are prohibitively expensive, and no vaccine against HCV is currently available. Therefore, new therapeutic approaches to treat HCV infection would be greatly beneficial. The HCV is an enveloped positive-stranded RNA virus. HCV genome encodes a polyprotein precursor of 3012 amino acid residues, which is then processed by cellular and viral proteases to produce 10 active proteins for viral replication and assembling. These include structural proteins Core, E1 and E2, 6 nonstructural proteins, and p7. The HCV Core gene is highly conserved, and previous studies have shown that the Core protein is involved in various cellular processes, including cell proliferation and apoptosis. It performs specific functions upon localizing to important organelles such as the nucleus, ER, and mitochondria. However, the effects and mechanisms of the Core protein on mitochondrial functions are not clear. In this project, I constructed the HCV Core gene with a

mitochondrial targeting sequence, cloned into a CMV plasmid vector, and transformed into bacteria cells. The amplified recombinant plasmids were then transfected into human HepG2 cells, translated into recombinant Core protein, and translocate to the mitochondria. Measurements of mitochondrial respiration in intact cells show that cells expressing the Core protein have higher rates of basal respiration, ATP turnover, maximal respiration, and spare respiratory capacity. These observations are not evidence of mitochondrial dysfunction as reported by others, but I propose that the cells may have evolved mechanisms to cope with the stress imposed by the Core protein, and there is selection for cells with higher mitochondrial respiration efficiency and capacity. These results provide new insight into the pathogenesis of hepatitis C, and further investigation is required to confirm my hypothesis.

The Effects of HCV Core Protein on Mitochondrial Respiration

By

Shiyin Jiao

William Lewis, M.D.

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Biology

2014

Acknowledgements

This project was supported by NIDA (NIH/DHHS) grant 1RO1DA030996 to Dr. William Lewis in the Department of Pathology and Laboratory Medicine at Emory University School of Medicine. I would like to thank my advisor Dr. Lewis for providing guidance and support throughout my project over the course of two years, approving my work and acknowledging my progress. I also appreciate the entire Lewis Lab for their kindly help offered to me as a young undergraduate student in this scientific endeavor. I owe my great gratitude to my academic advisor Dr. Ronald Calabrese and my previous course instructor Dr. Rachelle Spell for serving on my thesis committee without hesitation, and providing helpful suggestions and valuable insights into my project, as well as my academic career. I am very fortunate to be a student in the Biology Department of Emory University, where I have been offered wonderful opportunities to enrich my experience and make progress toward my goals. Finally, I am deeply grateful to my family and friends for their love and unconditional support throughout my college years.

Table of Contents

Introduction

Hepatitis C virus (HCV) infection is one of the major causes of chronic hepatitis, which may contribute to cirrhosis, liver failure and hepatocellular carcinoma. According to the World Health Organization, more than 170 million people worldwide are estimated to be affected . HCV infection has been identified as the most common cause of liver transplantation in adults . Although not as deadly as other hepatitis viruses, the greater prevalence of HCV infection poses a serious public health problem and has stimulated research in this field. Many promising antiviral drugs including small-molecule protease inhibitors, RNA polymerase inhibitor, and nucleotide inhibitors are under investigation, but since HCV pathogenesis is a very complex phenomenon, many efforts are needed to achieve a satisfactory viral response . Treatment with pegylated interferon (PegIFN) combined with ribavirin (Rbv) is currently the most common regimen, which was considered the only option for end stage infection , but only 40%-50% of genotype-1 patients and 80% of genotype-2 or -3 patients achieved a sustained virus response . Newly approved drugs are prohibitively expensive, taking Sofosbuvir as an example, which costs €3500 per week of treatment . No vaccine against HCV is currently available because of a lack of suitable cell culture model and the high variability of HCV genotypes . Therefore, new therapeutic approaches to treat HCV infection would be greatly beneficial.

HCV is an enveloped positive-stranded RNA virus, as shown in Figure 1 (Anzola and Burgos 2003). HCV genome encodes a polyprotein precursor of

approximately 3000 amino acid residues, which is then processed by cellular and viral proteases to produce 10 active proteins for viral replication and assembling. These include structural proteins Core, E1 and E2, 6 nonstructural proteins, and p7 . The HCV Core gene is highly conserved across different genotypes, making it an ideal candidate to be included in a vaccine . Previous studies showed that the Core protein is involved in various cellular processes, including cell proliferation and apoptosis . It has been found to perform specific functions upon localizing to important organelles such as the ER membrane, the surface of lipid droplets , the mitochondrial membrane , as well as the nucleus . It was proposed that the Core protein traffics from the ER to both the mitochondria and lipid droplets via membrane bridges, and the targeting of Core to the mitochondria is regulated by an AA sequence in Core . Within the mitochondria, the expression of Core protein could lead to inhibition of electron transport at complex I, increased reactive oxygen species production, and decreased mitochondrial glutathione . However, exact mechanisms for the Core protein disturbing the mitochondria have not been clearly elucidated.

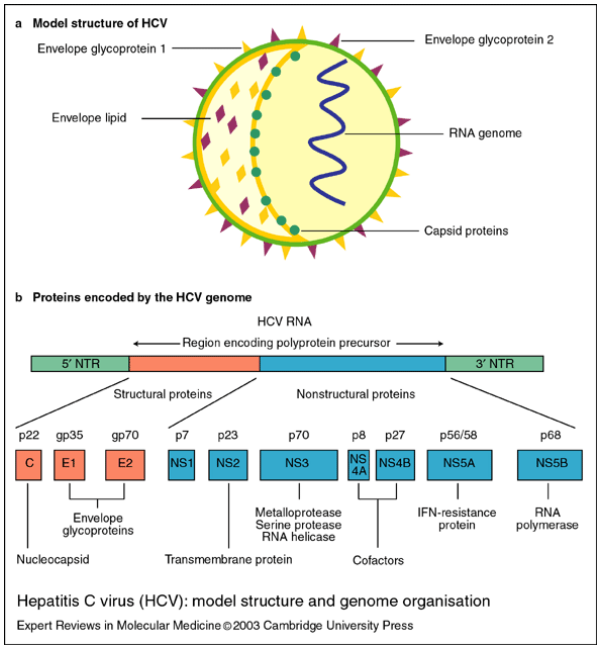

Figure 1. Viral structure and genome composition of HCV (Anzola and Burgos 2003)

The mitochondria play a crucial role in cellular metabolism, and mitochondrial dysfunction is a common factor in a wide range of degenerative and metabolic diseases . Four major functions performed by the mitochondria that could potentially be affected by disease pathogenesis are: a) ATP production, b) reactive oxygen species generation, c) cytosolic calcium ion regulation, and d) apoptosis regulation. ATP production in the mitochondria occurs through oxidative phosphorylation at the electron transport chain in the inner mitochondrial membrane, which consists of five complexes. As electrons are transferred down the ETC, energy is released and used to pump protons out across the mitochondrial inner membrane through complexes I, III, and IV. Potential energy from this proton gradient is used by ATP synthase (complex V) to convert ADP to ATP . Oxygen molecule is the final electron acceptor, which gets reduced to H_2O at complex IV .

