APOLIPOPROTEIN-AI REGULATES HEPATIC VLDL SECRETION BY CONTROLLING INTRACELLULAR VLDL-TRAFFICKING

by

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ABSTRACT

Cardiovascular diseases cause 17 million deaths annually, which is estimated to increase to 23 million deaths by the year 2030. One of the major risk factors for the pathogenesis of cardiovascular diseases is increased secretion of very-low density lipoproteins (VLDL) by the liver; however, reduced VLDL-secretion causes fatty liver disease. Synthesis and secretion of VLDL by the liver plays an important role in maintaining overall lipoprotein homeostasis. Assembly of VLDL occurs along with the expression of apolipoproteinB-100 (apoB100) and its lipidation at the endoplasmic reticulum (ER) level. Once formed in the ER lumen, the nascent VLDL is transported to the Golgi for its maturation. In the Golgi compartment, the nascent VLDL acquires apolipoproteinAI (apoAI), more triglycerides, and its apoB100 undergoes phosphorylation and glycosylation. These modifications are necessary for VLDL-exit from the trans-Golgi network (TGN) and this step is mediated by post-Golgi VLDL transport vesicle (PG-VTV). The transport of mature VLDL from the TGN to the plasma membrane (PM) is required for its secretion by the liver but remains to be studied. Our group has shown that the nascent VLDL particles do not contain apoAI, however, VLDL acquires apoAI in the *cis*-Golgi compartment. Interestingly, apoAI comes off the VLDL as soon as VLDL is secreted into the blood. We hypothesised that apoAI plays an important role in post-TGN VLDL trafficking and thus controls VLDL secretion by the liver. To determine the role of apoAI in the formation of PG-VTV and VLDL secretion, we knocked down apoAI in the hepatocytes using apoAI specific siRNA. The deficiency of apoAI did not have any effect on the expression of apoB100 and other apolipoprotein synthesis that are involved in VLDL synthesis; however, VLDL secretion was significantly reduced. Next, we overexpressed apoAI using plasmid with apoAI gene sequence and checked for the effects in VLDL secretion from the hepatocytes. We observed a significant increase in VLDL secretion from apoAI-overexpressing hepatocytes which is consistent with knockdown results. To determine the role of apoAI in post-TGN trafficking of the mature VLDLs, we isolated sub-cellular organelles from apoAI knockout (apoAI KO) and control mice. Subsequently, we

performed in vitro PG-VTV budding assays to assess the effect of apoAI silencing on PG-VTV formation from the TGN. Our results strongly suggest that the deficiency of apoAI increases PG-VTV formation (i.e. TGN-exit of mature VLDL) but significantly reduces VLDL-triglyceride secretion from the hepatocytes. We conclude that apoAI controls VLDL secretion by the liver by regulating post-TGN trafficking of mature VLDL.

ACKNOWLEDGMENTS

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CHAPTER 1: INTRODUCTION

The main function of all living organisms is to maintain homeostasis and any deviation causes disease state. The liver maintains lipid homeostasis in the body by synthesis, secretion and uptake of various lipoproteins. The liver converts toxic fatty acids to triglycerides or cholesteryl esters (neutral lipids) and packages them in the lipoprotein particles for their secretion into blood. The packaging of these neutral lipids (hydrophobic) is necessary for their secretion into aqueous blood environment. These lipoproteins have an important role of providing energy in the form of neutral lipids to different tissues in the body. These lipoproteins are metabolised to various remnant forms once they transfer the energy to different tissues. Finally, these remnant forms are taken up by the liver and degraded or recycled. One of the distinct feature of these lipoproteins is the presence of apoproteins on them. Some apoproteins are unique to a set of lipoproteins, whereas others are present on most of the lipoproteins[1][2][13].

Very low density lipoprotein (VLDL) synthesis and secretion occurs mainly from the liver. Once secreted into the blood, VLDL then undergoes metabolic changes to form Intermediate density lipoprotein (IDL) and Low density lipoprotein (LDL), cholesterol rich lipoproteins. Increased LDL content in blood is one of the major cause of atherosclerosis. Aberrant synthesis and secretion of VLDL is directly related to dyslipidemia, cardiovascular diseases and fatty liver disease. Cardiovascular diseases cause 17 million deaths annually and this number will increase to 23 million by the year 2030. More than $316 billion are spent directly or indirectly in treatment of cardiovascular and heart diseases[1][2][3].

Apolipoprotein AI

ApolipoproteinAI (ApoAI) is a 28kDa protein synthesised and secreted majorly by the liver and other tissues[12]. ApoAI is mostly known for its atheroprotective functions, it transports excess cholesterol from extra-hepatic tissues and atherosclerotic plaques back to the liver[15]. The role of apoAI in the liver is not

clearly understood, viz., the transport, lipidation, overall secretion of apoAI is not well characterised. The ratio of apoB100 to apoAI in blood/plasma determines the risk of developing atherosclerosis[4].

VLDL Biosynthesis

Synthesis and secretion of VLDL from the hepatocytes is a complex and regulated step. VLDL synthesis starts with the expression of ApolipoproteinB100 (ApoB100), a 515kDa marker protein for VLDL. ApolipoproteinB100 is 44.8 kb gene present on chromosome 2p which encodes for ApoB100 protein (4563 amino acids). ApoB100-mRNA is partially translated and the complex (mRNA-Ribosome) transported to the rough-ER for its further translation. The complex is then transported to the ER-lumen where it's concurrently translated and lipidated by microsomal triglyceride transfer protein (MTP). MTP has three functional domains, viz., lipid transfer, membrane binding and apoB binding domains. All three domains are involved in apoB100-VLDL synthesis at the ER level in hepatocytes. apoB binding domain binds to the newly translated apoB sequence and lipidates it by the lipid transfer domain. MTP adds neutral as well as polar lipids to apoB100 for the formation of nascent-VLDL, each VLDL contains only one apoB100. Once the nascent-VLDL is formed, it is then ready to be transported to the Golgi compartment for its maturation and secretion[2][5].

The ER to Golgi transport of Nascent-VLDL

Nascent-VLDL is transported from the ER to Golgi in distinct vesicles called VLDL transport vesicles (VTVs) and each VTV contains one nascent-VLDL. These vesicles differ from the ER-derived protein transport vesicles (PTVs) in terms of size, density and protein composition. VTVs are lighter in density and larger in size (100-120nm in diameter) compared to PTVs. VTVs formation requires COPII (Coat complex II) proteins, Sar1 protein, cytosolic factors, ATP and optimum temperature (37°C). The transport of VTV from the ER to Golgi is the rate-limiting step for VLDL secretion. VTVs contain v-SNARE

(vesicle-soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor) protein required for its docking and fusion in the *cis*-Golgi compartment. This v-SNARE (Sec22b) interacts with Syntaxin 5, rBet1 and GS28 (target-SNARE protein on *cis*-Golgi) and form a four-member α-helix coiled-coil structure for the fusion of VTV into the *cis*-Golgi. One of the important findings is that VTVs do not contain apoAI but VLDL acquires apoAI in the *cis*-Golgi[2][6][7][8][9].

Maturation of VLDL in the Golgi

Once the VTV fuses within the *cis*-Golgi, nascent-VLDL undergoes further modifications. VLDL size increases due to addition of more triglycerides, apoB100 is phosphorylated and glycosylated. Finally, an important step of apoAI acquisition[9][11].

Post-Golgi transport of mature-VLDL

Mature-VLDL exits the *trans*-Golgi network (TGN) in distinct vesicles called post-Golgi VLDL transport vesicle (PG-VTV). PG-VTV formation requires optimum temperature (37°C), cytosolic factors and energy in the form of ATP/GTP. PG-VTVs differ from other Golgi-derived protein transport vesicles in size, density, protein composition. These vesicles are larger in diameter (300-350 nm) and contain more than one mature-VLDL. It is shown that PG-VTV transport is unidirectional and can fuse only with the cytosolic side of plasma membrane (PM) [9][10].

VLDL and apoAI

It is shown that apoAI is absent in VTVs but VLDL acquires apoAI in the *cis*-Golgi compartment of the hepatocytes. VLDL retains apoAI in the Golgi compartment and in post-Golgi VLDL transport vesicle. ApoAI finally detaches from VLDL once secreted from the hepatocytes (Figure 1) [9].

