Role of Phospholipid Transfer Activity of MTP in Promoting Lipoprotein Assembly; Molecular Mechanisms and Structural Implications

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I dedicate this entire work to my family for their continued support and love throughout this long journey towards accomplishing this goal and for always being there. Besides them there are many other people I want to thank, without them this endeavour could not have been completed. First of all, I would also like to express my appreciation and sincere thanks to my advisor, Dr. Hussain for his guidance, support and suggestions throughout this research project in the lab. I have learnt a lot during this entire period from him and by working in his lab. I also like to extend my thanks to my committee members for their support, encouragement and constructive suggestions through this entire period at graduate school and taking their time to make this process successful. Besides that I also express my sincerest appreciation for the support from Dr. Susan Schwartz Giblin, Dr. Stewart, Dr. Chirico and the graduate school.

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KEYWORDS AND ABBREVIATIONS

Keywords

abetalipoproteinemia, apolipoprotein B, triglycerides (TG), phospholipids (PL), lipoprotein assembly, microsomal triglyceride transfer protein, MTP

Abbreviations

hMTP, Human MTP; dMTP, Drosophila MTP; ApoAI, apolipoprotein A1; ApoB, apolipoprotein B; ApoE, apolipoprotein E; BSA, bovine serum albumin; OA, oleic acid; MTP, microsomal triglyceride transfer protein; SFM, serum-free medium; L-MTP$^{-/-}$, Liver specific MTP knockout;HDL, high density lipoproteins; VLDL, very low density lipoprotein; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; EM, Electron microscopy; ER, Endoplasmic reticulum; WT, wild-type; Ad-Luc, Recombinant adenovirus expressing luciferase; Ad-hMTP, Recombinant adenovirus expressing FLAG tagged hMTP; Ad-dMTP, Recombinant adenovirus expressing FLAG tagged dMTP; PDI, Protein disulfide isomerase; VTG, Vitellogenin; LV, Lipovitellin; ABL, Abetalipoproteinemia

ABSTRACT

ApoB, MTP and lipids are essential for apoB-lipoprotein assembly and secretion. Deficiency in any of these three factors affects lipoprotein production. The main function of apoB-lipoproteins is to deliver fat to other tissues for normal physiologic activities; but their excess accumulation in the plasma induces atherosclerosis and an array of metabolic complications such as Type 2 diabetes and obesity. It is evident that approaches to reduce apoB-lipoprotein production would help treat dyslipidemias. Although the exact mechanism of apoB-lipoprotein assembly and secretion is unknown, it is believed that microsomal triglyceride transfer protein (MTP) transfers neutral lipids onto nascent apoB while it is co-translationally translocated into the endoplasmic reticulum (ER) to form primordial lipoproteins. The goal of this study was to obtain structure and function of MTP to elucidate the role of different lipid transfer activities of MTP in the biosynthesis of apoB-lipoproteins. We used two independent approaches. In the first approach, we used evolutionarily diverse MTP orthologs that differ in their lipid transfer properties. Second, we used missense mutations reported in human that display different disease phenotype to elucidate the importance of different amino acids, α-helices and β-sheets in the MTP molecule that are crucial for different lipid transfer properties.

Our evolutionary studies indicate that MTP evolved as a phospholipid transfer protein and acquired triglyceride transfer activity during a transition from invertebrate to vertebrate. Comparative *in vitro* studies revealed that *Drosophila* MTP (dMTP) could transfer phospholipids but does not transfer triglyceride. In contrast, human MTP (hMTP) transfers both phospholipids and triglycerides. The importance and exact role of these lipid transfer activities in apoB-lipoprotein assembly is not known. We set out to find the physiological gains endowed on MTP as it acquires triglyceride transfer activities. To delineate *in vivo* roles of these transfer activities, we expressed these orthologs in liver-specific MTP deficient mice that have low plasma and high hepatic lipids. MTP orthologs reduced hepatic lipids and partially restored plasma lipids in these mice. dMTP mice produced apoB100/apoB48 VLDL and phospholipid-rich apoB48 HDL lipoproteins. Lower plasma lipids in dMTP mice, compared to hMTP were due to increased degradation of apoB. hMTP increased association of triglycerides with apoB in the endoplasmic

reticulum membranes, inhibited proteasomal degradation, and formed triglyceride-rich VLDL. These studies demonstrate that MTP's phospholipid transfer activity prevents hepatosteatosis and maintains low plasma lipids by synthesizing triglyceride-poor VLDL and phospholipid-rich HDL particle. We posit that triglyceride transfer activity of MTP is dispensable and its specific inhibition could be used to lower plasma lipids.

Our missense mutation studies show that complete loss of phospholipid and triglyceride transfer activities abolish apoB secretion. This is consistent with the absence of apoB-lipoproteins in abetalipoproteinemia. Analysis of missense mutations found in hypobetalipoproteinemia showed that these mutants lacked phospholipid transfer activity and exhibited partial triglyceride transfer activity. These mutants supported secretion of apoB-lipoproteins but to a lower extent compared to normal MTP. Thus partial loss of triglyceride transfer activity and selective loss of phospholipid transfer activity in MTP could support apoB-lipoprotein assembly and secretion but to lesser extent thereby resulting in a less severe phenotype in individuals resembling heterozygous hypobetalipoproteinemia which is primarily caused by mutations in apoB rather than abetalipoproteinemia. Therefore, hypobetalipoproteinemia phenotype could be due to inefficient assembly and secretion of apoB-lipoproteins. It is likely that genetic screening for mutations in MTP besides apoB could potentially explain hypobetalipoproteinemia in subjects that do not carry mutations in apoB.

In short, our evolutionary and mutation studies suggest that both phospholipid and triglyceride transfer activities of MTP are required for optimum apoB-lipoprotein assembly. MTP has been a therapeutic target to lower hyperlipidemia; however available drugs globally inhibit MTP leading to adverse events. Recognition of different roles for phospholipid and triglyceride transfer activities suggested that these activities could be separated and targeted for differential inhibition. Domain specific antagonists rather than global inhibition of MTP could help identify better therapeutic approaches to combat hyperlipidemia, obesity and atherosclerosis.

CHAPTER I

INTRODUCTION

High plasma concentrations of apolipoprotein B (apoB)-containing lipoproteins are a risk factor for various cardiovascular and metabolic disorders such as atherosclerosis, obesity, diabetes, and metabolic syndrome (1-4). In mammals, liver is an important organ in maintaining lipid homeostasis by playing an important role in lipid metabolism. One of the major functions of liver is the synthesis of apoB lipoproteins by assembling hydrophobic lipids such as triglycerides and cholesteryl esters into a hydrophobic core surrounded by an amphipathic monolayer of phospholipids and free cholesterol with a single molecule of apolipoprotein B (apoB). Although it is known that microsomal triglyceride transfer protein (MTP) is an essential chaperone for assembly of apoB containing lipoproteins (5-9); but the exact molecular mechanisms of apoB-lipoprotein assembly are not fully elucidated (10).

Apolipoprotein B (apoB): ApoB is a large structural protein that exists in plasma as apoB48 or apoB100 originating from intestine and liver respectively (11). Mice liver secretes both apoB48 and apoB100 (12). Among all plasma apolipoproteins, apoB is unique because of its hydrophobic nature, high molecular weight and inability to transfer between lipoproteins (13). ApoB100 consists of 4536 amino acids and apoB48 corresponds to 48% of N-terminal portion of apoB100 (Fig 1). Both apoB 100 and apoB48 are encoded by the same gene (14) but cotranscriptional editing of codon 2153 into a stop codon results in premature termination forming apoB48 (15). *In vivo*, larger apoB containing lipoproteins, referred as nascent particles are secreted as VLDL and chylomicrons by the liver and intestine (16), respectively. ApoB is amphipatic in nature and this allows it to interact with the hydrophilic environment of the plasma as well as the exceptionally hydrophobic core of the lipoprotein particle allowing it to associate with hydrophobic lipids (Fig 2) (17). In humans, mutations leading to formation of truncated or non-functional apoB polypeptides causes hypobetalipoproteinemia in which there is moderate to significant reduction in apoB-lipoproteins and plasma lipids (18;19). Similar plasma lipoprotein profiles are seen in mice expressing truncated apoB forms. Although mice harboring homozygous deletions of apoB die *in utero*, viable heterozygotes have reduced apoB-lipoproteins and plasma lipids. Thus, apoB is an essential component of lipoproteins and any alteration in apoB expression affects plasma lipoprotein and lipid levels.

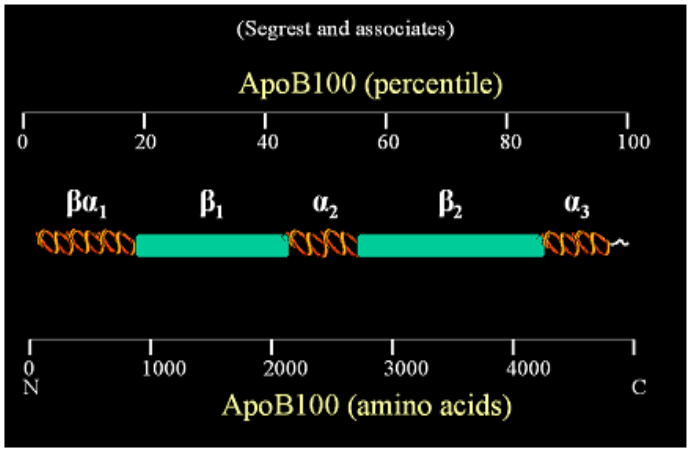

Figure 1. Structural domains in apoB100: Bottom line shows number of amino acids in apoB that correspond to middle line showing secondary structure of apoB. The middle panel shows α-helices and β-sheets present in apoB (Segrest et al, 2003). The top line displays percentile nomenclature of apoB100.

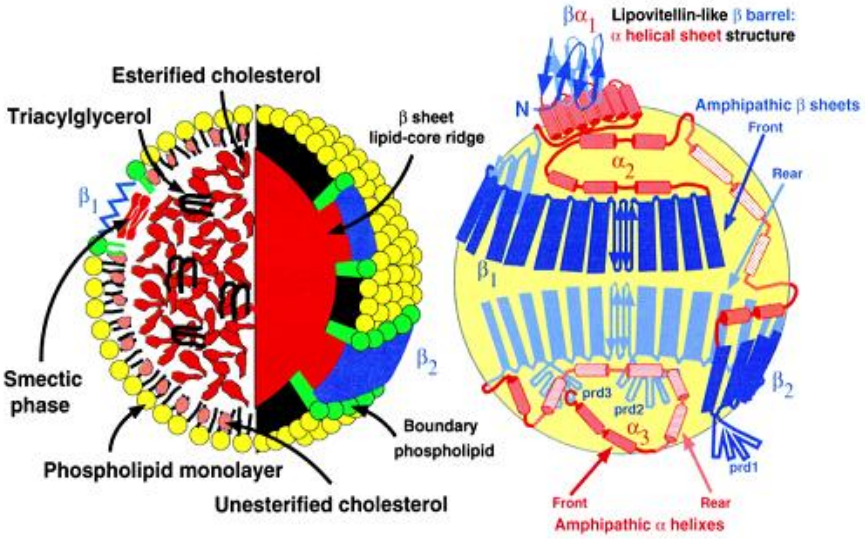

Figure 2. Schematic diagram of LDL: The left sphere demonstrates the organization of lipids including the lipoprotein core. The right sphere shows the proposed organization of apoB100 on the particle surface. (Adapted from Segrest et al, 2003)

Microsomal Triglyceride Transfer Protein (MTP): MTP was originally discovered in bovine microsomes and catalyzed transport of triglycerides (TG), cholesteryl ester (CE) and phospholipids (PL) between membranes in *in vitro* assays (20;21). Later on, this activity was also identified in rat liver and intestine. Further characterization following purification showed that MTP is a heterodimer of a large 97 kDa M subunit and a 55 kDa smaller subunit. The latter

was identified as the multifunctional protein, protein disulfide isomerase (PDI) (21;22), an abundant protein in the lumen of the endoplasmic reticulum (ER) of most tissues which plays an important role in maturation of resident ER and secreted proteins.

Structure of MTP: The amino acid sequence of the large subunit of MTP is highly conserved among species. Human MTP is composed of 894 amino acids, including an 18-amino acid signal peptide (8). The molecular mass of the mature protein is 97 kDa. Structure prediction of the large subunit of MTP based on sequence similarities with insect lipovitellin (LV) by Shoulders and group and later by Hussain and colleagues suggested that MTP is a member of the vitellogenin (VTG) family. MTP 97 kDa M subunit has been predicted to contain N-terminal β-barrel region (green), followed by a central α-helical domain (cyan), and a C-terminal lipid binding cavity containing two β-pleated sheets (red, A-sheet and blue, C-sheet cavity) (Fig 3) (23) (24;25). The predicted functional domains based on independent reports are, PDI binding (26), apoB binding sites, (26;27) and a lipid binding domain (Fig 3) (23;28).

Lipid transfer activity of MTP: The major defined function of MTP is its ability to transport lipids between unilamellar vesicles *in vitro*, which are conferred on to the protein complex by the large subunit. Kinetic studies with model membranes suggest that MTP transfers several lipids by a shuttle mechanism and that there are two binding sites for triglycerides and phospholipids in MTP (29-31). Each MTP molecule is proposed to interact transiently with a membrane, extract lipid molecules, dissociate from the membrane, bind transiently with another membrane, deliver lipids rapidly to the second membrane, and become available for another cycle of lipid transfer. MTP enhances the rate of lipid transfer between vesicles. The lipid transfer activity of MTP is classically measured using radiolabeled lipids. Later on, Hussain and colleagues developed a simple, rapid and sensitive assay to measure triglyceride transfer activity of MTP in which fluorescent triglycerides were incorporated into donor vesicles and incubated with acceptor vesicles in the presence of MTP source. MTP removes quenched fluorescent lipids from donor vesicles exposing the fluorescent moiety during transfer, which is measured as an increase in fluorescence detected by a fluorimeter. This increase in lipids transferred by MTP can be monitored in real time. Similarly phospholipid and cholesteryl ester transfer by MTP can be measured (32;33).

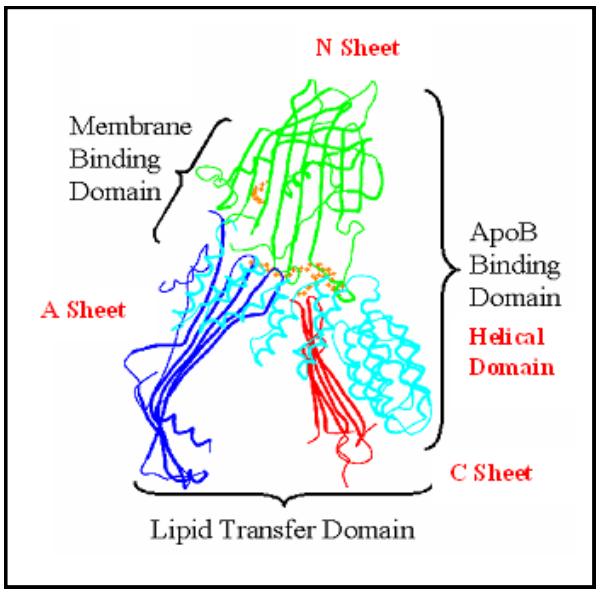

Figure 3: Predicted structure of MTP with putative domains and their role: Proposed three-dimensional structure of MTP-M, large 97 kDa subunit based on sequence homology with lipovitellin (*Hussain et al, 2003*).

MTP and Abetalipoproteinemia (ABL): Bassen and Kornzweig described clinical manifestations of abetalipoproteinemia as lipid malabsorption, acanthocytosis, pigmenatry degeneration of the retina, and ataxia with hypercholesterolemia (34). Based on these symptoms it was suggested that the disease results from an inborn error of lipid metabolism that leads to deleterious effects on the erythrocytes and nerve cells. The name of the disease was coined based on absence of lipoproteins with β-electrophoretic mobility. More than 100 ABL cases have been reported so far and for many cases of ABL, mutations in MTP have been established following sequencing (35;36). Infants with ABL are asymptomatic but with diets rich in lipids digestive disturbances appear within first few months. Plasma levels of vitamin E and β-carotene are extremely low in ABL due to fat-soluble vitamin deficiency resulting from chronic fat malabsorption. But vitamin D, K and A are only diminished to low levels due to the presence of alternate modes of transport besides plasma transport by apoB-lipoproteins. During the 1st and 2nd decade, the vitamin deficiencies result in neuro-opthalmologic complications that determine morbidity of ABL. There are numerous downstream ramifications resulting from absence of MTP ranging from neurological to cardiac complications (37).

Intestinal and hepatic ultrastructural studies of ABL patients showed intracellular accumulation of lipid droplets without formation of lipoprotein particles suggesting defects in apoB-lipoprotein

assembly. Wetterau et al first established link between MTP and ABL showing that 97 kDa M subunit of MTP was absent in intestinal biopsies from ABL patients and they also lacked lipid transfer activity (5). These data together with two other studies established that the large subunit gene of MTP is involved in ABL (8;36). The gene was localized to 4q22-24. Many patients since then have been studied at the molecular level and most of the mutations were demonstrated on both alleles in majority of the cases. The mutations range from frameshift causing truncations of the large subunit of MTP to missense with single amino acid substitutions (38).

Role of MTP in apoB-lipoprotein assembly and secretion: Once MTP was established as an essential cofactor for lipoprotein production, efforts were focused on elucidating the precise role of MTP in the phenomenon. Lipoprotein assembly and secretion is complex process. Although it is well established that both apoB and MTP are essential for assembly, but the exact mechanism and different steps of assembly and secretion of apoB-lipoproteins are not fully elucidated. There is considerable controversy regarding the assembly process and exact mechanism but a general consensus for the major pathway has been established from independent reports. As apoB is co-translationally translocated across ER membrane, simultaneously lipids are added. A small, dense, lipid-poor precursor primordial lipoprotein particle is formed referred to as the first step of lipoprotein assembly. Bulk addition of triglycerides occurs either continuously or in a second step by fusion of triglyceride-rich lipid droplet within ER lumen by ‘core expansion’ of the primordial lipoproteins to nascent apoB. MTP binding to nascent apoB (39;40) initiates lipidation of nascent apoB by its neutral lipid transfer activity thereby preventing its degradation.

Several published observations confirm that MTP plays an important role in the early stages of lipoprotein assembly. Cell culture studies showed that small apoB particles with density of HDL and LDL can be secreted from hepatocytes and enterocytes. However these particles are absent in ABL patients (7) indicating that block in lipoprotein synthesis that occurs in the absence of MTP occurs at a point in the assembly process before the formation of these particles . This defines inevitability of MTP for the first step of lipoprotein assembly. This was further

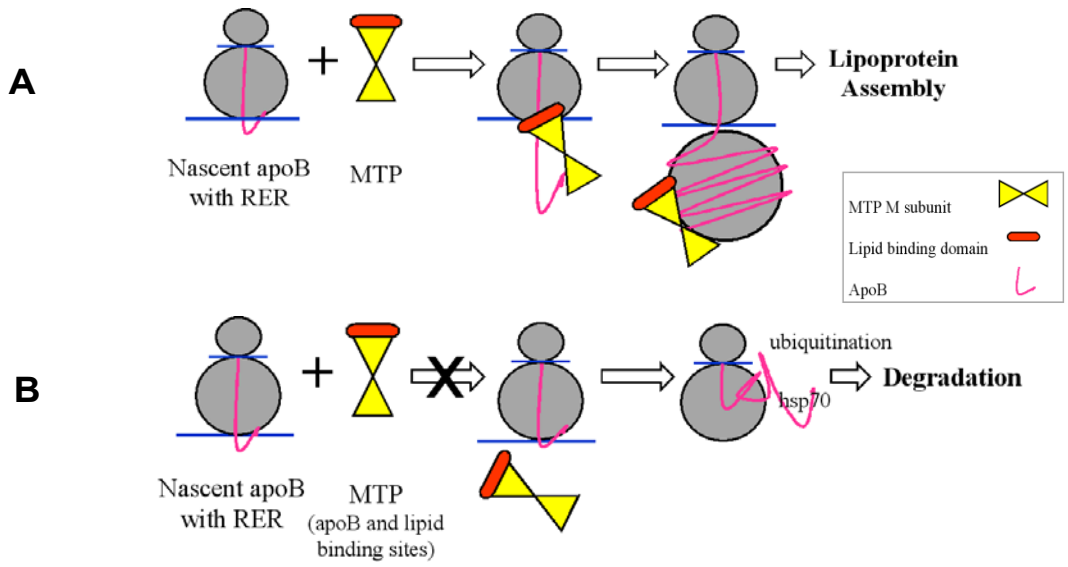

Figure 4. MTP and its role in prevention of apoB degradation by lipidation of nascent apoB: (A) Nascent apoB interacts with the inner leaflet of the ER membrane and interacts with MTP resulting in lipoprotein assembly and secretion. **(B)** Abrogation of apoB-MTP binding by the use of inhibitors or mutations in the binding site leads to apoB degradation involving hsp70 binding, ubiquitination, and proteosomal degradation. (*Adapted from Hussain et al, 2003*)

confirmed from cell culture studies, which normally do not make lipoprotein particles. Coexpressing apoB and MTP in cells lacking endogenous expression of both showed that MTP is required for lipoprotein biosynthesis (9;41). Co-expression of apoB and MTP in COS and HeLa cells secreted small dense lipoprotein particles which were not secreted when MTP was absent.

Transfecting cells that express neither MTP nor apoB with different apoB polypeptides showed that secretion of N-terminal 1000 or fewer amino acids of apoB polypeptides does not require MTP (40;42). These shorter apoB polypeptides secreted are primarily associated with phospholipids with a density greater than 1.23 g/ml. On the other hand, secretion of larger apoB polypeptides with a density under 1.21 g/ml requires MTP. Domains in proximity of N-terminal of apoB interact with MTP and are essential for the formation of lipoprotein complex. Studies have also concluded that MTP is required during the early stages of assembly to prevent the aberrant folding of apoB and its degradation by proteasomes (Fig 4) (43-45).

Expression of different length isoforms of apoB in hepatoma cells and co-expression of both apoB and MTP in non-hepatoma cells results in secretion of lipoprotein particles of different

densities, which have been referred as primordial lipoprotein particles (46-51). In contrast to these cells in culture, *in vivo* liver and intestine synthesize larger apoB containing lipoproteins VLDL and chylomicrons, respectively (15;52). Thus transition from the higher density HDL like primordial particle to a mature lipoprotein like VLDL with low density is unclear.

Role of MTP in later stages of lipoprotein assembly remains elusive owing to lack of experimental evidence. Independent studies have also shown that neutral lipid transfer activity of MTP is responsible for lumenal accretion of neutral lipids (53) which is presumed to act as a source of lumenal lipids that might associate with preformed primordial particles to form large nascent lipoproteins (54). Thus it is proposed that formation of larger nascent lipoproteins could be achieved by fusion of primordial particles with these lumenal neutral lipid-rich droplets by "core expansion"(16). There are two proposed mechanisms of how neutral lipids may be added to the core of the nascent lipoprotein precursor formed during the early step of assembly. Either individual lipid molecules are continuously added probably by MTP lipid transfer activity or fusion of the primordial particle with lipid-rich droplet (Fig 5). However, definitive experimental evidence outlining this phenomenon is lacking.

MTP and Steatosis: Several reports in humans have indicated cellular fat accumulation (steatosis) associated with decreased MTP expression (24)**.** Hepatic steatosis has also been reported with MTP deficiency (55)**.** Large fat droplets in hepatocytes were revealed by microscopic examination (56). Besides genetic defects, there is an association between low MTP expression due to a polymorphism in the promoter sequence and liver steatosis in type 2 diabetes patients (57;58). Hepatitis C virus type 3 infected subjects show reduced MTP activity and mRNA levels and high degree of steatosis (59;60). Several animal studies also indicate a relationship between decreased MTP expression and steatosis (6;56). Chronic alcohol abuse causes fatty liver and is associated with decreases in MTP (61). Transgenic expression of Hepatitis C virus core protein inhibits MTP activity, reduces VLDL secretion, and causes steatosis (59). Similarly, inhibition of MTP in cells also results in triglyceride accumulation. Therefore, significant reduction in MTP activity is usually associated with steatosis.

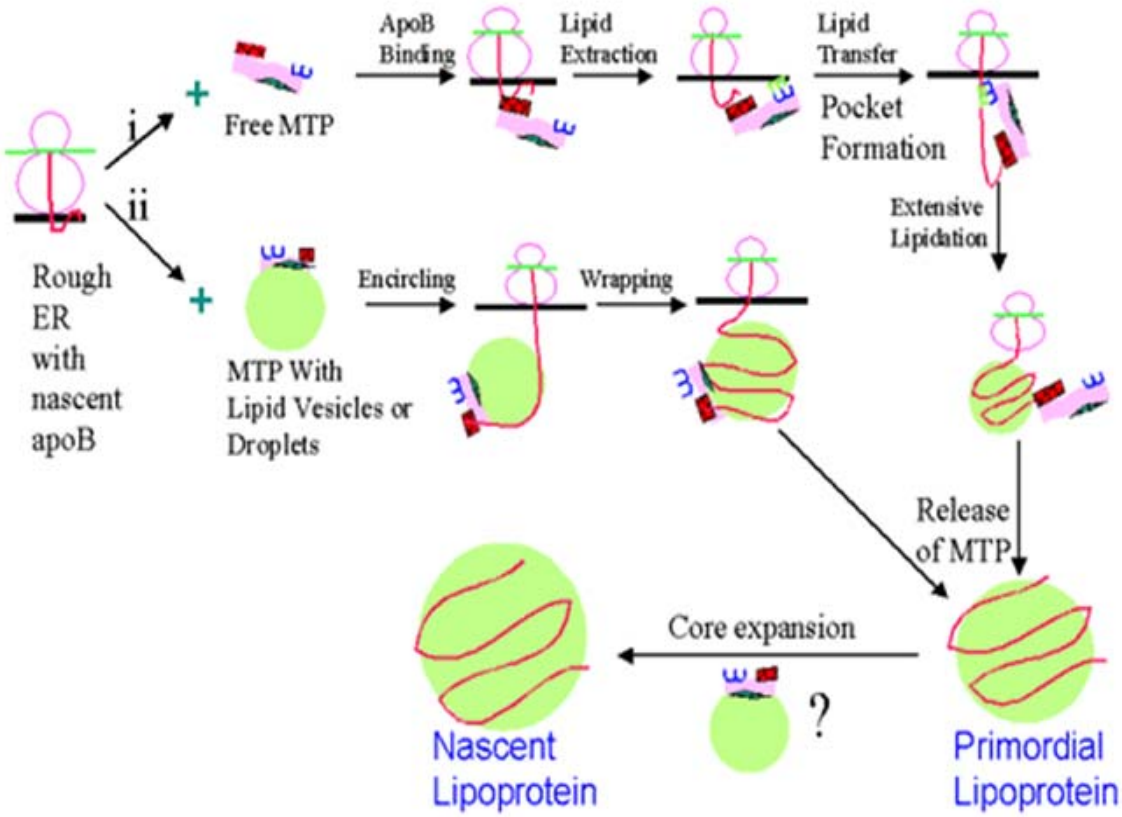

Figure 5. Lipidation of nascent apoB by MTP: Lipidation of apoB is predicted to occur by two pathways. (i) Free MTP may bind to apoB that is not associated with lipid droplets or vesicles. This apoB bound MTP can extract lipid molecules from the membrane and transfer them to nascent apoB. Multiple repetitions of this process will result in extensive lipidation of apoB. MTP molecules that are not physically associated with apoB can further assist in this step. (ii) MTP associated with lipid vesicles may bind to apoB and provide a lipid core for the nascent apoB to encircle and wrap around it. This would result in an efficient and faster assembly of apoB-containing lipoproteins and may predominate during excess lipid availablility. Release of MTP would result in formation of secretion-competent primordial lipoproteins. Formation of larger, nascent lipoproteins could be achieved by extensive lipidation of primordial lipoprotein by 'core-expansion' that could result from fusion with TG-rich droplets in the ER lumen. However, experimental evidence to support this process is lacking. (*Hussain et al, 2003*)

MTP as a Therapeutic Target: Hyperlipidemia is a risk factor for atherosclerosis. Significant progress in lowering plasma lipids has been made with statins by increasing their catabolism. However, attempts to control lipoprotein production have not yet been successful. ApoB and MTP are prime candidates to curb lipoprotein production. ApoB lacks lipid transfer activity therefore therapeutic interventions use siRNA-mediated approach to lower its production.

On the contrary, MTP unlike apoB has lipid transfer activity. Hence it has been a favorite target to identify small molecule inhibitors and to lower plasma lipids. MTP antagonists further reestablish that MTP is essential for lipoprotein assembly and secretion. Use of MTP antagonists *in vitro* inhibited lipid transfer activity and is associated with decreased apoB secretion *in vivo* (62-64). Furthermore, cell culture studies showed that wild type MTP can rescue apoB secretion but mutated proteins cannot. These results establish that the lipid transfer activity plays a key role in the assembly process.

Indeed several MTP antagonists have been identified that decrease lipoprotein production and plasma lipids (6;63;65-67) in humans. Unfortunately these drugs have been associated with side effects thereby complicating the treatment potentials. The first side effect is related to the inhibition of chylomicron assembly by enterocytes and manifests as gastrointestinal disturbances such as steatorrhea and diarrhea. These disturbances have been successfully avoided by administering MTP inhibitors 4 h after the supper. The second side effect is related to the toxicities associated with inhibition of hepatic lipoprotein assembly and secretion due to MTP inhibition. Thus, there is a critical need for novel approaches to inhibit MTP without causing steatosis.

MTP evolution: Dedicated systems for transporting fat, fat-soluble vitamins and other hydrophobic proteins are present in all animal species. Translocation of lipids and nutrients from extraovarian tissue to the developing oocyte of oviparous vertebrates and invertebrates by vitellogenins (VTG) is one of the ancient lipid transport systems. VTGs in present day species exhibits conservation in both sequence and function inspite of the evolutionary divergence from nematodes through vertebrates (27;28). Both ApoB and MTP are member of this gene family (17;23).

While MTP is required for the assembly of apoB lipoproteins, it is clear that this protein is not restricted to organisms that utilize apoB to transport extracellular lipids. MTP orthologs have been reported in nematodes, insects, fish, birds and mammals (68). No MTP orthologs were identified in organisms that diverged earlier than nematodes suggesting that MTP may have evolved during emergence of nematodes or earlier precursor shared minimal homology. The

conservation of MTP in insect and nematodes reflects that a property other than triglyceride transfer must be ancient and required for survival. Rava and Hussain have shown that insect MTP can transfer phospholipids (69). Thus the ancient activity may be the phospholipid transfer activity and its initial biological role in phospholipid metabolism.

Functional studies involving expression of different MTP orthologs in COS cells that do not express endogenous MTP indicated that *Drosophila* MTP can transfer phospholipids but cannot transfer triglycerides (69;70). In contrast, human MTP can transfer both phospholipids and triglycerides (69). Based on these studies, it was concluded that MTP evolved as a phospholipid transfer protein and acquired triglyceride transfer activity during evolution from invertebrates to vertebrates. Even though it lacked triglyceride transfer activity, dMTP was able to assist in the secretion of apoB lipoproteins indicating that phospholipid transfer activity of MTP is sufficient to support apoB-containing primordial lipoprotein secretion (69).

Major function of MTP is to assist in the dietary fat and sterols via chylomicron assembly and secretion in mammals. Dietary lipids are transported by lipid transfer particles, lipophorins in insects. Insect fat body, which performs the equivalent function to vertebrate liver and adipose tissue synthesizes and secretes HDL into the hemolymph. These particles acquire lipids from the midgut and fat body via the action of extracellular lipid transfer particle (LTP) and become low density lipophorins. These lipophorin particles then interact with cell surface receptors to deliver lipids to their target tissues (24). Thus phospholipid transfer activity of MTP in insects probably plays a crucial role in the intracellular assembly of high-density lipophorins by the fat body (24;70) and that this process may involve the phospholipidation of nascent apolipophorin by MTP (Fig 6).

Thus it was concluded that phospholipid transfer activity might have been evolutionarily the earliest transfer activity. Triglyceride transfer was acquired as an adaptation to accommodate expanding requirement of triglyceride transport in vertebrates.