The oxygen consumption rate directly correlates to the efficiency of mitochondrial function, and thus can be monitored to show changes in mitochondrial respiration in the face of viral infection. Blocking specific sites of the ETC by a chemical can alter oxygen consumption rate to different levels, from which we can potentially determine which part of the ETC is affected by the introduced viral protein.

Materials and Methods

Part I. Cloning and Expression of HCV Core Protein in HepG2 Cells

PCR Amplification. PCR was performed to amplify the HCV Core gene using a Platinum Pfx DNA polymerase. An HCV plasmid containing all the HCV genes was used as the template. PCR primers were designed to add the mitochondrial targeting sequence from ornithine transcarbamylase to the N-terminus of the HCV Core gene as well as an HA tag to the C-terminus region. Recognition sites of restriction enzymes (*EcoR*V and *Xho*I) and the necessary components for gene expression were also added to the gene. Gel Electrophoresis was performed to examine the PCR product on a 1% agarose gel, stained by ethidium bromide and visualized under UV light.

Gel Extraction. The PCR product of 5 reactions containing the same mixture was run on a 1% gel. The DNA band was excised from the agarose gel under UV light and DNA was extracted using the QIAEX II Gel Extraction Kit. The DNA concentration was determined using the 260 nm absorbance.

Restriction Digest and Ligation. The extracted MTS-HCV Core DNA and the pCMV-Script Vector (Agilent) were digested for one hour separately using *EcoR*V and *Xho*I restriction enzymes (Thermo Scientific). The restricted products were ligated using T4 DNA Ligase, at two experimental ratios of insert to vector (5:1 and 10:1).

Transformation. The ligation mixture was transformed into competent D5α *E. coli* cells and diluted 10-fold in SOC media. 100 µL of each dilution were spread onto LB-agar plates containing 50 µg/mL kanamycin.

Colony Selection. Colonies were isolated and PCR genotyped using primers designed to amplify regions on the CMV vector that flank the inserted MTS-HCV Core gene. PCR amplicons with an approximate 600 base-pair band shift are considered positive for the MTS-HCV Core plasmid.

Plasmid Extraction. The positive clones were cultured overnight in LB medium, and the recombinant plasmid was extracted using the QIAprep Spin Miniprep Kit, and confirmed by restriction enzyme digestion and PCR. The CMV vector plasmid was also extracted from overnight cultures of bacteria glycerol stock, and verified by PCR using specifically designed primers.

Plasmid Sequencing. Samples of pCMV + MTS + HCV Core plasmid and 4 primers that are about 600 bp apart from each other were sent to Beckman Coulter

Genomics for sequencing reactions. The sequencing data were sent back and the obtained 4 sequences were aligned with the designed sequence using the MegAlign program. The clone that aligned properly to the reference sequence was chosen for subsequent experiments.

Transfection. HepG2 cells were seeded into six p100 dishes. X-tremeGENE 9 DNA Transfection Reagent (Roche) was mixed with serum free media, and same amount of Vector and MTS-Core plasmid DNAs were each added to two sets of transfection reagent, at two ratios of transfection reagent to plasmid DNA (3:1 and 6:1). Incubated transfection complexes were added to the cells in a dropwise manner, and after incubation and switching to media containing G418, the cells were split into new p100 dishes at a dilution of 1:50.

Cell Culture and Plasmid DNA Verification. Cells were trypsinized once a week and media were changed every other day. At the time cell colonies were visible, big colonies were selected using cloning cylinders, trypsinized, and added to G418 media in 6-well plates for better growth. After 2 weeks of growth, DNA was isolated from samples of the transfected cells using DNA lysis buffer and ethanol precipitation, and the plasmid taken up by the cells was verified by PCR using primers specifically designed for each insert.

SDS-PAGE, Western Blotting, and Protein Detection. Cells with pCMV vector and cells with MTS-Core clones were each lysed using RIPA buffer (Sigma-Aldrich) to

obtain the nuclear and mitochondrial fractions. Protein concentration of each sample was determined by Bradford Protein Assay. Following a standard western blot protocol, 20 μg of each sample were run on a pre-made polyacrylamide gel, electrophoretically transferred to a membrane, and incubated overnight in a blocking solution of 5% non-fat dried milk and 0.1% Tween 20. Primary and secondary anti-SHDB (mitochondrial marker, Novus Biologicals, dilution of 1:5000) and anti-HA antibodies (MTS-Core marker, Thermo Scientific, dilution of 1:500) were added, and West Pico Chemiluminescent Substrate (Thermo Scientific) was used for protein detection. The membrane was exposed for 15 minutes on a film.

Part II. Measurement of Mitochondrial Respiration

Seahorse Analysis. To measure mitochondrial respiration rates, an XF24 Analyzer from Seahorse Bioscience was used. Cells transfected with Vector, MTS-Core Clone #1 and #2 were seeded in a 24-well plate at the density of 30,000 cells/well, and incubated for 72 hours. Oligomycin, FCCP, and Rotenone were added to the cells through a pre-hydrated flux kit at designated times, and oxygen consumption rates were measured about every 8 minutes. The data were collected in an Excel sheet and represented in graphs. Cell numbers were counted again upon completion of the assay to exclude cell density as a factor.

Results

PCR Amplification and Cloning

Synthesized HCV Core gene was amplified by PCR using designed primers that successfully added a mitochondrial targeting sequence (MTS) to the N-terminus of the Core gene, and an HA tag intended for protein detection to the C-terminus. The Kozak sequence was added since it is required by the ribosome to initiate translation (De Angioletti, et al. 2004). Recognition sites of *EcoR*V and *Xho*I enabled subsequent ligation to a CMV vector plasmid. A diagram of the PCR product is shown in Figure 2. A temperature gradient was used to optimize the reaction (Figure 3), and 5 PCR products annealed at the optimized temperature (68°C) were subjected to gel electrophoresis and extracted. Ethanol precipitation of the DNA resulted in a concentration of 49.1 µg/mL.

Figure 2. Construct of MTS-HCV Core gene. MTS is derived from ornithine transcarbamylase, which directs mitochondrial import across both the outer and inner membranes. The Kozak sequence plays a major role in the initiation of the translation process. Recognition sites of restriction enzymes *EcoR*V and *Xho*I are intended for ligation to a vector plasmid.