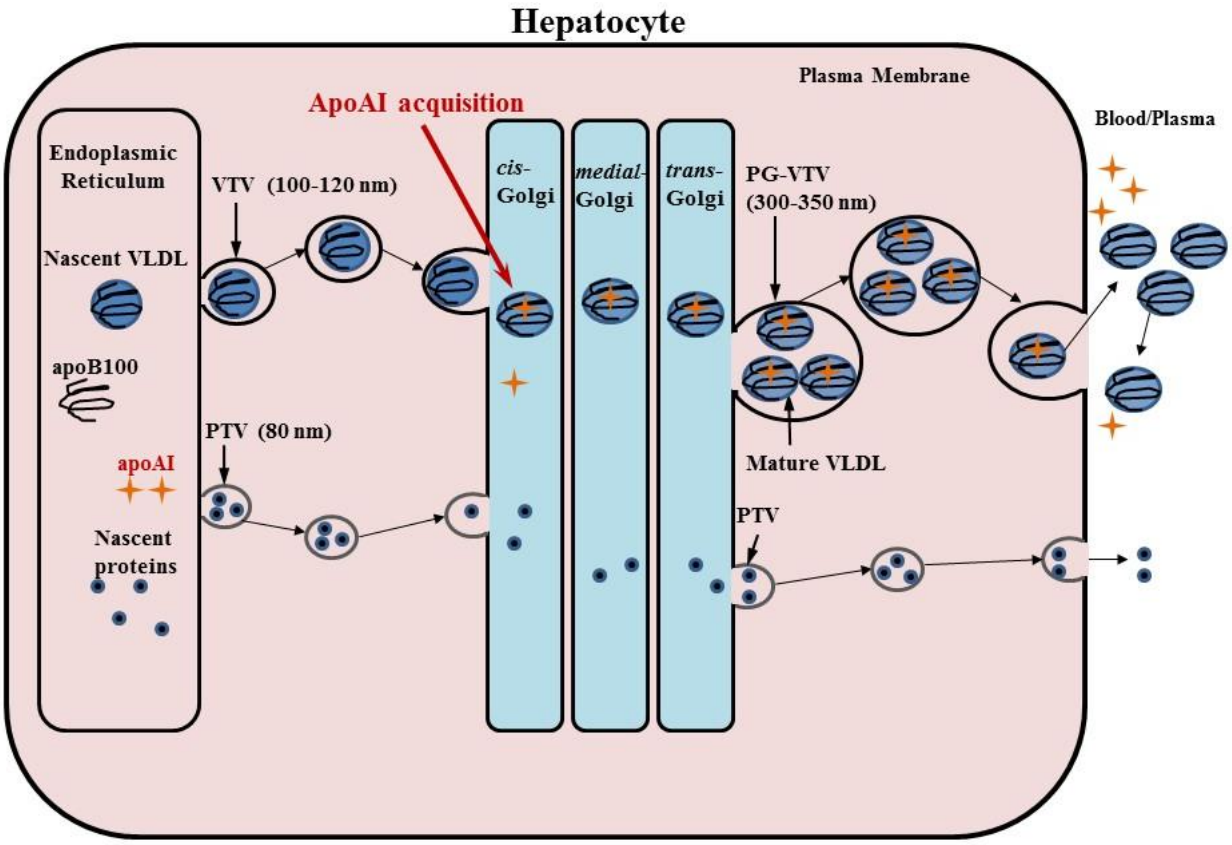

Figure 1: Intracellular trafficking and secretion of VLDL.

Synthesis of VLDL starts at the ER level in the hepatocytes, nascent-VLDL is transported to the *cis*-Golgi in VLDL transport vesicle (VTV). VLDL matures and acquires apoAI in the *cis*-Golgi, apoAI is retained on VLDL from the *cis*-Golgi till its secretion into blood. Mature-VLDL is transported in post-Golgi VLDL transport vesicle (PG-VTV) from the *trans*-Golgi network to the plasma membrane for its secretion into the blood.

CHAPTER 2: MATERIALS AND METHODS

Materials

McA-RH7777 (Rat hepatoma) cell line, Trypsin-EDTA, *Escherichia coli* DH52/pZB101 A1024230 (DH52 cells) and Fetal Bovine Serum (FBS) were purchased from American type cell culture (ATCC). [^{3}H]-Oleic acid (45.5 Ci/mM) was purchased from PerkinElmer Life Sciences. Bovine Serum Albumin (BSA)-Oleic acid, Penicillin-Streptomycin and Phosphate Buffered Saline (PBS) were purchased from Sigma Aldrich. Protease inhibitor cocktail tablets (catalogue number 04693116001) were purchased from Roche Applied Sciences. Gel electrophoresis experiments, immunoblotting reagents were purchased from Bio-Rad; enhanced chemiluminescence (ECL) reagents were purchased from GE Healthcare; other reagents used were of analytical grade and purchased from local companies. ApoAI$^{-/-}$ (Apoa1^{tm1Unc}) and Wild type (C57BL/6) mice were purchased from The Jackson Laboratory®. siRNAs were purchased from Thermo Fisher Scientific: siapoAI (catalog number 4390771) and si-negative control (catalog number 4390843). Plasmids were purchased from Genscript: apoAI overexpressing plasmid and control plasmid. UltraPure™ Ethidium Bromide (catalog number 15585011), BlueJuice™ Gel Loading Buffer (10X) (catalog number 10816015), LB Agar powder (catalog number 22700025), Miller's LB Broth Base® (LB Broth) (catalog number 12795027) Lipofectamine® RNAiMAX (catalog number 13778075) and Lipofectamine® 2000 (catalog number 11668027) were purchased from Thermo Fisher Scientific, Dulbecco's Modified Eagle Medium (DMEM) (catalog number 11965092), opti-Minimal Essential Medium (opti-MEM) (catalog number 31985070), BLOCK-iT™ Alexa Fluor® Red Fluorescent Control (catalog number 14750100) and Radioimmunoprecipitation (RIPA Lysis and Extraction Buffer) (catalog number 89900) buffer were purchased from Thermo Fisher Scientific. QIAGEN Plasmid Mini, Midi, and Maxi Kits (catalog number 12123 and 12125) purchased from QIAGEN. The experiments involved with

animal handling were strictly followed according to the University of Central Florida's Institutional Animal Care and Use Committee (IACUC) procedures and guidelines.

Antibodies

Mouse polyclonal antibodies to β-Actin; rabbit polyclonal anti-ApolipoproteinAI (ApoAI) antibody; goat polyclonal anti-ApolipoproteinAIV (ApoAIV) antibody, anti-ApolipoproteinE (ApoE) were all purchased from Santa Cruz Biotechnology, Inc. anti-ApoB antibody was gifted by Dr. Larry Swift.

Cell Culture Experiments

McA-RH7777 cell line was maintained in 5% FBS-DMEM with 1% penicillin/streptomycin at 37°C.

siRNA Transfection: McA-RH7777 cells ($0.3x10^6$ cells/well) were plated in 6-well plate in 5%FBS-DMEM media. These cells were then transfected with different concentrations of either with apoAI-siRNA or negative control-siRNA (negative control) or Alexa Fluor® (positive control) using lipofectamine® RNAiMAX for 24 hours at 37°C. Images of Alexa Fluor® containing set of cells were taken to confirm the transfection. The media was then discarded, cells were washed and collected using RIPA buffer. These were sonicated, centrifuged at 13,500xg for 15 mins at 4°C and the supernatants (cell lysates) were collected and checked for their protein concentration using Bradford Assay.

Pulse-Chase Experiment for siRNA transfected samples: McA-RH7777 cells were first transfected using siRNA (25nM) as described earlier. 24 hours post-transfection, cells were pulse labelled with [^{3}H]-Oleic acid-BSA complex (2μCi/well) for 1 hour at 37°C. The media was then dicarded, cells were washed with 1%FBS-DMEM media, fresh media (1%FBS-DMEM) was added and media samples were collected

at different time-points (0, 1, 2, 4, 6, 8, 24, 36 and 48 hours). Cell lysates were prepared for 24, 36 and 48 hour time-points and check protein concentration using Bradford Assay.

Plasmid Transformation: ApoAI overexpressing plasmid and control plasmid were first diluted to 50 ng/µL concentration (working condition). DH52 cells were incubated with 2µL of plasmid for 30 mins at 4°C. These cells were then heat shocked at 42°C for 40 seconds and then kept on ice for 2 minutes. 500µL of fresh LB Broth media was then added and these cells were then incubated at 37°C, 200 rpm for 1 hour. After 1 hour, cells were plated on an agar plate containing ampicillin and grown overnight at 37°C. Next day, one of the well grown colonies on agar plates was added to 15mL LB Broth containing ampicillin and incubated at 37°C for 6 hours at 200 rpm. This broth was added to 100 mL of fresh LB Broth containing ampicillin and cells were allowed to grow overnight at 37°C at 200 rpm.