Present Studies: The goal of this study was to obtain structure and function of MTP to elucidate the role of different lipid transfer activities of MTP in the biosynthesis of apoB-lipoproteins. We used two independent approaches. In the first approach, we used evolutionarily

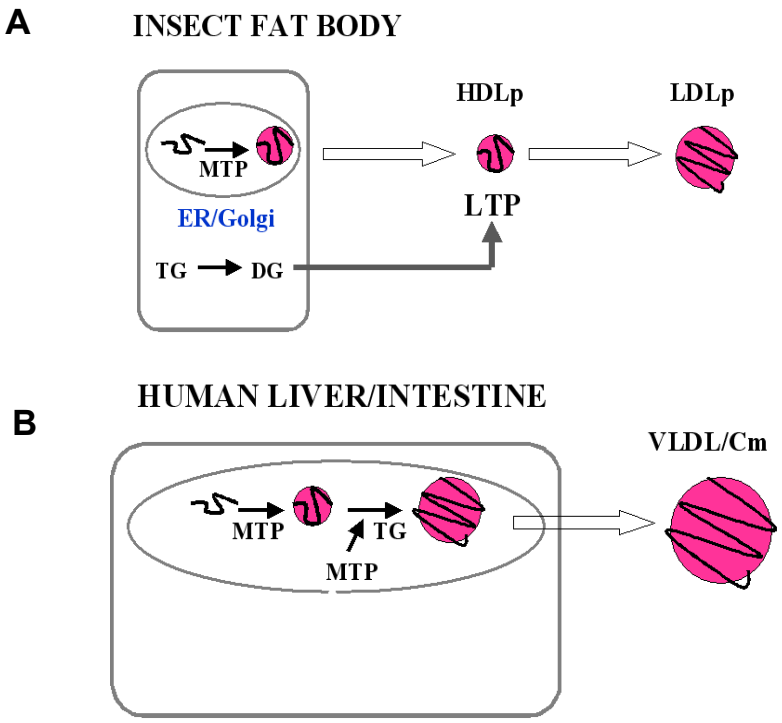

Figure 6. Schematic illustration of lipid transport systems in different compartments in insects and humans: Lipid is stored as triglyceride in fat body tissue in insects. Triglyceride undergoes partial hydrolysis to diacylglycerol following hormonal stimulation. This is transported across from plasma membrane to an acceptor lipoprotein, high density lipophorin (HDLp) by LTP. Probably, insect MTP could help in the formation of nascent HDLp in the insect fat body that is secreted in the hemolymph. (1) Conversion of HDLp to LDLp occurs in extracellular space in insects through transfer of diacylglycerol from fat body to HDLp . (2) In humans, both precursor apoB apoB-containing lipoprotein formation and second step core-expansion of the lipoprotein precursos by subsequent extensive lipidation occurs in the secretory pathway. (*Adapted from Shoulders and Shelness, Curr. Topics in Med. Chem., 2005*)

diverse MTP orthologs that differ in their lipid transfer properties. Second, we used missense mutations reported in human that display different disease phenotype to elucidate the importance of different amino acids, α-helices and β-sheets in the MTP molecule that are crucial for different lipid transfer properties.

We set out to explore what roles phospholipid and triglyceride transfer activities of MTP play in different steps of apoB-lipoprotein assembly. Availability of these human and drosophila MTP orthologs provide us with a unique opportunity to delineate the role of different lipid transfer activities of MTP in lipoprotein assembly. Our goal is to find out the type of particles synthesized by dMTP and what gains hMTP made in acquiring triglyceride transfer activity during evolution. In addition, we intended to know which lipid transfer activity of MTP is critical for this process. Our working hypothesis was that different lipid transfer activities of

MTP carry out different functions. We evaluated all possible mechanisms for the differences in apoB biosynthesis owing to distinct lipid transfer activity and anticipated that answering these fundamental questions will help us to understand the mechanistic role of MTP in apoB-lipoprotein assembly. Our evolutionary and mutation studies suggest that both phospholipid and triglyceride transfer activities of MTP are required for optimum apoB-lipoprotein assembly. Recognition of different roles for phospholipid and triglyceride transfer activities suggested that these activities could be separated and targeted for differential inhibition.

Contributions:

Chapter II: The study aims and hypotheses were conceived by MMH and IK. IK and MMH designed all the experiments. IK developed, optimized and conducted all the experiments and drafted the paper. MMH edited the paper drafts. IK performed RT-PCR experiments with help from JI. IK did all the recombinant adenoviral preparations and amplifications for the *in vivo* studies throughout the project using the protocol provided by MW and DC at UAB. One pair of MTP floxed mice and MTP floxed Albumin cre mice was obtained from SZ and RB from Harvard Medical School. GS is long-term collaborator of MMH. dMTP plasmids were initially obtained from GS for studies by Rava.

Chapter III: MTP mutations studies were not proposed as part of thesis for this study. IK prepared all the stable cell lines, designed and performed all the experiments. The retroviruses were generated by using the plasmids constructed by AEC. IK drafted the paper and MMH edited it.

Note: Although the papers have been included as chapters in the thesis. They have been formatted to accommodate thesis requirements. Much more detailed and extensive information including figures has been provided in the course of rewriting compared to the original manuscript to meet the requirements of a thesis document.

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CHAPTER II

The phospholipid transfer activity of MTP is sufficient to produce apoB-lipoproteins and reduce hepatosteatosis, while maintaining low plasma lipids in mice

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Running title: Phospholipid transfer activity of MTP is sufficient for in vivo lipoprotein assembly and avoiding steatosis

Abbreviations used: Ad, adenovirus; apoB, apolipoprotein B; apoAI, apolipoprotein AI; ER, endoplasmic reticulum; MOI, multiplicity of infection; MTP, microsomal triglyceride transfer protein; hMTP, Human MTP; dMTP, Drosophila MTP; TG, triglycerides; PL, phospholipids; HDL, high-density lipoprotein; VLDL, very low density lipoprotein; PFU, Plaque forming units; EM, Electron microscopy.

SUMMARY

Microsomal triglyceride transfer protein (MTP) is essential for apoB-lipoprotein biosynthesis, whose overproduction contributes to hyperlipidemia, an atherosclerosis risk factor. We showed that *Drosophila* MTP (dMTP) transfers phospholipids whereas human MTP (hMTP) transfers phospholipids and triglycerides. To delineate *in vivo* roles of these transfer activities, we expressed these orthologs in liver-specific MTP deficient mice that have low plasma and high hepatic lipids. MTP orthologs reduced hepatic lipids and partially restored plasma lipids in these mice. dMTP mice produced apoB100/apoB48 VLDL and phospholipid-rich apoB48 HDL lipoproteins. Lower plasma lipids in dMTP mice, compared to hMTP were due to increased degradation of apoB. hMTP increased association of triglycerides with apoB in the endoplasmic reticulum membranes, inhibited proteasomal degradation, and formed triglyceride-rich VLDL. These studies demonstrate that MTP's phospholipid transfer activity prevents hepatosteatosis and maintains low plasma lipids by sysnthesizing triglyceride-poor VLDL and phospholipid-rich HDL particle. We posit that triglyceride transfer activity of MTP is dispensable and its specific inhibition could be used to lower plasma lipids.

INTRODUCTION

High plasma concentrations of apolipoprotein B (apoB)-containing lipoproteins are a risk factor for various cardiovascular and metabolic disorders such as atherosclerosis, obesity, diabetes, and metabolic syndrome (1-4). Both over production and decreased catabolism of apoB lipoproteins contribute to hyperlipidemic states. Microsomal triglyceride transfer protein (MTP) is the essential chaperone for the assembly and secretion of apoB-lipoproteins as evidenced by the absence of these lipoproteins in the plasma of abetalipoproteinemia subjects that have mutations in the *MTTP* gene (5-9). Although the exact mechanism of apoB-lipoprotein assembly is still undefined, it is believed that MTP transfers neutral lipids onto nascent apoB while it is co-translationally translocated into the endoplasmic reticulum (ER) to form primordial lipoproteins (10-14). These are subsequently lipidated by a second step 'core-expansion' involving fusion of primordial lipoproteins with apoB-free triglyceride-rich droplets to form mature lipoproteins (15;16). MTP is also implied to be essential for the partitioning of triglyceride in the ER lumen (14;17).

In vitro studies indicate that MTP transfers lipids by a ping-pong bi-bi shuttle mechanism where MTP interacts with donor membranes and extracts lipids, and then associates with acceptor membranes to deposit them (18;19). Triglyceride transfer is fast, whereas phospholipid transfer is biphasic consisting of fast and slow phases. Based on these studies, it has been proposed that MTP has two different lipid transfer sites; a high affinity-binding site for triglycerides and phospholipids, and a second low affinity binding site for phospholipids only (18-20). But, it is unknown whether MTP directly transfers lipids onto apoB *in vivo* and, if it does, whether both neutral and polar lipid transfer activities of MTP are critical for lipoprotein assembly.

A breakthrough in understanding of the lipid transfer properties of MTP came with the identification of *Drosophila* MTP (dMTP) ortholog that was shown to transfer both fluorescent and radiolabeled phospholipids but was unable to transfer triglycerides (21-23). Although it lacked triglyceride transfer activity, dMTP was able to assist in the secretion of small apoB48- and apoB52-lipoproteins in cell culture studies (21-23). Evolutionary studies showed that MTP evolved as a phospholipid transfer protein and acquired triglyceride transfer activity during a transition from invertebrates to vertebrates (23). It is unknown whether dMTP can support lipoprotein assembly in mammals and why the triglyceride transfer activity was acquired during evolution.

MATERIALS AND METHODS

Materials: Restore western blot stripping buffer (Thermo scientific), 4-15% Ready Tris-HCl gels and precision-plus protein ladder (Bio-Rad Laboratories), ß-mercaptoethanol (Invitrogen), Calcium chloride, Ethylene diamine tetraacetic acid, Hepes, Kanamycin, Magnesium chloride, Molecular weight markers, Oleic acid, Phosphotungstate, Tween-20 and Triton X-100 (Sigma). Cesium chloride, Glycerol, Heparinized micro-hematocrit capillary tubes, Sodium Dodecyl Sulfate, and Sucrose (Fisher chemicals).

Cell culture and apoB secretion: COS7 cells were grown in Dulbecco's modified Eagle's medium (CellGrow) containing 10% fetal bovine serum supplemented with L-glutamine and antibiotics. ApoB expression vectors were transfected into COS7 cells using FuGENE 6 (Roche Applied Sciences) according to the instructions provided by the manufacturer. The cells were detached from the plate after 8-10 h using trypsin and were plated in 6-well plates (400,000 cells/well). These cells were then transduced with Ad-Luciferase, Ad- hMTP-FLAG or Ad-dMTP-FLAG. Media was replaced after 48 h post-transduction of recombinant adenoviruses with 1 ml of DMEM containing 0.4 mM oleic acid complexed with 1.5% BSA and 1mM glycerol. Media and cells were collected for ELISA and functional assay, respectively, after 16 h of incubation in the lipid-supplemented media. Protease inhibitors (Sigma-Aldrich) were added to the media. Cells were harvested and cell-debris was pelleted after brief spinning. ApoB contents in the media were measured by ELISA. Cells were used for analyzing lipid transfer as a functional assay for MTP.

Immunofluoroscence: COS7 cells were grown on coverslips in 12-well cell culture plates and sequentially transfected with apoB48 expression plasmid followed by Ad-hMTP-FLAG or Ad-dMTP-FLAG. Cells were fixed, blocked and probed with primary and secondary antibodies as described earlier (22). Similarly, liver and intestine tissues were prepared for immunofluoroscence (24). Tissues were fixed in 4% paraformaldehyde, embedded in Tissue-Tek O.C.T. compound (Fisher Scientific). Tissues were sectioned onto Tissue Tack slides (Polysciences, Niles, IL) and incubated with 0.05% Tween 20 in 10 mM sodium citrate buffer (pH 6.0) at 37^{o}C for 15-20 min. PBS containing 1mM $MgCl_2$, 0.5 mM $CaCl_2$, 3%BSA and 1% horse serum was used as blocking reagent. Primary and secondary antibody dilutions were made in the same buffer. The cells were incubated for 1 h with 1:100 dilution of mouse anti-FLAG M2 (Sigma) and Topro 3 iodide

(Molecular Probes) for 1 h with 0.1% Triton-X to increase the permeability. Subsequently, Alexa Fluor 488-conjugated goat anti-mouse IgG1 (Green fluorescence) and Alexa Fluor 594-conjugated goat anti-rabbit IgG1 (Red fluorescence) (Molecular Probes) were incubated for 1 h following primary antibody treatment. The coverslips were mounted in anti-fade (Vectashield) to prevent fluorescent bleaching and analyzed with a laser-scanning microscope (Model LSM 510, Carl Zeiss). Images were imported and analyzed with Photoshop 6.0 before exporting to Illustrator CS2 (Adobe).

Lipid transfer assays: MTP activity in COS7 cells was determined by triglyceride transfer assays as described earlier (22). MTP activity in tissues was determined using triglyceride and phospholipid transfer assays as a measure of functionality of human and drosophila MTP. Liver and proximal small intestinal pieces were extensively washed in ice-cold PBS. Tissues were then homogenized in 1 ml of ice-cold homogenizing buffer (Tris-HCl, pH7.6; 1mM EGTA, and 1 mM MgCl2) containing protease inhibitor cocktail (Sigma) using a glass homogenizer. Microsomal fractions were obtained by centrifuging the homogenates in SW55 Ti rotor at 50,000 rpm for 1 h at 10^{o} C. Supernatants were used for MTP lipid transfer assays in triplicates as described (25). Fluorescence values containing no MTP were subtracted from samples containing MTP proteins and divided by the total fluorescence of vesicles to determine percentage of lipid transfer. Similarly phospholipid transfer assays were performed (26;27).

Animals: *Mttp*$^{fl/fl}$ (WT) breeding pairs were obtained from Harvard medical school and crossed with *Alb-cre* (The Jackson Laboratories) mice to obtain *Alb-cre-mttp*$^{fl/fl}$ mice with liver specific ablation of MTP (L-MTP$^{-/-}$). Mice were kept on a 07:00/19:00-h lighting schedule with free access to water and standard laboratory chow. For steady state levels, mice were fasted for 6 h before sacrifice. Mice were anesthetized and blood was collected from heart. Liver and intestine were collected, washed extensively with ice-cold PBS and used for lipid extraction. Tissue homogenates were used for lipid extraction using Bligh and Dyer method (28). Triglyceride (Infinity triglyceride kit), total and free cholesterol (Wako Chemicals GmbH) and phospholipid (Phospholipid C, Wako) levels in tissues and plasma were determined according to manufacturer's instructions and concentrations were calculated from standard curves prepared in parallel. Esterified cholesterol in tissues was estimated by subtracting free cholesterol from total cholesterol.

Generation of recombinant adenovirus and *in vivo* delivery: The full-length hMTP or dMTP FLAG tagged fusion proteins were cloned into pShuttle vector (Stratagene). To generate a recombinant adenovirus backbone containing the orthologs, pShuttle vectors containing hMTP-FLAG or dMTP-FLAG sequences were co-transfected with pAdEasy-1 (Adenovirus backbone) into BJ5183 cells (Stratagene) to allow recombination. Potential adenoviral recombinants containing MTP genes were screened using restriction digest according to manufacturers' suggestions. The recombinant adenoviral backbone was linearized and transfected in 293 A cells (QBiogene) and overlayed with agar. Single plaques were selected and small-scale virus amplification was done for initial screening by western blot and functional assay. Large-scale amplification was carried out and virus were purified and concentrated by double Cesium Chloride (CsCl) gradient centrifugation. Viral stock titers were determined using $TCID_{50}$ assay, which is a modified plaque forming unit assay (Stratagene).

Adenoviral vectors were diluted in sterile PBS to deliver doses of $1X10^7$, $1X10^9$ or $1X10^{10}$ pfu of Ad-Luciferase, Ad-hMTP-FLAG or Ad-dMTP-FLAG per mouse. The dose of Ad-Luciferase was similar to the MTP ortholgs to control for any adverse effects contributed by adenoviral vectors *in vivo*. A total of 100-150 µl were injected into the tail vein of mice immobilized by restrainer (BrainTree Scientific). The other control was for tail vein injections and vehicle control in which the animals received sterile PBS.

For mechanistic experiments, on the third day following injections, mice were fasted for 4 h, injected intraperitoneally with Poloxamer 407 (P407) (30 mg/mice) to block plasma lipoprotein hydrolysis and clearance (29) and fasted for another 2 h before sacrifice allowing to measure lipoprotein production by preventing plasma clearance. Blood was collected in EDTA containing tube by cardiac puncture and plasma was separated by centrifugation. Tissues were collected and snap frozen in liquid nitrogen and stored at -80^0 C.

Plasma lipid and lipoprotein analysis: Plasma cholesterol and triglyceride levels were measured using colorimetric enzymatic assays according to manufacturers' recommendations (Infinity triglyceride and cholesterol). Free glycerol (Free glycerol determination kit, Sigma) in plasma were subtracted from total triglyceride values to represent triglyceride only. Phospholipid levels were measured with Phospholipids C assay that measures choline-phospholipids according to

manufacturer's protocol (Wako Chemicals). Plasma high-density lipoproteins were isolated after the precipitation of apoB-lipoproteins with phosphotungstate/$MgCl_2$ reagent (Roche) (25) and lipids were measured as mentioned above. HDL lipid values from the totals were subtracted to obtain non-HDL apoB-lipoprotein triglyceride and cholesterol levels. The distribution of lipids in the plasma lipoprotein fractions was determined by fast-performance liquid chromatography (FPLC). Triglycerides, cholesterol and phospholipids were measured in fractions corresponding to VLDL and HDL lipoproteins as described above. In addition, VLDL and HDL fractions were pooled to measure lipids in VLDL and HDL lipoproteins.

Western blot analysis: Small intestine and liver tissues from mice were homogenized and microsomal proteins were isolated as described previously (30;31) and separated by SDS-PAGE. Western blots were developed to determine expression of endogenous MTP protein levels using purified mouse anti-MTP (BD Transduction Laboratories) in liver and intestine and MTP-FLAG expression in liver induced by *in vivo* delivery of recombinant adenoviruses using mouse anti-FLAG M2 (Sigma) and GAPDH (Abcam Inc), β-actin (AbCam Inc) or albumin (Santa Cruz) as loading controls. Plasma samples were subjected to western blot analysis and apoB was detected using rabbit anti-mouse apoB antibody (Biodesign), and goat polyclonal apoAI (BD Biosciences).

Hepatic triglyceride production rate: Adenoviral vectors were diluted in sterile PBS to deliver doses of $1X10^{10}$ pfu of Ad-Luciferase, Ad-hMTP-FLAG or Ad-dMTP-FLAG in L-$MTP^{-/-}$ mice. In addition, wild type and L-$MTP^{-/-}$ were injected with PBS as control. After 72 h of adenoviral delivery mice were fasted for 4 h. Blood was drawn from tail to measure the basal level of triglycerides. Blood was drawn at different time points from tail after blocking lipoprotein clearance by P407 administration to measure triglyceride content (29). Total plasma triglycerides and VLDL-triglycerides were measured and production was plotted as function of time in X-Y plot. Triglyceride secretion rates were calculated from the difference in plasma triglyceride levels over different time points following P407 injection from the basal state and expressed as micromoles of lipids produced per hour per gram of body weight assuming a plasma volume of 3.5% (liters per kilogram).

Determining hepatic apoB production: Adenoviral vectors were diluted in sterile PBS to deliver doses of $1X10^{10}$ pfu of Ad-Luciferase, Ad-hMTP-FLAG or Ad-dMTP-FLAG in L-$MTP^{-/-}$ mice. In

addition wild type and L-MTP$^{-/-}$ mice were injected with PBS as control. After 72 h mice were fasted for 4 h and 1mCi of of [^{35}S] Easy Tag Protein labeling Mix, ([^{35}S] promix) (Perkin Elmer) was injected via tail vein to achieve hepatic labeling of proteins, in this case apoB. P407 was injected intraperitoneally in these mice after 30 minutes to block clearance of plasma lipoproteins thereby enabling to measure hepatic production of newly synthesized apoB over time. Blood was collected at different time points following collection at basal time point at 5 min and plasma was isolated. SDS-PAGE sample buffer was added to each sample and boiled, run on 5% SDS-PAGE gel, incubated with Amplify (GE Healthcare), dried and fluorographed. ApoB100 and apoB48 levels in all groups at different time points were quantified by densitometry using NIH image software. Radioactivity in apoB48 and apoB100 were determined in the bands cut from the gel and dissolved in soluene (Perkin Elmer) (32). Amounts of radioactivity in apoB100 and apoB48 bands were counted using scintillation counter, corrected for 5 min apoB counts and secretion rates were calculated as cpm/µl/h (32).

Analysis of VLDL and HDL apolipoproteins: As described above, mice in each group were fasted and metabolically labeled with [^{35}S] promix via tail vein. Plasma samples from each group were pooled and size fractionated using FPLC. Size fractionated samples of plasma into VLDL and HDL were obtained. VLDL lipoprotein fractions were subjected to immunoprecipitation for apoB using rabbit anti-mouse apoB antibody with immunoprecipitation buffer containing 0.5 M Tris-HCl, pH 7.4; 1.5 m NaCl, 500mM EDTA, 1% (w/v) SDS (33). ApoB complexes were pulled down by protein A-Sepharose beads (10). SDS-PAGE sample buffer (6mM Tris-HCl, 10% Glycerol, 2% SDS and 5% β-mercaptoethanol) were added to the immunoprecipitated apoB and boiled. Samples were separated on 5% SDS-PAGE gels, fixed and incubated with amplify for 30 min and dried.

To analyze apoAI free HDL lipoproteins, HDL lipoproteins were incubated with anti-mouse apoAI antibody and protein A-sepharose beads. The mixture was spinned down briefly to obtain the supernantant devoid of apoAI and immunoprecipitated with apoB as described above. Samples were pelleted, boiled and run on 5% SDS-PAGE. Gels were incubated with Amplify, dried and fluorographed. ApoB100 and ApoB48 levels in all groups were quantified by densitometry using NIH image software. Radioactivity in apoB48 and apoB100 were also quantified in the bands cut from the gel.

Histology and electron microscopy: Tissues were fixed in 4% paraformaldehyde, embedded in Tissue-Tek O.C.T. compound (Fisher Scientific), sectioned onto Tissue Tack slides (Polysciences, Niles, IL), and stained with hematoxylin and eosin (H&E). Lipids were stained with Oil-Red O and examined by light microscopy. For electron microscopy, liver tissues were fixed in 2% glutaraldehyde and 4% paraformaldehyde solution followed by 1% osmium tetraoxide solution. Tissues were dehydrated through graded series of 70, 90 and 95 percent ethanol and embedded in epoxy (Epon A12) resin. Thicker sections (1μm) were cut and stained with toluidine blue for light microscopy. For ultrastructural analyses, 50 nm sections were viewed using scanning electron microscopy after positively staining with uranyl acetate and lead citrate.

For negative staining and electron microscopy of lipoproteins, VLDL and HDL fractions following gel-filtration were pooled. Respective lipoproteins were concentrated using centricons (Millipore) according to manufacturers instructions, mixed 1:1 (v/v) with 2% phosphotungstate for 1 min (34). This sample was then applied to a carbon-formvar grid and excess sample was removed by blotting. The grid was air-dried and viewed under the electron microscope. For analysis of apoAI-free HDL, HDL fractions were pooled from FPLC, apoAI was immunoprecipitated using apoAI antibody (Biodesign). Supernatants devoid of apoAI were concentrated using centricons as described above and subjected to negative staining and electron microscopy.

Metabolic labeling of lipids and proteins in isolated murine primary hepatocytes: Primary hepatocytes were isolated and cultured from *Alb-cre-Mttp*$^{fl/fl}$ (L-MTP$^{-/-}$) mice transduced with 10^{10} pfu of FLAG tagged hMTP or dMTP adenoviral vectors after 3 days. Hepatocytes were isolated following a collagenase I (Worthintgton Biochemicals) perfusion of the repective mouse. Using a Percoll (GE Helathcare) gradient, living cells were spun down and resuspended in William's E (Cellgro), washed and following a brief spin were resuspended in 10% FBS supplemented DMEM (33). Cells were seeded in collagen-coated plates and allowed to attach for 2 h before labeling with radioactive precursors for individual experiments. L-MTP$^{-/-}$ primary hepatocytes from hMTP or dMTP infected L-MTP$^{-/-}$ mice were pulse labeled with ^{3}H-oleic acid (1μCi/ml) for 30 min, washed with PBS to remove unbound radioactivity, and homogenized in sucrose buffer (250 mM sucrose, 300 mM imidazole, pH 7.4). Homogenates were centrifuged at 500 X *g* for 10 min. Post nuclear supernatant were centrifuged at 100, 000 g X *g* for 60 min. Microsomal pellet were collected and luminal contents were released using 0.1 M Sodium carbonate (pH 11). This mixture was spun at

100,000 X *g* for 90 min at 21°C to separate membrane from lumen content. Microsomal lumen (supernatant) and membrane (pellet) were collected and immunoprecipitated with apoB under non-denaturing conditions without detergent to isolate apoB-bound newly synthesized lipids (35). These lipids were extracted using chloroform and methanol by Bligh and Dyer method. Phospholipids and triglycerides were separated by thin layer chromatography (36). Bands corresponding to each lipid species were scraped and counted.

For proteasomal degradation experiments, cells were labeled with [^{35}S] methionine (150 µCi/ml) in the presence or absence of proteosomal degradation inhibitor, MG132 (Sigma) in methionine free media for represented time points in the experiments. Intracellular and secreted apoB were analyzed by fluorography following separation on SDS-PAGE after immunoprecipitation of newly synthesized apoB from cell lysates and media respectively (33).

Statistical analyses: One-way ANOVA (one way analysis of variance, Newmans-Keul test) was done for 3 independent comparisons to compare differences between groups. Comparisons were made between *Mttp*$^{fl/fl}$ (WT) and all knockout groups and significant differences were depicted by # to compare loss of function after MTP ablation, gain of function after ortholog re-expression. Statistically significant differences between PBS injected L-MTP$^{-/-}$ control and all other L-MTP$^{-/-}$ treated groups were denoted by *. Moreover, comparisons between hMTP and dMTP groups were shown by @. Comparisons between two groups were performed using Student's t-test (unpaired t-test) as indicated in the figure legends with equal number of variants for each comparison. Data represents average of triplicate values of each mice and average of those values for respective n values per group ± SEM. Data are representative of two to three independent experiments.

RESULTS

Human and *Drosophila* MTP support apoB48-lipoprotein assembly and secretion in COS7 cells

Previously, we have shown that plasmid-mediated expression of apoB48 with either dMTP or hMTP in COS7 cells results in the secretion of apoB48 indicating that both hMTP and dMTP support biosynthesis of apoB-lipoproteins; however, dMTP-expressing cells secreted less apoB48 (22). To delineate the role of these different MTP orthologs in lipoprotein biosynthesis in mice, we

made adenoviruses expressing hMTP-FLAG or dMTP-FLAG. Prior to *in vivo* studies, the recombinant adenoviruses were introduced in COS7 cells and characterized for expression and functional activity. Immunofluorescence analysis for the FLAG epitope in these cells illustrated dMTP and hMTP expression (Fig 1A). We then studied the effect of these recombinant adenoviruses on apoB48 secretion by these cells. Transduction of increasing amounts of hMTP or dMTP augmented apoB48 secretion (Fig 1B). Furthermore, apo48 secretion increased with viral titer. However, the amounts of apoB48 secreted by cells expressing dMTP were always less than those secreted by cells expressing hMTP (Fig 1B). Next we analyzed whether the respective recombinant adenovirus mediated expression of hMTP or dMTP were functional or not. COS7 cells treated with PBS as control did not show any triglyceride transfer activity. However hMTP expressing cells showed triglyceride transfer activity and this activity increased with time, unlike dMTP expressing cells which did not show any triglyceride transfer and were more similar to PBS treated control cells (Fig 1C). This lipid transfer activity profile was consistent with earlier published observations with plasmid mediated transfection studies (22). These studies show that adenoviral mediated expression of hMTP and dMTP supports apoB48 secretion similar to plasmid mediated expression of these proteins. Moreover, hMTP shows robust triglyceride transfer activity in contrast to dMTP, which was unable to transfer triglyceride.

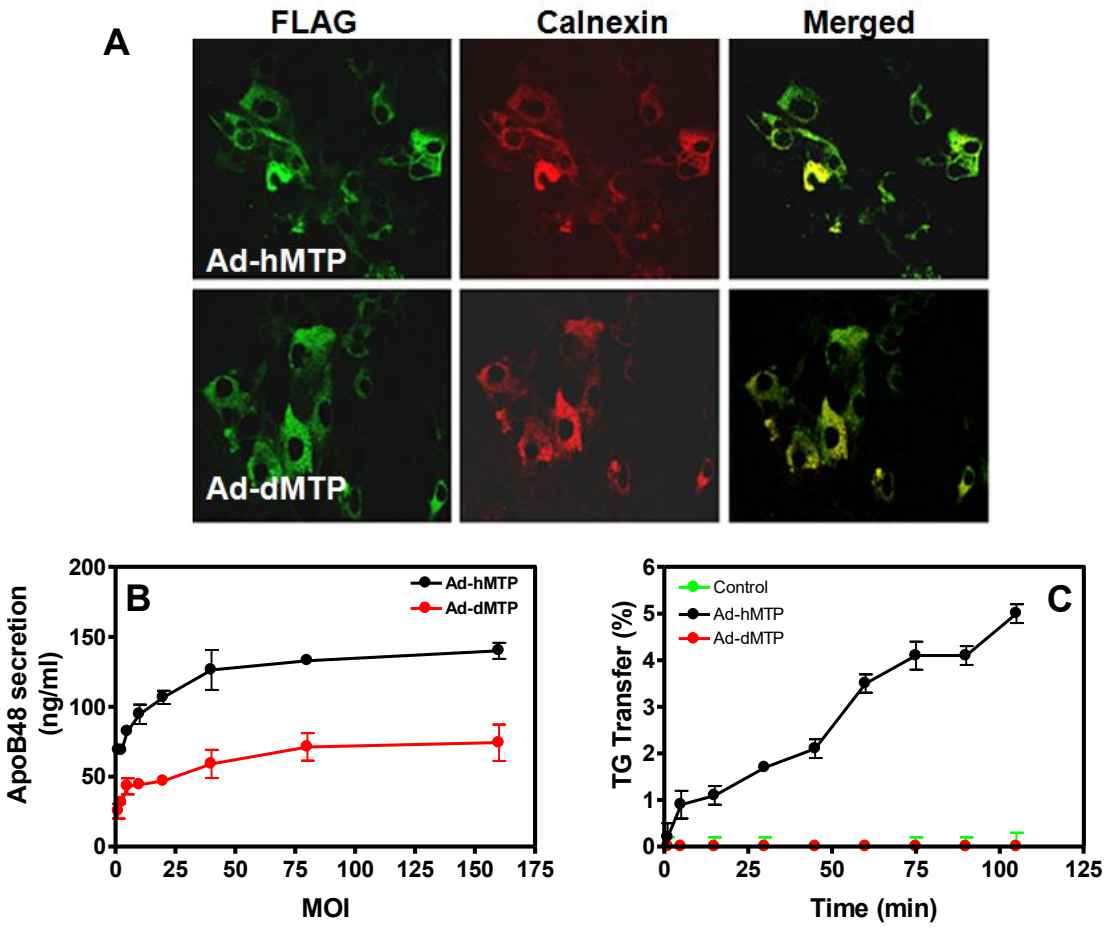

Figure 1. Expression of recombinant adenoviruses in COS 7 cells:
(A) COS 7 cells were grown on coverslips in 12-well cell culture plates and sequentially transfected with ApoB48 expression plasmid followed by Ad-hMTP-FLAG or Ad-dMTP-FLAG transduction. Cells were fixed, blocked and probed with primary and secondary antibodies. Immunofluoroscence showing FLAG expression in hMTP (Top panel) and dMTP (Bottom panel) expressing cells. Middle panel shows Calnexin, marker for ER expression. Right panel shows merged images illustrating colocalization of ER marker calnexin and hMTP or dMTP.
(B) ApoB48 secretion in COS 7 cells expressing different titers of Ad-dMTP and Ad-hMTP was measured in the media by ELISA. Transduction of increasing amounts of hMTP and dMTP supported apoB48 secretion. The amounts of apoB48 secreted by cells expressing dMTP were lower than those secreted by cells expressing hMTP.
(C) Percentage of triglyceride transfer was measured in homogenized extracts of COS-7 cells expressing Ad-dMTP, Ad-hMTP or control cells. Only Ad-hMTP shows triglyceride transfer activity.

Characterization of hepatic and plasma lipids with individual lipid transfer activity of MTP during normal lipid availability

After establishing that Ad-dMTP-FLAG and Ad-hMTP-FLAG characterization were consistent with earlier characterization in COS7 cells, to delineate the role of MTP orthologs with distinct lipid

transfer property *in vivo*, a mice model lacking MTP in liver were used since total MTP ablation in mice is lethal. MTP deficient models reported so far have been based on acute ablation of MTP (37;38); in this study we use a chronic model of liver specific deletion of MTP. Liver specific MTP deletion (L-MTP$^{-/-}$) was obtained by crossing Alb-Cre mice with *Mttp* floxed mice that results in MTP ablation only in the liver due to liver specific albumin promoter.

Hepatic expression of hMTP-FLAG and dMTP-FLAG in L-MTP$^{-/-}$ mice: To understand the physiologic dichotomy extended by different lipid transfer properties of MTP, *Drosophila* ortholog with phospholipid transfer and human MTP with both phospholipid and triglyceride transfer properties were introduced in the livers of L-MTP$^{-/-}$ mice. In addition, control L-MTP$^{-/-}$ mice received sterile PBS injections. MTP $^{fl/fl}$ (WT) counterparts were used as positive control, which received PBS injections as well. Expression of endogenous MTP in the liver of L-MTP$^{-/-}$ mice was barely detectable compared to their WT counterparts as revealed by western blot analysis (Fig 2A) consistent with other studies indicating successful loss of the endogenous *Mttp* gene (7). Western blot analysis showed the presence of FLAG-tagged ~ 97 kDa proteins in Ad-hMTP-FLAG and Ad-dMTP-FLAG transduced animals (Fig 2A) which were expressed in comparable levels (Fig 2B). Furthermore, immunohistochemical analysis showed presence of dMTP and hMTP in the livers (Fig 2C). The FLAG staining did not co-localize with nuclear staining. These studies indicate successful and comparable hepatic expression of dMTP and hMTP.