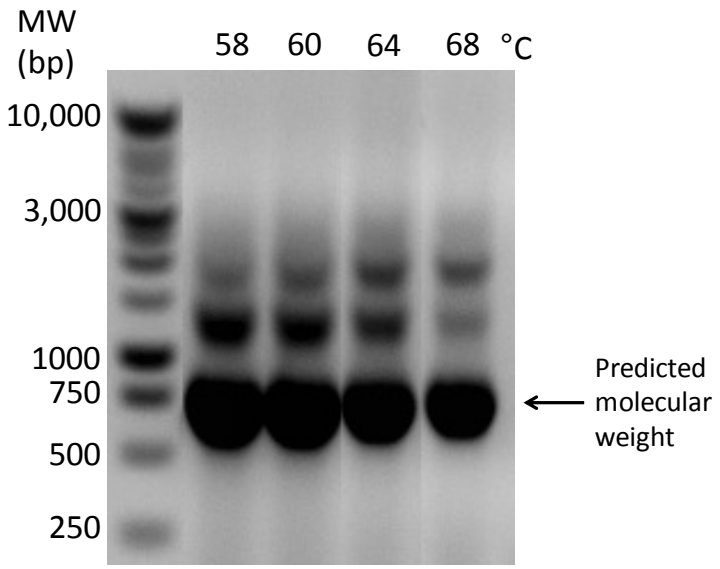

Figure 3. Gel electrophoresis of PCR products amplified from HCV Core gene. A temperature gradient – 58, 60, 64, and 68 °C – was used to obtain the optimal annealing temperature. The predicted molecular weight of the PCR product is about 600 base pairs, while the other bands of longer molecular weight are non-specific bands.

The obtained MTS-HCV Core DNA and pCMV vector were restriction digested and ligated to each other, forming a recombinant plasmid as shown in Figure 4 A. The restriction enzymes *EcoR*V and *Xho*I were selected from those present in the pCMV-Script multiple cloning site region, because of their high fidelity and absence of restriction sites in the MTS, HCV Core, and HA tag. The recombinant plasmid was transformed into competent D5α *E. coli* cells and grown on LB-agar plates containing kanamycin. Only those cells that were able to grow colonies had successfully taken up the recombinant plasmid, which contains the kanamycin resistance gene.

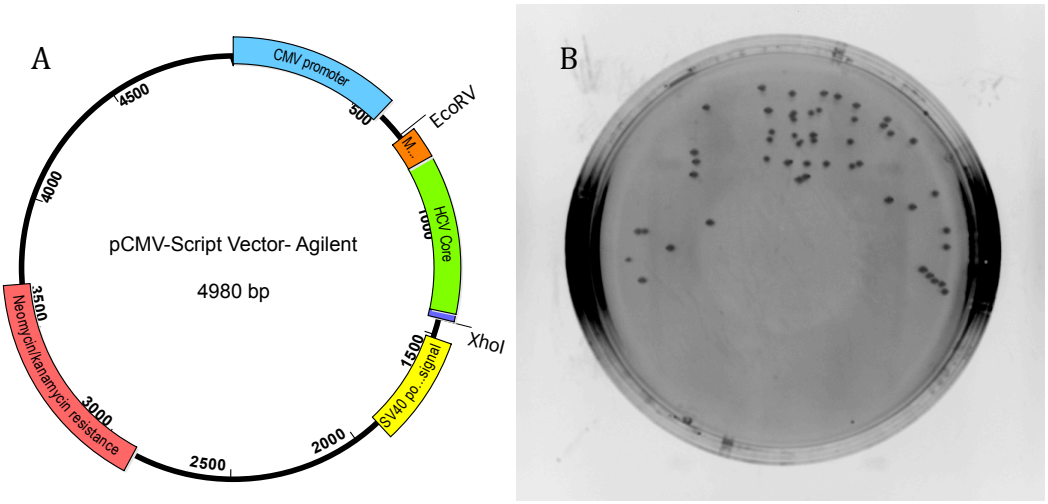

Figure 4. (A) Recombinant plasmid CMV-MTS-Core. PCR amplified MTS-HCV Core DNA was extracted from agarose gel, restriction digested by *EcoR*V and *Xho*I, and ligated to pCMV vector. **(B) Transformation of the recombinant plasmid into competent *E. coli* cells.** Cells were selected for on plates containing kanamycin.

PCR genotyping of 51 bacteria colonies identified 13 positive clones with plasmid containing an MTS-Core insert (Figure 5). Plasmid was extracted from overnight cultures of the positive clones and verified by enzyme digestion and PCR. For a negative control, CMV vector plasmid with no insert was extracted from overnight cultures of bacteria glycerol stock and verified by PCR as well.

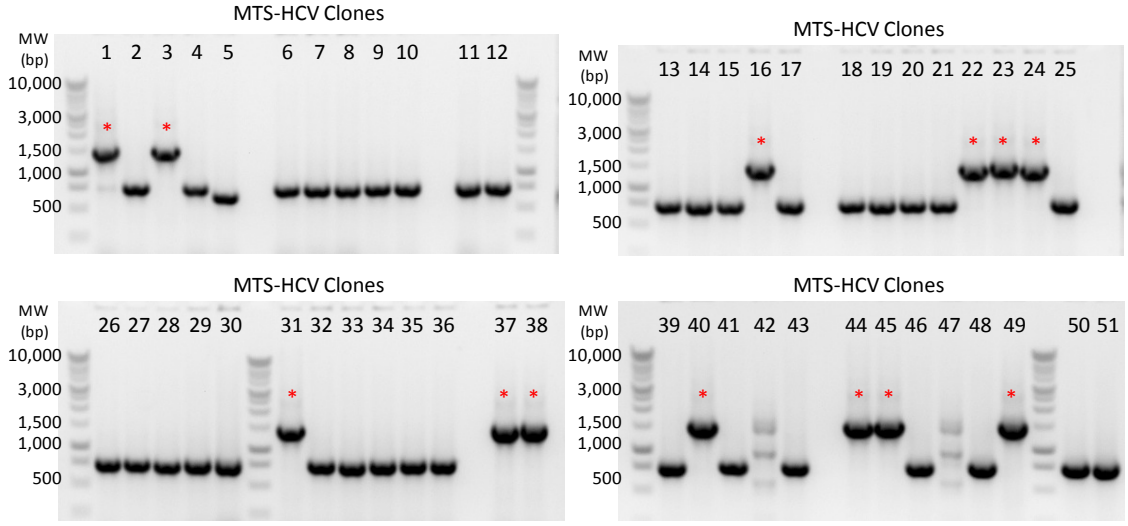

Figure 5. PCR genotyping selecting for clones transformed with MTS-Core plasmid. Amplicons with a band shift of about 700bp were identified as positive clones, labeled *.

Sequencing data from Beckman Coulter Genomics were retrieved, and the four individual sequences obtained were aligned to the designed CMV-MTS-Core sequence using the MegAlign program. Clone #40 in Figure 5 turned out to align perfectly to the reference sequence without any gaps. The verified CMV-MTS-Core plasmid as well as the CMV vector plasmid were each transfected into HepG2 cells and grown into 3 pure cell populations, 2 transfections for the MTS-Core, and 1 for the vector.