Plasmid Isolation and purification: QIAGEN buffers: P1, P2, P3, QBT, QC and QF were used to isolate and purify the plasmid. The cells were first kept at 4°C for 30 mins and centrifuged at 3380xg at 4°C for 10 mins. The pellete was then resuspended using 10mL P1 buffer (resuspension buffer) at 4°C, the ratio of broth to P1 buffer should be 1:10 respectively, 10mL P2 buffer (Lysis buffer) was mixed with the resuspended solution and kept at 25°C for 10 mins (solution changed to blue colour). 10mL P3 buffer (Neutralisation buffer) was mixed in the solution (solution changed from blue to white colour) and incubated at 25°C for 5 mins. This was then centrifuged at 11180xg at 4°C for 20 mins, supernatant was collected and 10mL QBT buffer (Equilibration buffer) was added and the solution was filtered using QIAGEN filtration kit. Plasmid bound to the column was washed twice with 30mL QC buffer (wash buffer). 15mL of QF buffer (elution buffer) was used to elute the purified plasmid from the column. 10mL Iso-propanol was added to the eluted sample and was centrifuged at 11180xg at 4°C for 25 mins,

supernatant was discarded and pellete (plasmid) was dried and re-suspended in 500µL of RNAse and DNAse free water.

Plasmid purity: The re-suspended plasmid was analysed for its purity and concentration in Nanodrop analyser (absorbance at 260nm and 280nm). Once the concentration of plasmid in the solution was identified, a part of solution was diluted to get the plasmid concentration of 0.5µg/µL.

Plasmid Overexpression: McA-RH7777 cells ($0.1x10^6$ cells/well) were plated in 6-well plate in 1%FBS-DMEM media. These cells were then transfected with different concentrations of apoAI overexpressing plasmid or control plasmid using lipofectamine®2000 for 48 hours at 37°C. The media was then discarded, cells were washed and collected using RIPA buffer. These were sonicated, centrifuged at 13,500xg for 15 mins at 4°C and the supernatants (cell lysates) were collected and checked for their protein concentration using Bradford Assay.

Pulse-labelling Experiment for plasmid-transfected cells: McA-RH7777 cells were first transfected with plasmid (1.25µg/well) as described earlier. 48 hours post-transfection, cells were pulse labelled with [^{3}H]-Oleic acid-BSA complex (2µCi/well) for 1 hour at 37°C. The media was then dicarded, cells were washed with 1%FBS-DMEM media, fresh media (1%FBS-DMEM) was added and media samples were collected at different time-points (0, 1, 2, 4, 6, 8, 24, 36 and 48 hours). Cell lysates were prepared for 24, 36 and 48 hour time-points and check protein concentration using Bradford Assay.

Liquid scintillation counts

100µL of sample was mixed with 5 mL of scintiverse in scintillation vials and the mixture was analysed for [^{3}H]-disintegrations per minute (d.p.m.) in the liquid scintillation counter.

ER and Golgi Isolation

The protocol for hepatic- ER and Golgi isolation, Cytosol preparation and PG-VTV budding assay is standardised and published in current protocols in cell biology[10]. Livers from mouse models were first isolated and perfused with saline. The liver was chopped into pieces of $1cm^2$ in buffer A (Table 2), add [^{3}H]-oleic acid-BSA complex (50µCi [^{3}H]-oleic acid in 500µL of BSA-oleic acid) and incubate it at 37°C (water bath) for 35-40 mins by gently swirling it in every 5 mins. After incubation wash the pieces with 2% BSA, centrifuge at 600 x g for 5 mins at 4°C, re-suspend the pellet and homogenise it in buffer B (Table 2) using Parr cell disruption vessel at 1,100 psi for 40 mins at 4°C. Collect the homogenate and spin at 600 x g for 10 min in a Sorvall centrifuge using Fiberlite F21S-8x50y rotor at 4 °C. Collect the post-nuclear supernatant (PNS) and centrifuge the PNS at 100,000 x g for 95 min at 4 °C using a Beckman 70 Ti rotor. Re-suspend the pellet in ice-cold 0.25 M sucrose solution containing protease inhibitor mixture (PI). Adjust the density of this solution to 1.22 M sucrose using ice-cold 2.1 M sucrose solution in 10 mM Hepes buffer. Transfer 3 mL of this solution (1.22 M sucrose) in 12-mL polyallomer centrifuge tube and overlay it with 2.6 mL of sucrose buffers: 1.15 M, 0.86 M and 0.25 M. Centrifuge the step gradient at 82,000 x g for 3 hours at 4 °C using a Beckman SW41 Ti rotor. Carefully collect the *trans*-Golgi network (TGN) at the 0.25 M/0.86 M interface, *cis*-Golgi at 0.86/1.15 M interface, 1.22 M sucrose layer as smooth ER and the pellet as rough ER. Use Bradford assay to determine the protein content of these samples and aliquot (100–150 µL) these samples to store them at -80°C.

Cytosol Preparation

Isolate and chop the liver in small pieces (1cm^2) in cold buffer C (Table 2). Pellet the pieces using centrifugation (600 x *g*) at 4 °C. Re-suspend the pellet in cold buffer C (25–30 ml) containing PI and homogenise them using Parr cell disruption vessel at 1,100 psi for 40 min at 4 °C. Collect homogenate in a centrifuge tube and spin at 600 x *g* for 10 min in Sorvall centrifuge using Fiberlite F21S-8x50y rotor at 4 °C. Collect the post-nuclear supernatant (PNS) and centrifuge the PNS at 100,000 x *g* for 95 min at 4 °C in a Beckman 70 Ti rotor. Collect the supernatant (cytosol) carefully (avoid the layer on the top) using a glass Pasteur pipette. Dialyse the cytosol against cold buffer C for 6–8 hours at 4 °C in a 10 kDa cut-off membrane and concentrate until the cytosol volume is reduced to 10 ml. Collect the cytosol and place it in centricon tubes (YM-10 membrane) and centrifuge at 4000 x *g* at 4 °C until the protein concentration of cytosol is ~10–15 mg/ml. Use Bradford assay to determine the protein concentration and aliquot the cytosol in 100–150 μL and store it at −80 °C until use. Check the purity of cytosol by immunoblotting for marker proteins.

Budding Assay and Isolation of PG-VTVs

Thaw TGN membranes and cytosol at 37 °C, add all the reactants as described in Table 3 in a pre-chilled glass tube. The negative control contains cytosol buffer instead of cytosol or the reaction is performed at 4 °C. Mix the reactants gently by shaking the tubes with hand, incubate the mix at 37 °C water bath for 30–32 min. Make sure that the temperature of water bath is maintained and shake the tubes very gently occasionally. Post-incubation, place the tubes on ice and add 700 μL of ice-cold 10 mM Hepes buffer to stop the reaction. Set the density of the reaction mix to 0.1 M using ice-cold 10 mM Hepes buffer. Prepare a sucrose continuous density gradient (0.1 M–0.86 M) (for every sample) in a 12-ml polyallomer centrifuge tube, using a two-chambered gradient maker. Carefully overlay reaction mix (~ 1.2 ml) on top of a continuous density gradient (0.1 M–0.86 M). Keep gradient tubes at 4 °C and do not disturb the

gradient. Centrifuge these gradients at 115,000 x g (Beckman SW41 Ti rotor) for 2 hours at 4 °C in a pre-chilled rotor. Using a glass Pasteur pipette, carefully discard the top 100 µL fraction that contain cytosolic proteins and collect 500-µl fractions from the top of the tube for further analysis.

SDS-PAGE and Western blotting

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used to separate proteins, proteins in gel were transblotted onto nitrocellulose membranes, probed against specific proteins and detected using ECL reagents. Resolving gel and stacking gel (4%) were prepared based on the required concentrations (see Table 1 for different concentrations), protein samples were loaded in different wells of the gel. 20-30 mA current was applied to run the gel and the gel was then set on to western transfer assembly with nitrocellulose membrane. The transfer was kept at 50 mA at 4°C for 16 hours, the membrane was then removed, washed with PBS and kept on blocking (10% milk in PBS-T) for 6 hours at room temperature (RT). Post-blocking, the membrane was washed with PBS-T, membrane was then incubated with primary antibody (antibody against protein of interest) for 1 hour at RT. The membrane is then washed with PBS-T, incubated with secondary antibody (against primary antibody) for 1 hour at RT. Post-incubation membrane is washed with PBS-T/PBS and ECL reagent is added to the membrane. Photographic films are used to detect the chemiluminisence and the blot was developed in the film development machine.