Lipid transfer activities after MTP gene deletion and re-expression of its orthologs: Next, lipid transfer activities of the transgenes were examined in hepatic lumenal contents to characterize functionality of the orthologs following expression in MTP deficient livers. L-MTP$^{-/-}$ mice injected with PBS or Ad-luciferase showed a significant ~ 2 fold decrease in phospholipid transfer activity compared to WT (Fig 2D). hMTP and dMTP expression restored this activity to the levels seen in WT animals (Fig 2D). Triglyceride transfer analysis revealed that MTP gene deletion was accompanied by ~80-85% reduction in triglyceride transfer activity (Fig 2E). Expression of luciferase in MTP deficient livers did not cause any further effect. However, robust triglyceride activity was present in hMTP-FLAG expressing liver homogenates but not in dMTP (Fig 2E). On the contrary, no such changes in intestinal MTP triglyceride and phospholipid transfer activities were found (Fig 3A-B) indicating a liver specific effect. These studies indicate that hMTP restores

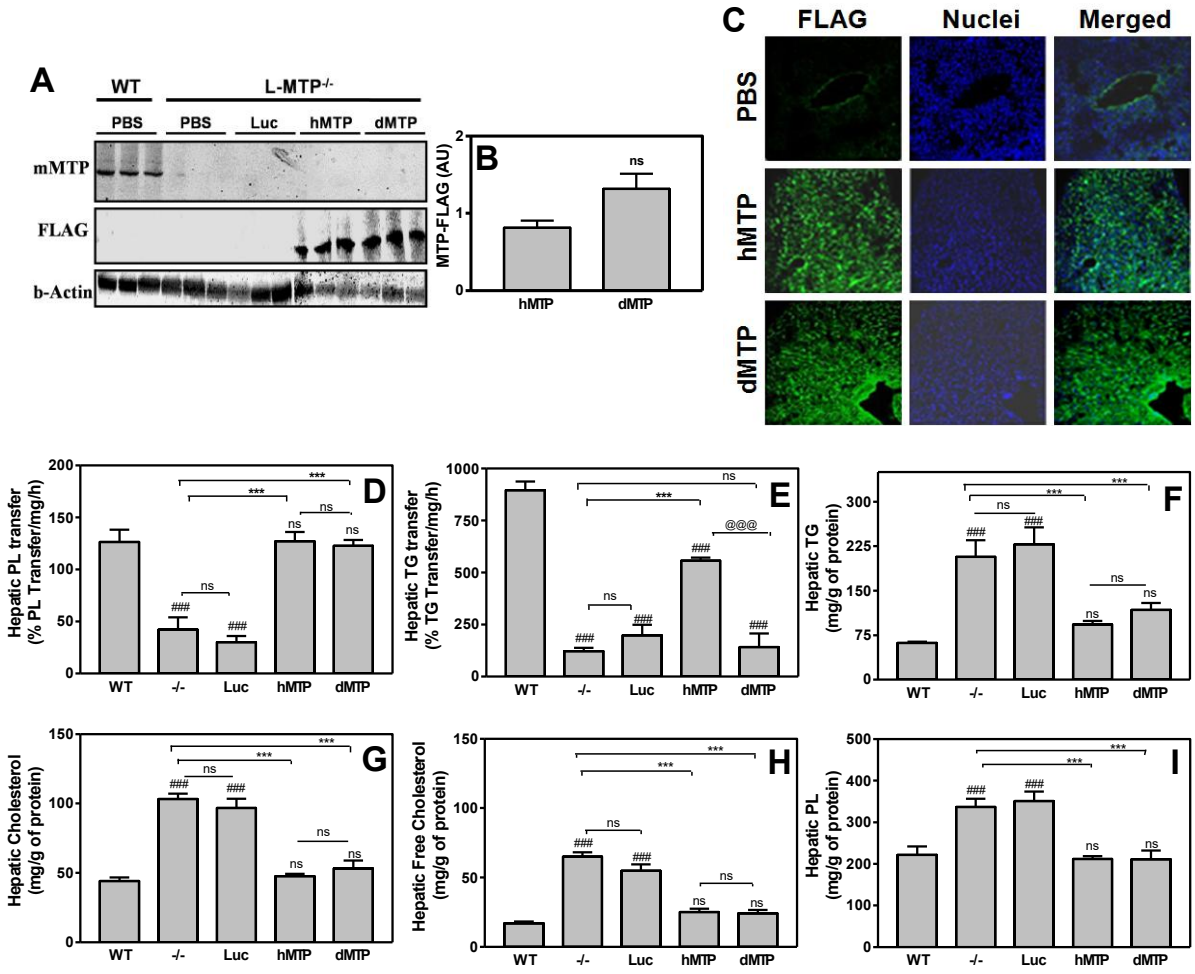

Figure 2. Expression and hepatic lipid profiles after expression of MTP orthologs: Recombinant adenoviruses (10^{10} pfu) were transduced into L-MTP$^{-/-}$ mice via tail vein. In addition, L-MTP$^{-/-}$ (-/-) and MTP$^{fl/fl}$ (WT) mice received sterile PBS injections for control. Plasma and tissue were collected following 6 h of fasting after 72 h of adenoviral delivery. Data are representative of two independent experiments, Mean ± SEM, n=3-6. Comparisons between WT and all knockout groups are designated as ###, comparisons between PBS injected L-MTP$^{-/-}$ and all other L-MTP$^{-/-}$ mice transduced with different viruses are designated ***. Comparisons between hMTP and dMTP are designated @. ***p < 0. 001, **p < 0. 01, *p < 0. 05 and ns not significant.
(A) Western blot analysis showing expression of endogenous mouse MTP (mMTP, top panel). FLAG expression (middle panel) was seen in the liver of mice transduced with Ad-hMTP-FLAG (hMTP) and Ad-dMTP-FLAG (dMTP). β-actin expression (bottom panel) was used as loading control.
(B) Quantification of hMTP and dMTP expression by Image J from NIH.
(C) Immunohistochemical analysis of liver sections showing expression of FLAG (left panel), nuclei staining with topro blue (middle panel), and merged images (right panel).
(D-E) Liver homogenates from different mice (n=3) were prepared as described in methods and were used to measure **(D)** phospholipid and **(E)** triglyceride transfer activities of MTP in triplicate.
(F-I) Liver homogenates from different mice (n=6) were prepared as described in methods and were used to measure Hepatic lipids were extracted and used to measure **(F)** triglyceride **(G)** total cholesterol **(H)** free cholesterol and **(I)** phospholipids.
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triglyceride transfer activity but dMTP does not in L-MTP$^{-/-}$ mice. In contrast, both hMTP and dMTP reinstate phospholipid transfer activity.

Changes in hepatic lipids after MTP gene deletion and re-expression of its orthologs: We then studied changes in hepatic lipids in these mice. There was increased accumulation of hepatic total triglyceride (~2 -3 fold; Fig 2F), total and free cholesterol (~ 2 fold; Fig 2G-H), and phospholipids (~1.5 fold; Fig 2I) in L-MTP$^{-/-}$ mice compared to WT and similar observations were made in mice injected with Ad-Luciferase (Fig 2F-I). By contrast, expression of human MTP significantly lowered hepatic triglyceride (Fig 2F), total and free cholesterol (Fig 2G; 2H) as well as phospholipid (Fig 2I) levels in L-MTP$^{-/-}$ mice. Surprisingly, expression of dMTP also reduced the elevated hepatic triglyceride, cholesterol and phospholipids in L-MTP$^{-/-}$ mice. On the contrary, no such differences were observed in intestinal lipid contents in these groups (Fig 3C-E). Thus, hepatic lipids were elevated with liver specific ablation of MTP. Overall conclusion from these studies was that dMTP lowers hepatic triglyceride similar to hMTP. Thus, introduction of hMTP or dMTP in the L-MTP$^{-/-}$ abrogates accumulations of hepatic triglyceride, free cholesterol, total cholesterol and phospholipids (Fig 2F-I) associated with *Mttp* gene deletion.

Structural changes in hepatocytes after MTP gene deletion and expression of different MTP orthologs: Histological analysis showed presence of numerous vacuoles indicating a fatty liver in L-MTP$^{-/-}$ mice injected with luciferase compared to WT mice (Fig 4A, Top panel). Expression of both hMTP and dMTP reduced these vacuoles (Fig 4A, Top panel). Oil Red O staining showed significant accumulation of lipids in L-MTP$^{-/-}$ livers compared to WT. These lipids were reduced after hMTP and dMTP expression (Fig 4A, Middle panel). Osmium tetraoxide staining of lipids also revealed a similar profile as seen with Oil Red O staining (Fig 4A, Bottom panel). These studies indicated that expression of both hMTP and dMTP ameliorates steatosis associated with *Mttp* gene ablation. We further did ultrastructural analysis to detect whether chronic MTP ablation is associated with any subcellular structural alterations. Electron microscopic analysis of liver revealed microvesicular steatosis characterized by numerous lipid droplets in the cytoplasm of hepatocytes with no displacement of nuclei to the periphery in L-MTP$^{-/-}$ mice compared to WT (Fig 4B). Mitochondrial structural alterations such as swelling, rarefication of cristae, and hypodense matrix, were common in L-MTP$^{-/-}$ mice transduced with Ad-luciferase. In addition, loss of distinct ER and Golgi structural complexes were also observed in MTP deficient hepatocytes.

Mitochondria were numerous and more heterogeneous (both normal and swollen) in hMTP and dMTP expressing livers. hMTP and dMTP expression showed reductions in lipid droplets, appearance of numerous ER (red arrow) and Golgi complexes (black arrow). In addition livers from hMTP and dMTP showed more cytosolic spaces, which could be due to mobilization of cytosolic lipid droplets (Fig 4B). These studies indicate that *Mttp* gene ablation leads to significant structural changes in subcellular organelles that are partly ameliorated by transient expression of hMTP and dMTP.

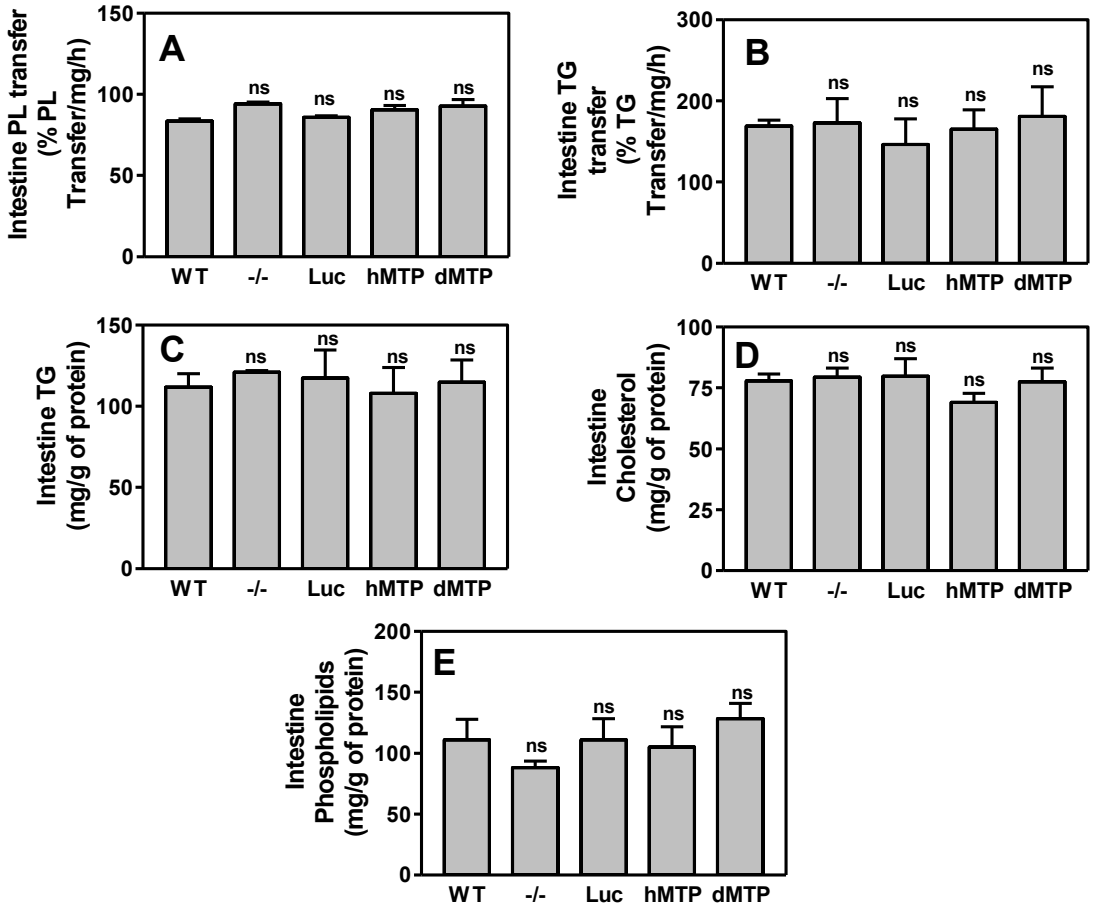

Figure 3. Intestinal MTP activity and lipids in different mice: (A-B) Intestinal phospholipid and (A) and triglyceride (B) transfer activity in WT, L-MTP$^{-/-}$ mice injected with PBS (-/-), adenoviruses expressing luciferase (Luc), hMTP-FLAG, or dMTP-FLAG. (C-E) Lipids were extracted from jejunum (Bligh and Dyer, 1959) to measure triglyceride (C), cholesterol (D), and phospholipid (E). Data represents Mean ± SEM, n=3.

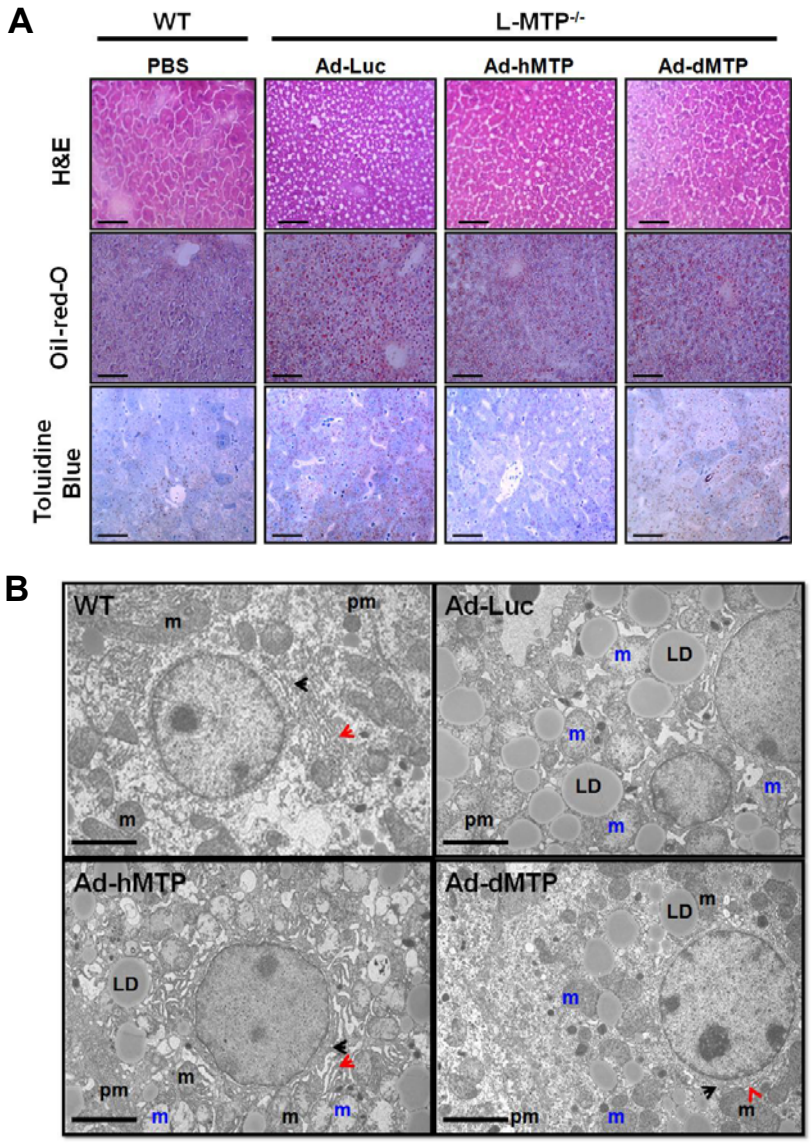

Figure 4. Histological and ultrastructural changes with expression of hMTP and dMTP in L-MTP$^{-/-}$ mice: Recombinant adenoviruses (10^{10} pfu) were transduced into L-MTP$^{-/-}$mice via tail vein.MTP$^{fl/fl}$ (WT) mice received sterile PBS injections for control.Tissue were collected following 6 h of fasting after 72 h of adenoviral delivery. Images shown here are representative of several fields.

(A) Light microscopy of liver sections stained with hematoxylin and eosin (H&E) for histological analysis (top panel), Oil Red O staining to detect lipids and counterstaining with hematoxylin for nucleus (middle panel), and osmium tetraoxide followed by counterstaining with toluidene blue for ultrastructural analysis by electron microscopy (Bottom panel). Magnifications, Bars = 50 μm.

(B) Liver from represented groups were processed as described in methods and embedded in epoxy resin and sectioned. **(WT)** Single hepatocyte has numerous mitochondria (m) with dense matrix and well-formed cristae, and plasma membrane (pm) separating the adjoining hepatocyte. Golgi complex (black arrowhead), numerous RER in the vicinity of mitochondria (red arrowhead). **(Luc)** Two adjoining hepatocytes in L-MTP$^{-/-}$ mice transduced with Ad-Luc showing numerous cytosolic lipid droplets (LD) in both cells, swollen mitochondria (m-letter in blue) with hypodense matrix and deformed crista. Cells also show overall reduction in mitochondrial numbers with numerous instances of mitochondrial fusions and loss of intact ER and Golgi structures. **(hMTP)** hMTP expression in L-MTP-/- liver showing lesser cytosolic lipid droplets in one hepatocyte. Abundant ER and Golgi complex in the cytosol, and several heterogeneous mitochondria ranging from normal to abnormal including swelling and fusion are evident. Numerous intracytosolic spaces are seen resembling vacuoles possibly due to removal of cytosolic lipids. **(dMTP)** dMTP expression in MTP deficient livers showing abundance of cytosolic lipid droplets lesser than knockout but more than hMTP. Several heterogeneous population of mitochondria is seen as in hMTP. Magnifications, Bars = 1 μm.

Changes in plasma lipids after re-expression of MTP orthologs in L-MTP$^{-/-}$ mice: Next, we studied the effect of liver-specific MTP deletion on steady state fasting plasma lipids and also analyzed modulations following MTP orthologs expression (Fig 5A). L-MTP$^{-/-}$ mice were hypolipidemic due to significant reductions in plasma triglyceride, cholesterol and phospholipid (Fig 5A-C). Ad-luciferase injected L-MTP$^{-/-}$ mice had no further effect on plasma lipids that were similar to the L-MTP$^{-/-}$ mice treated with PBS. Expression of hMTP restored plasma triglycerides to levels seen in WT mice; dMTP expression also (Fig 5A-C) raised plasma triglyceride in L-MTP$^{-/-}$ mice but these levels were significantly lower than both hMTP and WT (Fig 5A) mice. Total cholesterol levels in the L-MTP$^{-/-}$ were ~2 fold lower compared to WT mice; expression of hMTP rescued these levels (Fig 5B). dMTP also rescued these lower plasma cholesterol in L-MTP$^{-/-}$ mice associated with hepatic MTP gene deletion. Plasma phospholipids were reduced by ~2.5-3 fold in the L-MTP$^{-/-}$ and expression of different MTP orthologs rescued their levels partially but not significantly compared to the WT group (Fig 5C). Western blot analysis following agarose gel electrophoresis showed significant reductions in pre-β and β-lipoproteins (Fig 5D) in luciferase expressing L-MTP$^{-/-}$ mice compared to WT counterparts. Expression of hMTP in these mice rescued both pre-β and β-lipoproteins to almost similar levels as WT mice. In dMTP there was slight increase pre-β and β-lipoproteins compared to luciferase expressing L-MTP$^{-/-}$ mice but these levels were significantly lower than hMTP. No such changes in α-lipoproteins were observed (Fig 5D).

Thus, introduction of hMTP or dMTP in the L-MTP$^{-/-}$ mice abrogates accumulations of hepatic triglyceride, free cholesterol and total cholesterol that is associated with significant reduction in lipid droplets. Plasma triglyceride levels in the L-MTP$^{-/-}$ mice were partially restored by both hMTP and dMTP compared to WT mice; however, rescue of lower plasma lipid levels associated with hepatic MTP ablation in L-MTP$^{-/-}$ mice were higher in hMTP compared to dMTP expressing mice (Fig 5A). Therefore, there is a significant cause and effect relationship between MTP ablation/expression and changes in hepatic triglyceride/free cholesterol and plasma lipids.

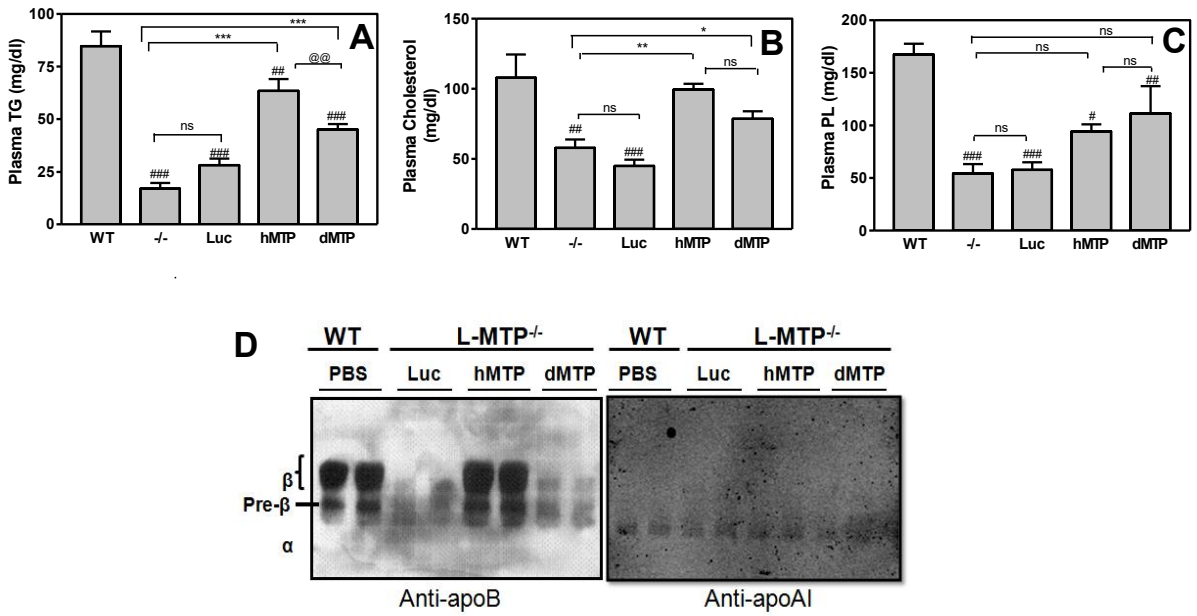

Figure 5. Expression of hMTP and dMTP alters plasma lipid profiles in L-MTP$^{-/-}$ mice: Recombinant adenoviruses (10^{10} pfu) were transduced into L-MTP$^{-/-}$ mice via tail vein. In addition, L-MTP$^{-/-}$ (-/-) and MTP$^{fl/fl}$ (WT) mice received sterile PBS injections for control.Plasma and tissue were collected following 6 h of fasting after 72 h of adenoviral delivery. Data are representative of two independent experiments. One-way ANOVA was performed to compare differences between groups. Comparisons between WT and all knockout groups and designated as ###; PBS injected L-MTP$^{-/-}$ control and all other L-MTP$^{-/-}$ mice transduced with different viruses shown as ***; comparison of hMTP and dMTP groups shown as @. ***p < 0. 001, **p < 0. 01, *p < 0. 05 and ns not significant.
(A-C) Fasting plasma **(A)** triglycerides (n=5), **(B)** cholesterol (n=5) and **(C)** phospholipids (n=3) were measured.
(D) Plasma samples from different groups were separated by agrose gel electrophoresis, western blotted for mouse apoB (left panel) and mouse apoAI (right panel). Distribution of beta, pre-beta and alpha lipoproteins are shown.

Characterization of hepatic and plasma lipids during higher lipid availability with individual lipid transfer activity of MTP

So far we observed that plasma triglycerides and VLDL-triglycerides in dMTP expressing mice are lower at all times in chow-fed mice. It is also important to compare the capacity of dMTP and hMTP during excess lipid availability. Therefore, we examined lipid metabolism in L-MTP$^{-/-}$ fed western-diet and transduced with different MTP orthologs. Our aim was to learn whether hMTP may be able to handle higher lipid loads more efficiently by supporting more apoB lipoprotein biosynthesis due to its triglyceride transfer activity. This is important to eliminate the possibility that differences in dMTP and hMTP are secondary to lipid availability. More importantly, we wanted to learn whether phospholipid transfer activity of MTP supports the assembly of larger primordial particle when fat availability is not limiting.

Hepatic expression and functional gain following hMTP-FLAG and dMTP-FLAG expression in western-diet fed L-MTP$^{-/-}$ mice: To further assess efficacy of different MTP orthologs in reducing hepatic lipids, we expressed these orthologs in L-MTP$^{-/-}$ mice fed a western-diet. Liver homogenates were subjected to western blot analysis for FLAG-tagged proteins in Ad-hMTP-FLAG and Ad-dMTP-FLAG transduced mice (Fig 6A) and hepatic expression of FLAG tagged MTP orthologs were normalized to GAPDH (Fig 6B). Phospholipid transfer activities were significantly reduced in L-MTP$^{-/-}$ mice injected with Ad-luciferase compared to WT; expression of both hMTP and dMTP restored this activity to levels seen in WT mice, however there was significantly more activity from dMTP expressing livers possibly due to more expression than hMTP (Fig 6C). *Mttp* gene deletion reduced triglyceride transfer activity by ~ 4-5 fold (Fig 6D, Luc). Although robust triglyceride transfer activity was present in hMTP, but it was absent in dMTP expressing livers (Fig 6D). These studies indicate that hMTP restores triglyceride transfer activity but not dMTP in L-MTP$^{-/-}$ mice. In contrast, phospholipid transfer activity is restored similarly by both hMTP and dMTP in the liver.

Changes in hepatic lipids after hMTP-FLAG and dMTP-FLAG expression in western-diet fed L-MTP$^{-/-}$ mice: Hepatic lipid analysis revealed increased accumulation of hepatic triglyceride (~ 4 fold; Fig 6E); total cholesterol (~ 2-2.5 fold; Fig 6F); and phospholipids (~1.5 fold; Fig 6G), in Ad-Luciferase expressing L-MTP$^{-/-}$ mice compared to WT. The levels of hepatic triglyceride accumulations in WT mice fed western-diet were higher compared to chow-fed conditions as seen earlier although no significant accumulations in cholesterol were found (31). By contrast, expression of hMTP in L-MTP$^{-/-}$ livers significantly lowers hepatic triglyceride (Fig 6E), total cholesterol (Fig 6F), and phospholipid (Fig 6G). Expression of dMTP also reduced the elevated hepatic triglyceride, cholesterol and phospholipids (Fig 6E-G). However, dMTP expression in hepatic deficient livers showed less removal of accumulated hepatic cholesterol compared to hMTP (Fig 6F). These studies indicate that expression of both hMTP and dMTP ameliorates steatosis associated with *Mttp* gene ablation in L-MTP$^{-/-}$ mice fed a western diet.

H&E staining of liver sections showed extensive vacuolation of hepatic tissue indicating steatotic liver in L-MTP$^{-/-}$ mice injected with luciferase compared to WT mice (Fig 6H, Top panel). Expression of both hMTP and dMTP reduced these vacuoles considerably (Fig 6H). Oil Red O staining showed significant accumulation of lipids in L-MTP$^{-/-}$ livers compared to WT. These lipids

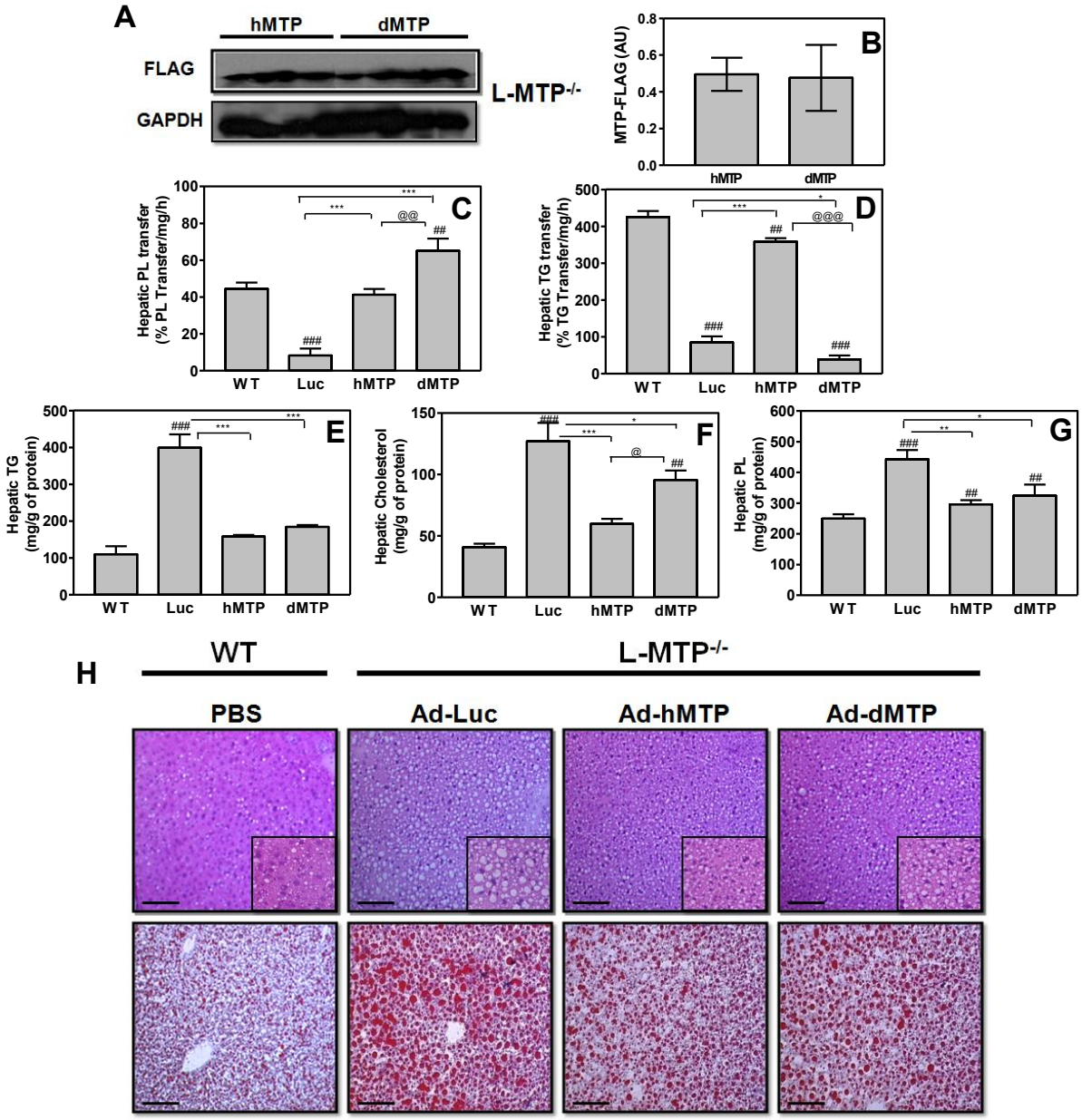

Figure 6. MTP ortholog changes hepatic lipid profiles during higher lipid availability: $MTP^{fl/fl}$ (WT) mice and L-$MTP^{-/-}$were fed western type diet (Harlan Teklad Adjusted Calories Diet, 42% from Fat) for 15 days ad libitum. Different recombinant adenoviruses (10^{10} pfu) were transduced into L-$MTP^{-/-}$ mice via tail vein. In addition, L-$MTP^{-/-}$(-/-) received Ad-Luciferase as viral control and $MTP^{fl/fl}$ (WT) mice received sterile PBS injections for control. Mice were continued on western-diet for additional 5 days, fasted for 6 h, and tissues were collected. Plasma and tissue were collected for lipid profile analysis. Data values, Mean ± SEM, n=5.
(A) FLAG expression in liver homogenates from FLAG tagged hMTP and dMTP transduced mice via adenoviral delivery as descibed above and **(B)** relative quantification of expression are shown.
(C-D) Lipid transfer assays in microsomal fractions of liver showing **(C)** Phospholipid transfer and **(D)** Triglyceride transfer in respective groups as shown.
(E-G) Lipids were extracted from liver homogenates, measured and calculated per milligram of liver tissue. Hepatic **(E)** Triglycerides **(F)** Cholesterol and **(G)** Phospholipids are shown in different groups.
(H) Representative images of liver sections from each group as indicated stained with H&E (Top panel) showing histology of the hepatic tissue, Oil Red O staining showing lipid content of liver in each group (Bottom panel). Magnifications, Bars = 50μm.

were reduced after hMTP and dMTP expression (Fig 6H, Bottom panel), however the reductions achieved were higher in hMTP compared to dMTP. Therefore, expression of hMTP and dMTP reduces hepatic lipid accumulation in L-MTP$^{-/-}$ mice fed a western-diet.