Presence of Core Protein in HepG2 Cells

To test for expression of HCV Core protein in the mitochondria of HepG2 cells, western blot analysis was performed on nuclear and mitochondrial fractions (Figure 6). SDHB (succinate dehydrogenase iron-sulfur subunit) is a protein that is only expressed in the mitochondria, and is thus anti-SDHB antibody was used as a mitochondrial marker. Anti-HA antibody would only bind to HA-tagged MTS-Core protein. Due to failure in loading the CMV vector cell nuclear fraction into the first lane of the gel, the presence of SDHB or HA protein in the mitochondria of vector cells cannot be determined. Both SDHB as a mitochondrial marker and HA as an MTS-Core marker were detected in the nucleus of MTS-Core cells, which contradicts with my expectation of an absence of SDHB in the nucleus. In addition, no SHDB or HA protein was detected in the mitochondrial fraction of either vector or MTS-Core cells, indicating that the nuclear fractions were most likely whole cell lysates resulting from unsuccessful subcellular fractionation. Regardless, it is confirmed that the Core protein was successfully expressed in HepG2 cells transfected with the

CMV-MTS-Core plasmid, in an amount that was detectable by immunoblotting. Intensity of the protein bands indicates that expression level of HCV Core protein in MTS-Core Clone #2 was higher than that in MTS-Core Clone #1.

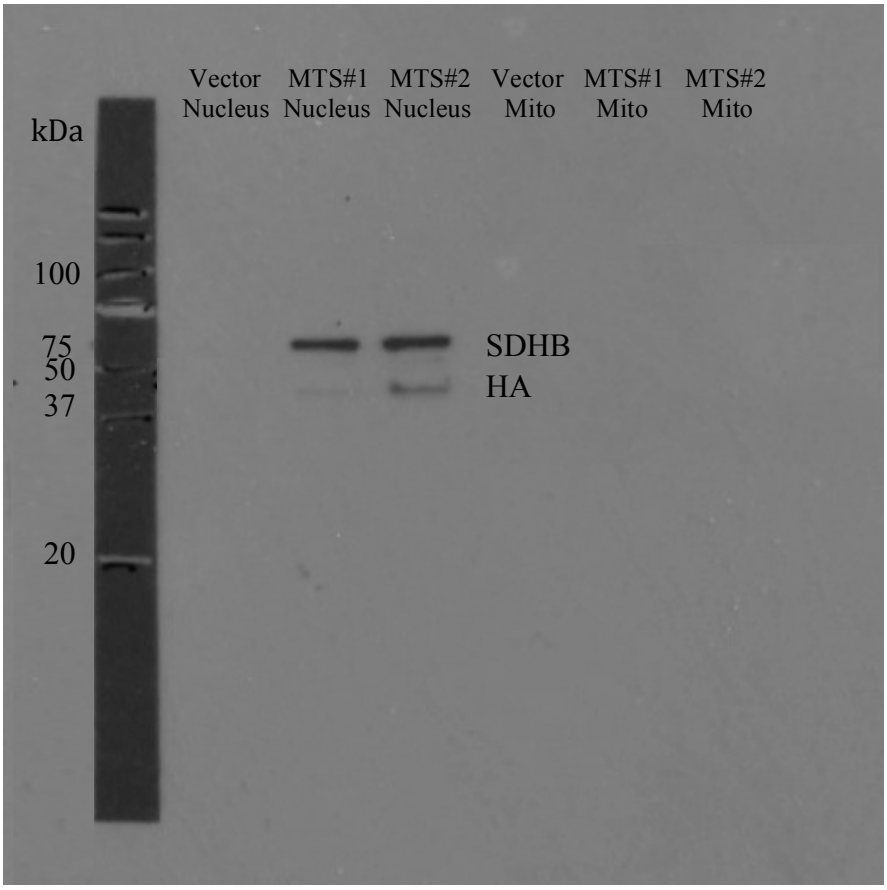

Figure 6. Expression of HCV Core protein in HepG2 cells. Western blot was performed on nuclear and mitochondrial fractions extracted from Vector, MTS-Core Clone #1, and MTS-Core Clone #2 cells. 20 μg of protein were loaded into each well. Most of the sample in the first lane was lost in the loading process. SDHB and HA proteins were both detected in the nuclear fractions of MTS-Core clones, but not in the mitochondrial fractions. Expression level of HCV Core protein was higher in MTS-Core Clone #2 than Clone #1. The observed band sizes for anti-SDHB and anti-HA are both longer than the predicted molecular weight, 32kDa for SDHB and 26kDA for MTS-Core-HA. Post-translational modification could be a possible reason for this inconsistency.

Effects of HCV Core Protein on Mitochondrial Respiration

To assess mitochondrial respiration levels in the three HepG2 cell-populations, I used an XF24 analyzer from Seahorse Bioscience, which allowed direct measurement of the rate of change of O_2 and pH in the media immediately surrounding living cells cultured in a microplate. *In vivo* assessment provides the advantage of maintaining a normal environment for the mitochondria that is close to the inside of human liver, which is of great physiological relevance . In the cell respiratory control experiment, identical number of cells for the 3 populations – Vector, MTS-Core #1, and MTS-Core #2 – were seeded in 5 wells for each and grown in complete media for 72 hours. From the beginning, oxygen consumption was measured every ~8 minutes, while cells were exposed sequentially to oligomycin, FCCP, and rotenone. A graphical view of the oxygen consumption rate (OCR) change as the experiment proceeded is shown as in Figure 7. From the measured OCRs at some time points, eight parameters can be further determined – basal respiration, ATP turnover, proton leak, coupling efficiency, maximum respiration rate, apparent respiratory control ratio, spare respiratory capacity and non-mitochondrial respiration. As we can see in the graph, cells expressing HCV Core protein have a higher OCR than cells with vector only (negative control) at the starting point. Prior to any drug treatment, the baseline respiration rate moderately decreases in time for all three cell-populations, suggesting that mitochondria slows down oxygen uptake as nutrients in the media decreases. Addition of oligomycin to the cells causes an abrupt decrease in OCR, resulting from the block of the proton channel of the ATP synthase (Complex V), which cuts down oxygen reduction at Complex IV

though a negative feedback mechanism. FCCP acts as a protonophoric uncoupler that disrupts the H^+ gradient by transporting H^+ across the inner mitochondrial membrane back to the intermembrane space. Thus the cells must overcome this additional counteracting force by consuming significantly more oxygen to pump the excess protons, which gives rise to the sharp increase of OCR. Adding rotenone to the cells inhibits the transfer of electrons from iron-sulfur centers in Complex I to ubiquinone, which stops the flow of electrons in the Electron Transport Chain, leading to a drastic drop in OCR. The major shifts in OCR due to drug treatment are very similar in trend for all three cell-populations, but cells expressing HCV Core protein have a higher respiration level than the negative control (vector) throughout the experiment. Upon completion of the assay, samples of the three cells populations were collected and cell numbers were counted again, which turned out to be approximately equal, ruling out cell density as a confounding factor.