CHAPTER 3: RESULTS

ApolipoproteinAI knockdown efficiency and VLDL secretion

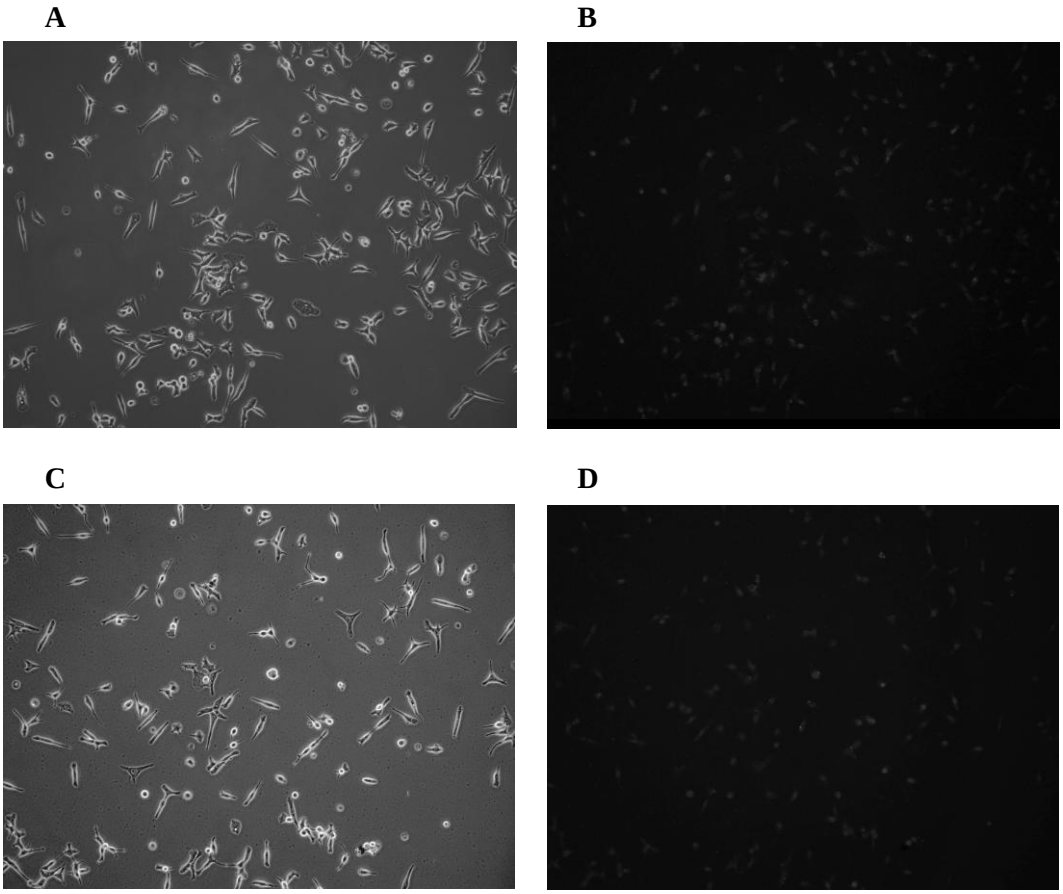

Figure 2: siRNA transfection of McA-RH7777 cells.

Microscopic Images of transfected (Alexa Fluor®) cells. Cells transfected with 25nM of Alexa Fluor® (A and B); A: cells under white light, B: cells under fluorescence (red). Cells transfected with 50nM of Alexa Fluor® (C and D); C: cells under white light, D: cells under fluorescence (red).

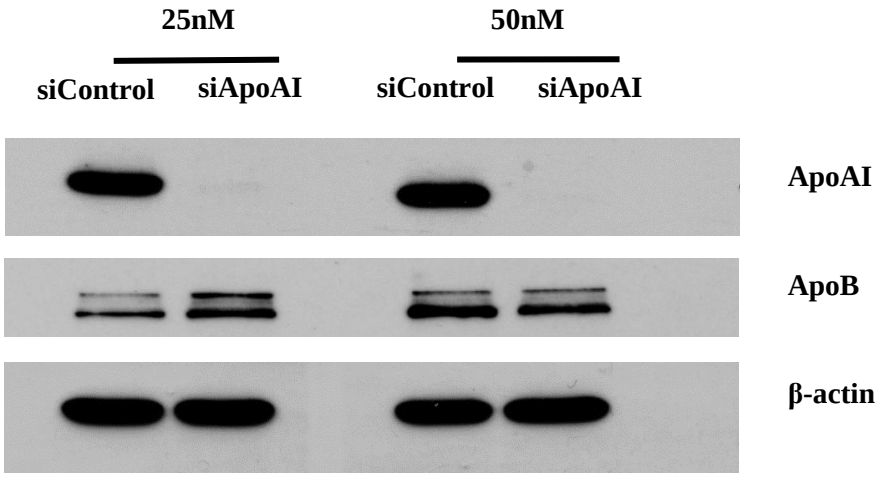

Figure 3: Immunoblots for siRNA transfected McA-RH7777 cell lysates.

Protein levels of apoB, apoAI and β-actin (loading control) in McA-RH7777 cell lysates transfected with either control siRNA (siControl) or apoAI siRNA (siapoAI). Cell lysates prepared from transfected cells were evenly loaded on 12% SDS-PAGE, transblotted on nitrocellulose membrane then probed for the proteins (apoB, apoAI and β-actin) using specific antibodies and detected using ECL reagent.

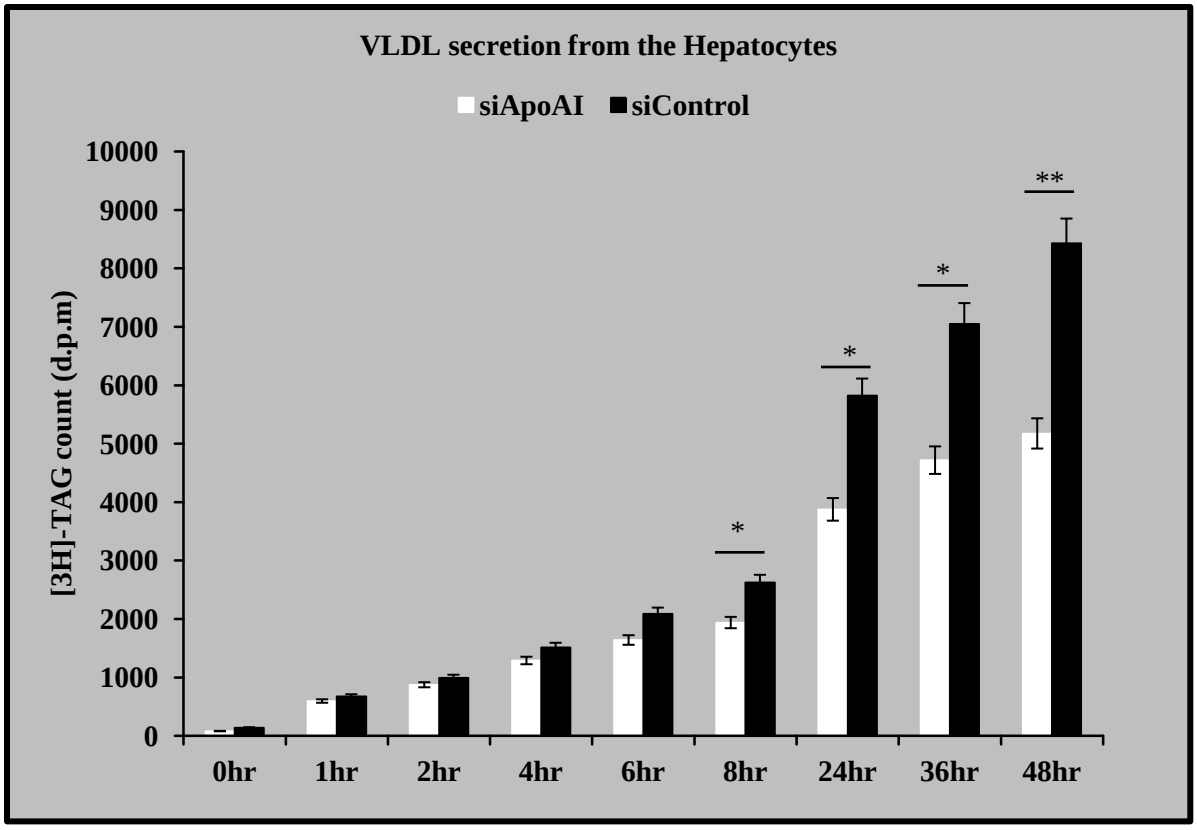

Figure 4: [^{3}H]-Triglyceride(TAG) secretion at different time-points from transfected McA-RH7777 cells.

McA-RH7777 transfected with siControl or siApoAI were incubated with [^{3}H]-Oleic acid-BSA complex for an hour, fresh media was added and media samples were collected at different time-points. These time-points were then analysed for [^{3}H]-TAG counts disintegrations per minute (d.p.m.) using liquid scintillation counter (LSC).