Changes in plasma lipids after hMTP-FLAG and dMTP-FLAG expression in western diet fed L-MTP$^{-/-}$ mice: Ad-luciferase transduced L-MTP$^{-/-}$ mice had low plasma lipids compared to WT mice (Fig 7A). Compared with standard chow, a western-diet significantly increased plasma triglyceride and cholesterol in WT mice similar to the results of earlier studies (31;39). Expression of hMTP partially restored plasma triglycerides; dMTP expression also raised plasma triglyceride in L-MTP$^{-/-}$ mice but these levels were significantly lower than hMTP and WT (Fig 7A). Total plasma cholesterol levels in the L-MTP$^{-/-}$ mice were ~ 4 fold lower than WT mice fed a western-diet and expression of hMTP and dMTP slightly increased these levels (Fig 7B). Plasma phospholipids were reduced by ~3-4 fold in the L-MTP$^{-/-}$ mice and expression of both MTP orthologs partially rescued their levels (Fig 7C); plasma phospholipids were higher in dMTP expressing mice compared to hMTP. Plasma apoB levels were significantly reduced in L-MTP$^{-/-}$ compared to WT mice (Fig 7D). hMTP expression led to significant increase of both apoB100 and apoB48 in the plasma. Similarly, dMTP also rescued both apoB100 and apoB48 in the plasma of L-MTP$^{-/-}$ mice but these levels were lower than hMTP (Fig 7D). Agarose gel electrophoresis showed significant reductions in pre-β and β-lipoproteins (Fig 7E) in luciferase expressing L-MTP$^{-/-}$ mice compared to WT counterparts. On the other hand expression of hMTP as well as dMTP in these mice rescued both pre-β and β-lipoproteins to almost similar levels as WT mice. No such changes in α-lipoproteins were observed (Fig 7E). Compared to steady state conditions, dMTP had significantly higher β-lipoproteins in western-diet fed conditions. Distribution of lipids by FPLC in plasma VLDL and HDL lipoproteins and also studies performed later in this study showed that dMTP assembles a triglyceride-poor, smaller VLDL lipoprotein. The smaller VLDL particle would be hydrolysed faster in the plasma into smaller LDL, β-lipoproteins. These levels were higher in this case because overall plasma lipid levels rescued by dMTP in L-MTP$^{-/-}$ mice were higher than chow-fed steady state conditions.

Mechanism of lipoprotein biosynthesis with distinct lipid transfer activity of MTP

After primary characterization of mice transduced with dMTP and hMTP under both normal and higher lipid loads, we focused to understand the role of these orthologs in hepatic lipoprotein

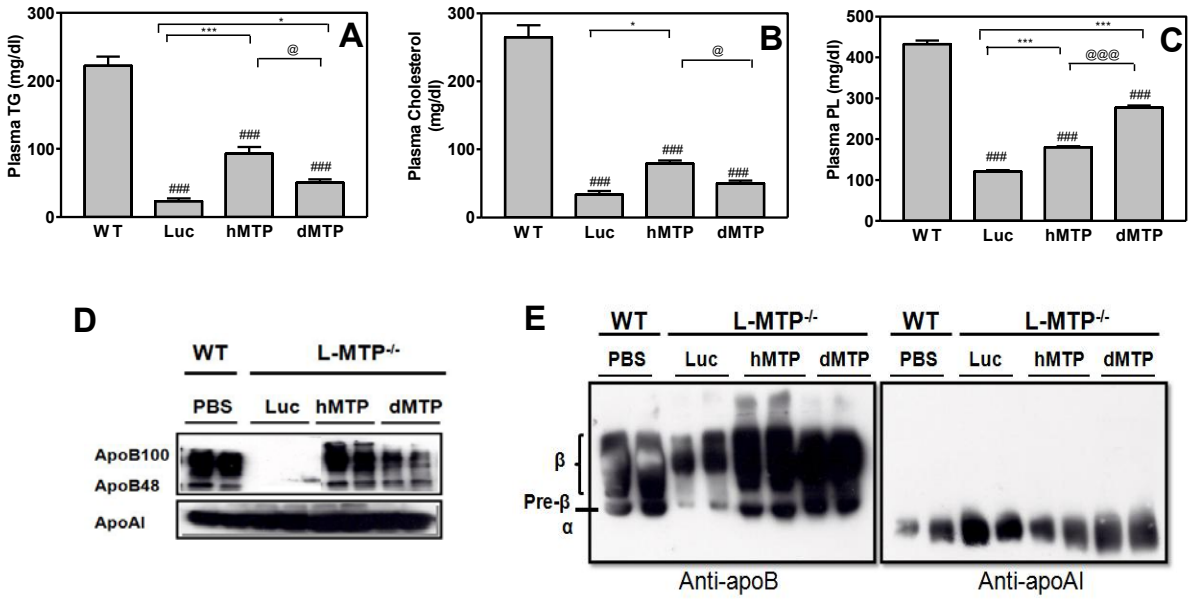

Figure 7. MTP ortholog changes plasma lipid profiles during higher lipid availability: $MTP^{fl/fl}$ (WT) mice and L-$MTP^{-/-}$ (-/-) were fed western type diet (Harlan Teklad Adjusted Calories Diet, 42% from Fat) for 15 days ad libitum. Different recombinant adenoviruses (10^{10} pfu) were transduced into L-$MTP^{-/-}$ mice via tail vein. In addition, L-$MTP^{-/-}$ (-/-) received Ad-Luciferase as viral control and $MTP^{fl/fl}$ (WT) mice received sterile PBS injections for control. Mice were continued on western diet for additional 5 days, fasted for 6 h on the final day and sacrificed. Plasma and tissues were collected for lipid profile analysis. Data values, Mean ± SEM, n=5.
(A-C) Fasting plasma from western type diet fed mice in each group as indicated showing plasma total **(A)**Triglycerides **(B)** Cholesterol and **(C)** Phospholipids.
(D) Fasting plasma samples from different groups were separated on 4-15% SDS-PAGE gel, transferred onto membrane and western blotted for apoB (Top panel) and apoAI (Bottom panel).
(E) Plasma samples from respective groups separated on agarose gel, western blot developed for mouse apoB (left panel) and apoAI (right panel) showing distribution of pre-β, β and α-lipoproteins after membrane transfer of proteins.

assembly and secretion. Characterization of liver derived lipoproteins was difficult in normal plasma because of their low plasma concentrations, contamination of intestinal lipoproteins, and complications associated with possible differential catabolism. To minimize contribution of intestinal lipoproteins, all studies were performed in 6 h fasted animals. To avoid complications associated with differential catabolism of lipoproteins synthesized by these orthologs, we inhibited lipoprotein lipase activity by injecting P407 (29).

Dose dependent changes in plasma and hepatic lipids after the expression of increasing amounts of MTP orthologs:

Changes in hepatic lipids: To understand the mechanistic aspect of lipoprotein biosynthesis with respect to MTP, we performed dose-response studies. Western blot analysis showed that mice transduced with 10^7 pfu had low MTP levels compared to those transduced with higher viral

particles (Fig 8A). Western blot analysis showed that mice transduced with 10^7 pfu had low MTP levels compared to those transduced with higher viral particles.

Next, we studied the effect of different concentrations of viruses on hepatic lipids. Values in WT, L-MTP$^{-/-}$, and L-MTP$^{-/-}$ transduced with Ad-luciferase were obtained in parallel and are plotted as bar graphs for comparison. Phospholipid transfer activities significantly increased to similar extents in a concentration dependent manner with both dMTP and hMTP expression (Fig 8B). Triglyceride transfer activity was significantly enhanced in the liver with increased expression of hMTP obtained with introducing higher titers of recombinant vectors (Fig 8C). In contrast, transduction of different amounts of Ad-dMTP-FLAG did not show any increase in triglyceride transfer activity (Fig 8C). Hepatic triglycerides were reduced in L-MTP$^{-/-}$ mice after the transduction of hMTP and dMTP expressing viruses but not with viruses expressing Ad-luciferase (Fig 8D). Hepatic cholesterol accumulations in L-MTP$^{-/-}$ mice were lowered by both orthologs in a dose-dependent manner (Fig 8E). Similarly, expression of either hMTP or dMTP reduced hepatic phospholipids (Fig 8F). These studies indicate that increasing expression of hMTP as well as dMTP in L-MTP$^{-/-}$ mice lower hepatic lipid levels reaching to levels seen in WT mice.

Changes in plasma lipids: Next changes in plasma lipids were studied pursuant to expression of different amounts of hMTP and dMTP in L-MTP$^{-/-}$ mice. L-MTP$^{-/-}$ mice showed progressive increase in plasma triglycerides with corresponding increases in the expression of both MTP orthologs but levels in hMTP expressing animals tended to be higher than those seen in dMTP expressing mice (Fig 9A) mainly due to augmentations in plasma non-HDL (Fig 9B); albeit, increases were higher in hMTP expressing mice (Fig 9A-C). The reasons for lower plasma triglyceride in dMTP expressing mice were explored later. Plasma cholesterol in L-MTP$^{-/-}$ mice were increased with the expression of different orthologs to similar levels primarily due to corresponding increases in non-HDL cholesterol, no significant changes were seen in HDL-cholesterol (Fig 9D-F) within groups. Plasma phospholipids were also rescued upon hMTP and dMTP re-expression in the MTP deficient livers (Fig 9G-I). Non-HDL phospholipids were increased with corresponding increase in expression, though increases in hMTP were higher than dMTP. Interestingly, HDL-phospholipids increased in dMTP in a concentration dependent manner in contrast to hMTP.

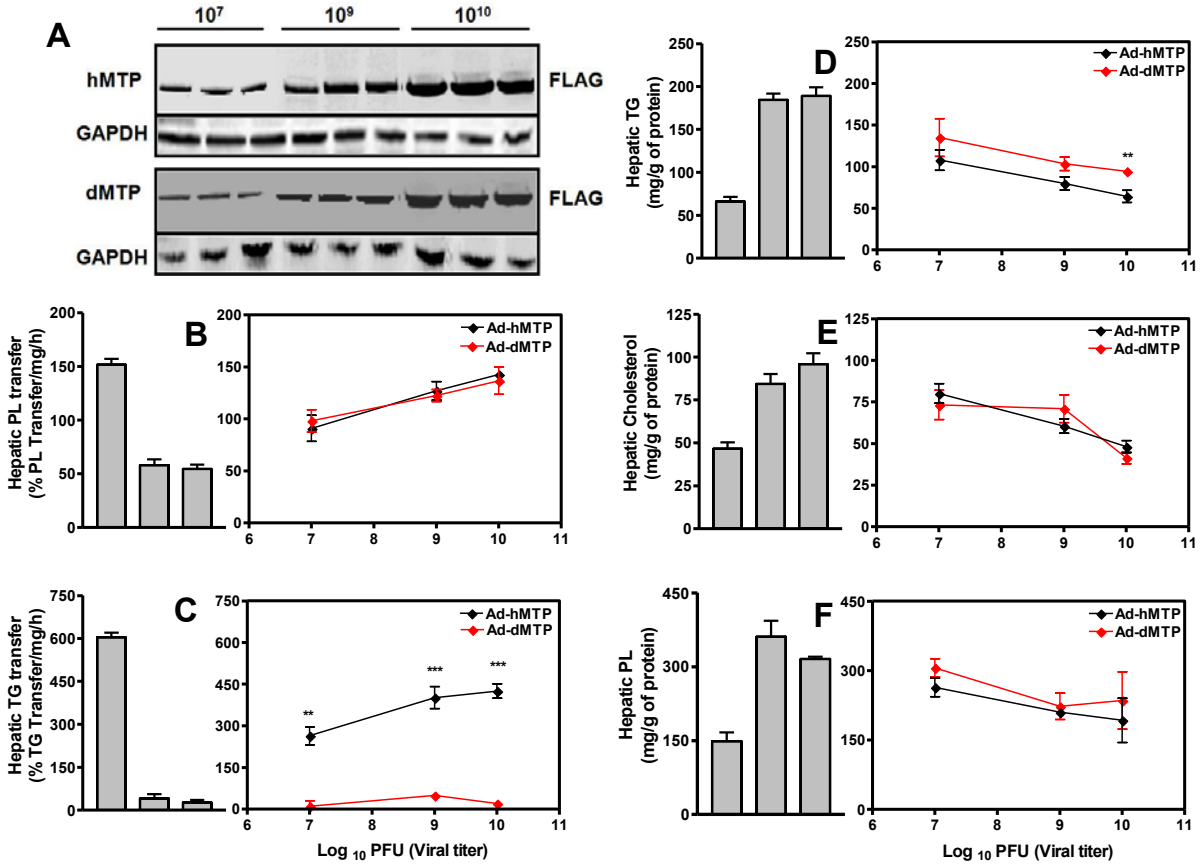

Figure 8. Effect of transducing different amounts of MTP orthologs on hepatic lipids: L-MTP$^{-/-}$ mice were transduced with 10^7, 10^9, and 10^{10} pfu of Ad-Luciferase, Ad-hMTP-FLAG or Ad-dMTP-FLAG. After 3 days, mice were fasted for 4 h, injected with P407, and plasma was collected after another 2 h of fasting.
(A) Western blots showing hepatic FLAG expression in mice transduced with different amounts of viruses. GAPDH is shown as loading control.
(B-F) Data from WT, L-MTP$^{-/-}$and L-MTP$^{-/-}$ mice transduced with Ad-Luciferase (10^{10} pfu) are shown as bar graphs. Effects of expression of different amounts of hMTP and dMTP are shown as line graphs. Lipid transfer activities in hepatic microsomal fractions indicating **(B)** Phospholipid and **(C)**Triglyceride transfer activities in L-MTP$^{-/-}$ mice expressing different amounts of hMTP or dMTP. Hepatic lipid content measured in liver homogenates after lipid extraction showing **(D)** Triglycerides **(E)** Cholesterol and **(F)** Phospholipids in respective groups. Data (Mean ± SEM, n=3) are representative of two to three independent experiments. Student's t-test was performed to compare differences between dMTP and hMTP at different concentrations. ***p < 0. 001, **p < 0. 01, *p < 0. 05 and ns not significant.

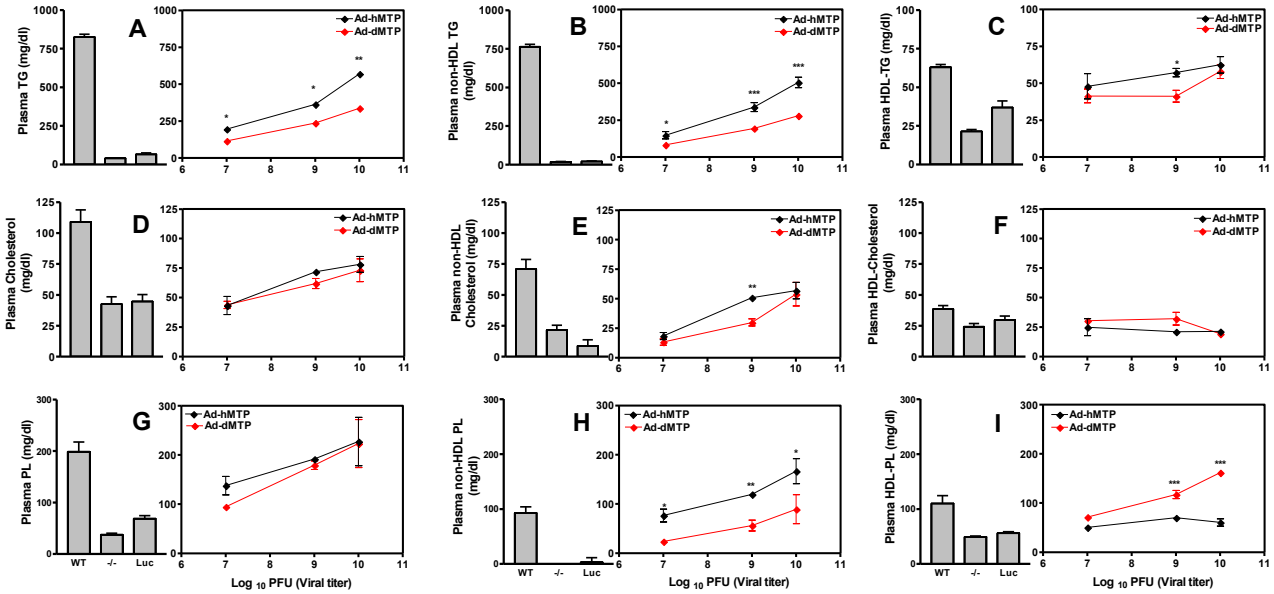

Figure 9. Effect of transducing different amounts of MTP orthologs on plasma lipids: L-MTP$^{-/-}$ mice were transduced with 10^7, 10^9, and 10^{10} pfu of Ad-Luciferase, Ad-hMTP-FLAG or Ad-dMTP-FLAG. After 3 days, mice were fasted for 4 h, injected with P407, and plasma was collected after another 2 h of fasting. Data from WT, L-MTP$^{-/-}$and L-MTP$^{-/-}$ mice transduced with Ad-Luciferase (10^{10} pfu) are shown as bar graphs. Effects of expression of different amounts of hMTP and dMTP are shown as line graphs.
(A-C) Plasma total **(A)**, non-HDL **(B)**, and HDL **(C)** triglycerides,
(D-F) Plasma total **(D)**, non-HDL **(E)**, and HDL **(F)** cholesterol,
(G-I) Plasma total **(G)**, non-HDL **(H)**, and HDL **(I)** phospholipids are shown in respective groups.
Data (Mean ± SEM, n=3) are representative of two to three independent experiments. Student's t-test was performed to compare differences between dMTP and hMTP at different concentrations. ***p < 0. 001, **p < 0. 01, *p < 0. 05 and ns not significant

Distribution of lipids in lipoproteins by Fast Performance Liquid Chromatography (FPLC):

Lipid changes in different lipoproteins were further substantiated by FPLC. The recovery of lipids during FPLC from mice transduced with 10^7 pfu was low; therefore data from mice transduced with 10^9 and 10^{10} pfu of hMTP and dMTP are shown. FPLC analyses of plasma revealed that increasing concentrations of Ad-hMTP increased plasma triglyceride in VLDL/LDL fractions (Fig 10A). Cholesterol was in VLDL and HDL fractions in L-MTP$^{-/-}$ mice injected with the lowest concentrations of Ad-hMTP (Fig 10B). Injections of higher amounts of Ad-hMTP enhanced cholesterol levels in VLDL/LDL fractions. Similarly, analyses of phospholipids showed increases in VLDL/LDL fractions (Fig 10C). These studies indicate that expression of increasing amounts of hMTP enhances triglycerides, cholesterol and phospholipids in VLDL/LDL fractions suggesting efficient assembly and secretion of triglyceride-rich lipoproteins. Similar analyses in plasma of mice

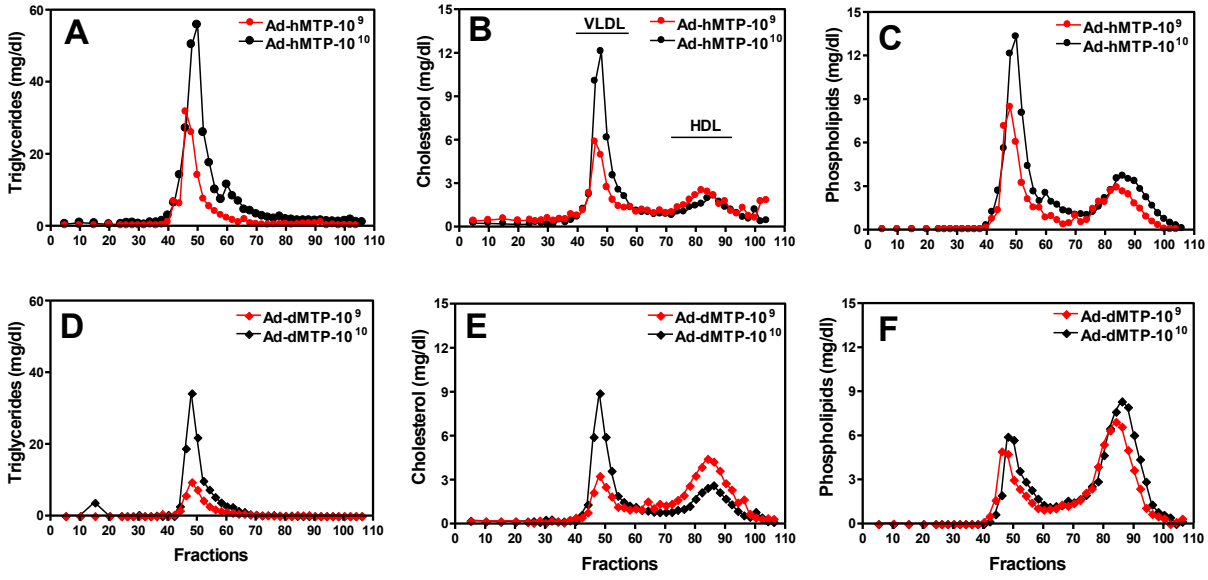

Figure 10. Plasma lipoprotein analysis: L-MTP$^{-/-}$ mice were transduced with different titers of adenoviruses. After 3 days mice were fasted for 4 h and injected with P407, plasma was collected after another 2 h of fasting. Plasma from each group were pooled and subjected to FPLC for size fractionation by gel filtration. 150 μl of 106 fractions were collected and lipids were analyzed in fractions to demonstrate distribution of different lipids in VLDL and HDL lipoprotein population.
(A-C) Lipid analysis showing **(A)** Triglyceride **(B)** Cholesterol and **(C)** Phospholipid distribution in different lipoproteins of L-MTP$^{-/-}$ mice expressing different amounts of Ad-hMTP-FLAG are shown.
(D-F) (D) Triglyceride **(E)** Cholesterol and **(F)** Phospholipid distribution in lipoproteins obtained from mice expressing different amounts of Ad-dMTP-FLAG are shown.

transduced with increasing amounts of dMTP showed enhanced amounts of triglycerides (Fig 10D), cholesterol (Fig 10E) and phospholipids (Fig 10F) in VLDL/LDL fractions. However, these increases were lower than those seen in hMTP expressing animals. A major surprise was the

phospholipid changes in the HDL fractions. Transductions of 10^9 and 10^{10} pfu of Ad-dMTP significantly enhanced phospholipids in these fractions. These studies demonstrated that phospholipid transfer activity alone in dMTP is enhancing phospholipids in this HDL in a concentration dependent manner suggesting dMTP might help assemble a novel HDL particle. We therefore, asked what kind of particles is synthesized by dMTP expressing mice in terms of apolipoprotein and size, which also reflects composition.

Characterization of lipoproteins assembled and secreted by distinct lipid transfer activity of MTP:

Negative staining and electron microscopy of lipoproteins in the plasma of dMTP and hMTP expressing mice: To further characterize the lipoproteins in the plasma of hMTP and dMTP expressing mice, plasma lipoproteins were separated by FPLC, stained with phosphotungstate and observed by scanning electron microscopy (34). VLDL obtained from L-MTP$^{-/-}$ mice expressing hMTP were larger in size compared to the VLDL from dMTP expressing mice (Fig 11A). The diameters of VLDL particles were 61.9 ± 19.7 nm in WT, 19.1 ± 4.5 nm in luciferase, 47.7 ± 19.6 nm in hMTP, and 30.2 ± 7.1 nm in dMTP expressing L -MTP$^{-/-}$ mice (Fig 11B). This study showed that dMTP makes a smaller VLDL particle compared to hMTP. To observe and characterize the type of HDL population present in dMTP expressing mice, apoAI containing HDL were pulled down by incubations with agarose beads and anti-mouse apoAI antibody since apoAI is the primary component of HDL lipoproteins. ApoAI free supernatant were mixed 1:1 (v/v) with 2% phosphotungstic acid and fixed for 1 min. The fixed sample was then applied onto a carbon-formvar grid and excess sample removed. The grid was air-dried and viewed under the electron microscope. Western blot analysis was done to confirm the separation of apoAI in the pellet and apoAI free supernatant which was analyzed by EM. ApoB containing HDL sized particles were only seen in dMTP of 8.7 ± 2.5 nm in size after depleting apoAI-containing particles (Fig 11C). Western blot analysis confirmed removal of apoAI from HDL supernatants following inmunoprecipitation. Top panel shows western blot for apoB in pellet and supernatant for each group with apoB48 present only in dMTP apoAI depleted supernatant (Fig 11D). Bottom panel showing apoAI in pellet only of each represented group and not in ApoAI depleted HDL (supernatant) (Fig 11D). These studies substantiate the existence of new HDL particle in dMTP expressing mice. Next we further

confirmed these particles by demonstrating hepatic synthesis and secretion of apoB in VLDL and HDL lipoproteins in these groups.

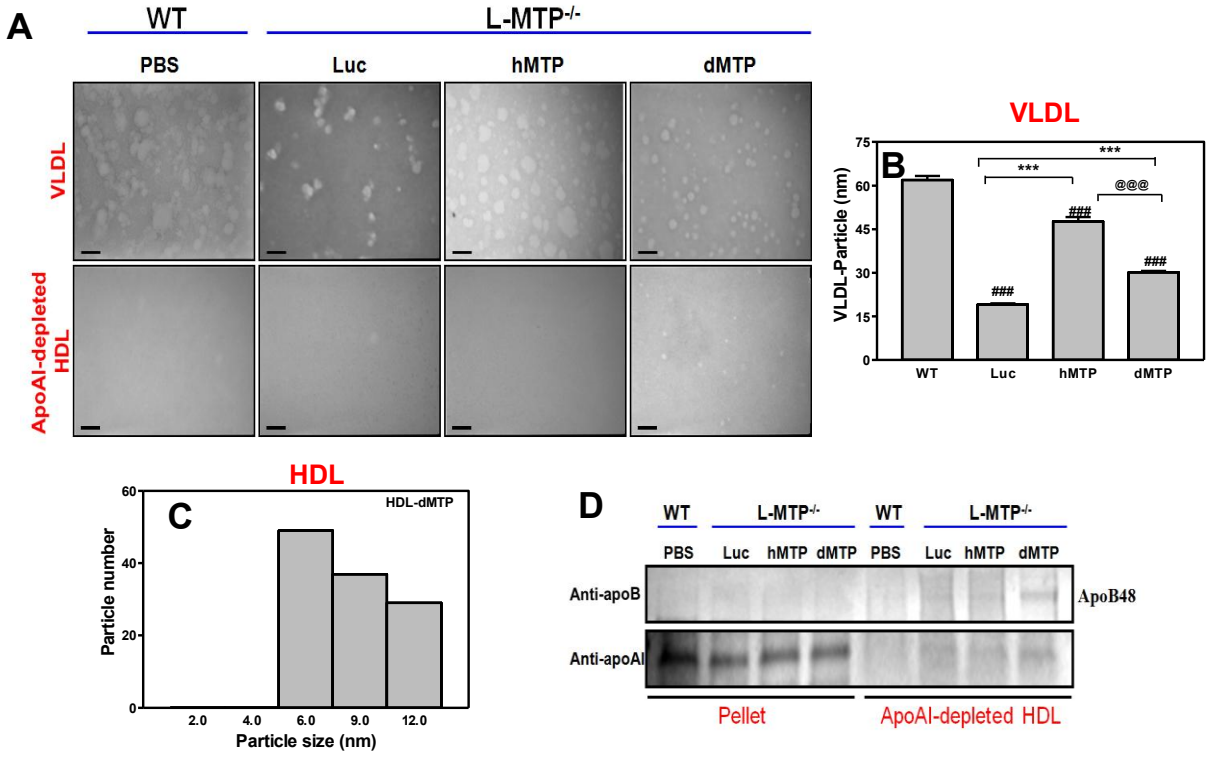

Figure 11. Plasma lipoprotein analysis: L-MTP$^{-/-}$ mice were transduced with different titers of adenoviruses: After 3 days mice were fasted for 4 h and injected with P407, plasma was collected after another 2 h of fasting. Plasma from each group were pooled and subjected to FPLC for size fractionation by gel filtration. VLDL fractions were pooled and concentrated as described in methods. Pooled HDL fractions were immune-depleted for apoAI.
(A) Negative staining and electron microscopy of VLDL (top panel) and apoAI depleted HDL (bottom panel) from hMTP and dMTP expressing mice. Magnifications, VLDL Bars = 50 nm, HDL Bars = 100 nm.
(B) Average size of VLDL particles from each mice as indicated (Mean ± SEM, n=150-200).
(C) Average size of apoAI free HDL particles only seen in dMTP expressing L-MTP$^{-/-}$ mice.
(D) Western blot analysis of HDL fractions after immunoprecipitation with apoAI, pellet containing apoAI and supernatant devoid of apoAI were immunoblotted for apoB (top panel) and apoAI (bottom panel). ApoB is only present in the supernatant only in dMTP (Top panel) which corresponds to the particles seen exclusively in dMTP by EM in panel A. ApoAI is only seen in pellet and not in supernatant indicating complete removal of apoAI from the supernatant following immunoprecipitation (bottom panel).

Identification of newly synthesized hepatic lipoproteins assembled by each ortholog:

The above studies show differences in types of lipoproteins in hMTP and dMTP expressing mice. To study their hepatic synthesis, 10^9 pfu of Ad-dMTP or Ad-hMTP were injected into L-MTP$^{-/-}$ mice. After 72 h mice were fasted for 4 h to avoid intestinal contribution, metabolically labeled intravenously with ^{35}S-methionine (1mCi/mice) to achieve a hepatic specific protein labeling and

injected with P407 to block their clearance from plasma. Plasma samples were collected after another 2 h of fasting and subjected to FPLC. The goal was to measure amounts of radioactivity in apoB100 and apoB48 bands in VLDL and HDL, which would demonstrate newly, synthesized hepatic apoB in different lipoprotein population. This would indicate a hepatic specific contribution of apoB biosynthesis and secretion. Furthermore results would also dissect the nature of newly synthesized apoB particles in VLDL vs HDL lipoproteins assembled and supported by hMTP or dMTP. There was substantial reduction in the hepatic synthesis and secretion of apoB48 and apoB100 VLDL in L-MTP$^{-/-}$ mice (Fig 12A-C) compared to WT mice. hMTP expression resulted in the synthesis of both apoB48 (Fig 12A-C) and apoB100 (Fig 12A-C) but levels were ~1.5 to ~2 fold

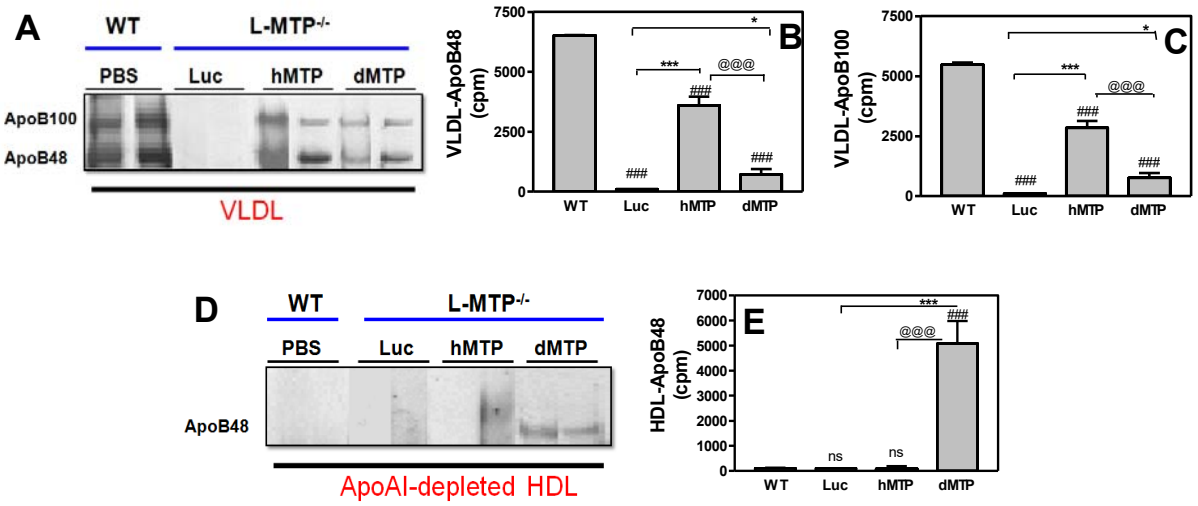

Figure 12. Plasma apoB characterization of lipoproteins: L-MTP$^{-/-}$ mice were transduced with different titers of adenoviruses. Mice were fasted for 4 h on day 3 following adenoviral delivery and metabolically labeled with 1 mCi of [^{35}S] promix via tail vein. Plasma were collected after another 2 h fast and subjected to FPLC for size fractionation by gel filtration. Data from one experiment performed in duplicate are shown, Mean ± SEM, n=4.
(A-C) VLDL fractions were pooled immunoprecipitated using rabbit anti-mouse apoB antibodies in 0.5M Tris-HCl, pH 7.4, 1.5 M NaCl, 500 mM EDTA, 1% SDS, separated on 5% SDS-PAGE and **(A)** imaged by autoradiography. ApoB100 and apoB48 bands in VLDL were excised and counted on scintillation counter for **(B)** VLDL-apoB48 and **(C)** VLDL-apoB100.
(D-E) HDL were pooled, ApoAI containing lipoproteins were immune-depleted, supernatants were precipitated with anti-apoB antibodies, proteins were separated on 5% SDS-PAGE and **(D)** imaged by autoradiography. Bands were excised and counted on scintillation counter for **(E)** HDL-apoB48.

lesser compared to WT. Similarly, dMTP also supported the synthesis of both VLDL apoB48 and apoB100 secretion but these levels were considerably lower than both hMTP and WT (Fig 12A-C). In dMTP expressing mice, we had observed the HDL population (Fig 11). To characterize this population, the major apolipoprotein associated with HDL, apoAI-containing particles were

removed using anti-apoAI antibodies, apoB was immunoprecipitated in the apoAI free supernatants, autoradiographed and apoB48 HDL was observed only in dMTP expressing mice (Fig 12D-E). We interpret these data to suggest that hMTP supports the formation of a triglyceride-rich VLDL particle that is larger in size. In contrast, dMTP makes smaller, triglyceride-poor VLDL particles. In addition, dMTP supports formation of a phospholipid-rich apoB48-containing HDL-size particle.