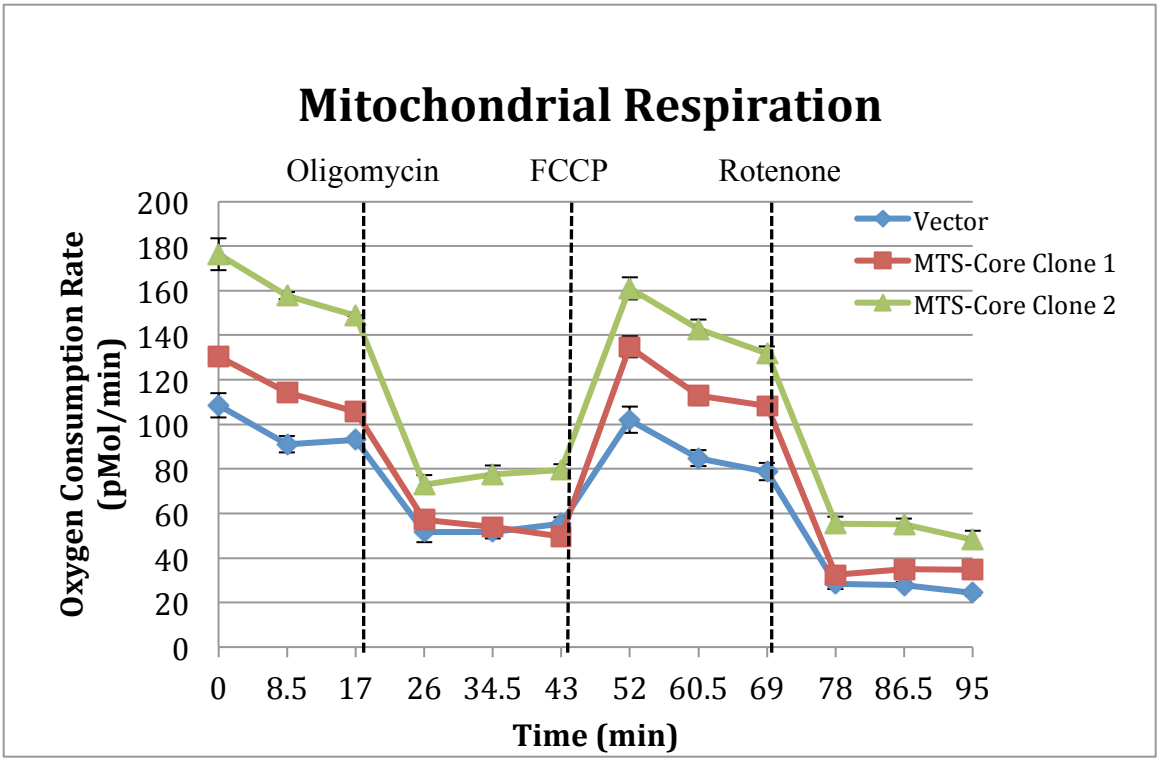

Figure 7. Change in oxygen consumption rate (OCR) as the cell respiration control experiment proceeds. OCR measurement was made for cells in each of 5 wells, and one data point represents the average of the 5 measurements ±SEM. Following a period of baseline respiration, oligomycin (ATP synthase inhibitor), FCCP (uncoupling agent) and rotenone (electron transport inhibitor) were added to the cells sequentially.

Discussion

The expression of HCV Core protein in the mitochondria has been reported to interfere with the Electron Transport Chain, especially causing an increase in ROS production at complex I (Korenaga, 2005) . Using a transgenic mouse model, the Korenaga group has shown the effects of the Core protein on the mitochondria by directly interacting a recombinant Core protein with isolated mitochondria from mouse liver. Santolini *et al*. investigated the biosynthesis and properties of the Core protein and found that it binds to the ER in vitro and in transfected cells (1994).

Examined by confocal laser scanning microscopy, the Core protein was also seen in the nucleus . In this project, I developed a simple model system that allows me to study the effects of the Core protein exclusively on the mitochondria of human hepatocytes. Instead of direct incubation of isolated mitochondria with HCV Core protein, I transfected human HepG2 cells with a recombinant HCV Core plasmid containing an MTS, and utilized a newly developed technology to quantify mitochondrial function in intact cells. This new method offers the advantage of avoiding artifacts associated with cell lysis and mitochondrial isolation prior to performing assays, and thus is of greater physiological relevance, which is important for disease studies.

At many points throughout the long process of PCR amplification, cloning, plasmid transformation and transfection, the constructed CMV-MTS-HCV Core sequence with other necessary components was verified to make sure that the expressed Core protein could be properly targeted to the mitochondria of HepG2 cells. The mitochondrial matrix enzyme ornithine transcarbamylase, from which the MTS was derived, has long been reported to translocate across both the outer and inner mitochondrial membranes . As for the Core protein itself, there is no conclusive proof of whether it behaves as an outer membrane associated protein, or enters the mitochondria and interacts with components of the ETC, or both. With the addition of an MTS, there is still some uncertainty about the translocation of the Core protein into the mitochondria. Western blot shows the coexistence of the mitochondrial protein SDHB and the HA-tagged MTS-Core protein, in my MTS-Core Clone #1 and #2. Although there is not enough evidence that a successful

translocation of the Core protein to the mitochondria because SDHB was not detected in the mitochondrial fraction, the Core protein is still very likely to have been expressed in the mitochondria, but further tests would be necessary to demonstrate this. It is also notable that the HA-tagged protein band in MTS-Core Clone #2 is darker than that in Clone #1, indicating two different expression levels, which can be correlated with their mitochondrial respiration levels.

A representative cell respiratory control experiment is shown in Figure 8, which is obtained from Seahorse Bioscience official website. Compared to this standard curve, my results in Figure 7 follow a similar trend, but the maximal respiration rates for all three cell-populations are relative low, hardly leaving any space for spare respiratory capacity. It is possible that the cells were in a less than ideal condition, perhaps due to the selection by the antibiotic G418 in the cell culture media, and the mitochondria could not produce extra ATP to overcome stress from the uncoupling agent FCCP.