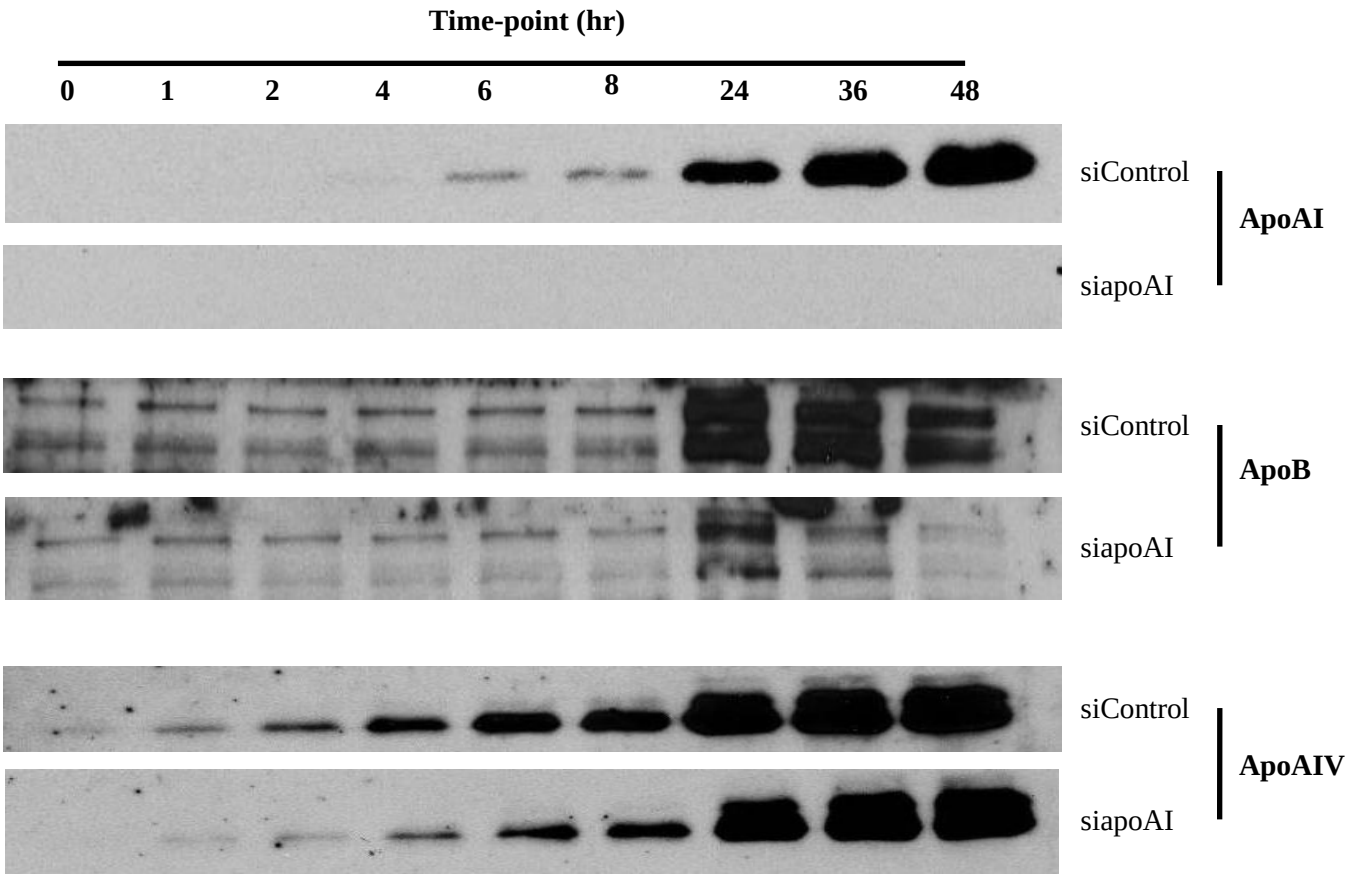

Figure 5: Secretion of apoproteins at different time-points from transfected McA-RH7777 cells.

The media samples collected at different time-points from transfected McA-RH7777 cells were then analysed for apoprotein levels using immunoblots. These samples were evenly loaded on 12% SDS-PAGE, transblotted on nitrocellulose membrane then probed for the proteins (apoAI, apoB and apoAIV) using specific antibodies and detected using ECL reagent.

ApolipoproteinAI-overexpression and VLDL secretion

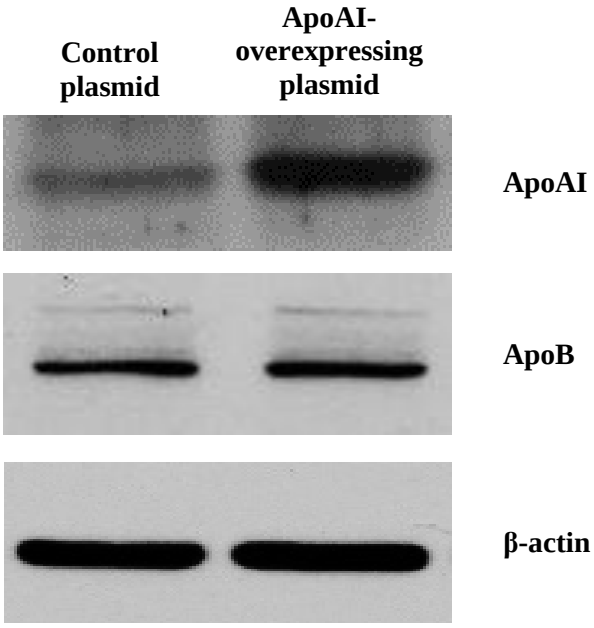

Figure 6: Immunoblots for plasmid transfected McA-RH7777 cell lysates.

Protein levels of apoAI, apoB and β-actin (loading control) in McA-RH7777 cell lysates transfected with either control plasmid or apoAI over-expressing plasmid. Cell lysates prepared from transfected cells were evenly loaded on 12% SDS-PAGE, transblotted on nitrocellulose membrane then probed for the proteins (apoAI, apoB and β-actin) using specific antibodies and detected using ECL reagent.

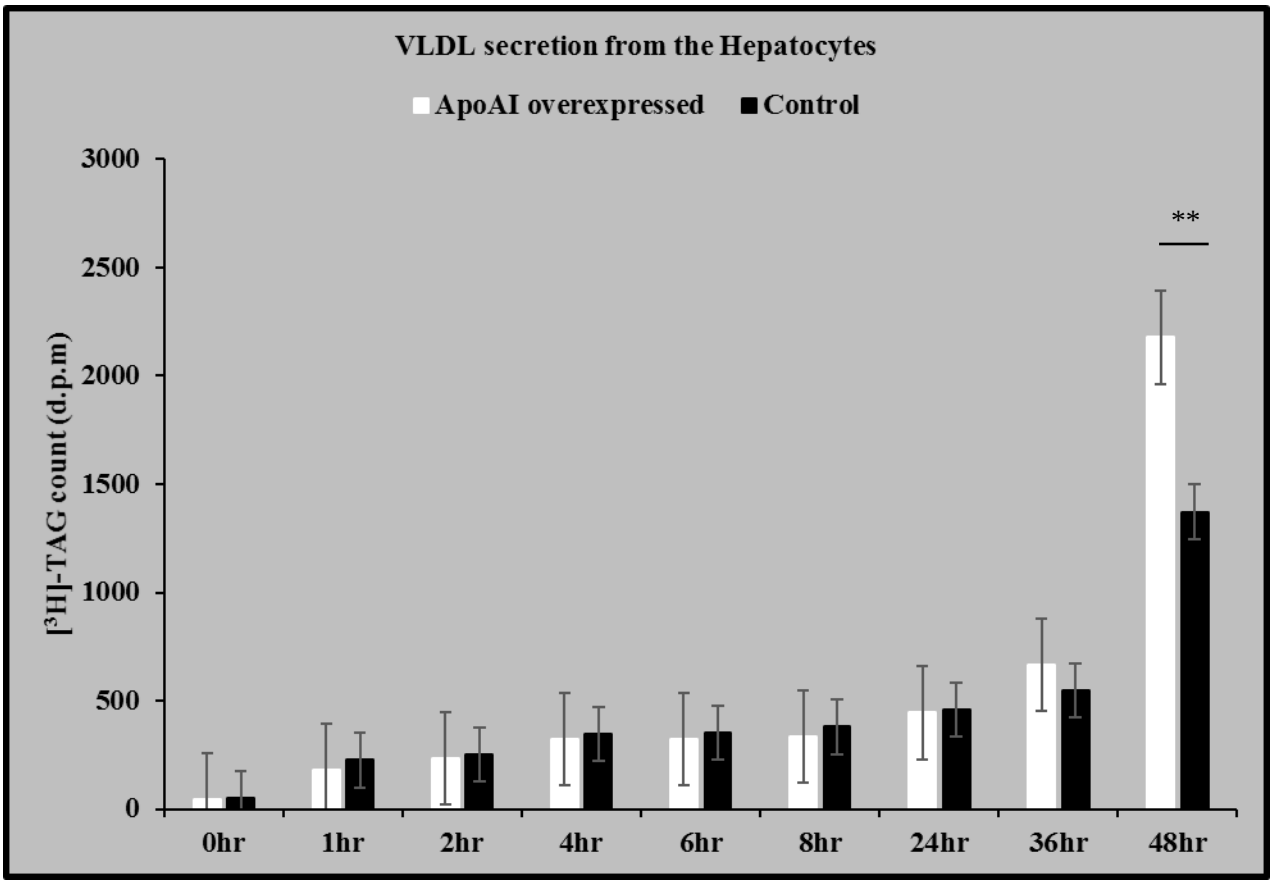

Figure 7: [^{3}H]-Triglyceride(TAG) secretion at different time-points from transfected McA-RH7777 cells.