Mechanisms for differences in hMTP and dMTP mediated hepatic lipoprotein biosynthesis

So far the studies confirm that hMTP expression supported assembly and secretion of VLDL containing higher triglyceride thereby also larger in size unlike dMTP expressing mice. Changes in VLDL-triglyceride production could be due to changes in the amount of triglycerides per VLDL particle, the number of VLDL particles secreted, or both. One possibility is that hMTP promotes higher triglycerides in VLDL to facilitate the loading of triglyceride onto nascent VLDL particles in the liver thereby resulting in the assembly and secretion of triglyceride-enriched VLDL particles. Alternatively, hMTP could increase VLDL-triglycerides by increasing the number of secreted VLDL particles. Since one apoB molecule is secreted per VLDL particle, VLDL-apoB production provides an estimate of number of secreted VLDL particles. Therefore, to understand whether hMTP increases the amount of triglyceride per VLDL particle or the net number of VLDL particles, we measured VLDL-triglyceride and hepatic apoB production *in vivo*.

Hepatic triglyceride secretion rates: Triglyceride production in L-MTP$^{-/-}$ mice injected with luciferase as control was very low at all time points compared to WT mice (Fig 13A). Introduction of hMTP and dMTP in L-MTP$^{-/-}$ resulted in time dependent increases in plasma total (Fig 13A) and non-HDL triglycerides (Fig 13B), but these values were lower in dMTP expressing mice (Fig 12B). Total triglyceride (Fig 13C) and non-HDL triglyceride (Fig 13D) secretion rates were significantly reduced in L-MTP$^{-/-}$ mice compared to WT. hMTP and dMTP expression significantly increased total triglyceride and non-HDL triglyceride secretion rates (Fig 13C-D); although these rates were significantly lower in dMTP expressing mice. These studies show that expression of different MTP orthologs partially rescues total and non-HDL triglyceride secretion rates. However, restoration of hepatic VLDL-triglyceride secretion rates by dMTP in L-MTP$^{-/-}$ mice were lower compared to hMTP (Fig 13C-D). Thus, higher plasma VLDL-triglycerides in hMTP expressing mice could be

secondary to increased hepatic triglyceride secretion rates.

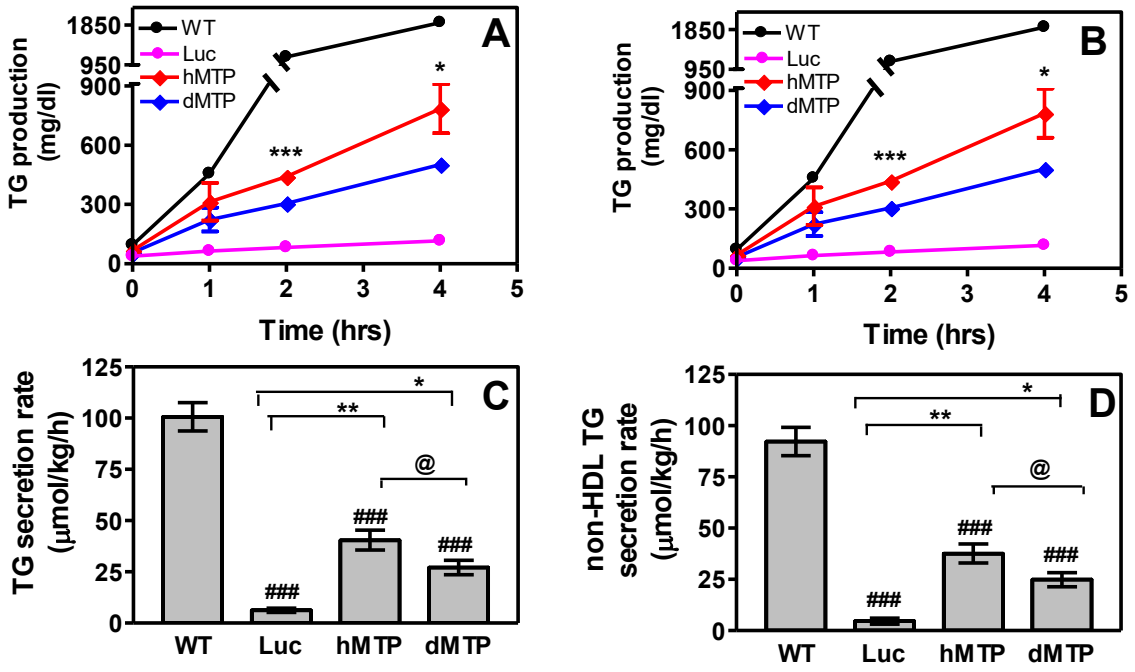

Figure 13. Hepatic triglyceride secretion rates: L-MTP$^{-/-}$ mice wre transduced (10^{10} pfu) with Ad-Luciferase, Ad-hMTP-FLAG or Ad-dMTP-FLAG. After 72 h mice were fasted for 4 h, blood was drawn from tail to measure the basal level of triglycerides, and injected with P407. Blood was drawn at different time points to measure triglyceride. Data are representative of two independent experiments.
(A-B) Total plasma and VLDL triglycerides were measured after precipitating HDL lipoproteins. Changes in plasma **(A)** total and **(B)** non-HDL triglyceride production over time are shown.
(C-D) Increases in triglyceride secretion rates were calculated after subtracting levels before the injection of P407 and expressed as micromoles of lipids produced per hour per gram of body weight assuming a plasma volume of 3.5% (liters per kilogram). **(C)**Total and **(D)** non-HDL triglyceride secretion rates were calculated using time points between 2 and 4 h.

Hepatic ApoB synthesis rates: Next, we studied hepatic apoB biosynthesis and secretion, which would give an estimate of VLDL particle number as each VLDL particle contains one apoB molecule. These metabolic labeling studies will provide rates of apoB secretion and establish if there are any differences. L-MTP$^{-/-}$ mice expressing luciferase had very little radiolabeled apoB in the plasma compared to WT (Fig 14A-C). hMTP expressing mice showed significant time dependent increases in the amounts of newly synthesized apoB100 and apoB48 (Fig 14A-C). Surprisingly, dMTP expressing L-MTP$^{-/-}$ mice also supported hepatic synthesis and secretion of both apoB48 and apoB100 that increased with time. However, these levels were lower than hMTP.

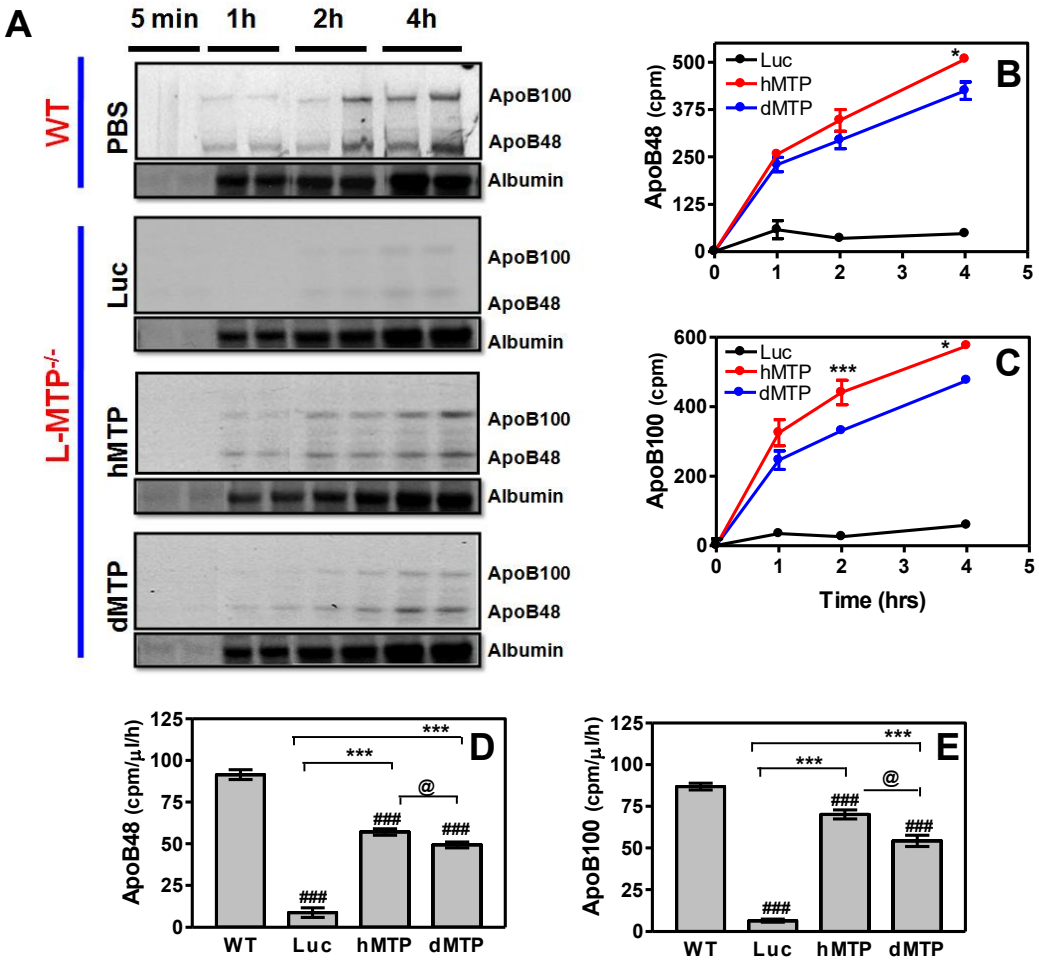

Figure 14. Hepatic apolipoprotein B secretion rates: Different adenoviral vectors (10^{10} pfu) were delivered to L-MTP$^{-/-}$ mice. In addition, MTP$^{fl/fl}$ (WT) and L-MTP$^{-/-}$ mice were injected with PBS as control. After 72 h, mice were fasted for 4 h and injected with 1 mCi [^{35}S] promix intravenously. After 30 min, Poloxamer 407 (P407) was injected intraperitoneally, blood was drawn from tail at 5 min, and 1, 2 and 4 h. Plasma was run on 5% SDS-PAGE, incubated with amplify fluorographic reagent (GE Life Sciences), dried and imaged by autoradiography. Results are representative of three experiments.
(A) Autoradiograph showing newly synthesized and secreted apoB from liver in plasma of mice from respective groups over time. Albumin is shown as loading control.
(B-E) Radioactivity in apoB48 and apoB100 were determined in the bands after dissolving in soluene (Perkin Elmer). The counts obtained at 5 min were used as background and subtracted from apoB counts obtained at different time points and plotted as X-Y plot showing increase in hepatic **(B)** apoB48 and **(C)** apo100 sythesis over time. Hepatic secretion rates of **(D)** apoB48 and **(E)** apoB100 are calculated using values from 2 and 4 h time points in cpm/µl/h.

These studies for the first time show that dMTP with only phospholipid transfer activity can support the biosynthesis of apoB100-lipoproteins (Fig 14D-E).

Mechanism for differences in hepatic triglyceride secretion rates and apoB synthesis

Subcellular distribution of newly synthesized lipids: To further understand the mechanisms for reduced secretion of triglycerides and compositional differences, we extended our studies in primary hepatocytes to analyze synthesis and subcellular distribution of newly synthesized lipids (Fig 15A-H) using ^{3}H-oleic acid (1μCi/ml) as described in methods (17;33). However, we observed that the synthesis of phospholipids (Fig 15A) and triglycerides (Fig 15B) was similar in hepatocytes expressing hMTP and dMTP excluding the possibility that lipid synthesis was limiting in dMTP expressing cells with only phospholipids transfer activity. There were no significant differences in the amounts of phospholipids associated with apoB in microsomal membranes and lumen (Fig 15C-D); however dMTP-expressing hepatocytes contained lesser amounts of triglycerides associated with apoB (Fig 15E-F). A major difference was in the amounts of triglycerides associated with apoB-lipoproteins present in the microsomal lumens (Fig 15F). No such changes were seen in triglycerides not associated with apoB in the microsomal membrane and lumen (Fig 15G-H). These data indicate that triglyceride transfer activity might be more critical in adding triglycerides to nascent membrane bound and lumenal apoB particles.

Regulation of apoB by proteosomal degradation: It is known that apoB secretion is mainly controlled at translational and post-translational levels mainly due to alterations in the degradation of newly synthesized apoB. Therefore, we hypothesize that differences in dMTP and hMTP might arise from their differential abilities to rescue nascent apoB from proteasomal degradation. This hypothesis was based on the results obtained in aim 6 showing differences of VLDL-apoB secretion rates. To test this hypothesis we studied the synthesis of apoB in primary hepatocytes isolated from L-MTP$^{-/-}$ transduced with hMTP or dMTP. Cells were pulse labeled with [^{35}S]-cysteine/methionine for 30 min in the absence and presence of inhibitor that blocks proteasomal degradation, MG132. Amounts of apoB in cells and secreted to media were quantified after immunoprecipitation.

Continuous Pulse Study: Continuous pulse study showed intracellular accumulation of apoB that was not degraded over time in both hMTP and dMTP. ApoB synthesis and accumulation at early time points of label incorporation were analyzed to check significant amount of co-translational degradation of nascent apoB as it is synthesized. hMTP expressing hepatocytes showed apoB degradation in the absence of MG132 which could be explained as earlier studies have shown that significant amount of apoB is degraded prior to secretion from hepatic cells (40-43). The amounts of apoB synthesized by hMTP were greater than those synthesized by dMTP expressing hepatocytes

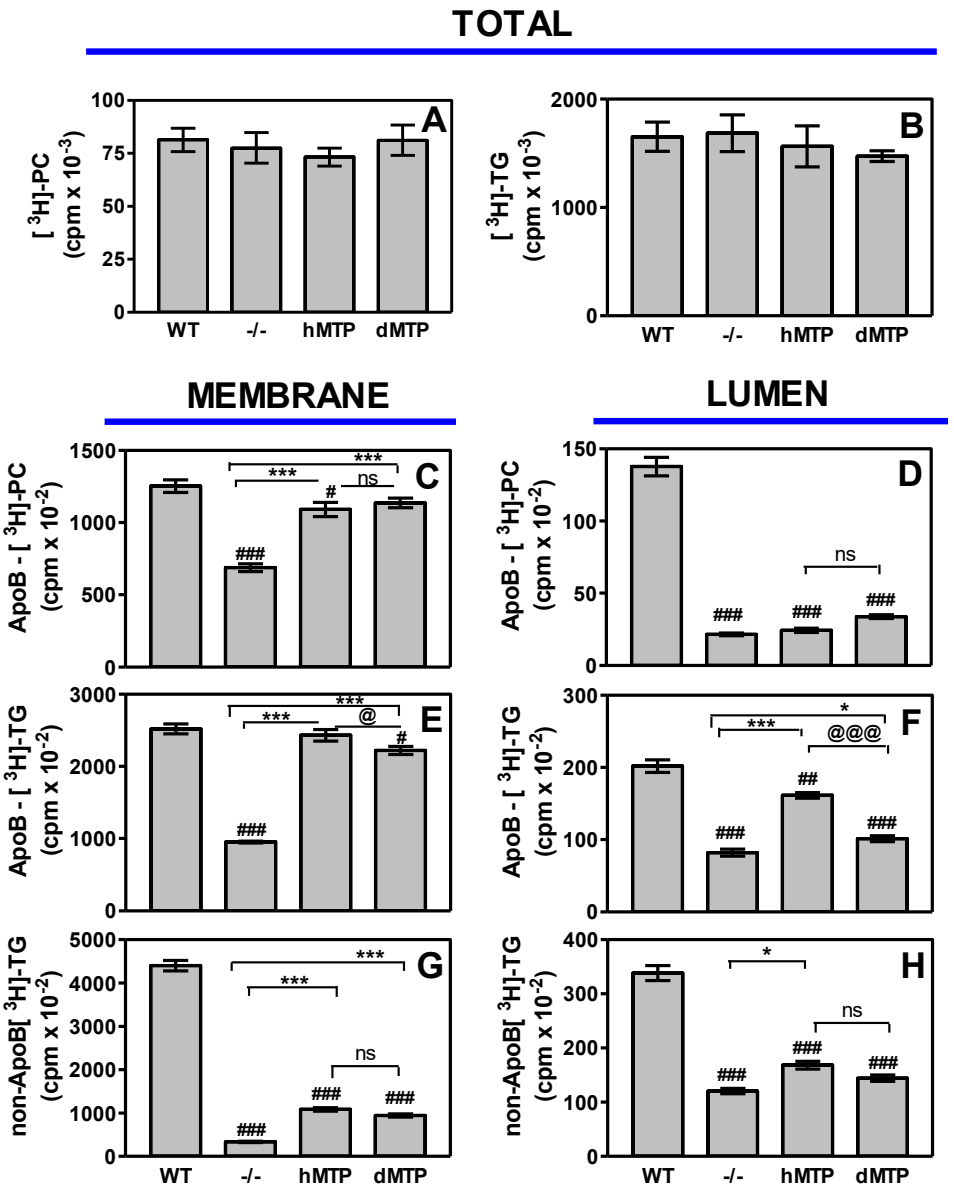

Figure 15.Subcellular lipid distribution and partitioning of newly synthezised lipids: Primary hepatocytes were isolated from L-MTP$^{-/-}$ mice transduced (10^{10} pfu) with Ad-hMTP-FLAG or Ad-dMTP-FLAG and incubated with [^{3}H] oleate (10 µCi/ml) for 30 minutes. Microsomal lumenal and membrane were isolated as described in methods and incubated with anti-mouse apoB antibody at 4º overnight. Protein-A Sepharose was added and incubated for 2 h and apoB-protein A complex was pulled down and apoB-associated lipids were extracted using bligh and dyer method (23).The entire immunoprecipitation were done under non-denaturing condition in the absence of detergent described in methods for apoB associated lipid studies. Lipids were separated on TLC in by (chloroform/methanol/acetic acid/formic acid/water; 70:30:12:4:2 v/v) solvent system first to separate phospholipids followed solvent system (hexane/dipropyl ether/acetic acid; 65:35:2 v/v) to separate neutral lipids (8;31). Bands corresponding to phosphatidylcholine (PC) and triglyceride (TG) were scraped and counted.
(A-B) Newly synthesized total lipid content in hepatocytes represented by **(A)** total PC and **(B)** total TG.
(C-H) Subcellular lipid distribution showing apoB associated **(C)** microsomal lumenal PC and **(D)** membrane PC. Corresponding apoB associated neutral lipid distribution showing **(E)** lumenal TG and **(F)** membrane TG. Non-apoB associated neutral lipid distribution showing **(G)** lumenal TG and **(H)** membrane TG in respective groups. Data (Mean ± SEM, n=3) are representative of three independent experiments. One-way ANOVA was performed to compare differences between groups.

in the absence of proteosomal inhibitor. Under abrogation of proteosomal degradation in the presence of MG132, dMTP expressing hepatocytes had intracellular apoB levels similar to that of hMTP (Fig 16A). Percent apoB degradation in dMTP expressing hepatocytes was ~ 3 and ~1.5 fold higher for apoB48 and apoB100 respectively compared to hMTP (Fig 16B-C). In contrast, these differences were abolished in the presence of proteaosomal degradation inhibitor, MG132 (Fig 16D-E).

Pulse Chase Study: Next, we studied the post-translational degradation of apoB in these hepatocytes (Fig 17A). Percent intracellular apoB levels in hMTP did not show any difference with proteosomal inhibition and in the absence of proteosomal inhibition (Fig 17A, Top panel, 16B-E) indicating that apoB is more sensitive to co-translational degradation as an early check point to control early steps of apoB lipoprotein assembly. Furthermore, percent changes in corresponding levels of secreted apoB in media did not show much difference in hMTP as well as dMTP (Fig 17A, Bottom panel, 17B-E). No such changes were observed in apoAI with presence or absence of proteasomal inhibitor at each time point (Fig 17A). These studies indicate that triglyceride transfer activity of MTP is critical in avoiding primarily the early pre-secretory degradation of nascent apoB during early translation and not post-translationally.

Hepatic Adaptive Mechanisms: Comparative gene expression studies were performed in hepatic tissue to analyze any homeostatic adaptation by liver that could possibly contribute to the differential effect by the orthologs. Thus relative gene expression of different key target genes responsible for β-oxidation, lipogenesis and VLDL assembly were analyzed between hMTP and dMTP groups (Fig 18A, C, D). No significant differences were found in transcription factors, PPARα and PPARγ and their target genes, ACC and CPT1 that are involved in β-oxidation (Fig 18A). SCD1 and FAS, which are key genes in de novo lipogenesis, also did not show any differences (Fig 18C). Genes that are involved directly or indirectly in VLDL assembly such as LDLr, L-FABP, DGAT1 and DGAT2 did not show any differences either (Fig 18D). Furthermore, lipid peroxidation assays were performed and no differences were found between hMTP and dMTP groups, neither under normal lipid loads nor at higher lipid availability (Fig 18B).

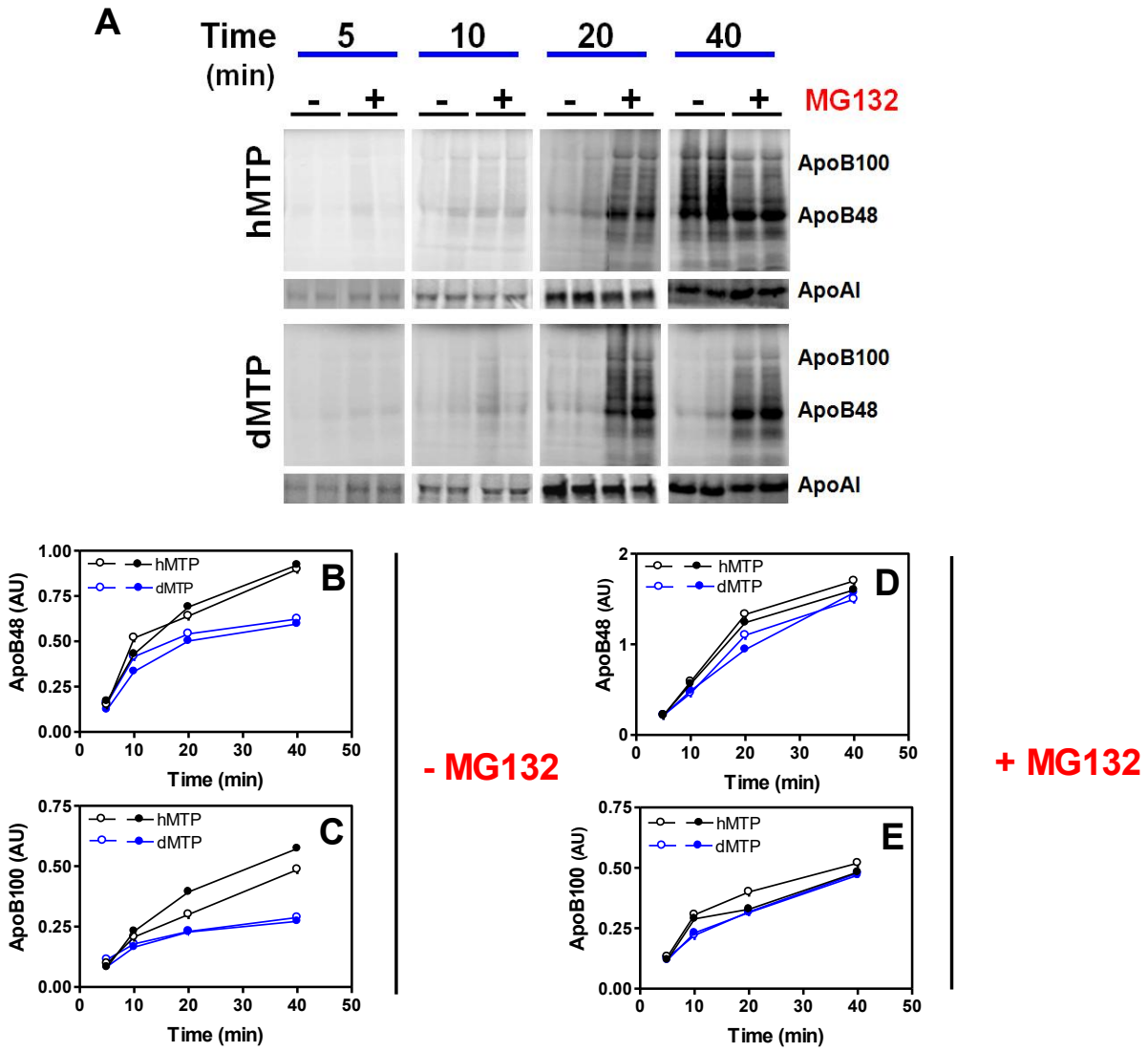

Figure 16. Kinetics of apoB synthesis and secretion; ApoB continuous pulse proteosomal degradation study: Primary hepatocytes were isolated from L-MTP$^{-/-}$ mice transduced (10^{10} pfu) with Ad-hMTP-FLAG or Ad-dMTP-FLAG and incubated with methionine free medium with DMSO (-MG132) or presence of MG132 (10 μM) to inhibit proteasomal degradation. Cells were labeled with 150 μCi/ml [^{35}S] methionine for indicated time points in the presence or absence of MG132. Proteosomal degradation of intracellular apoB was analyzed by immunoprecipitaing apoB from cell homogenates using anti-mouse apoB antibody and protein-A Sepharose beads. ApoB-protein A complexes were pulled down and apoB was solubilized in laemmali buffer. Samples were boiled and separated on 5% SDS-PAGE gels. Gels were dried and fluorographed. Continuous pulse labeling indicating co-translational degradation of apoB are shown. Results are representative of two independent experiments. Results from two samples for each condition from one experiment are shown.
(A) Autoradiograph showing intracellular apoB in the absence and presence of MG132 in hMTP and dMTP expressing hepatocytes respectively.
(B-C) (B) ApoB 48 and **(C)** apoB100 bands were quantified and synthesis over time is plotted in the absence of MG132.
(D-E) (D) ApoB 48 and **(E)** apoB100 bands were quantified and synthesis over time is plotted in the presence of MG132.

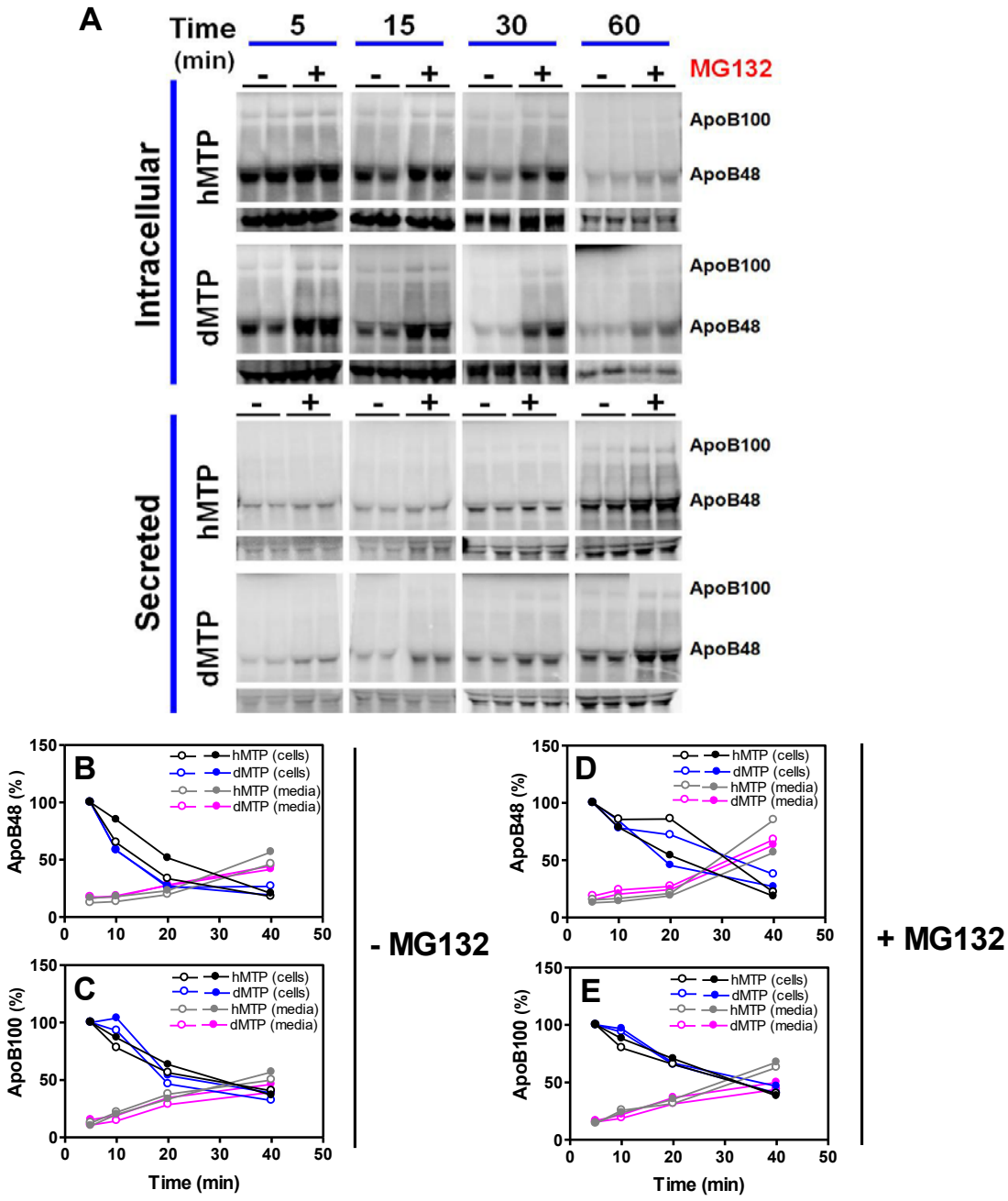

Figure 17. Kinetics of apoB synthesis and secretion; ApoB pulse-chase proteosomal degradation study: Primary hepatocytes were isolated from L-MTP$^{-/-}$ mice transduced (10^{10} pfu) with Ad-hMTP-FLAG or Ad-dMTP-FLAG and incubated with methionine free medium with DMSO (-MG132) or presence of MG132 (10 μM) to inhibit proteasomal degradation. . Pulse-chase study showing sensitivity of apoB to proteasomal degradation. Cells were incubated with 150 μCi/ml [^{35}S] in the presence or absence of MG132 for 1 h. Cells were washed and chased for 5, 15, 30 and 60 min. Results are representative of two independent experiments. Results from two samples for each condition from one experiment are shown.
(A) Cells and media were collected at indicated time points and intracellular (Top panel) and secreted apoB (Bottom panel) from duplicate samples for each condition were run on SDS-PAGE and autoradiographed.
(B-E) Percent **(B)** apoB48 degradation and **(C)** apoB100 degradation in cells and media were calculated from band quantification at each time point was calculated in the absence of MG132 in cells and media are shown. Percent **(D)** apoB48 degradation and **(E)** apoB100 degradation in cells and media were calculated from band quantification at each time point shown in the presence of MG132 in cells and media.