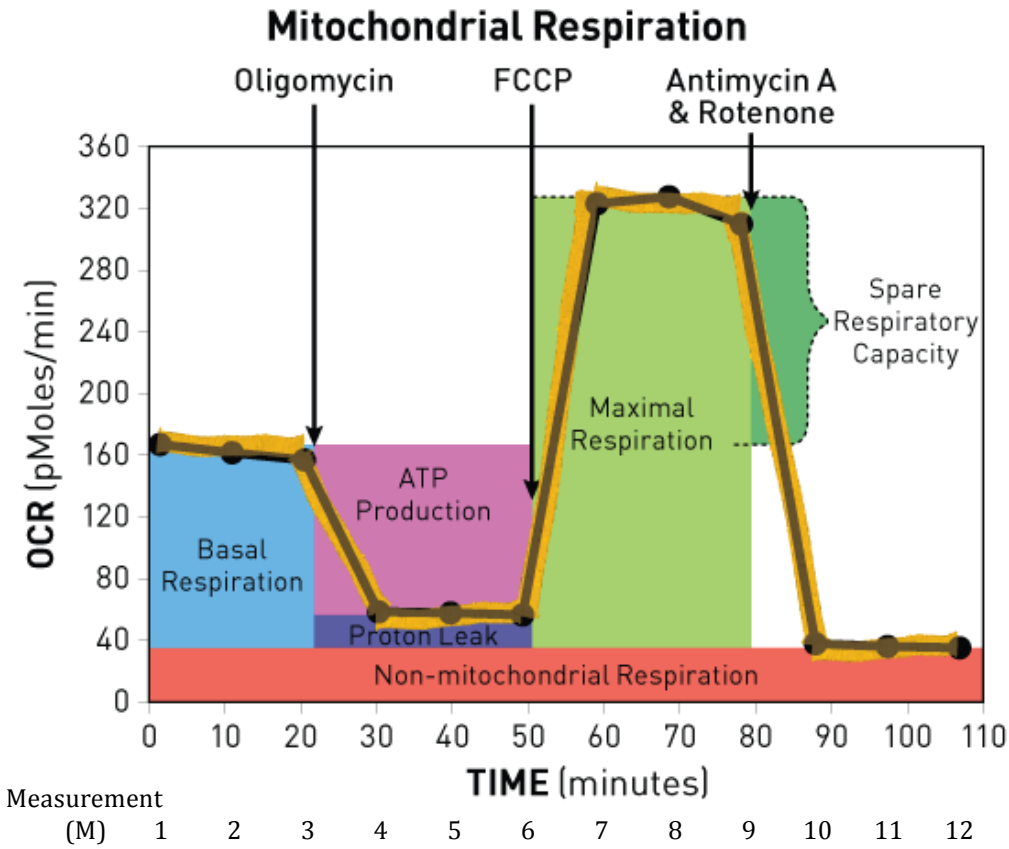

Figure 8. A representative cell respiratory control experiment. 12 measurements of OCR are made over a course of about 100 minutes. Oligomycin is added immediately after the 3rd measurement, FCCP after the 6th, Rotenone after the 9th. Five parameters can be determined as differences between two measurements, as shown in color blocks (XF Cell Mito Stress Test Profile 2013).

For a more detailed view, Figure 9 shows the inhibitory effects of the three drugs used in the experiment on specific ETC components. By the sequential additions of the three drugs to the cells causing OCR shifts, various respiration rates can be determined, and shown as column graphs in Figure 10.

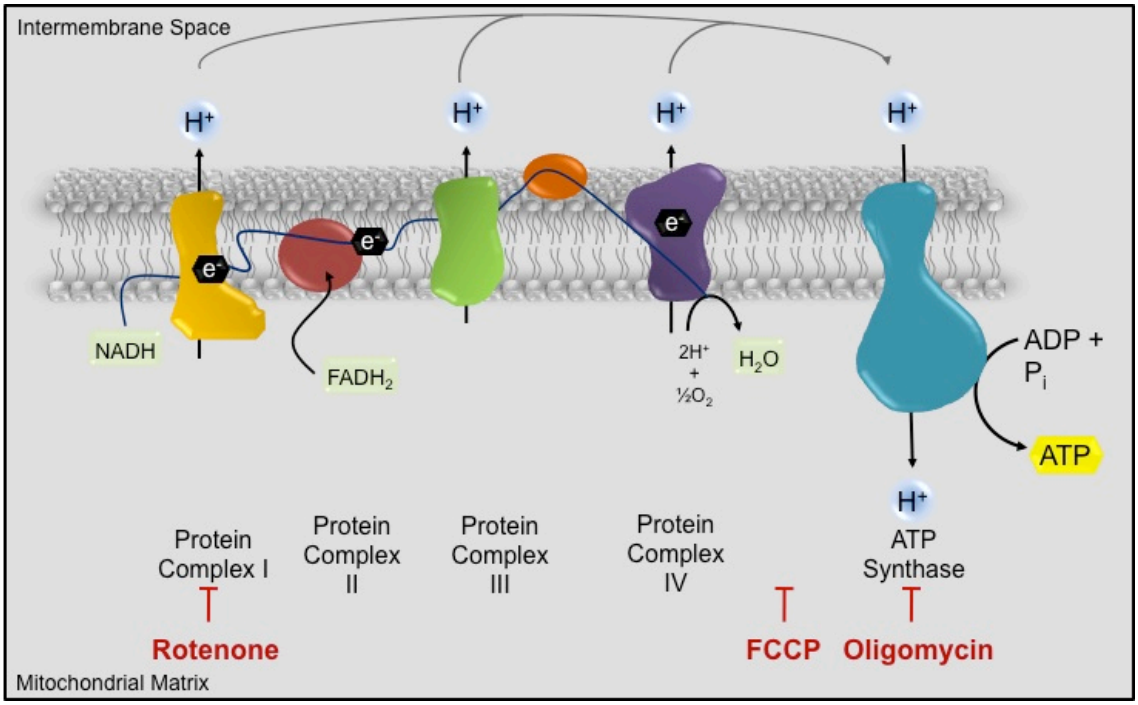

Figure 9. Electron Transport Chain in the mitochondrion and the effects of ETC disrupting drugs. Five Complexes reside in the inner mitochondrial membrane, transporting electrons and protons to drive ATP synthesis. Oligomycin is an inhibitor of ATP Synthase (Complex V). FCCP is an uncoupling agent that transports protons from the intermembrane space back to the mitochondrial matrix, disrupting the proton gradient used for oxidate phosphorylation. Rotenone is an inhibitor of complex I, specifically blocking electron transfer from iron-sulfur centers in Complex I to ubiquinone.

Basal respiration (Figure 10A) is mostly controlled by the need of ATP production and partly by proton leak, as we can see in Figure 8. A change in basal respiration rate usually indicates a change in ATP demand, such as an inhibition of the ATP synthase activity by oligomycin. Cells expressing the MTS-Core protein have a higher basal respiration rate than the negative control. This difference in absolute rate is difficult to interpret because of differences in cell size or mitochondrial density among the three cell-populations. Quantifying mitochondrial DNA would be one way to normalize respiration rates to copy numbers of mtDNA. However, it may be argued that transfection of HCV Core into the cells has stimulated mitochondrial

growth for the benefit of viral protein replication, giving rise to a higher basal respiration rate than cells without the viral protein. MTS-Core Clone #2 shows an even higher level of basal respiration, perhaps because of a higher expression level of the Core protein (Figure 6),

ATP turnover (Figure 10B) can be estimated from the decrease in OCR upon inhibition of the ATP synthase by oligomycin. The relative levels of ATP turnover for the 3 cell-populations are about the same as basal respiration, just on a smaller scale, which agrees with that fact that ATP turnover accounts for the most part of basal respiration.

Proton leak (Figure 10C) is directly measured as the respiration rate in the presence of oligomycin. Since the ATP synthase activity is completely blocked by oligomycin, proton leak would be the only source of energy and thus very sensitive to uncoupling agents but insensitive to a change in ATP demand. MTS-Core clones have an even lower proton leak rate than the negative control, indicating that there is not only no apparent damage to the mitochondria, but on the contrary, a smaller amount of the Core protein (as in Clone #1) might have promoted the mitochondrial efficiency for ATP production.

Coupling efficiency (Figure 10D) is defined as the fraction of basal respiration that is used for ATP production and is thus sensitive to a change in ATP demand. The coupling efficiency of MTS-Core #1 is slightly lower that the other two, but the differences are not significant.

