CRITICAL FACTORS INVOLVED IN
INTESTINAL CHYLOMICRON ASSEMBLY

by

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ABSTRACT

Assembly of intestinal chylomicron particles (lipid-protein complexes) is the fundamental mechanism by which we absorb dietary fat. Two intestinal lipid transporters, Cluster of Differentiation 36 (CD36) and fatty acid-binding protein 1 (FABP1), have been shown to play a role in lipid absorption, however, it remains unclear how knockdown of these proteins leads to aberrant intestinal chylomicron secretion. In an enterocyte-like cell culture model, Caco-2 cells, we hypothesized that knockdown of CD36 or FABP1 using short-hairpin RNA interference techniques would impair triacylglycerol (TG) and apolipoprotein B (apoB) secretion. Surprisingly, knockdown of these lipid transporters lead to an increase in TG and apoB secretion that was associated with an increase in fatty acid synthase and fatty acid transport protein 4 (FATP4) protein levels. De novo fatty acid synthesis was slightly increased in CD36-, but not FABP1-knockdown Caco-2 cells. This study highlights the importance of fatty acid targeting in regulating chylomicron production.

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LIST OF ABBREVIATIONS

ApoB Apolipoprotein B
APS Ammonium persulfate
BSA Bovine serum albumin
β-Me β-Mercaptoethanol
DTT Dithiothreitol
DGAT Diacylglycerol acyltransferase
ECL Enhanced chemiluminescence
EDTA Ethylenediaminetetraacetic acid
ER Endoplasmic reticulum
FABP Fatty acid binding protein
FFA Free fatty acid
HDL High density lipoprotein
HRP Horseradish peroxidase
KBr Potassium bromide
mRNA messenger RNA
MGAT Monoacylglycerol acyltransferase
MTP Microsomal triglyceride transfer protein
PBS Phosphate-buffered saline
PI Protease inhibitor cocktail
PPAR Peroxisome proliferators activated receptor
PVDF Polyvinylidene fluoride
SDS Sodium dodecyl sulfate
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TG Triacylglycerol
TEMED N,N,N’,N’-tetra-methyl-ethylenediamine
VLDL Very low density lipoprotein
CHAPTER 1

1. INTRODUCTION

1.1. The Metabolic Syndrome

Within the past century, a global trend towards a more sedentary lifestyle and an increase in caloric intake has increased the prevalence of chronic disorders such as obesity and type 2 diabetes. The rise in obesity and type 2 diabetes is so overwhelming that it is now deemed a worldwide “epidemic” (Zimmet et al., 2001). The metabolic syndrome, formerly referred to as insulin resistance syndrome, is the culmination of the complications of obesity and insulin resistant states. Furthermore, it is characterized by pathologies such as glucose intolerance, insulin resistance, obesity, dyslipidemia, and hypertension (Avramoglu et al., 2006). It has been predicted that, unless preventative measures are taken, the total number of people with diabetes will rise to 366 million in less than 30 years (Meetoo et al., 2007). The development of complications associated with diabetes such as retinopathy, nephropathy, neuropathy, cardiovascular diseases, peripheral vascular diseases and stroke, will undoubtedly lead to major socioeconomic costs (Meetoo et al., 2007).

The metabolic syndrome is a common pathological state that include a number of complications such as obesity, insulin resistance, glucose intolerance, dyslipidemia, and hypertension (Avramoglu et al., 2003). This cluster of risk factors significantly increases the risk of cardiovascular disease and type 2 diabetes (Klein et al., 2002; Malik et al., 2004). Insulin resistance, a decreased response to circulating plasma insulin levels, usually develops as the first indicator of type 2 diabetes. At this stage, the pancreas becomes stressed as it tries to keep up with the increasing demand for insulin due to tissue insulin resistance in order to maintain blood glucose levels. Type 2 diabetes manifests when the pancreas fails and can no longer produce enough insulin (Avramoglu et al., 2003).
1.2. **Metabolic Dyslipidemia: A Common Component of the Metabolic Syndrome**

The dyslipidemia that is observed in insulin resistance and type 2 diabetes is the most common and important risk factor associated with the development of cardiovascular disease and atherosclerosis (reviewed in (Avramoglu et al., 2006)). Metabolic dyslipidemia is characterized by an abnormal lipoprotein profile with elevated plasma TG, elevated small dense LDL particles, and a decrease in HDL particles (Krauss, 2004; Taskinen, 2005). This abnormal fasting lipemia is indicative of the magnitude of the postprandial dyslipidemia (Annuzzi et al., 1989).

1.3. **Postprandial Hyperlipidemia in Insulin Resistant States**

Patients with type 2 diabetes present with abnormal plasma lipoprotein profiles in the postprandial state (Mero et al., 1998). A study by Annuzzi, et al. investigated the role of insulin resistance on the development of postprandial dyslipidemia in type 2 diabetic patients (Annuzzi et al., 2004). Their sample consisted of type 2 diabetic patients with good control over blood glucose, normal fasting lipoprotein profiles, yet they displayed an exaggerated postprandial response (Rivellese et al., 2004). A hyperinsulinemic glycemic clamp ensured that the glucose and insulin remained at similar levels between groups, such that the only difference was the level of insulin sensitivity (Annuzzi et al., 2004). Independent of circulating glucose and insulin levels, insulin resistant patients displayed abnormal postprandial lipoprotein profiles, pointing to a direct role for insulin resistance in the development of the postprandial hyperlipemia observed in the metabolic syndrome (Annuzzi et al., 2004).
### 1.4. Factors Affecting Postprandial Metabolism

The mechanism responsible for aberrant postprandial metabolism in insulin resistant conditions is unclear. There is evidence of a role for lipoprotein lipase (Lpl), the enzyme that catalyzes the hydrolysis of TG-rich lipoproteins for uptake into peripheral tissues (reviewed in (Goldberg et al., 2009)). In a study by Annuzzi, et al. 2008, adipose tissue LpL activity was significantly lower in diabetic subjects compared to control subjects, both in the fasted and postprandial states (Annuzzi et al., 2008). These results suggest that LpL may play a role in the exaggerated postprandial response observed in diabetic subjects (Annuzzi et al., 2008).