McA-RH7777 transfected with control plasmid or apoAI overexpressing plasmid were incubated with [^{3}H]-Oleic acid-BSA complex for an hour, fresh media was added and media samples were collected at different time-points. These time-points were then analysed for [^{3}H]-TAG counts disintegrations per minute (d.p.m.) using liquid scintillation counter (LSC).

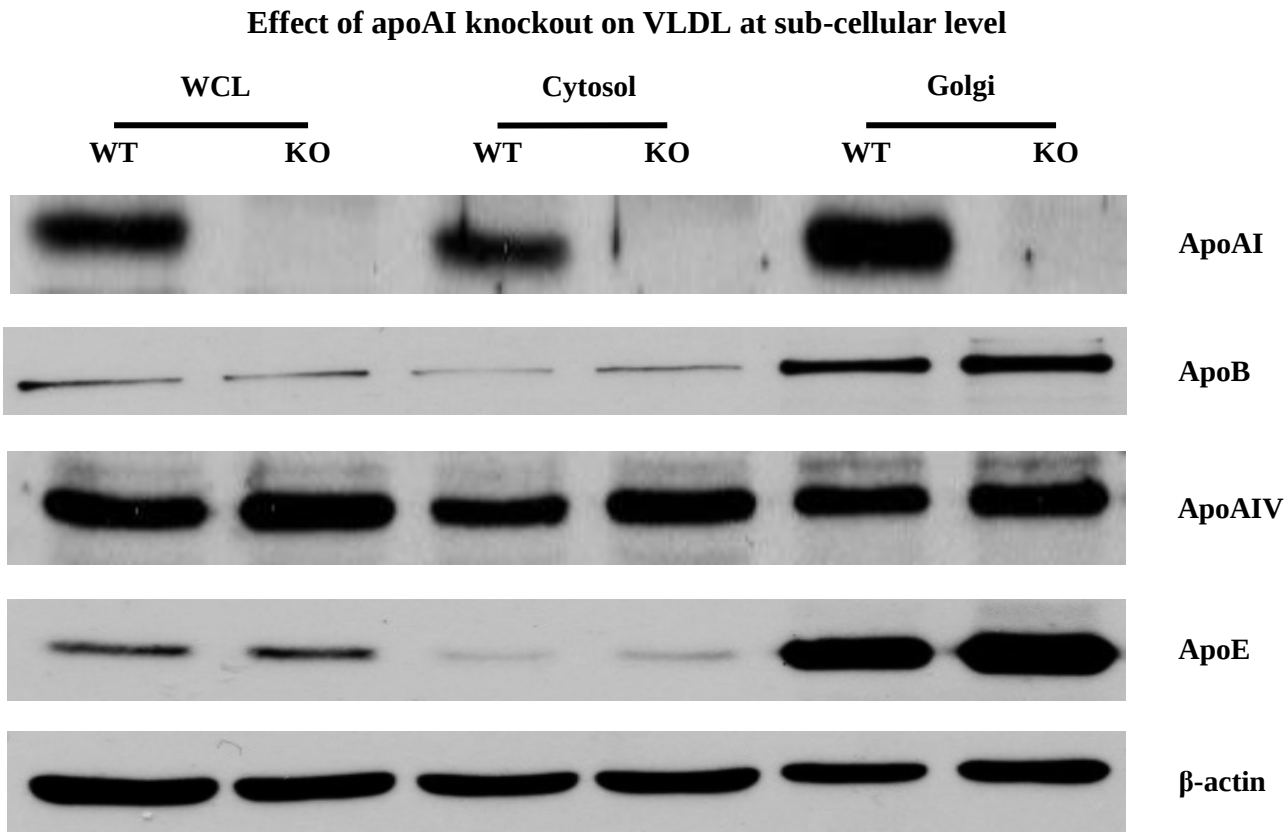

Figure 8: Immunoblots of proteins at different sub-cellular levels in wild-type (WT) and apoAI knockout (KO) mouse liver samples.

Protein levels of apoAI, apoB, ApoAIV, ApoE and β-actin (loading control) in Whole cell lysates (WCL), Cytosol and Golgi samples of wild-type (WT) and apoAI knockout (KO) mouse liver samples. Livers from 8-weeks old mice (WT and KO) were isolated. Cytosol, Golgi and whole cell lysates (WCL) were prepared using the protocol mentioned in the materials and methods section. These samples checked for protein content, then evenly loaded on 12% SDS-PAGE, transblotted on nitrocellulose membrane then probed for the proteins (apoAI, apoB, ApoAIV, ApoE and β-actin) using specific antibodies and detected using ECL reagent.

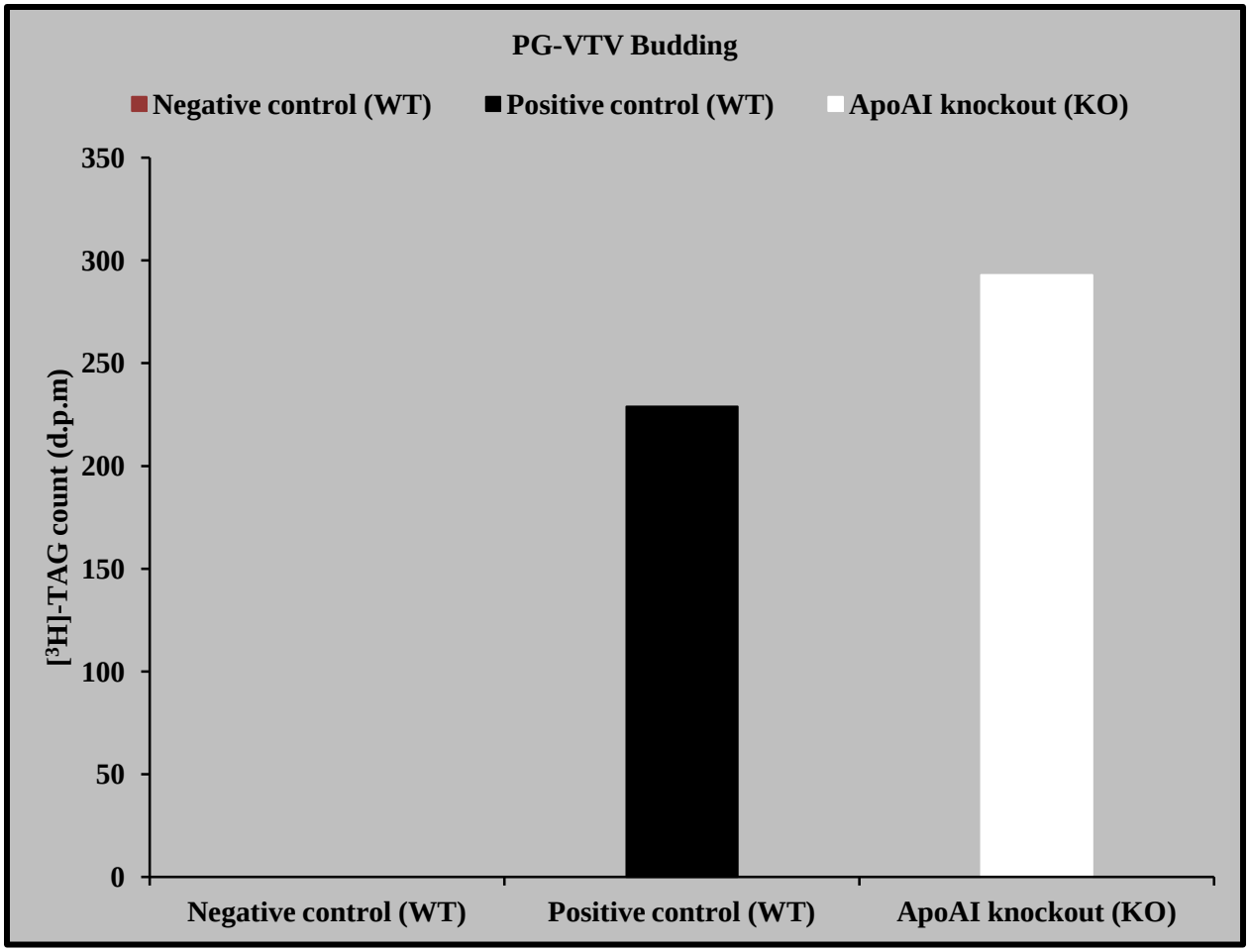

Figure 9: Cell-free *in-vitro* post-Golgi VLDL Transport Vesicle (PG-VTV) budding assay for wild-type (negative and positive controls) and apoAI knockout (KO) samples.