Maximal respiration (Figure 10E) is stimulated by addition of FCCP, an uncoupling agent. The cells must overcome the proton leak across the inner

mitochondrial membrane, and OCR increases drastically as more oxygen is consumed to pump the excess protons back to the intermembrane space. A decrease in maximal respiration rate indicates potential mitochondrial dysfunction. However, interestingly, both MTS-Core clones have a higher maximal respiration rate than the negative control, and clone #2 that expresses more of the Core protein functions slightly better. Since intact cells were used for the assay, the artificial energy demand might not be able to stimulate a high response as it would be in isolated mitochondria, for the cells are likely to have other mechanisms to counteract the effect. But we can propose that expression of the Core protein in HepG2 cells helps to cope with increased energy demand, possibly through an adaptive mechanism that enhances mitochondrial function.

Spare respiratory capacity (Figure 10F) is determined by the difference between maximal and basal respiration, which represents the ability of the cells to respond to a increase in energy demand. A small spare respiratory capacity is an indicator of a mitochondrial dysfunction that might not be as obvious under basal ATP production. MTS-Core #2 and negative control have similarly small spare capacities, but MTS-Core #2 is a little better. Spare capacity of MTS-Core #1 is almost three times as large as the other two, meaning these cells are operating fairly far away from its energy limit. In comparison, MTS-Core #2 has less respiratory capacity to spare when ATP demand increases, and the negative control barely had any spare capacity. These results do not mean that control cells have mitochondrial dysfunction but may well show that cells containing the HCV Core protein have moderate advantage over control cells.

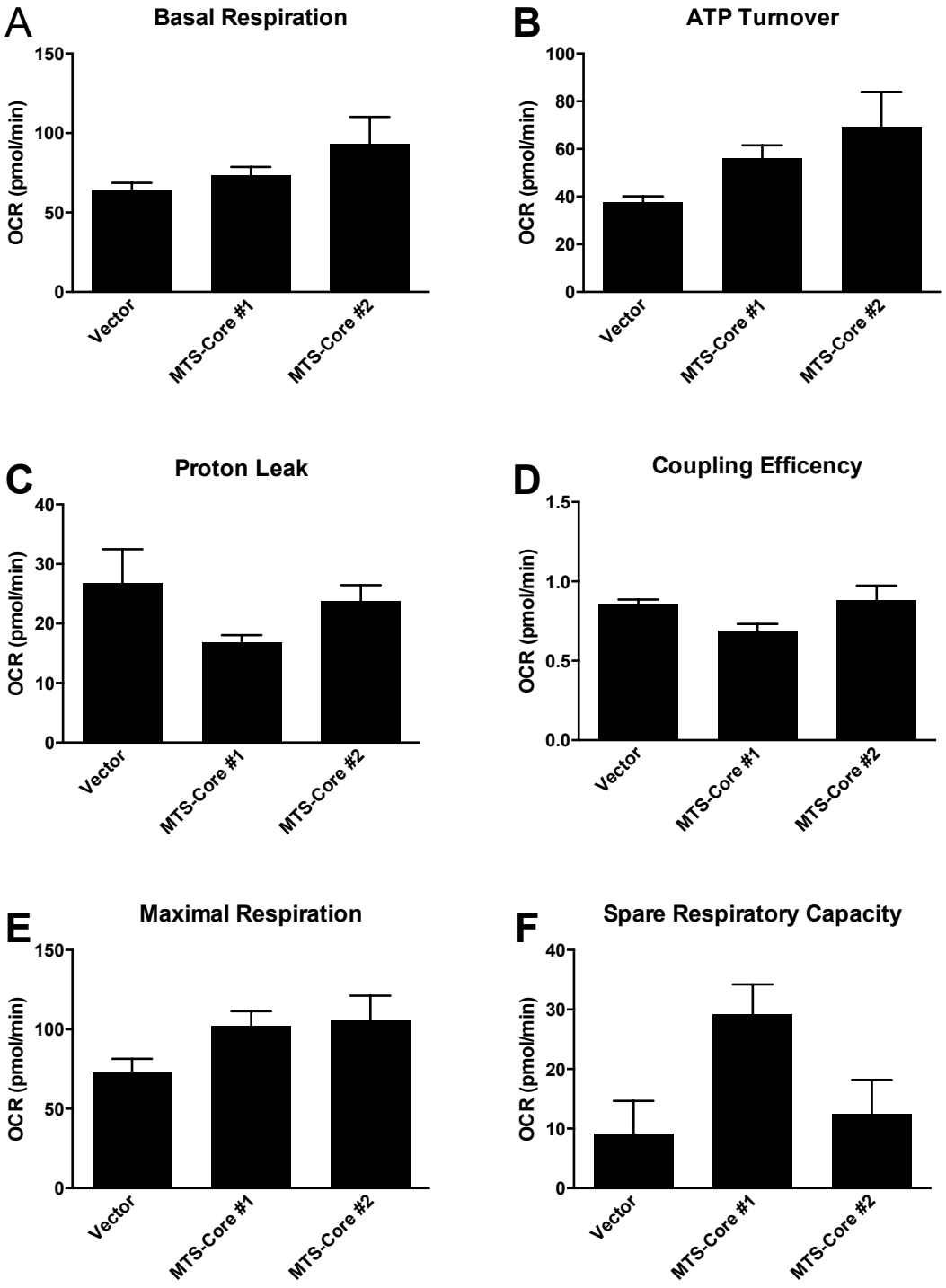

Figure 10. Measurements of oxygen consumption in the cell respiratory control experiment. (A) Basal Respiration = M3–M10. ATP Turnover = M3–M6. (C) Proton Leak = M6–M10. (D) Coupling Efficiency = (M3-M6)/(M3-M20). (E) Maximal Respiration = M7-M10. Spare Respiratory Capacity = M7-M3. Each bar represents the mean of 5 measurements ± SEM.

In conclusion, I successfully developed a model system by amplification and cloning of a recombinant HCV Core plasmid with a mitochondrial targeting sequence, and transfecting the plasmid into HepG2 cells. HCV Core protein expression can be detected, and mitochondrial respiration was assessed using a Seahorse analyzer that has never been used in HCV studies. Compared to the negative control, cells containing the Core protein show higher rates of basal respiration, ATP turnover, maximal respiration, and spare respiratory capacity. Furthermore, a higher expression level of the Core protein seems to correlate with even higher rates of basal respiration, ATP turnover, and maximal respiration. These results are inconsistent with what I expected to be an inhibitory effect of the HCV Core protein on mitochondrial functions. However, most of the results of previous studies come from *in vitro* experiments on isolated mitochondria, lacking interactions with the substrates and other organelles in the cytoplasm as the natural environment for the mitochondria. Using the Seahorse analyzer allows direct measurement of mitochondrial respiration in intact cells, which is of greater physiological relevance. Considering the complexity of the interactions among organelles and molecules surrounding the mitochondria, we are still not able to mimic the complete setting for mitochondrial activities, without using an intact animal or human model.