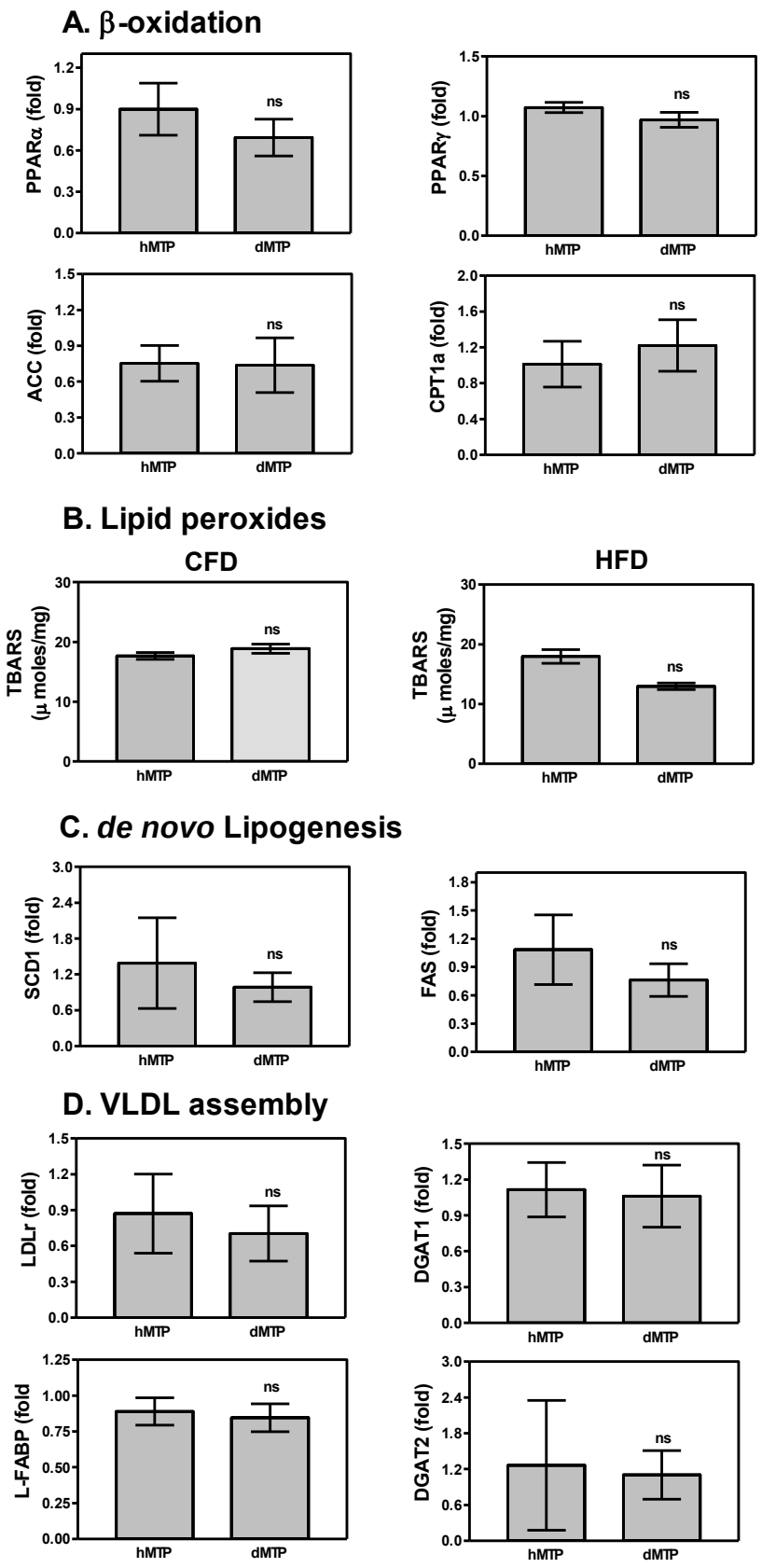

Figure 16. Relative hepatic mRNA expression levels in mice expressing different MTP orthologs: Hepatic mRNA were extracted from each group using TRIzol (Invitrogen). Genes are represented as fold change relative to hepatic ARPp0 expression level. (A) Hepatic mRNA levels of transcription factor peroxisome proliferator-activated receptor (PPAR) n=3, PPARα and PPARγ did not differ significantly in hMTP and dMTP expressing mice. Similarly, genes involved in ß-oxidation (n=3) that are controlled by PPARs such as acyl-CoA carboxylase (ACC) and carnitine palmitoyl transferase-1a (Cpt1a) were not significantly different in these mice indicating that ß-oxidation is probably not different in these mice.
(B) Lipid peroxidation in liver was monitored by measuring thiobarbituric acid reactive substrates (TBARS Assay Kit, Cayman Chemicals). Lipid peroxides were similar in hMTP and dMTP mice, n=4.
(C) Expression levels of genes involved in de novo lipogenesis (n=3) such as fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1) were also similar in hMTP or dMTP expressing mice. (D) Genes that directly or indirectly affect VLDL assembly (n=3) such as LDLr, DGAT1, DGAT2 and L-FABP did not exhibit any changes in mice expressing different MTP orthologs.

DISCUSSION

Role of phospholipid transfer activity of MTP in plasma and hepatic lipid homeostasis

The data presented here show that L-MTP$^{-/-}$ mice expressing dMTP have low plasma triglyceride, and synthesize lower amounts of apoB100 and apoB48 lipoproteins compared to hMTP. Expression of dMTP and hMTP was similar and differences in plasma triglyceride persisted when mice were expressing different amounts of these orthologs or fed different diets. Metabolic studies revealed that absence of triglyceride transfer activity in dMTP results in the synthesis of fewer triglyceride-poor, smaller size VLDL particles. Hence, phospholipid transfer activity of dMTP is sufficient for *in vivo* biosynthesis of apoB-containing VLDL. The lower plasma lipids are contributed by both reductions in size and decrease in number of apoB-lipoproteins in dMTP expressing mice.

By contrast, dMTP mice had higher plasma phospholipids than hMTP; albeit these levels were significantly lower than in WT. Mechanistic studies revealed that this was secondary to the synthesis of novel phospholipid/cholesterol-rich apoB48-containing HDL-sized lipoproteins. Absence of apoB100-HDL size particles could be due to an absolute need for neutral lipids for its successful lipidation into secretion-competent particles or minimal phospholipidation of apoB100 might yield a particle with gel filtration properties similar to VLDL particles.

Another surprising observation was that dMTP-expressing mice had normal hepatic lipids seen in WT and hMTP expressing mice. Due to the absence of triglyceride transfer activity in dMTP and low levels of plasma apoB-lipoproteins, we had anticipated that mice expressing this ortholog would accumulate significant amounts of lipids as in L-MTP$^{-/-}$ mice. Low hepatic phospholipids and cholesterol could be attributed to the assembly of apoB48 HDL. Low hepatic triglyceride could be due to increased ß-oxidation and/or reduced de novo lipogenesis. These possibilities were excluded since no significant differences in transcription factors (PPARα and PPARγ) and their target genes (ACC and CPT1a) involved in ß-oxidation, TBARS, and de novo lipogenesis were seen in dMTP and hMTP mice. A clue to the possible reasons for the low hepatic triglycerides came from the observations that, while VLDL particles secreted by dMTP had lower levels of triglyceride, these particles were still able to assemble a neutral lipid core. This was achieved with no significant change in expression of genes involved in VLDL assembly. We posit

that transport of low levels of triglycerides with VLDL over time might be sufficient to avoid hepatic triglyceride accumulation and conclude that phospholipid transfer activity of MTP is not only sufficient for lipoprotein biosynthesis, but it also ameliorates hepatosteatosis while maintaining low plasma lipids.

Role of different lipid transfer activities of MTP in lipoprotein biosynthesis

Analysis of secreted lipoproteins showed that dMTP supports assembly and secretion of phospholipid-rich apoB48-containing HDL and triglyceride-poor apoB100 and apoB48 VLDL. To explain the role of different lipid transfer activities in the biosynthesis of these particles, we propose that apoB-lipoprotein biosynthesis occurs at different "nucleation sites" in the inner leaflet of the ER (Fig 19). Formation of a simple nucleation site might involve interaction of newly translated apoB peptide with the phospholipid monolayer. Phospholipid transfer activity might bring more phospholipids to these sites helping in the phospholipidation of nascent apoB peptide. Desorption of apoB from these nucleation sites might lead to the synthesis of phospholipid-rich HDL size apoB48-containing primordial lipoprotein particles. A second type of nucleation site may contain some neutral lipids that arrive at these sites by lateral diffusion from their site of synthesis. Formations of such triglyceride-containing nucleation sites are necessary in the biosynthesis of apoB100-containing particles. Desorption of lipoproteins from these nucleation sites results in the formation of triglyceride-poor VLDL size particles. We further posit that phospholipid transfer activity might transfer phospholipids to apoB in the formation of nucleation sites and is essential for desorption of particles from these nucleation sites (Fig 19A). The presence of triglyceride transfer activity helps in the transfer of triglycerides to various nucleation sites leading to protection of nascent apoB from degradation and formation of triglyceride-rich VLDL (Fig 19B).

Triglyceride transfer activity of MTP is dispensable

Based on the observations that dMTP expressing mice have low plasma and normal hepatic lipids, we hypothesize that phospholipid transfer activity is sufficient for normal hepatic lipid homeostasis and that the triglyceride transfer activity may be dispensable. Compounds that inhibit triglyceride transfer activity of MTP and spare its phospholipid transfer activity are expected to lower plasma lipids with no associated steatosis. Furthermore, these compounds are not expected to affect the

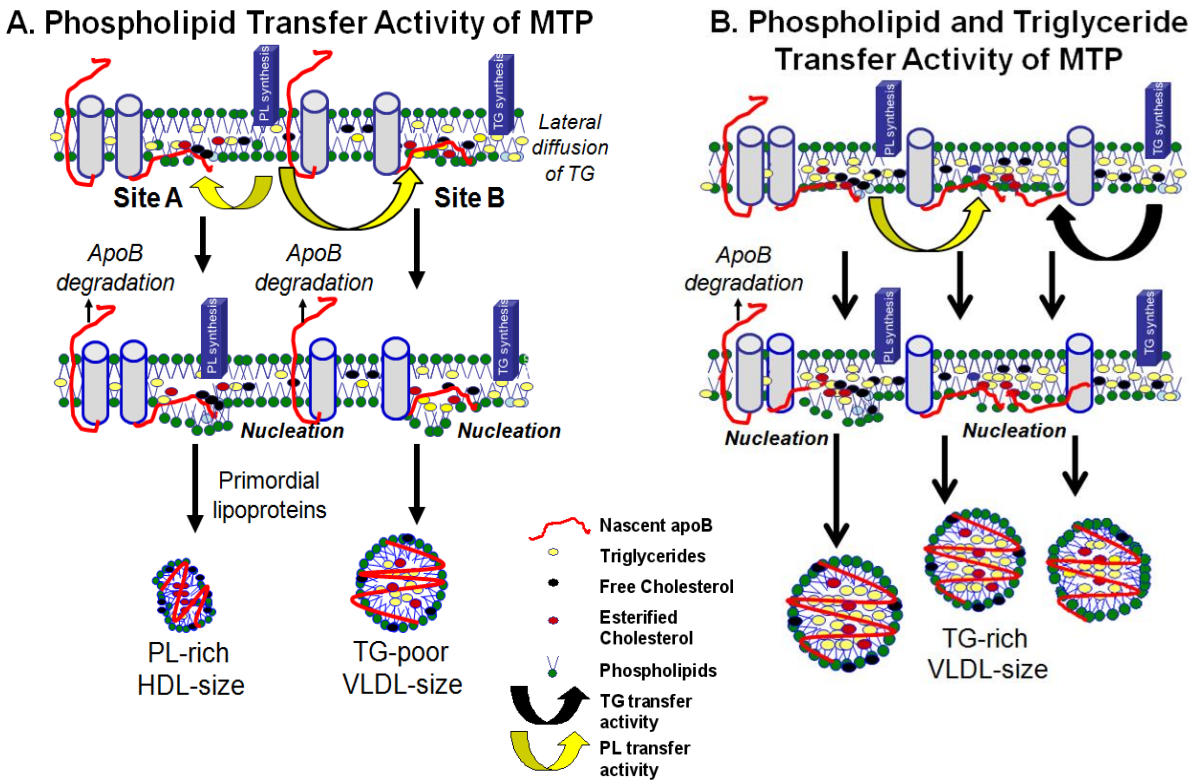

Figure 19. Model of lipoprotein biosynthesis:
(A) Biosynthesis in the absence of triglyceride transfer activity by dMTP: Nascent apoB (red) is shown to traverse the ER membrane and interact with inner leaflet to form "nucleation sites" or retro-translocated for degradation. Phospholipid transfer activity in dMTP can bring phospholipid molecules to nascent apoB (yellow arrows) and help in the formation of these nucleation sites. Nucleation site A has fewer triglycerides. Desorption from this site may yield phospholipid-rich apoB48-containing HDL size particles. At site B, some triglyceride molecules are shown to arrive via lateral diffusion. Desorption of apoB-lipoproteins from this site can give rise to triglyceride-poor apoB100 and apoB48-containing VLDL size lipoproteins. Besides forming nucleation sites, phospholipid transfer activity of MTP might play an essential role in desorption as well as phospholipidation of desorbed particles to render apoB secretion competent. Other phospholipid transfer proteins do not compensate for this activity probably because they are unable to physically interact and bind with apoB or not expressed at the site of lipoprotein assmebly.
(B) Biosynthesis in the presence of triglyceride transfer activity by hMTP: Triglyceride transfer activity (black arrow) can bring neutral lipids creating triglyceride enriched nucleation sites, lipidate nascent apoB, and prevent proteasomal degradation. Desorption from these nucleation sites might yield heterogeneous VLDL size particles containing apoB100 and apoB48.

biosynthesis of CD1d, another molecule that is phospholipidated by MTP (44) and would not compromise immune function (45). Hence, domain specific antagonists rather than global inhibition of MTP might be a better therapeutic approach to combat hyperlipidemia.

There is significant evolutionary (21;23), kinetic ((19)and structural (23) evidence that phospholipid transfer activity might be separate from triglyceride transfer activity. Our studies indicate that MTP evolved as a phospholipid transfer protein and later acquired triglyceride transfer

activity during a transition from invertebrates to vertebrates (23). Structural analyses based on homology with lipovitellin show that hMTP contains two phospholipid binding sites (9;20). Studies by Atzel and Wetterau (19) suggest that mammalian MTP has two sites for the transfer of lipids (46); one high affinity site that transfers triglycerides as well as phospholipids and the other low affinity site that only transfers phospholipids. Therefore, it is likely that small molecules can be identified that significantly inhibit triglyceride transfer activity while only partially lowering phospholipid transfer activity of MTP. Hence, we advocate for the identification of selective inhibitors of triglyceride transfer activity that have no effect on or only partially inhibit phospholipid transfer activity of mammalian MTP to lower plasma lipids.

The molecular bases for differences in lipid transfer activities in dMTP and hMTP are not obvious. Both proteins have similar primary, secondary, and tertiary structures (23). We have previously reported that helix A known to be critical for triglyceride transfer activity (47;48) is not conserved in dMTP and hMTP (21). Hence, specific mutations rather than domain acquisition, perhaps, played a role in the gain of triglyceride transfer activity.

The present comparative study using dMTP as an invertebrate ortholog and hMTP as a vertebrate ortholog with distinct lipid transfer activity provides important insights into evolutionary gains made by MTP during transition from invertebrates to vertebrates. Acquisition of triglyceride transfer activity might have allowed mammals to efficiently transport neutral lipids by inhibiting proteasomal degradation of apoB and packaging more lipids in the core of these particles. This might have provided an advantage in the assimilation of energy at the time of food scarcity. In modern era of food abundance, this activity might be superfluous.

Acknowledgement

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CHAPTER III

Selective loss of phospholipid transfer activity due to mutations in microsomal triglyceride transfer protein causes hypobetalipoproteinemia

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Running title: Hypobetalipoproteinemia due to MTP mutations

Abbreviations used: apoB, apolipoprotein B; ER, endoplasmic reticulum; MTP, microsomal triglyceride transfer protein; hMTP, Human MTP; TG, triglycerides; PL, phospholipids; PDI, Protein Disulfide Isomerase; VTG, Vitellogenins; LV, Lipovitellin; ABL, Abetalipoproteinemia.

SUMMARY

Apolipoprotein B (ApoB) and microsomal triglyceride transfer protein (MTP) are essential for apoB-containing lipoprotein assembly and secretion. Mutations in apoB and MTP cause familial hypobetalipoproteinemia (FHBL) and abetalipoproteinemia (ABL), respectively. New MTP missense mutations have been reported in ABL and FHBL patients but biochemical basis for different diseases in these patients have not been elaborated. In this study, we characterized the effect of various MTP missense mutations found in patients with respect to their expression, subcellular location, PDI interaction, triglyceride transfer activity of MTP and apoB secretion. R540H and N780Y that are known to lack triglyceride transfer activity, also lacked phospholipid transfer activity and did not support apoB secretion. Mutants S590I and G746E were unable to transfer both triglycerides and phospholipids and did not support apoB secretion. In contrast, H297Q and G661A lacked phospholipid transfer activity but were able to transfer triglyceride at a reduced level compared to normal hMTP. They were partially effective in secreting apoB. These studies explain that lack of both phospholipid and triglyceride transfer activity leads to ABL, whereas absence of phospholipid transfer with reduced triglyceride transfer activity manifests as heterozygous FHBL phenotype with less severe symptoms compared to homozygous FHBL or ABL. Thus it is likely that genetic screening for mutations in MTP besides apoB could be employed in subjects diagnosed with heterozygous FHBL.

INTRODUCTION

Microsomal triglyceride transfer protein (MTP) is a heterodimer of a large 97 kDa M subunit and a 65 kDa Protein disulfide isomerase (PDI), P subunit held together by non-covalent interactions (1;2), located in the microsomal lumen that is critical for assembly of VLDL and chylomicrons from liver and intestine, respectively (3). PDI helps to maintain the solubility of MTP (1). The M-subunit of MTP is a single peptide of 894 amino acids. Based on its homology with lipovitellin (VTG gene product), an ancient transport and storage lipoprotein found in egglaying vertebrates, structural modeling predicts that MTP contains three major structural domains: N- terminal β-barrel (22-297), central α-helical region (298-603) and C-terminal domain (604-894) (4-7). Based on mutagenesis followed by functional studies, Mann et al suggested three functionally independent domains in MTP; N-terminal domain mediates apoB binding (6), central α-helical region mediates interaction with both PDI and apoB (8) and C-terminal domain is involved in lipid binding and lipid transfer (6). MTP facilitates transfer of lipid molecules to nascent apoB as it is co-translationally translocated across the ER lumen thereby aiding in the assembly of primordial lipoprotein particle and preventing apoB from presecretory proteosomal degradation (9). Kinetic studies predict that MTP has two lipid binding sites, fast and slow. The fast one is implicated in triglyceride and phospholipid transfer whereas the slow site is involved in phospholipid transfer (10;11).

Apolipoprotein B (ApoB) and MTP (9;12-14) are critical proteins required for the biosynthesis of triglyceride-rich VLDL and chylomicrons by the liver and intestine. Mutations in these gene results in familial hypobetalipoproteinemia (FHBL, OMIM#107730) and abetalipoproteinemia (ABL, OMIM#200100) respectively. Key features of inherited disorders, FHBL and ABL are primary deficiency of low-density lipoprotein (LDL) and apoB. These disorders are due to the defects in apoB-lipoprotein assembly and secretion in liver and intestine (15;16). FHBL is an autosomal, co-dominant disorder characterized by plasma levels of total cholesterol, LDL-cholesterol and apoB below the fifth percentile of distribution in the general population (16). FHBL has been primarily linked to codominant mutations in apoB gene carried on chromosome 2

(17;18). Heterozygous FHBL in individuals have half the normal levels of β-lipoproteins whereas homozygotes have less than 50 mg/dl. On the other hand, ABL is a recessive disorder with LDL and VLDL are almost absent in patients. It is caused by homozygous autosomal recessive mutations in the MTP gene. Plasma triglycerides are typically < 0.23 mmol/L, cholesterol < 1.16 mmol/L, LDL and apoB are undetectable. Acanthocytosis of red blood cells (RBC) is a distinguishing feature on blood smear (19). Diagnosis is made by the absence of plasma apoB and intestinal biopsies showing lack of MTP activity. The severity of symptoms varies and their subsequent downstream array of complications depends on early diagnosis and treatment. During the first and second decade, first symptoms to be noticed are fat malabsorption, steatorrhea and failure to thrive. The other ramifications of the disease are fat-soluble vitamin deficiency specifically vitamin E (20) and vitamin A since VLDL and LDL distribute these vitamins to peripheral tissues. Most prominent and deabilitating clinical manifestation of ABL in the second decade are neurological disorders caused by the deficiency of vitamin E and progressive degeneration of central nervous system involvement leading to eventual death. Symptoms observed in homozygous ABL are indistinguishable from those with homozygous FHBL arising from molecular defects in apoB (21;22). Obligate ABL heterozygotes (parents of ABL patients) have no symptoms and no evidence of reduced plasma lipid levels.

Majority of mutations that were reported following sequence analysis of the MTP gene or cDNA in ABL subjects were frameshift, non-sense and splice site mutations that are predicted to encode truncated forms of MTP completely devoid of function (23-31). Ricci et al reported a nonsense mutation, G865X that caused 30 amino acid truncation at the C-terminal β-sheet of the 97 kDa MTP subunit (32;33). The translated protein lost the ability to interact with PDI and was unable to transfer lipids indicating that C-terminal β-sheet may play a role in the formation of the active complex. However, few missense mutations, R540H, S590I, N780Y, D384A, G661A, G746E and H297Q have been reported in MTP and it is likely that they will provide important structure-function information about MTP. Indeed, N780Y (28) has been suggested to be involved in interacting with membranes and extracting lipids during transfer, whereas, R540H, has been suggested to form an internal salt bridge that is necessary to keep the central α-

helical domain in proper conformation for PDI binding. Not much is known about other missense mutations. S590I and G746E have been shown to result in severe form of ABL in subjects (27) but no biochemical evidence has been provided for such a phenotype. Leo et al have reported a compound heterozygote individual for three missense mutations, D384A, H297Q and G661A, in highly conserved regions of MTP that had plasma lipid profile similar to that seen in heterozygous FHBL rather than the expected ABL phenotype (34). This patient was also homozygous for apoε2 allele. They speculated that hypobetalipoproteinemia in this subject might be due to mild MTP deficiency that may or may not be associated with

TABLE I. The mutations in abetalipoproteinemia (ABL) and hypobetalipoproteinemia (HBL) subjects that were reported are summarised with the author as well as the type which ranged from simple to compound with other mutations as represented under type.

Mutations	Type	Reports in ABL
H297Q	Compound heterozygote D384A, R540H	Rehberg et al, 1996
D384A	Compound heterozygote H297Q, R540H	Rehberg et al, 1996
R540H	Compound heterozygote H297Q, D384A	Rehberg et al, 1996
	Homozygous	Wang et al, 2000
S590I	Homozygous	Wang et al, 2000
	Homozygous	Al-Shali et al, 2003
G746E	Compound heterozygote nonsense mutation,A2524T K842X	Wang et al, 2000
N780Y	Homozygous	Ohashi et al, 2000
	Type	**Reports in HBL**
G661A H297Q D384A	Compound heterozygote	DiLeo et al, 2004

apoε2 allele. It is anticipated that knowledge about these missense mutations might provide new information concerning structure and function of MTP. Therefore, we explored the effects of these missense mutations (Table I) on various aspects of cellular, molecular and biochemical properties of MTP.

MATERIALS AND METHODS

Materials: 4-15% Ready Tris-HCl gels and precision-plus protein ladder were obtained from Bio-Rad Laboratories. ß-mercaptoethanol and restore western blot stripping buffer were purchased from Invitrogen and Thermo Scientific, respectively. Calcium chloride, ethylene diamine tetraacetic acid, Hepes, kanamycin, magnesium chloride, molecular weight markers, oleic acid, Tween-20 and Triton X-100 were bought from Sigma. Glycerol, heparinized micro-hematocrit capillary tubes, sodium dodecyl sulfate, and sucrose were obtained from Fisher chemicals. Fugene (Roche) and lipofectamine were obtained from Invitrogen. Antibodies M2 FLAG, anti-human apoB, anti-human PDI, anti-human GAPDH, Alexa Fluor 488 and 594 were obtained from molecular probes.

Site directed mutagenesis: Single amino acid mutations for each missense mutation reported in humans were introduced in hMTP-FLAG sequence using site-directed mutagenesis. Later these mutations were cloned in pMFG retrovirus vector.

Cloning and generation of recombinant retroviruses: Human kidney derived cell lines 293GPG, modified cell lines generated for efficiency were used as retroviral pacakaging cell line. pMFG retrovirus vector carrying normal and mutant MTP were transiently transfected using lipopfectamine 2000 (Invitrogen) according to manufacturer's instructions. Viruses were harvested 48-72 h following transfection in tet-off media to initiate viral production. The viral supernatants were collected and amplified on large scale and subsequently used to generate stable cell lines.

Generation of stable cell lines expressing MTP mutations: For generating stable cell lines, 2 x 10^5 Monkey kidney cells, COS7 were seeded. Cells were transduced with recombinant retroviruses and allowed to grow till confluency. This infection step was

repeated three times and cells were stored in liquid nitrogen. Since all the proteins generated were FLAG tagged, FLAG expression was quantified by western blot analysis to compare protein levels.

Cell culture and ApoB secretion studies: Stable cell lines of COS7 cells that were expressing different mutants were grown in Dulbecco's modified Eagle's medium (CellGrow) containing 10% fetal bovine serum supplemented with L-Glutamine and antibiotics. ApoB expression vectors were transfected into these cells using FuGENE 6 (Roche Applied Sciences) according to the instructions provided by the manufacturer. The cells were detached from the plate after 8-10 h using trypsin and were plated in 6-well plates (400,000 cells/well). During the final 16 h of 48 h post transfection, media was replaced with either 1 ml of 10% DMEM with 1.5% BSA or 10% DMEM containing 0.4 mM oleic acid complexed with 1.5% BSA and 1mM glycerol. Media and cells were collected for ELISA and functional assay, respectively, after 16 h of incubation in the lipid supplemented media. Protease inhibitors (Sigma-Aldrich) were added to the media and cell-debris were pelleted after brief spinning. Secreted apoB was measured by ELISA in the supernatant and cells were used for analyzing lipid transfer activity of MTP as described previously (35;36)**.** Cells were also used for western blot analyses to compare protein expression.

Immunofluoroscence: Stable cell lines were grown on coverslips in 12-well cell culture plates. Cells were fixed, blocked and probed with primary and secondary antibodies as described earlier (12). Cells were incubated with 0.05% Tween 20 in 10 mM sodium citrate buffer (pH 6.0) at 37^{o}C for 15-20 min. PBS containing 1mM $MgCl_2$, 0.5 mM $CaCl_2$, 3%BSA and 1% horse serum was used as blocking reagent. Primary and secondary antibody dilutions were made in the same buffer. The cells were incubated for 1 h with 1:100 dilution of mouse anti-FLAG M2 (Sigma) and Rabbit anti-PDI (AbCam) or Rabbit anti-Calnexin (SantaCruz) for 1 h with 0.1% Triton-X to increase the permeability. Alexa Fluor 488-conjugated goat anti-rabbit IgG1 (Green fluorescence) and Alexa Fluor 594-conjugated goat anti-mouse IgG1 (Red fluorescence) (Molecular Probes) were incubated for 1 h following primary antibody treatment. The coverslips

were mounted in anti-fade (Vectashield) to prevent fluorescent bleaching and analyzed with a laser-scanning microscope (Model LSM 510, Carl Zeiss). Images were imported and analyzed with Photoshop 6.0 before exporting to Illustrator CS2 (Adobe).

Lipid transfer assays: MTP triglyceride and phospholipid transfer activity in COS7 stable cell lines expressing different mutants were determined by triglyceride and phospholipids transfer assays as described earlier (12;35). Cells were extensively washed in ice-cold PBS. They were then homogenized in 1 ml of ice-cold homogenizing buffer (Tris-HCl, pH7.6; 1mM EGTA, and 1 mM MgCl2) containing protease inhibitor cocktail (Sigma) using a Dounce homogenizer. Microsomal fractions were obtained by centrifuging the homogenates in SW55 Ti rotor at 50,000 rpm for 1h at 10^{o} C. To measure activity in purified proteins, microsomal fractions were incubated with M2 agarose beads from Sigma and eluted with FLAG. Supernatants were used for MTP lipid transfer assays in triplicates as described (37-39). Fluorescence values containing no MTP were subtracted from samples containing MTP proteins and divided by the total fluorescence of vesicles to determine percentage of lipid transfer. Similarly phospholipid transfer assays were performed (36).

Immunoprecipitation and western blot analysis: Total protein expression studies were based on isolating cell homogenates and subsequently microsomal fractions. Equal amounts of protein were reduced with SDS-PAGE Laemmali buffer, boiled and loaded onto SDS-PAGE precast 4-20% gels. Proteins were separated, transferred onto nitrocellulose membrane and western blot analysis was developed for mouse anti-FLAG, rabbit anti-PDI and goat anti-GAPDH as loading control. For co-immunoprecipitation studies for MTP and PDI, COS7 stable cell lines transduced with different mutants were grown. Cells were extensively washed with PBS, scraped and collected. Cells were homogenized as described above and protein content in homogenates was determined by Bradford assay. Equal amount of total proteins adjusted to 150 mM NaCl, pH 7.4 for each condition were incubated with mouse-anti-FLAG antibody according to manufacturers recommendations at 4^{o} overnight. Protein A/G agarose beads from Santa Cruz were added next morning and further incubated for 2 h. Beads were pelleted,

washed and resuspended in 1X SDS-PAGE Laemmali buffer. Beads were spun down after boiling and supernatant containing the immunopreciptated protein were loaded onto 4-20% precast SDS-PAGE gel (BioRad), separated, transferred onto nitrocellulose membrane and probed for protein expression, FLAG and PDI.

Statistical analyses: One-way ANOVA was done to compare differences between groups. Comparisons were made between WT COS7 cells and all other mutations and significant differences were depicted by *, differences between hMTP and all other groups are shown as #. Other comparisons are shown with bars connecting the two groups that were compared. Data represents average of triplicate values of each condition and average of those values for respective n values per group ± SEM. Data are representative of two to three independent experiments.

RESULTS

Location of MTP missense mutations in MTP protein: The amino acid sequence and thermodynamically feasible secondary structure of MTP is shown in Fig 1. Different missense mutations have been identified to highlight their location in different regions of the protein (Fig 1). A shortened schematic diagram is shown in Fig 2A. Further their locations on the tertiary ribbon structure have been identified (Fig 2B). Additionally, the ball and stick model residues that are mutated in different ABL patients (Fig 2C).

Subcellular Location of MTP mutants: In order to determine the effect of different missense mutation(s), site-directed mutagenesis was employed to create individual missense mutations into the coding sequence of wild type MTP 97-kDa subunit in the retrovirus expression vector pMFGhMTP-FLAG. Stable cell lines of COS7 were generated expressing these proteins. COS7 hMTP-FLAG were used as positive control and COS7 cells as the negative control which lack endogenous apoB and MTP and are unable to support lipoprotein assembly.

To determine whether single amino acid substitutions affect subcellular location of the peptide, we performed indirect immunofluoroscence studies in different stable cell lines.

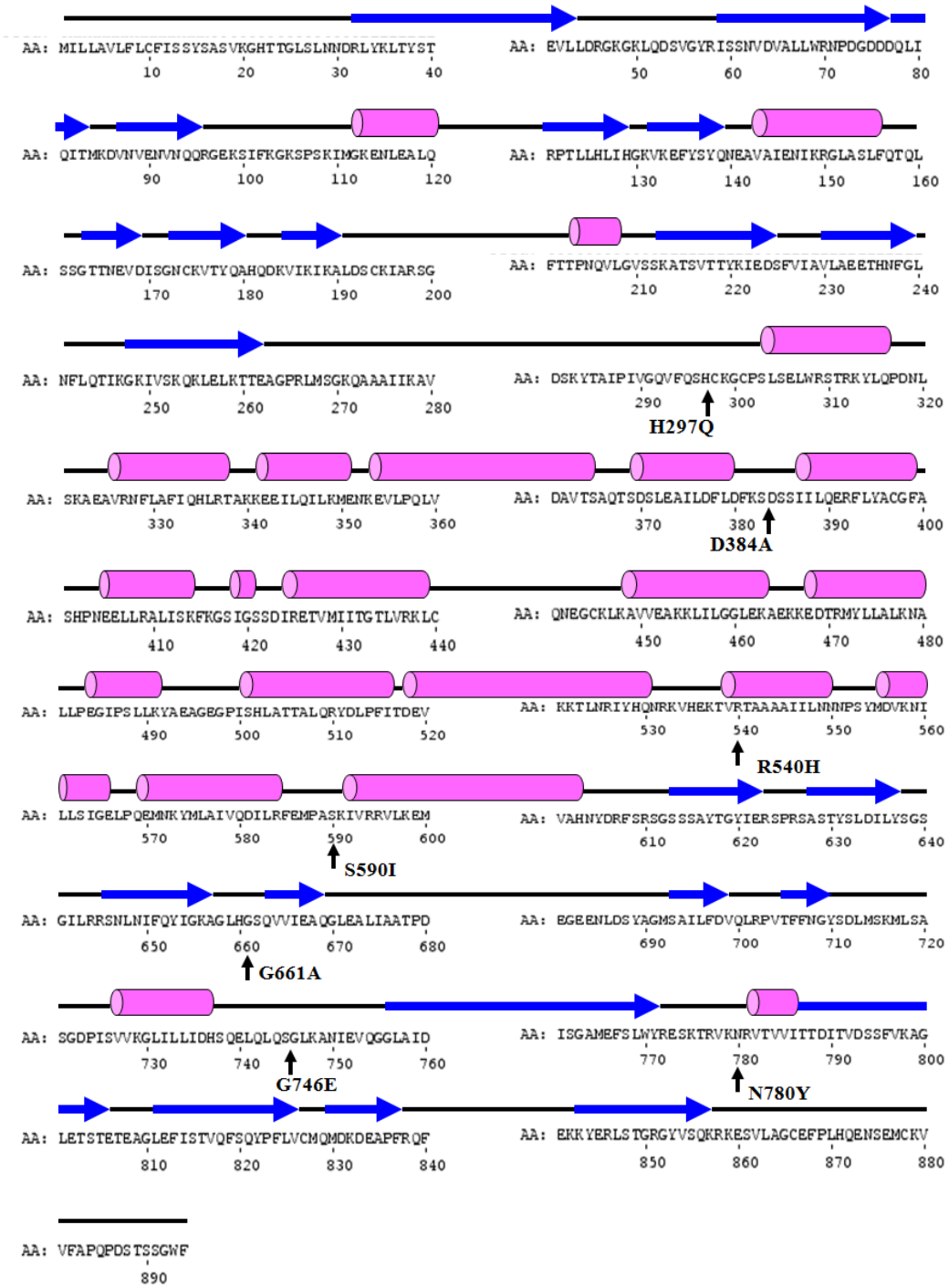

Figure 1. Amino acid alignment with secondary structure of MTP: Secondary structures of MTP in different domains drawn manually with predicted structure from PSIPRED and Hussain et al (JLR, 2003) throughout the entire length of 894 amino acid residues of the large subunit, 97kDa M subunit is shown. All the missense mutations and their precise location is marked with the single amino acid substitution with respect to their original residue. The top row depicts the secondary structure representing coiled region (straight black line), ß-sheet (Bold blue arrows) and α-helices (Pink barrels). The second row depicts amino acid residues in hMTP. The third row shows number of amino acid residues.