[^{3}H]-TAG labelled Golgi samples from WT and KO mice were incubated for 30 minutes at 37°C with ATP and respective cytosol samples (positive control and apoAI knockout) or cytosol buffer (negative control). To stop the reaction, samples were kept at 4°C and then centrifuged in 10mL sucrose continuous gradient (0.1M-0.86M) at 115,000xg for 2 hours at 4°C. 500µL fractions were collected separately and checked for [^{3}H]-TAG counts (d.p.m.) in liquid scintillation counter.

CHAPTER 4: DISCUSSION

Abnormal secretion of VLDL is related to dyslipidemia and other metabolic disorders. Increased VLDL secretion is directly related to pathogenesis and development of atherosclerosis[16]. The ratio of apoB100/apoAI in the blood determines the risk of developing atherosclerosis[14]. The two distinct markers of VLDL are apoB100 and triglycerides. Synthesis and secretion of VLDL is highly regulated by multiple factors in the liver. Nascent-VLDL is synthesised at the ER level and transported to the *cis*-Golgi in VTV. The transport of VLDL to the Golgi compartment is required for its maturation and secretion from the hepatocytes. In the Golgi compartment, VLDL increases in size by addition of triglycerides, apoB100 is phosphorylated and glycosylated. Also, VLDL acquires apoAI in the *cis*-Golgi compartment of the hepatocytes; however VTVs do not contain apoAI (Figure 1). Mature-VLDL retains apoAI till its secreted into from the hepatocytes. On the other hand, a significant portion of apoAI in the blood is secreted by the hepatocytes. apoAI forms high density lipoprotein (HDL) on lipidation. apoAI has atheroprotective functions in the blood[4][15]. Studies have been done previously on the role of apoAI and apoB100 in the blood but the direct interaction of apoAI and VLDL in the liver was first identified by our group. This addition of apoAI on VLDL in the Golgi compartment of hepatocytes indicated a major role of apoAI in VLDL-maturation and secretion. In this study we focused on the role of apoAI on VLDL secretion and post-Golgi VLDL trafficking[2][6][8][9][11].

ApoAI deficiency causes reduced VLDL secretion from the hepatocytes

To check the effect of apoAI on VLDL secretion, we first transfected sets of McA-RH7777 cells with either apoAI-siRNA or negative control siRNA or Alexa Fluor® (positive control) for 24 hours. The efficiency of transfection was determined by identifying the fluorescence (Alexa Fluor®) in the cells under the fluorescence microscope (Figure 2). The cells were efficiently transfected as observed in Figure

2, cell lysates were prepared, protein was estimated for all the sets. Immunoblots for these cell lysates was performed to check the knockdown of apoAI and effect on marker protein (apoB100) of VLDL. ApoAI knockdown was confirmed in the cells transfected with apoAI-siRNA and there was clear indication apoAI in negative control sample. This knockdown was very specific and had no effect on apoB100 protein expression. The samples were evenly loaded as seen in β-actin blot

Once the knockdown of apoAI was confirmed, we then checked for VLDL secretion using one of the marker component of VLDL, Triglycerides. We pulse-labelled (protocol in Cell Culture Experiments section) the transfected cells with [^{3}H]-Oleic acid-BSA complex for one hour at 37°C so that the fatty acid ([^{3}H]-Oleic acid) gets assembled into triglyceride which is then added in VLDL. The amount of [^{3}H]-triglycerides secreted into the media will signify the amount of VLDL secreted at different time-points. As seen in Figure 4, apoAI knockdown cells had significantly reduced VLDL-triglycerides secretion starting from 8th hour post-incubation. The maximum reduction in VLDL-triglyceride secretion was observed at the 48th hour post-incubation.

To confirm reduced VLDL secretion from apoAI knockdown cells, we performed western blots for the same media samples and checked for the secretion of apoB100, a marker protein of VLDL. Control cells secreted apoAI but apoAI knockdown cells did not. ApoB100 secretion in media proved to be consistent with our VLDL-triglyceride secretion results. ApoB100 secretion was significantly reduced in apoAI knockdown cells starting from 8th hour post-incubation. A significant reduction was observed at 48th hour post-incubation. The knockdown had no effect on other apoprotein secretion (apoAIV and apoE). The reduction in VLDL secretion was only due to the knockdown of apoAI (Figure 5).

ApoAI overexpression causes increased VLDL secretion from the hepatocytes

The knockdown of apoAI had significant effect on VLDL secretion, so we wanted to check if excess of apoAI has any effect on VLDL secretion. So we transfect sets of McA-RH7777 cells either with control or apoAI overexpressing plasmid. To confirm overexpression of apoAI in desired set of cells, we performed western blots on the cell lysates of cells transfected with control and apoAI-overexpressing plasmids. Cells transfected with apoAI-overexpressing had more apoAI compared to control cells and no effect on apoB100 expression. The samples were evenly loaded as seen in β-actin blot (Figure 6).

ApoAI-overexpression was confirmed, we checked for VLDL secretion using one of the marker component of VLDL, Triglycerides. We pulse-labelled (protocol in Cell Culture Experiments section) the transfected cells with [^{3}H]-Oleic acid-BSA complex for one hour at 37°C so that the fatty acid ([^{3}H]-Oleic acid) gets assembled into triglyceride which is then added in VLDL. The amount of [^{3}H]-triglycerides secreted into the media will signify the amount of VLDL secreted at different time-points. Compared to control, apoAI-overexpression caused significant VLDL-triglycerides secretion 48th hour post-incubation (Figure 7).

Apoprotein expression at sub-cellular levels in wild-type (WT) and apoAI knockout (KO) samples

It was confirmed that varying levels of apoAI in the hepatocytes affected VLDL secretion. So we wanted to check the effect of apoAI deficiency on VLDL trafficking at sub-cellular levels. Our lab discovered that apoAI is not present on VLDL in the VTVs but is added to the VLDL in the *cis*-Golgi compartment and is present on VLDL till its secretion from the hepatocytes. So we wanted to look for different protein levels involved in Golgi and post-Golgi VLDL trafficking and secretion.

We Isolated livers form 8-weeks old wild-type (WT) and apoAI knockout (KO) mice. Golgi (containing [^{3}H]-TAG), whole cell lysates (WCL) and Cytosol samples were prepared from these livers (protocol in ER and Golgi Isolation, Cytosol Preparation, Cell Culture Experiments section). We then performed western blots to check for levels of different proteins (apoAI, apoB, apoAIV, apoE and β-actin) in these samples. Proteins were evenly loaded in all the samples as shown in β-actin blot. WT samples expressed apoAI; whereas there was no apoAI expression in KO samples (confirming complete apoAI knockout). No significant difference in apoB100 and other apoproteins was observed at any of the sub-cellular levels (Figure 8).

Post-Golgi VLDL transport vesicle (PG-VTV) budding assay for wild-type (WT) and apoAI knockout (KO) samples

As there was no effect in apoprotein levels at the Golgi level, we went ahead and check for any difference in next step of VLDL-trafficking, the PG-VTV formation. Cell free *in-vitro* PG-VTV budding assay (protocol in Budding Assay section) determines the formation of PG-VTVs containing mature-VLDL for secretion into the blood. WT and KO Golgi samples were incubated with their respective cytosol samples in ATP re-generating system at 37°C for 30 mins, one of the WT sample contained cytosol buffer (no cytosol) and was used as negative control. Samples were then centrifuged in sucrose continuous gradient at 115,000 x g for 2 hours to isolate the vesicles (PG-VTVs) formed, fractions were collected and tested for marker component of VLDL, triglycerides. The budding activity was observed in the top three fractions of the samples. Negative control did not show any PG-VTV budding; whereas PG-VTV formation occurred in other samples. ApoAI knockout samples showed significant increase in PG-VTV budding compared to positive control (Figure 9).

CHAPTER 5: CONCLUSION

VLDL acquires ApoAI in the *cis*-Golgi organelle and retains apoAI till it is secreted from the hepatocytes. In this study we observed the effect of apoAI on intracellular VLDL trafficking and secretion. On overexpressing apoAI in the hepatocytes, VLDL secretion is significantly increased. Also, deficiency of apoAI in the hepatocytes causes significant reduction in VLDL secretion. These findings indicate that apoAI is required for normal VLDL secretion from the hepatocytes. The attachment of apoAI on VLDL in the *cis*-Golgi is required for the regulation of PG-VTV budding in the hepatocytes. ApoAI is a negative regulator of PG-VTV budding and post-Golgi mature-VLDL trafficking in the hepatocytes.