To confirm or refute the reported negative effects of HCV Core protein on mitochondrial respiration would require many more repeated Seahorse analyses. Based on results shown here, I propose that the expression of HCV Core protein in human hepatocytes may actually select for cells that can better cope with the stress imposed by Core and promote mitochondrial growth for the benefit of viral protein

replication. There have been many examples of viral proteins interacting with mitochondria, altering mitochondrial apoptosis pathways, and modifying host cell compartments to facilitate viral replication and dissemination (Korenaga, 2005). Similarly, HCV Core protein could sometimes promote viral infection by inhibiting apoptosis, preventing clearance of infected cells, and sometimes promoting viral dissemination by accelerating apoptosis. The exact mechanisms of HCV Core protein interacting with the mitochondria to cause differential effects requires further experiments and analyses. On the other hand, ATP production in the mitochondria is essential for so many cellular activities, so most likely the mitochondria have evolved compensatory or feedback mechanisms in response to stress by viral activity. Dysfunctional mitochondria might signal the nucleus to produce more mitochondrial proteins to maintain ATP production, which could cause elevated respiration rates in the long term.

This project has demonstrated successful cloning and expression of a target gene in an appropriate cell model, in which cell respiration rates can be monitored and compared for different cell populations. The study has shed some light on the effects of the highly conserved HCV Core protein on the mitochondria. Further understanding of the mechanisms of Core protein's interactions with the mitochondria calls for additional research efforts, which could possibly lead to new therapeutic approaches to relieve the burden of this widespread chronic disease.

Works Cited

"Global Surveillance and Control of Hepatitis C. Report of a Who Consultation Organized in Collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium." J Viral Hepat 6, no. 1 (1999): 35-47.

"XF Cell Mito Stress Test Profile." Seahorse Bioscience. 2013. http://www.seahorsebio.com/resources/downloads/mito-stress.pdf (accessed March 2014).

Anzola, Mónica, and Juan José Burgos. "Hepatitis C virus (HCV): model structure and genome organisation." Expert Reviews in Molecular Medicine. November 19, 2003. http://journals.cambridge.org/fulltext_content/ERM/ERM5_28/S1462399403006926sup002.htm.

Brand, M. D. and D. G. Nicholls. "Assessing Mitochondrial Dysfunction in Cells." *Biochem J* 435, no. 2 (2011): 297-312.

Colombo, M. "Treatment with Pegylated Interferon (Pegifn) Combined with Ribavirin (Rbv) Is the Only Option for Preventing Hcv-Related End Stage Liver Disease." *J Viral Hepat* 19 Suppl 1, (2012): 1-2.

De Angioletti, Maria, Giuseppina Lacerra, Vincenzo Sabato, and Clementina Carestia. "β+45 G → C: a novel silent β-thalassaemia mutation, the first in the Kozak sequence." British Journal of Haematology 124, no. 2 (January 2004): 224–231.

Hajarizadeh, B., J. Grebely and G. J. Dore. "Epidemiology and Natural History of Hcv Infection." Nat Rev Gastroenterol Hepatol 10, no. 9 (2013): 553-62.

Irshad, M., D. S. Mankotia and K. Irshad. "An Insight into the Diagnosis and Pathogenesis of Hepatitis C Virus Infection." *World J Gastroenterol* 19, no. 44 (2013): 7896-909.

Jones, D. M. and J. McLauchlan. "Hepatitis C Virus: Assembly and Release of Virus Particles." *J Biol Chem* 285, no. 30 (2010): 22733-9.

Khaliq, S., S. Jahan and S. Hassan. "Hepatitis C Virus P7: Molecular Function and Importance in Hepatitis C Virus Life Cycle and Potential Antiviral Target." *Liver Int* 31, no. 5 (2011): 606-17.

Korenaga, M., M. Okuda, K. Otani, T. Wang, Y. Li and S. A. Weinman. "Mitochondrial Dysfunction in Hepatitis C." *J Clin Gastroenterol* 39, no. 4 Suppl 2 (2005): S162-6.

Korenaga, M., T. Wang, Y. Li, L. A. Showalter, T. Chan, J. Sun and S. A. Weinman. "Hepatitis C Virus Core Protein Inhibits Mitochondrial Electron Transport and Increases Reactive Oxygen Species (Ros) Production." *J Biol Chem* 280, no. 45 (2005): 37481-8.

Petta, S., G. Cabibbo, M. Enea, F. S. Macaluso, A. Plaia, R. Bruno, A. Gasbarrini, A. Craxi, C. Camma and W. E. F. Study Group. "Cost-Effectiveness of Sofosbuvir-Based Triple Therapy for Untreated Patients with Genotype 1 Chronic Hepatitis C." *Hepatology*, (2014).

Polyak, S. J., K. C. Klein, I. Shoji, T. Miyamura and J. R. Lingappa. "Assemble and Interact: Pleiotropic Functions of the Hcv Core Protein." In *Hepatitis C Viruses: Genomes and Molecular Biology*, edited by S. L. Tan. Norfolk (UK), 2006.

Santolini, E., G. Migliaccio and N. La Monica. "Biosynthesis and Biochemical Properties of the Hepatitis C Virus Core Protein." *J Virol* 68, no. 6 (1994): 3631-41.

Sarrazin, C., C. Hezode, S. Zeuzem and J. M. Pawlotsky. "Antiviral Strategies in Hepatitis C Virus Infection." *J Hepatol* 56 Suppl 1, (2012): S88-100.

Shi, S. T., S. J. Polyak, H. Tu, D. R. Taylor, D. R. Gretch and M. M. Lai. "Hepatitis C Virus Ns5a Colocalizes with the Core Protein on Lipid Droplets and Interacts with Apolipoproteins." *Virology* 292, no. 2 (2002): 198-210.

Suzuki, R., S. Sakamoto, T. Tsutsumi, A. Rikimaru, K. Tanaka, T. Shimoike, K. Moriishi, T. Iwasaki, K. Mizumoto, Y. Matsuura, T. Miyamura and T. Suzuki. "Molecular Determinants for Subcellular Localization of Hepatitis C Virus Core Protein." *J Virol* 79, no. 2 (2005): 1271-81.

Sztul, E. S., J. P. Hendrick, J. P. Kraus, D. Wall, F. Kalousek and L. E. Rosenberg. "Import of Rat Ornithine Transcarbamylase Precursor into Mitochondria: Two-Step Processing of the Leader Peptide." *J Cell Biol* 105, no. 6 Pt 1 (1987): 2631-9.

Wallace, D. C., W. Fan and V. Procaccio. "Mitochondrial Energetics and Therapeutics." *Annu Rev Pathol* 5, (2010): 297-348.

Yasui, K., T. Wakita, K. Tsukiyama-Kohara, S. I. Funahashi, M. Ichikawa, T. Kajita, D. Moradpour, J. R. Wands and M. Kohara. "The Native Form and Maturation Process of Hepatitis C Virus Core Protein." *J Virol* 72, no. 7 (1998): 6048-55.