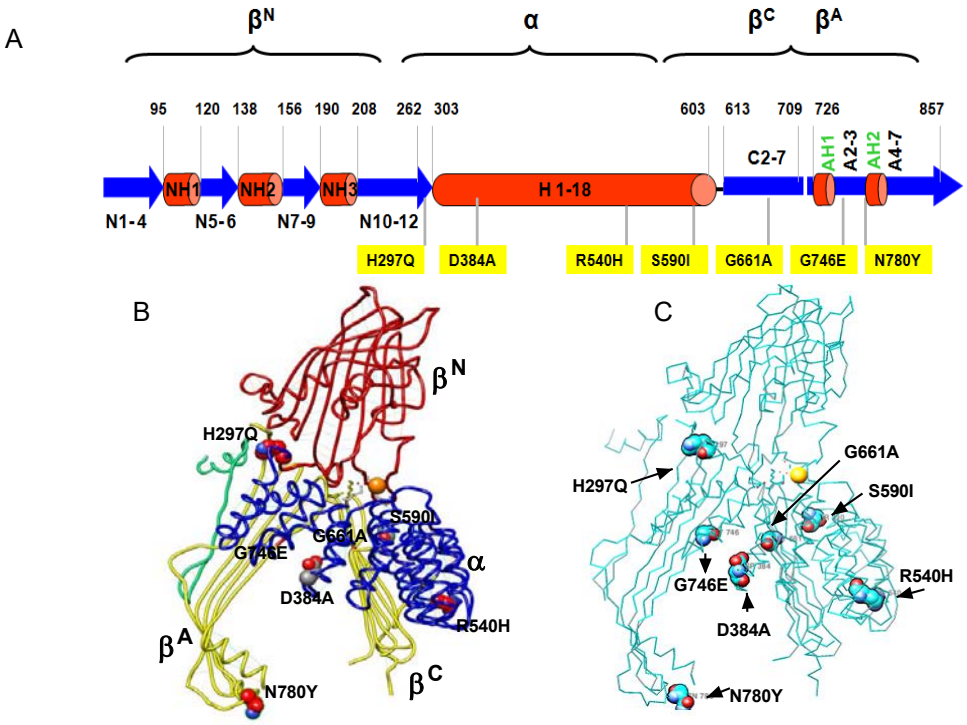

Figure 2. Location of different missense mutations in the secondary and tertiary structure of the MTP 97 kDa subunit: (A) Line diagram of secondary structure of MTP 97kDa subunit showing all the putative structural domains. N terminal ß-sheets (22-297), ß^{N}containing three helices depicted NH1, NH2 and NH3 and 12 ß-sheets (N1-12). The central α-helical domain (298-603) contains 18 helices, H1-18. The C-terminal end contains two ß-sheet domains (604-894), ß^{C}, C-sheet (C2-7) and ß^{A}, A-sheet (A2-7). Importantly, A-sheet also contains two short highly conserved a-helices, AH1 and AH2. Corresponding amino acid residues of the 894 residues are shown above the secondary structure. The missense mutations reported in different subjects are depicted below the secondary structure. These are mutants that were cloned and characterized in this study. (B) Tertiary structure of MTP built in chimera, color coded depicting different domains (ß^{N}-red, α-helix-blue, ß^{C}-yellow, ß^{A}-yellow) showing mutations with side chainshown as van der waal spheres, and (C) Ball and stick model of the tertiary structure showing mutations and atoms, nitrogen-blue, carbon-cyan, oxygen-red, c-alpha-cyan, dim gray.

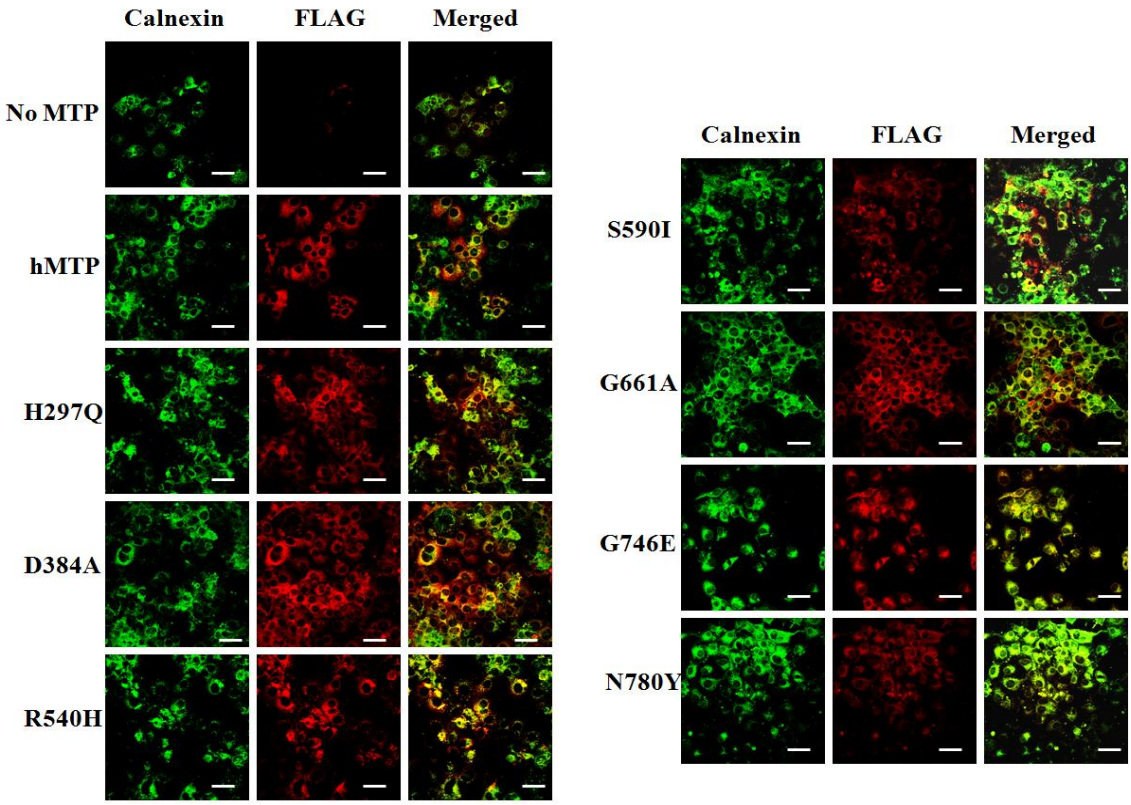

Figure 3. Subcellular localization of hMTP mutants: WT COS7 cells (no MTP) and COS7 stable cell lines expressing hMTP or different mutants were fixed, permeabilized, blocked and probed with primary antibodies for FLAG and Calnexin. Subsequently, they were probed with Alexa Fluor 488 (Calnexin) and Alexa Fluor 594 (FLAG). Immunofluoroscence was detected using Carl Zeiss confocal microscope. Images were overlapped to detect colocalization of both proteins. Several fields were scanned and duplicates for each sample were also processed and observed. Representative images are shown. The bar represents 50µm.

Calnexin (Alexa Fluor 488, Green), an ER marker was used to illustrate ER location. A reticular fluoroscent pattern (Alexa fluor 594, red) was detected in hMTP and all other mutants except WT COS7 that was not transfected with hMTP. Merging of the images showed overlap (yellow) demonstrating that mutant MTP proteins, reside in the ER similar to the intracellular localization in hMTP (Fig 3). This expression pattern is consistent with our MTP ortholog, human and Drosophila MTP study (Chapter II). This data demonstrate that single amino acid substitutions do not impact the subcellular location of the protein and they are still retained within ER.

MTP mutations and complexing with PDI: MTP 97 kDa, M subunit complexes with the smaller PDI subunit. This complex is required for lipid transfer activity, therefore, we performed immunofluoroscence and CO-IP analysis to study interactions between subunits in COS7 cells stably expressing different MTP mutants (Fig 3). Punctate staining was seen for both FLAG representing MTP protein (Red, Alexa Fluor 594) and PDI (Green, Alexa Fluor, 488) in hMTP and all other mutants. COS7 cells are shown as negative control in which compared to rest of the groups, very faint amount of FLAG staining reflecting background. Overlap of MTP-FLAG and PDI was evident as yellow in merged images in most cell lines. This was not seen in control cells and cells expressing R540H did not show extensive colocalization (Fig 4).

We next determined whether MTP mutants physically associate with PDI using co-immunopreciptation studies. Presence of both MTP and PDI were analyzed by western blotting after immunoprecipitation of FLAG proteins from COS7 cells expressing different mutants (Fig 5). FLAG expression representing the mutant MTP protein was recognized at ~100 kDa and PDI at ~55 kDa. No FLAG protein or PDI was detected in WT COS7 cells (No MTP). COS7 cells expressing D384A, G661A, H297Q, G746E, S590I and N780Y showed both FLAG and PDI (Fig 5A). On the contrary, the amounts of PDI precipitated with R540H were lower consistent with Mann et al in contrast to complete loss of PDI interaction reported by Rehberg (25). Densitometric quantifications to measure relative levels of MTP M subunit represented by FLAG tag and PDI subunit for each mutant are shown (Fig 5B). In most case, this ratio ranged between 0.7-1,

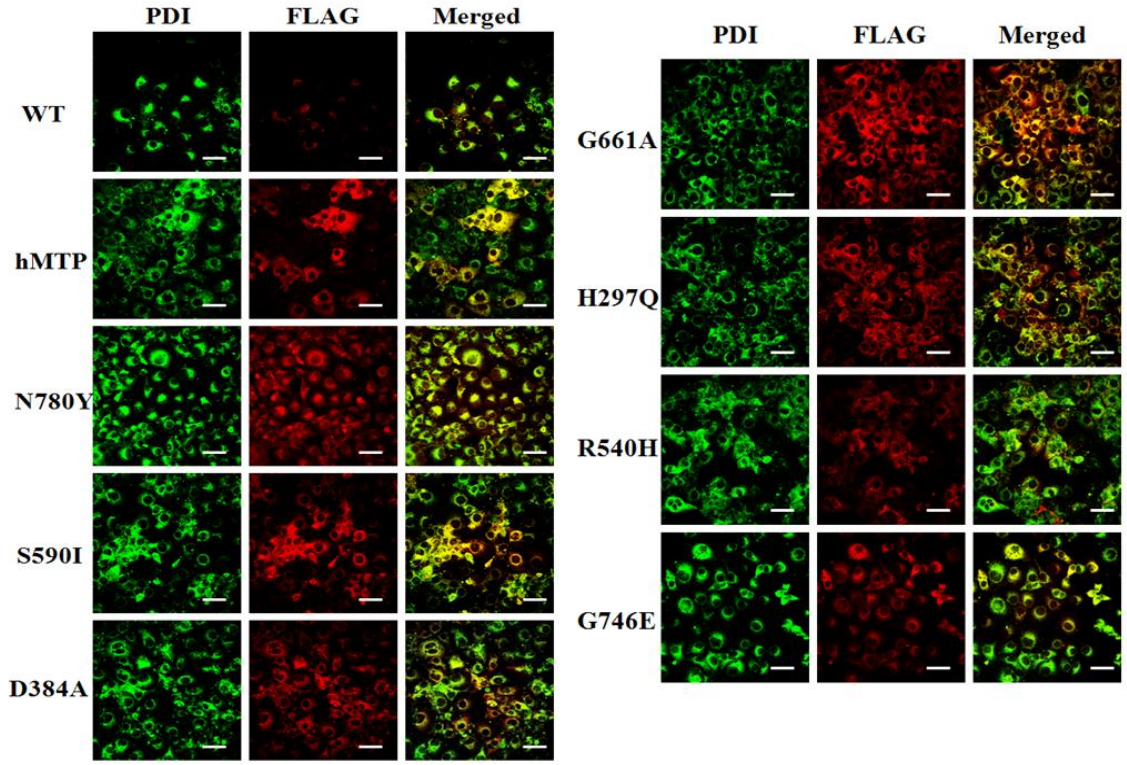

Figure 4. MTP and PDI interactions: WT showing COS7 cells (no MTP) and COS7 stable cell lines expressing hMTP or different mutants as shown in figure were processed as described in materials and methods. Subsequently, they were probed with Alexa Fluor 488 (PDI) and Alexa Fluor 594 (FLAG). Immunofluoroscence was detected using Carl Zeiss confocal microscope. Images were overlapped to detect colocalization of both proteins. Several fields were scanned and duplicates for each sample were also processed and observed. Representative images are shown. The bar represents 50μm.

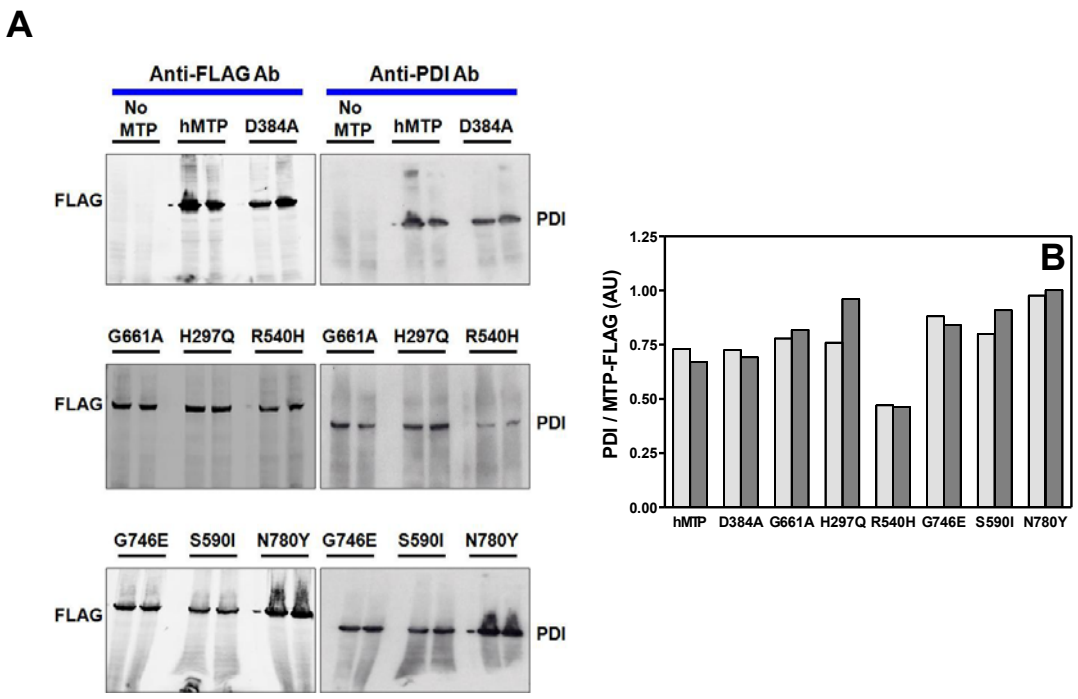

Figure 5. Co-immunoprecipitation studies showing MTP and PDI interactions: WT showing COS7 cells (no MTP) and COS7 stable cell lines expressing hMTP or different mutants as shown in figure were seeded and grown till confluency. **(A)** Microsomal fractions were prepared as described earlier, equal amounts of total proteins were immunoprecipitated using FLAG antibody as described in methods. Immunoprecipitated proteins were separated on 4-20% SDS-PAGE gels, transferred to a nitrocellulose membrane and western blot analysis were performed for FLAG tagged MTP proteins using M2 anti-FLAG antibody (left panel). Membranes were stripped and western blot analyses were performed for PDI proteins using rabbit anti-human PDI antibody (right panel). **(B)** Relative expression levels (ratio) of PDI and MTP (FLAG) for each lane (duplicates of each mutant) quantified from the gel obtained by co-immunoprecipitation of proteins are shown.

whereas this was significantly lower in R540H. We conclude that all mutants, except for R540H interact well with PDI.

Effect of different missense mutations on lipid transfer activities of MTP: The vertebrate MTP has higher affinity toward neutral lipid binding and their subsequent transfer. Purified proteins were used to assay the lipid transfer activities. Western blot analysis was performed in purified proteins (Fig 6A). The yield for G746E was lower which is consistently seen throughout all experiments. Since the 97 kDa, M subunit endows the lipid transfer activity to the protein complex; we tested triglyceride transfer as a measure of function (Fig 6B). No triglyceride transfer activity was obtained in microsomal fractions of N780Y, S590I, R540H and S590I expressing COS7 cells similar to WT COS7 cells (no MTP) (Fig 6B). Significant triglyceride transfer activity was

demonstrated by hMTP, which is the positive control for the experiment. D384A, G661A and H297Q were the only mutants that exhibited triglyceride transfer activity but these levels were significantly lower than hMTP, ~ 2 fold lower in D384A, ~1.5 fold in G661A and H297Q. Furthermore, D384A exhibited significantly less activity compared to both G661A and H297Q (Fig 6B). Besides triglyceride transfer, MTP also transfers phospholipids. Phospholipid transfer activity was exhibited by hMTP and D384A to almost similar levels. But no phospholipid transfer activity was demonstrated by N780Y, S590I, G661A, H297Q, R540H and G746E (Fig 6C). These studies identify two mutations; H297Q and G661A that exhibit no phospholipid transfer activity but retain low triglyceride transfer. D384A has lower triglyceride transfer activity but normal phospholipid transfer activity. N780Y, S590I, R540H were unable to transfer both triglycerides and phospholipids.

Ability of different missense mutations to support apoB secretion: Major function of MTP is to support apoB lipoprotein assembly and secretion. ApoB secretion was supported by hMTP, no secretion was seen in WT COS7 cells (no MTP) only. Mutants N780Y, S590I, R540H and G746E did not support any apoB secretions which were reflecting the results similar to the negative control, WT COS7 cells (no MTP). On the other hand, D384A, G661A and H297Q were able to support apoB secretion. However, the levels were significantly lower than hMTP. Among D384A, G661A and H297Q, D384A had highest amount of apoB secretion which seemed more concerted with hMTP than G661A and H297Q which were ~2 fold lower than D384A and ~2.5 fold lower than hMTP. No significant differences were found between G661A and H297Q (Fig 7A). We further wanted to investigate whether this effect still persists with higher lipid availability, whether the differences are abolished or they become more pronounced. To test this, cells were supplemented with oleic acid complexed with BSA and glycerol. ApoB secretion was enhanced with higher lipid availability, increase of ~40% in hMTP and also the other mutants compared to Fig 7A under normal lipid availability (Fig 7B). The differences within groups that were able to support apoB secretion, like hMTP, D384A, G661A and H297Q were still similar to that obtained under normal availability. No significant differences were found between G661A and H297Q, but they supported

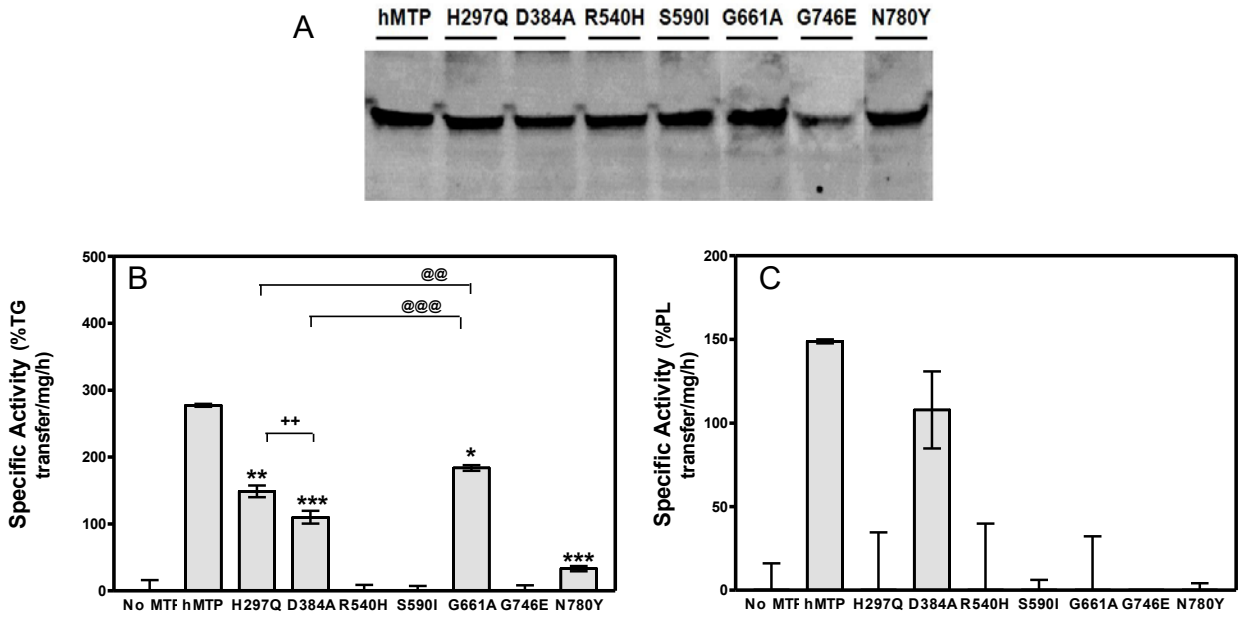

Figure 6. Lipid transfer activity of MTP mutants: Microsomal fractions from represented COS7 cells were analyzed for lipid transfer assays with equal amount of proteins. (A) Western blot analysis of the purified proteins are shown for the respective groups to depict FLAG expression. (B) Triglyceride transfer activity in purified proteins obtained from microsomal fractions isolated from represented stable cell lines expressing different mutations. WT represents COS7 cells only with no MTP, hMTP is used as positive control and all other single amino acid mutations in hMTP as shown. (C) Phospholipid transfer activity in purified proteins are shown as represented. Data are representative of two independent experiments. Mean ± SEM, n=3. One-way ANOVA was performed to compare differences between groups. ***p < 0. 001, **p < 0. 01, *p < 0. 05 and ns not significant.

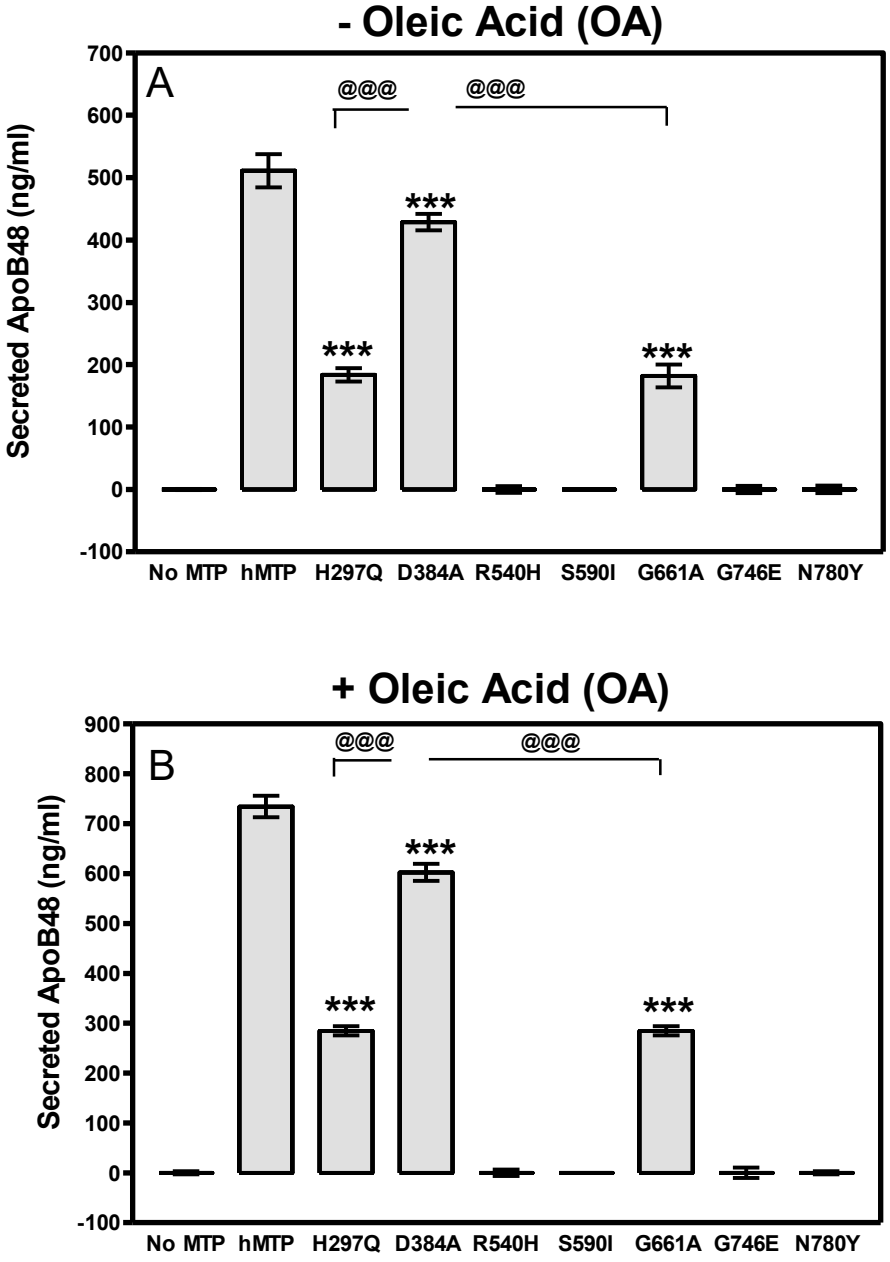

Figure 7. ApoB secretion studies supported by MTP mutants: ApoB expression plasmids were transiently transfected in COS7 stable cell lines expressing hMTP or MTP mutants as represented. Cells were seeded in equal density and treated equally for all the steps and processed as described in materials and methods. (A) ApoB secreted under normal conditions were studied by collecting media and detection of apoB content by indirect sandwich ELISA. (B) ApoB secretion supported by MTP mutants under higher lipid availability were studied by supplementing cells with 0.4 mM oleic acid complexed with 1.5% BSA and 1mM glycerol for the last 16 h of transfection. Secreted apoB were calculated from known standards and calculated as nanogram of apoB secreted per ml of media by each mutant. WT COS7 cells (no MTP) is shown as negative control where no apoB is detected in media. hMTP is shown as positive control to confirm feasibility and success of the experiment. Data represents, Mean ± SEM, n=3. One-way ANOVA was performed to compare differences between groups. ***p < 0. 001, **p < 0. 01, *p < 0. 05 .

apoB secretions which were significantly lower, ~2 fold lower than D384A and ~2.5 fold lower than hMTP. The other mutants N780Y, S590I, R540H and G746E were still unable to support apoB secretion.

DISCUSSION

Although it is physiologically established that MTP is indispensable for apoB lipoprotein assembly and secretion, gathering information about the structure and function of MTP has been difficult due to difficulties in obtaining a three-dimensional X-ray crytallographic structure. Demonstration of missense mutations in MTP 97 kDa subunit as the prime cause of ABL opened up a possible new approach to explore structure-function relationships. We found that D384A is a neutral MTP variant (25) with no impact on apoB secretion, ER retention, PDI interaction or lipid transfer activity (Table II). Hence, this is an innocuous polymorphism. This is surprising since a change from D to A is expected to cause significant changes. A reason for no effect on MTP activity could be due to its location on surface or in a region that is not critical for activity. Individuals with D384A homozygote have not been identified but it has been found to exist as compound heterozygote with other MTP mutations in ABL subjects (25;29). Therefore, physiologic effects of this mutation are unknown. Based on our studies, we predict that individuals with homozygous for D384A may not have any lipid abnormality.

All the mutants studied were retained in the ER (Table II). This is not surprising since these mutations were not in the signal peptide and did not affect PDI binding except R540H, which showed reduced PDI binding compared to hMTP. R540 is located within residues 520-598 that are responsible for maximum PDI binding (8) (Table III). These observations suggest that the sequence requirements for recognition of the 97 kDa subunit by PDI are quite rigid and that amino acid R540 within the helix 15 is critical for the association of this protein with PDI. Surface accessibility of represented residues using MSMS and based on score, accessibility was calculated (Table III). R540 is the only residue that was buried in contrast to all other residues, which were accessible from

the surface of the protein. In fact, it has been shown that R540 forms salt bridge that might be important in stabilizing the tertiary structure of the protein.

One of the most unique features of MTP is its lipid transfer activity. We measured both triglyceride and phospholipid transfer activities. S590I, R540H, G746E and N780Y lacked both triglyceride and phospholipid transfer activity. S590I, located in α-helical region (Table III), resulted in loss of lipid transfer activity. It has been suggested that I592 along with Y554, M555, and K558 is involved in PDI-binding as combined mutagenesis of Y554, M554, and I592 to alanine severely curtails PDI binding and lipid transfer activity by 86 and 78%, respectively (5). However, individual K591A, I592A, R594A, R595A, K598A, E599A mutations have very small effect on MTP activity (5). Thus, it was surprising to see that S590I resulted in complete loss of MTP activity. It has been previously pointed out that S590 could reside in a loop in between helices 16 and 17 of the central helical region and could form a hydrophobic patch at the edge of the helical domain along with F585, M587, and A589. We noticed that S590 could form a hydrogen bond with M587 and is capable of hydrophobic interactions with F585 and A589 forming a hydrophobic pocket between helices 17 and 18 (Fig 8). Further confirmation by mutagenesis of M587 and F585 to alanines in terms of MTP activity would indicate that this small hydrophobic pocket is critical for MTP activity. Corresponding lipovitellin (LV) residues F598, S593, N595 and E597 were also analyzed for the existence of such a hydrophobic loop (Fig 8). Previously, another mutation, R540H, in the helical region has been shown to be important for lipid transfer activity. R540 has been shown to be involved in internal salt bridge formation (25).