The deficiency of apoAI causes increased PG-VTV budding but the overall mature-VLDL secretion of is significantly reduced from the hepatocytes. On deficiency of apoAI, the PG-VTVs do not fuse with the plasma membrane causing accumulation of VLDL in the hepatocytes and eventually fatty liver disease. Other studies have also shown that deficiency of apoAI causes formation of fatty liver disease. Hence, we conclude that deficiency of apoAI causes increased PG-VTV formation but these vesicles are fusion incompetent causing these vesicles to accumulate in the hepatocytes leading to significant reduction in VLDL secretion (Figure 10).

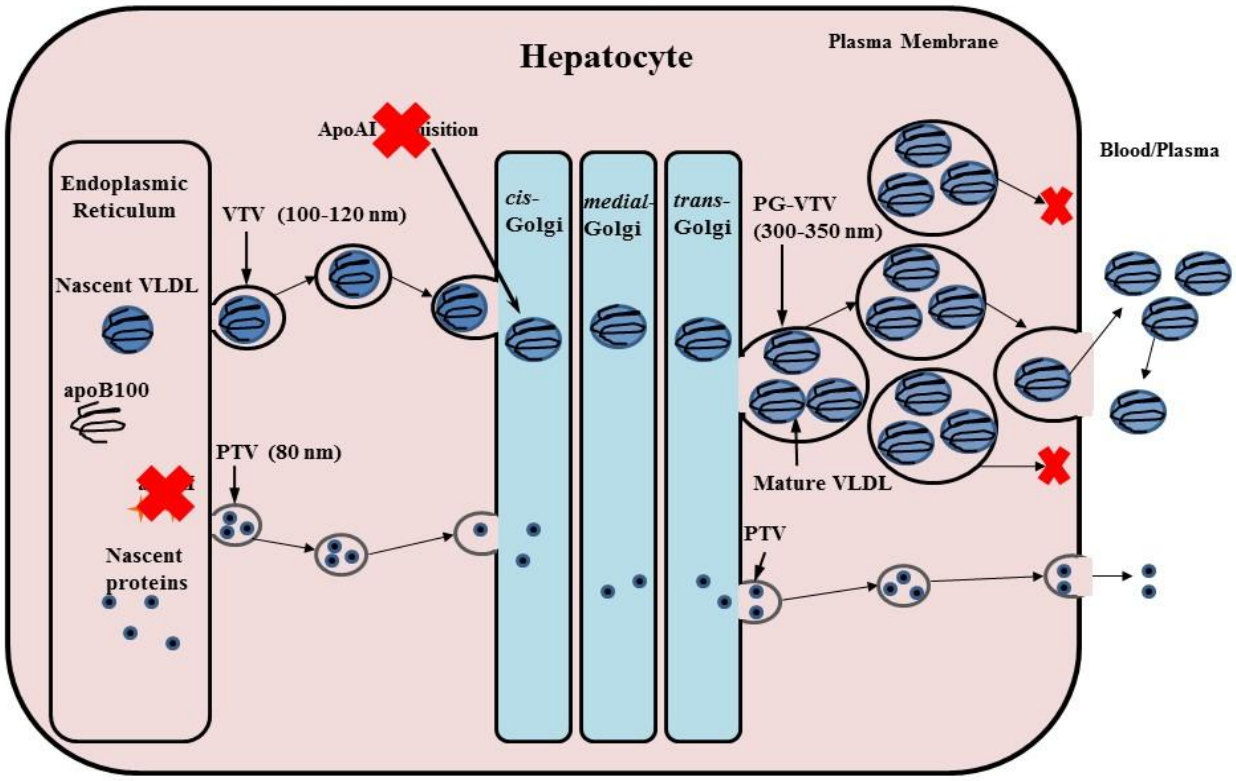

Figure 10: ApoAI deficiency causes reduced heaptic-VLDL secretion.

ApoAI deficiency causes increased PG-VTV formation but these vesicles (PG-VTVs) are fusion incompetent causing reduced VLDL secretion from the hepatocytes.

APPENDIX A: TABLES

Table 1: SDS-PAGE gel formulations

Percent Gel (%)	**DDI Water (mL)**	**30% Degassed Acrylamide/ Bis (mL)**	**Gel Buffer (mL)**	**10 % w/v SDS (mL)**
4	6.1	1.3	2.5	0.1
8	4.7	2.7	2.5	0.1
10	4.1	3.3	2.5	0.1
12	3.4	4.0	2.5	0.1

	10 % APS (µL)	Temed (µL)
Resolving Gel (10ml)	50	5
Stacking Gel (10ml)	50	10

Table 2: Buffers for ER/Golgi Isolation, Cytosol preparation and PG-VTV budding assay[10]

Buffer A	**Buffer B**	**Buffer C**
• 136 mM NaCl • 11.6 mM KH_2PO_4 • 8 mM Na_2HPO_4 • 7.5 mM KCl • 0.5 mM DTT • Adjust pH to 7.2 and store at 4◦C	• 0.25 M sucrose in 10mM Hepes (pH 7.2) • 5 mM EDTA • Protease Inhibitor cocktail • Store at 4 °C and use within 1–2 months.	• 25 mM Hepes (pH 7.2) • 125 mM KCl • 2.5 mM $MgCl_2$ • 0.5mM dithiothreitol (DTT) • 0.5 mM EGTA • 5 mM diethyl-p-nitrophenylphosphate (E600)

Transport Buffer:
• 30 mM Hepes (pH 7.2)
• 0.25 M sucrose
• 2.5 mM magnesium acetate
• 30 mM KCl

ATP Regenerating System
• 5 mM ATP in 10 mM Hepes (pH 7.2)
• 5 mM phosphocreatine
• 25 Units of creatine phosphokinase
• Store at −80 °C and use within a year

Table 3: PG-VTV budding recipe mixture[10]

Reactants	**Volume (µL)**	**Final concentration**
Rat hepatic TGN membranes containing [^{3}H]-TAG	40-50	200 µg protein
Rat hepatic cytosol	50-60	500 µg protein
ATP regenerating system	100	1 mM ATP/5mM phosphocreatine/5 units of creatine phosphokinase
50 mM GTP	10	1 mM
50 mM CaCl2	50	5 mM
50 mM MgCl2	50	5 mM
50 mM dithiothreitol (DTT)	50	5 mM
50 mM diethyl-p-nitrophenylphosphate (E600)	10	1 mM
Transport buffer	Make upto 500 µL	

APPENDIX B: IACUC APPROVAL LETTER

THE UNIVERSITY OF CENTRAL FLORIDA
INSTITUTIONAL ANIMAL CARE and USE COMMITTEE (IACUC)
Re-Approval to Use Animals

Dear Dr. Shadab Siddiqi,

Your application for IACUC Re-Approval has been reviewed and approved by the UCF IACUC Reviewers.

Approval Date: 12/10/2015

Title: A Cell Biological Approach to Hepatic Lipid Metabolism

Department: Biomolecular Science Center

Animal Project #: 15-14

Expiration: 3/31/2017

You may purchase and use animals according to the provisions outlined in the above referenced animal project. This project will expire as indicated above. You will be notified 2-3 months prior to your expriation date regarding your need to file another renewal.

Christopher Parkinson, Ph.D.
IACUC Chair

APPENDIX C: RADIOACTIVITY USE CERTIFICATE

University of Central Florida — Radiation Control Form

Form RC-3a

Radioactive Material Use Permit

Principle Investigator (please attach a current Curriculum Vitae if not already on file with EH&S)

Shadab A. Siddiqi	Assistant Professor	BSBS
Principle Investigator	Title	Department

1001	363	407-266-7041	shadab.siddiqi@ucf.edu
Building	Room	Phone Number	Email Address

Proposal Information

Duration of Proposal (5 year limit)—From: 2/2015 Through 2/2020

Location(s) of Use: 1001 (Building) 363 (Rooms)

(Building) (Rooms)

Radionuclides	Maximum Activity	Use and Chemical Form
1. ^{3}H– oleate	25 mCi	^{3}H– oleic acid/liquid
2. ^{14}C– oleate	5 mCi	^{14}C– oleic acid/liquid
3. ^{3}H– leucine	5 mCi	^{3}H– leucine/liquid
4. ^{32}P– ATP	10 mCi	^{32}P– ATP/ liquid
5.		

I hereby accept responsibility for the proper use, storage and disposal of the radioactive materials listed on this Permit. I am also responsible for radiation workers operating under my permit in ensuring they are fully trained and have experience in handling radioactive materials or will be provided training by myself or my Associate Investigators.

S. A. Siddiqi

Principle Investigator Signature

02/06/2015

Date

Associate Investigator Signature Date

Associate Investigator Signature Date

Associate Investigator Signature Date

Radiation Safety Approvals

Radiation Safety Officer

1/5/15

Date

R Chakrabarty

Chair, Radiation Safety Committee

1/5/15

Date

Form RC-3a — RAM Use Permit

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