C-terminal β-sheet of MTP has been suggested to be crucial in its lipid transfer activity. Within this C-sheet two α-helices, AH1 (residues 725-736) and AH2 (residues 781-786) have been proposed to play an important role in lipid transfer (6). This is based on the observation that N780Y missense mutation results in loss of lipid transfer activity (6;28). Evident from this study that G746E missense mutation is also accompanied with loss of triglyceride and phospholipid transfer activities and causes ABL reinforces the idea that this sheet is critical for lipid transfer activity of MTP. In fact, sequences in the lipid

binding cavity are highly hydrophobic and most likely critical in creating a hydrophobic pocket for the assimilation of lipids (Fig 9). Since G746E is located in the deep pocket of

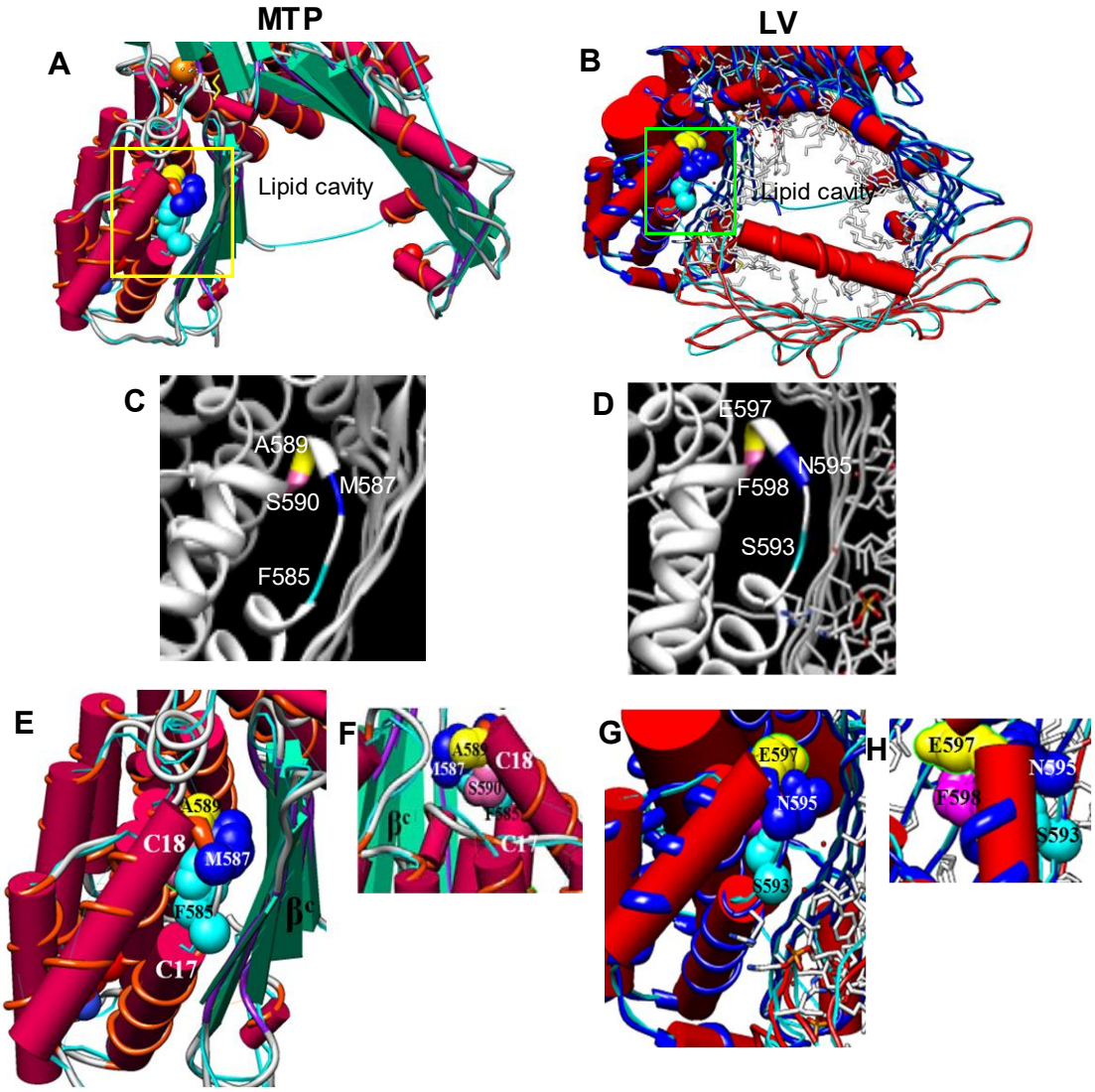

Figure 8. Molecular model showing expanded view of S590 in the central α-helices and adjacent β^c-sheets: Expanded view showing **(A)** location of S590, A589, M587, and F585 (spheres) in the central α-helical region (Barrels, red) and adjacent C-terminal β^c-sheet (green) flanking the central lipid binding cavity with C-terminal β^A-sheet (green) on the other side in MTP. **(B)** Corresponding homologous residues F598, E597, N595 and S593 (spheres) in lipovitellin (LV) shown in relation to the lipid binding cavity. The inset containing the residues with predicted hydrophobic pocket in (A) MTP and (B) LV is enlarged showing the ribbon diagram of the loop in between helices. **(C)** Predicted hydrophobic loop in MTP showing F585 (cyan), M587 (blue), A589 (yellow) and S590 (pink) with side chains. **(D)** Loop in LV showing corresponding residues in the same color, S593 (cyan), N595 (blue), E597 (yellow) and F598 (pink). **(E)** Detailed view showing residues F585, M587, A589 in the loop between helices C17 and C18. S590 is behind A589 which is shown from **(F)** a different angle. Adjacent β^c-sheet is also shown. The side chains of the residues are shown as van der waal spheres. S590 is predicted to form hydrogen bond with M587 with an estimated bond length of 2.996 A^o. F585 and S590 are within bonding distance to form hydrophobic interactions. **(G)** Homologous residues in LV in the same color showing S593 (cyan), N595 (blue), E597 (yellow) and **(H)** F598 at the back (pink) shown from a different angle. F598 and N595 have an estimated bond length of 3.042 A^o capable of hydrophobic interactions. S593, N595 and E597 are within bonding distance to form hydrogen bonds.

MTP lipid transfer domain, we predict that introduction of a charged residue in this hydrophobic pocket might severely affect neutral lipid interaction with this domain for lipid transfer activity. Thus, conversion of glycine to glutamic acid is expected to abolish the hydrophobic environment and create a hydrophilic environment untenable for the assimilation of hydrophobic lipids.

On the other hand, H297Q and G661A transferred lower amount of triglycerides compared to hMTP but were lacking phospholipid transfer activity. H297Q probably loses the ability to transfer phospholipids unlike hMTP because the second predicted phospholipid binding site is in the N-terminal β-sheet. On the other hand, glycine at 661 is highly conserved in vertebrates and G661 is located within 613-825 residues of MTP that are predicted to form the lipid-binding cavity (Table III).

The lipid transfer activity of MTP aids in performing the function of apoB assembly and secretion. R540H, S590I, G746E and N780Y were unable to support apoB secretion. The loss of apoB secretion results from lack of both triglyceride and phospholipid transfer activities in these mutants. These studies demonstrate that loss of both lipid transfer activities abolishes MTP's ability to support apoB secretion. On the other hand, loss of only phospholipid transfer activity and retention of partial triglyceride transfer activity in H297Q and G661A supports apoB secretion to a lesser extent than hMTP. These studies suggest that some missense mutations reduce but do not completely abolish lipid transfer activity of MTP resulting in a "mild" ABL phenotype that may resemble symptoms of heterozygous FHBL due to partial loss of function.

Furthermore, this study provides biochemical explanation for novel missense mutations, S590I, G746E, H297Q, G661A, D384A and expands the reasons for ABL in N780Y and R540H mutations. Apart from the absence of triglyceride transfer activity, our studies show that N780Y and R540H do not transfer phospholipids. We also found that S590I and G746E also lack both triglyceride and phospholipid transfer activities. Another novel observation made is that lack of phospholipid transfer activity and diminished

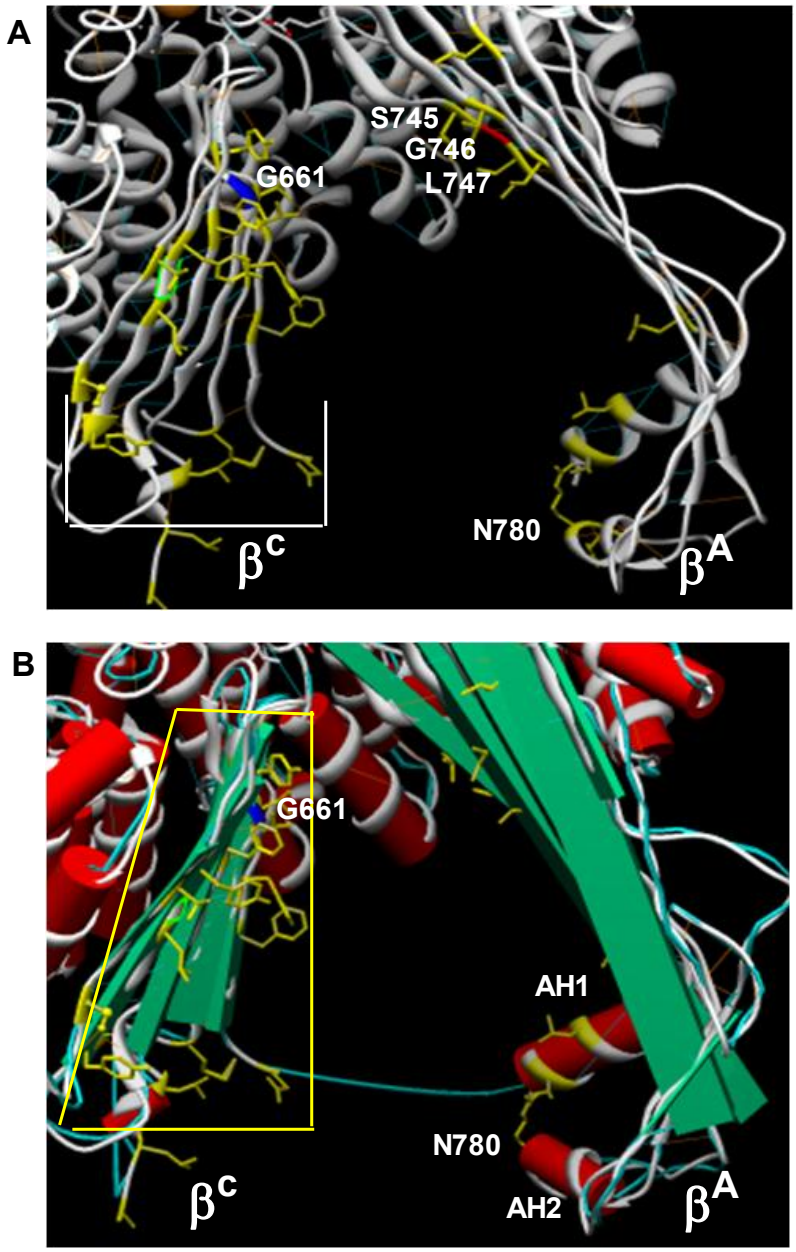

Figure 9. Hydrophobic lipid binding cavity of MTP: Ribbon diagram of lipid binding cavity of MTP flanked with C-terminal β^C-sheet and β^A-sheet is shown. **(A)** Hydrophobic residues (yellow) in β^C-sheet and β^A-sheet are shown whose side chains are exposed in the cavity which could potentially interact with neutral lipids influencing lipid binding capacity. G661 (blue) in β^C-sheet and G746 (red) in β^A-sheet flanked with hydrophobic residues S745 and L747 are also shown. **(B)** β^C-sheet (green planks) with exposed side chains of hydrophobic residues are shown (yellow inset). G661 (blue) is also exposed but has no side chains. G746 is not exposed to the surface due to lack of side chain but substitution to E (Glutamic acid) would introduce a charged residue. AH1 and AH2 helices (red barrel) in β^A-sheet are shown with hydrophobic residues with side chains exposed. N780 forms hydrogen bond with T783 with an estimated bond length of 2.901 A^o.

TABLE II. Summary of the characterizations of the mutations with the substituted residues is shown with respect to subcellular location, PDI binding, lipid transfer activities of the purified protein and apoB secretion studies reflective of both normal and higher lipid availability. Lipid transfer activity and apoB secretion supported by mutants are shown by +, +++ represents levels equal to hMTP (wild type counterpart), ++ reflects levels lower than hMTP, + represents further lower levels.

			Lipid transfer		
Mutations	ER localization	PDI binding	TG	PL	ApoB secretion
H297Q	Yes	Yes	++	No	+
D384A	Yes	Yes	+++	+++	+++
R540H	Yes	Partial	No	No	No
S590I	Yes	Yes	No	No	No
G661A	Yes	Yes	++	No	+
G746E	Yes	Yes	No	No	No
N780Y	Yes	Yes	No	No	No

triglyceride transfer activity in H297Q and G661A mutations leading to partial secretion of apoB lipoproteins. Interestingly the patient, a compound heterozygote for these mutations, a neutral variant, D384A (29), a common polymorphism, H297Q (30) and G661A, a rare non-conservative amino acid substitution in MTP was diagnosed as hypobetalipoproteinemia and not ABL (34). Therefore, the hypobetalipoproteinemia phenotype could be due to inefficient assembly and secretion of apoB-lipoproteins. It is likely that screening for mutations in MTP besides apoB could potentially explain hypobetalipoproteinemia in subjects that do not carry mutations in apoB. We have previously described that absence of triglyceride transfer activity in *Drosophila* MTP also results in partial assembly of apoB-lipoproteins. Hence, both triglyceride and phospholipid transfer activity of MTP are required for optimum apoB-lipoprotein assembly.

TABLE III. The mutations in ABL subjects that were characterized in this study are tabulated showing their respective location in regards to the tertiary structural domain, the corresponding functional domain and exact location in the secondary structure with respect to tertiary folded protein. Surface accessibility was calculated using MSMS to depict surface exposed residue or buried residue.

	Domains			
Mutations	Structural	Functional	Secondary structure	Surface accessibility
H297Q	Between N-terminal β^N and central α-helical domain	PDI binding	Between sheet N12 (247–262) of $\beta^N$and helix H1(303-316) of central α-helical domain	yes
D384A	Central α-helical domain	PDI binding	Between helices H5(369-380) and H6 (386-398) of central α-helical domain	yes
R540H	Central α-helical domain	Maximum PDI binding & ApoB binding	Within helix, H15 (537-550) of central α-helical domain	no
S590I	Central α-helical domain	Maximum PDI binding & ApoB binding	Between helices H17(569-584) and H18 (591-603) of central α-helical domain	serine -OH exposed
G661A	C-terminal β^C sheet domain	Lipid binding domain	Between C4(645-656) and C5(662-665) sheet of C-terminal β^C sheet domain	yes
G746E	C-terminal β^A sheet domain	Lipid binding domain	Within A2 (739-751) sheet of of C-terminal β^A sheet domain	yes
N780Y	C-terminal β^A sheet domain	Lipid binding domain	Between A3 (755-771) sheet and AH2 helix (781-786) of C-terminal β^A sheet domain	yes

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CHAPTER IV

DISCUSSION

ApoB, MTP and lipids are essential for apoB-lipoprotein assembly and secretion. Deficiency in any of these three factors affects lipoprotein production. The main function of apoB-lipoproteins is to deliver fat to other tissues for normal physiologic activities; but their excess accumulation in the plasma induces atherosclerosis and an array of metabolic complications such as Type 2 diabetes and obesity (1;2). It is evident that approaches to reduce apoB lipoprotein production would help treat dyslipidemias but the molecular mechanisms of apoB-lipoprotein assembly are not fully elucidated. It is known that microsomal triglyceride transfer protein (MTP) is an essential chaperone for their assembly. Our evolutionary studies indicate that MTP evolved as a phospholipid transfer protein and acquired triglyceride transfer activity during a transition from invertebrate to vertebrate. Our group has shown that *Drosophila* MTP (dMTP) transfers phospholipids whereas human ortholog (hMTP) transfers both phospholipids and triglycerides (3;4) but all these studies were performed in reconstituted cell system, COS7, monkey kidney cell line. The importance and exact role of these lipid transfer activities in apoB-lipoprotein assembly is not known. Availability of these two orthologs provides us with a unique opportunity to delineate *in vivo* role of different lipid transfer activities of MTP in lipoprotein assembly.

The goal of this study was to obtain structure and function of MTP to elucidate the role of different lipid transfer activities of MTP in the biosynthesis of apoB-lipoproteins. We used two independent approaches. In the first approach, we used evolutionarily diverse MTP orthologs that differ in their lipid transfer properties. Second, we used missense mutations reported in human that display different disease phenotype to elucidate the importance of different amino acids, α-helices and β-sheets in the MTP molecule that are crucial for different lipid transfer properties.

Phospholipid transfer activity maintains whole body lipid homeostsis

We studied effects of phospholipid and both phospholipid and triglyceride transfer activities on whole body lipid homeostasis, under normal lipid availability reflecting steady state conditions, higher lipid availability reflecting pathological situation and dose dependent changes. We found that rescue of MTP phospholipid transfer activity in MTP deficient livers by dMTP partially restores

plasma triglycerides and also lowers hepatic steatosis associated with hepatic MTP gene deletion in the L-MTP$^{-/-}$ mice compared to the wild type counterpart. In contrast, hMTP rescues both phospholipid and triglyceride transfer activity in the liver leading to partial restoration of plasma triglycerides compared to the wild type mice as well as lowering of hepatic steatosis associated with hepatic MTP gene deletion. Though both orthologs partially restored plasma lipids compared to the wild type mice but overall plasma levels rescued by dMTP were lower than hMTP. Interestingly phospholipid transfer activity alone lowered hepatic triglycerides associated with MTP gene deletion similar to hMTP inspite of the absence of triglyceride transfer activity in dMTP. The physiological effect obtained with dMTP were observed in both chow and western-diet fed mice indicating that this activity is sufficient to handle dietary fat challenges in avoiding liver steatosis and lowering plasma lipids.

Next the lipoproteins assembled and secreted by hMTP or dMTP were extensively characterized to understand the distribution of different lipid components in plasma VLDL and HDL. In addition, apolipoprotein composition of newly synthesized VLDL and HDL lipoproteins synthesized by the liver was also studied. Distribution of lipids were analyzed by gel-filtration and hMTP showed formation of triglyceride-rich VLDL lipoprotein. Cholesterol and phospholipids were distributed in both VLDL and HDL but their levels increased in VLDL not in HDL with higher expression of hMTP. This suggested that VLDL are the primary lipoproteins that are assembled and secreted by hMTP. Similarly dMTP was also able to increase triglycerides in VLDL but the levels were lower at all times compared to hMTP indicating that it makes a triglyceride-poor VLDL lipoprotein. Cholesterol and phospholipids were present in both VLDL and HDL but increases in cholesterol and phospholipids in VLDL were lower than hMTP. Interestingly, phospholipid distribution in dMTP showed higher amounts of HDL, which exhibited dose-dependent increase indicating for the presence of phospholipid-rich HDL particles.

We further characterized these VLDL and also the new HDL particle with electron microscopy following negative staining of lipoproteins. Comparison of VLDL lipoproteins synthesized by each ortholog showed that hMTP synthesizes a larger VLDL particle compared to dMTP. As a next step, newly synthesized lipoproteins from liver were analyzed by *in vivo* metabolic labeling studies.

hMTP supported assembly and secretion of both apoB100 and apoB48 containing VLDL lipoproteins from liver. dMTP also supported assembly and secretion of both apoB100 and apoB48 containing VLDL from liver but these levels were lower than hMTP. Biosynthesis studies showed the presence of apoB48-HDL. Therefore, characterization of the HDL following removal of apoAI, which is the abundant apolipoprotein in HDL showed apoAI-free HDL exclusively in dMTP plasma. Here we show for the first time that phospholipid transfer activity of MTP supports hepatic biogenesis of apoB100 containing VLDL lipoprotein. Besides, phospholipid transfer activity also promotes hepatic synthesis of apoB48 containing HDL in L-MTP$^{-/-}$ mice.

The possible reasons of higher plasma triglycerides could result from triglyceride content per particle that would affect the composition of the lipoprotein secreted or number of VLDL secreted assessed by apoB quantification since one apoB molecule is present per VLDL particle. We show that triglyceride transfer activity promotes higher hepatic triglyceride secretion rates and also increase in hepatic apoB100 and apoB48 secretion compared to phospholipid transfer activity alone in dMTP. Thus differences in lipoprotein biosynthesis between hMTP and dMTP are due to both composition of secreted particles supported by triglyceride secretion rate study and number of secreted VLDL particles shown by hepatic apoB synthesis rate.

Thus, these studies demonstrate that overall whole body lipid homeostasis is maintained by phospholipid transfer activity by supporting apoB-containing lipoprotein biosynthesis in the mouse liver. L-MTP$^{-/-}$ mice expressing dMTP have low plasma lipid levels, show no evidence of hepatosteatosis, and synthesize phospholipid-rich lipoproteins compared to mice that express hMTP or wild type mice with normal endogenous mouse MTP in liver. These observations indicate that phospholipid transfer activity of dMTP is sufficient for *in vivo* lipoprotein biosynthesis as well as in ameliorating steatosis associated with MTP deficiency while maintaining low plasma lipid levels. Thus phospholipid transfer activity is sufficient for apoB assembly and secretion under both low-fat (chow-diet) and high-fat (western-diet) conditions.

Evolutionary advantages of acquiring triglyceride transfer activity

Organisms use different lipoproteins for lipid transport. Nematodes and insect secrete VTG and lipophorins whereas vertebrates utilize apoB lipoproteins to transport lipids. Although apoB, VTG and apolipophorin share similar sequence but they are quite distinct from each other in the amount and type of lipid they transport (5-7). VTG and apolipophorins are phospholipid-rich lipoproteins and carry smaller amounts of neutral lipids. In contrast, apoB lipoproteins are neutral-lipid-rich particles and transport more than 1000 lipid molecules. ApoB emerged in fish and birds and was retained in mammals as primary mode of lipid transport. Studies from our group suggest that acquisition of triglyceride transfer by MTP and emergence of apoB as primary carrier of extracellular lipids co-evolved (8) to provide an efficient system to transport greater amounts of dietary neutral lipids. This also enhances endogenous lipid transport process. Within vertebrates fish, amphibians, and birds displayed 27%, 40% and 100% triglyceride transfer compared to mammals suggesting that this activity was not only acquired during evolution but also retained and became more efficient with corresponding increase in complexity of vertebrates (8). Percent identity between regions within structural domains of MTP protein shows decrease in identity between full length proteins and individual domains progressively from humans to other mammals, amphibians, fish, insects and nematodes (8). Thus evolutionary trend towards preferential conservation of α- helices and C terminal β-sheet domains in vertebrates but not in insect and nematode MTPs suggests that these regions might be critical for robust triglyceride transfer activity associated with vertebrate MTP.

The present comparative study using dMTP as an invertebrate ortholog and hMTP as a vertebrate ortholog with distinct lipid transfer activities provides important insights into evolutionary gains made by MTP during transition from invertebrates to vertebrates. The absence of neutral lipid transfer activity in dMTP ortholog does not prevent it from initial lipidation of nascent apoB to form primordial core within ER, however prevents bulk movement of lipids in secretory pathway. Hence the basic tenets required for apoB lipoprotein assembly were present prior to the emergence of apoB as primary transporter of lipids. The acquisition of triglyceride transfer by vertebrate enhanced efficiency of packaging neutral lipids in to apoB lipoproteins reflected by higher hepatic triglyceride secretion rates and hepatic apoB biosynthesis in hMTP compared to dMTP.

To answer how acquisition of triglyceride transfer activity helped in efficient packaging of lipids; mechanisms pertaining to different hepatic triglyceride secretion rates were dissected by subcellular partitioning studies of newly synthesized lipids after metabolic labeling of primary hepatocytes. Association of newly synthesized lipids with apoB was increased in both microsomal membrane and lumen by both hMTP and dMTP without any differences in lipid synthesis between groups. The association of triglycerides with apoB was higher in hMTP compared to dMTP. No such differences were found in the triglyceride pool not associated with apoB. These studies indicate increased association of triglycerides with apoB in the microsomal membrane in the presence of triglyceride transfer activity of hMTP reflecting apoB-associated triglyceride pool in nucleation sites. Subsequent accretion of these into the lumen reflects the secretion competent apoB-lipoprotein pools, which were higher in hMTP compared to dMTP.

Next, mechanisms leading to differences in hepatic apoB biosynthesis contributed by each ortholog were studied. ApoB secretion is regulated by proteasomal mediated presecretory degradation if not lipidated as it is co-translationally translocated across the endoplasmic reticulum membrane. Continuous pulse studies were performed in primary hepatocytes in presence and absence of proteasomal degradation inhibitor to assess co-translational degradation. Phospholipid transfer activity alone in dMTP showed more co-translational proteasomal degradation of apoB in contrast to hMTP which were abolished in the presence of proteasomal degradation inhibitor. These studies suggest that phospholipid transfer activity is less competent or efficient in lipidating nascent apoB as it is co-translationally translocated across the membrane thereby more apoB undergoes presecretory proteasomal degradation in contrast to hMTP. This explains the higher number of secretion competent apoB particles obtained by hepatic apoB biosynthesis studies in the presence of triglyceride transfer activity unlike phospholipid transfer activity alone. No differences in post-translational proteasomal degradation of apoB between hMTP and dMTP were seen. These studies indicate that apoB that is lipidated when it is co-translationally translocated, escapes presecretory proteasomal degradation and gains secretion competency. Post-translational degradation of apoB is not influenced by the presence or absence of triglyceride transfer activity.

These studies indicate that acquisition of triglyceride transfer activity aided in creating triglyceride enriched nucleation sites on the ER membrane implying more efficacious lipidation of nascent

apoB, therefore less apoB degradation leading to assembly of larger and more number of apoB-lipoproteins. Another advantage would be facilitating lipidation of nascent VLDL particles by enhancing subcellular partitioning of newly synthesized neutral lipids in ER membrane and lumen subsequently secreting triglyceride-rich particles unlike phospholipid transfer activity of MTP. These functional advantages resulting from the neutral lipid transfer activity gained by MTP with the emergence of vertebrates supports the basic differences in lipoprotein assembly in insects and humans primarily occurring in the secretory pathway in the latter. Perhaps this feature of neutral lipid accretion in the secretory pathway might have conferred the ability to efficiently handle bulk lipid load in a complex multicellular vetebrate system as opposed to invertebrates.

Implications in lipoprotein biosynthesis

Our studies further enhance understanding of the early steps in the biosynthesis of apoB-lipoproteins and the advantages gained by the acquisition of triglyceride transfer activity by MTP during evolution from invertebrates to vertebrates. The formation and secretion of a phospholipid-rich HDL-size particle predicts that it probably is the earliest secretion competent apoB48-lipoproteins synthesized by the liver. Furthermore, the phospholipid transfer activity is sufficient to assist in the formation of apoB100-containing VLDL particles. On the contrary, the triglyceride transfer activity of MTP helps in the assembly and secretion of triglyceride-rich lipoproteins. Further understanding of the molecular mechanisms pertaining to these differences with respect to different lipid transfer activity in the secretory pathway were provided by subcellular and apoB degradation studies.

Subcellular studies demonstrated that triglyceride transfer activity is not needed for the membrane triglyceride content, but it is required for the transfer of triglycerides into the lumen of the ER that is found associated with or without apoB-particles. Regulation of apoB degradation showed that lipid availability is not limiting for the assembly of nascent apoB in hepatocytes under normal conditions. Triglyceride transfer activity is important for the transfer of lipids onto nascent apoB to avoid proteasomal degradation. However, the intriguing information is that phospholipidation of nascent apoB by MTP inhibits proteasomal degradation to some extent and promotes lipoprotein assembly. These findings establish the concept of lower number of precursor primordial apoB particles formed

by phospholipid transfer activity result from inefficient lipidation of apoB compared to triglyceride transfer activity of MTP. This further explains the higher amounts of apoB-lipoproteins secreted by hMTP owing to efficient assembly of apoB-lipoproteins by the triglyceride transfer that involves prevention of degradation of nascent apoB and its enrichment with triglyceride in microsomal compartment.

It has been suggested that apoB lipoprotein assembly may occur in two steps. Based on this data, first step may involve phospholipidation of nascent apoB by MTP followed by budding of primordial particles from ER membrane. Lack of neutral lipid transfer activity affects both number and composition (size defined by neutral lipid content) of lipoproteins. However, dMTP VLDL apoB-particles acquire small amounts of triglyceride in the absence of neutral lipid transfer activity. It is known that neutral lipids have low solubility within phospholipid bilayers and tend to diffuse rapidly within the plane of the membrane. It is possible that after MTP dependent phospholipidation initiating domain of apoB inserts into the membrane and functions to trap neutral lipids in the form of small droplet between the bilayer. The other possibility could be spontaneous diffusion of triglycerides within the membrane due to a triglyceride synthesis site in the vicinity of lipid pockets where apoB phospholipidation has initiated with the co-translational translocation. At a critical stage apoB is released from the ER in the form of a nascent triglyceride core-containing emulsion particle.

Therapeutic implications in the treatment of cardiovascular disorders

Based on the observations that dMTP expressing mice have low plasma and normal hepatic lipids, we hypothesize that phospholipid transfer activity is sufficient for normal hepatic lipid homeostasis and that the triglyceride transfer activity is dispensable. It might be possible to inhibit triglyceride to achieve lower plasma lipid levels. Compounds that inhibit triglyceride transfer activity of MTP could also be used to avoid accumulation of triglyceride and free cholesterol in the liver. In contrast, studies using human mutations, H297Q and G661A revealed that absence of phospholipid transfer activity could also result in low plasma lipids. Therefore, inhibition of either of the lipid transfer activities could be beneficial in lowering plasma lipids. There is significant evolutionary, kinetic,

and structural evidence that phospholipid transfer activity might be separate from triglyceride transfer activity.

Studies by Atzel and Wetterau suggest that mammalian MTP has two sites for the transfer of lipids; one high affinity site that transfers triglycerides as well as phospholipids and the other low affinity site that only transfers phospholipids (9-11). The walls of the lipid binding cavity in MTP are presumed to be formed by the A and C sheets of C- terminal β-sheet domain that are located at the entrance of the lipid binding cavity for membrane binding similar to viral fusion peptides and lipid transfer activity of MTP (12;13). However the functional importance of helices A and B were predicted with respect to triglycerides interaction with Helix A and subsequently their acquisition by B but nothing was concluded with relevance to phospholipid acquisition and transfer from this binding site. Furthermore, sequence analysis revealed that Helix A (AH1) of Drosophila MTP is divergent (4;13) and lacks the amphipathic character required for the membrane binding which perhaps confers differential lipid transfer activities with respect to individual lipid transfer activity. In separate studies, structural analyses based on homology of MTP with LV predict that there are phospholipid binding sites in the molecule that are different from the major neutral lipid transfer domains (14). Therefore, it is likely that small molecules can be identified that significantly inhibit triglyceride transfer activity while only partially lowering phospholipid transfer activity of MTP. Alternatively, compounds can be found that inhibit phospholipid transfer activity with partial loss of triglyceride transfer activity. These compounds might be more useful in treating hyperlipidemia than compounds that globally inhibit all the lipid transfer activities of MTP.

At present, potent antagonists that inhibit both the triglycerides and phospholipid transfer activities of MTP and decrease apoB secretion have been identified (15-17). Most of these compounds have adverse effects, primarily related to hepatic lipid accumulation. Based on these data here, it might be useful to identify compounds that selectively inhibit either of the two lipid transfer activities. Compounds that inhibit only phospholipid transfer within first step would reduce the number of apoB precursor competent to acquire neutral lipids without perturbing MTP-dependent flux of triglyceride into the secretory pathway. Such classes of inhibitors may reduce total number of apoB lipoproteins secreted from the liver with minimal disruption in net export of triglyceride. Compounds that inhibit triglyceride transfer activity may allow synthesis and secretion of

primordial particles with small amounts of neutral lipids and help avoid toxicities found associated with MTP antagonists that globally inhibit MTP. It is unlikely that a compound can be found that significantly inhibits triglyceride transfer activity and has no effect on phospholipid transfer activity because triglyceride transfer domain may also transfer some phospholipids. Therefore, consideration should be given for the identification of compounds that significantly inhibit triglyceride transfer activity and only partially inhibit phospholipid transfer activity.

Conclusion

These studies provide a strong foundation for future structural and functional studies to identify specific residues that could have been responsible for acquiring triglyceride activity with MTP evolution during its transition from invertebrates to vertebrates. It is important because at the current moment not much is known about the structure and function (with respect to lipid transfer) of MTP due to poor homology between the invertebrate and vertebrate orthologs. Various biochemical and molecular approaches used in this study reveal novel molecular mechanisms by which different lipid transfer activities of MTP cooperate in the biosynthesis of nascent lipoproteins in context of molecular events and changes in lipid composition occurring in the microsomal membrane and lumen, which are the primary site of lipoprotein assembly. Evidences were gathered about the importance of triglyceride transfer activity in the mobilization of bulk lipids. Recognition of different roles for phospholipid and triglyceride transfer activities suggested that these activities can be separated and targeted for differential inhibition. Domain specific antagonists rather than global inhibition of MTP could help identify better therapeutic approaches to combat hyperlipidemia, obesity and atherosclerosis.

In summary my studies show that triglyceride and phospholipid transfer activities are required for optimum synthesis and robust mobilization of lipids. Thus presence of these activities might have provided significant advantage in assembly as well as mobilizing lipids. However, in this era of energy abundance, these activities may not be necessary. Hence, inhibiting any one of these activities might be beneficial in combating hyperlipidemia and atherosclerosis and other metabolic disorders.

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APPENDIX

- **Microsomal triglyceride transfer protein in plasma and cellular lipid metabolism.** Hussain MM, Rava P, Pan X, Dai K, Dougan SK, Iqbal J, Lazare F, **Khatun I.** *Curr. Opin. Lipidol. 19:3. 2008.*

  Contributions: Writing and editing the manuscript.

- **NR2F1 and IRE1beta suppress microsomal triglyceride transfer protein expression and lipoprotein assembly in undifferentiated intestinal epithelial cells.** Dai K, **Khatun I**, and Hussain MM. *Arterio. Throm. Vasc. Biol.* 30:568 2010.

  Contributions: The adenoviruses used for the study were constructed by IK. IK helped KD in performing the experiment for Fig 1H.

- **Acute suppression of apoB secretion by insulin occurs independently of MTP.** Sparks JD, Chamberlain J, O'Dell C, **Khatun I**, Hussain MM, Sparks, CE. *BBRC.* 2011

  Contributions: Constructed hMTP encoding recombinant adenovirus. Performed experiment and contributed to Fig 1B in the paper. Critical reading of the manuscript