The free fatty acids (FFAs) that are liberated from lipoproteins through Lpl mediated hydrolysis enter peripheral cells via passive diffusion or protein facilitated process. CD36, which will be discussed in greater detail in section 1.6.1, plays a role in fatty acid (FA) uptake in the heart, skeletal muscle and adipose tissue among other peripheral tissues (Coburn et al., 2000). Moreover, CD36 deficiency significantly impairs FFA uptake in these tissues (Coburn et al., 2000).

Lipoprotein TG is progressively hydrolyzed, forming what is referred to as a remnant lipoprotein. There is substantial evidence that intestinal lipoprotein remnants pose an atherosclerotic risk when they are chronically elevated (Havel, 2000; Karpe et al., 1994; Weintraub et al., 1996). A myriad of proteins and receptors have been linked to the clearance of chylomicron remnants, such as LDL receptor, LRP, SR-B1, hepatic lipase, and ABCA1 (reviewed in (Cianflone et al., 2008)).

### 1.5. Intestinal Lipoprotein Overproduction in Insulin Resistance

Although intestinal lipoprotein assembly is required for the absorption of dietary fat and fat-soluble vitamins (Hussain et al., 2005), it has long been regarded as a passive process. There is growing evidence that intestinal lipoprotein overproduction is a major contributor to the both
fasting and postprandial lipemia observed in these pathological conditions. The intestine contributes to the pathologies in a number of ways. Intestinally derived lipoproteins, particularly chylomicrons (CMs), can accelerate the development of obesity by delivering energy-rich TG to adipose depots (Williams, 2008). Due to an increased activity of lipoprotein lipase (LpL) in adipose and a decreased activity in muscle, there is a diversion of CM triacylglycerol (TG) from combustion in muscle, into adipose tissue for storage in sedentary individuals (Mead et al., 2002; Hamilton et al., 2007). As the CM delivers TG to peripheral tissues, it becomes a more dense, cholesterol (CH)-rich particle. These small dense particles enter the arterial wall and promote the formation of atherogenic plaques. Finally, CMs interfere with the catabolism of atherogenic hepatic lipoproteins, which also act as key mediators for the formation of atherosclerotic plaques (Karpe and Hultin, 1995).

1.6. Dietary FA Uptake

In the lumen of the small intestine, dietary fat is hydrolyzed into its components, monoacylglycerol (MG) and FFAs that are then dispersed in bile acids (Fig. 1.). In close proximity to the enterocyte surface, the pH is lower, causing protonation of the FAs. Free FAs then dissociate from the bile salt micelles and either passively diffuse or are transported across the brush border membrane by protein mediated transport. Several proteins have been implicated in the facilitated uptake of FAs by the enterocyte including cluster determinant 36 (CD36), fatty acid binding protein (FABPpm), and fatty acid transport protein family members (FATPs) (Abumrad et al., 1999). Both CD36 and FABPpm are thought to reside in specialized microdomains called lipid rafts (Ehehalt et al., 2006). Lipid rafts have a higher content of highly saturated hydrocarbon chain shingolipids, which pack more tightly than other phospholipids species, and are therefore more ordered microdomains of the plasma membrane (Ehehalt et al., 2006).
1.6.1. The Role of CD36 in FA Uptake

CD36 is a scavenger receptor that is expressed in many cell types such as megakaryocytes, erythroid precursors, platelets, monocytes, dendritic cells, adipocytes, myocytes, retinal and mammary epithelial cells, and endothelial cells of the microvasculature and the small intestine. CD36 deficiency is common in subpopulations that are at higher risk of developing type 2 diabetes (Abumrad et al., 1999). However, studies in humans and mice do not consistently present an association of CD36 deficiency and insulin responsiveness. Thus, the effect of CD36 deficiency on the development of pathological conditions may strongly depend on dietary conditions.

CD36 was initially recognized as a modulator of FA uptake in adipocytes (Abumrad et al., 1981). Subsequent labeling of the protein with membrane impermeable FA analogs lead to the identification of a membrane protein that was shown to have 85% identity to CD36 (Abumrad et al., 1993). Nada Abumrad’s group studied FA uptake and utilization in CD36\textsuperscript{-/-} mouse tissues using \[^{3}H\]-palmitic acid and a non-oxidizable FA analog, \(\beta\)-methyl 15-(p-iodophenyl) pentadecanoic acid (BMIPP) (Coburn et al., 2000). They showed that FA uptake was decreased in the fed state in the heart, skeletal muscle, and adipose tissues of CD36\textsuperscript{-/-} mice (Coburn et al., 2000), however, FA uptake in the intestine was not significantly different between CD36\textsuperscript{-/-} and wild type mice. There was a 2-fold reduction in BMIPP and \[^{3}H\]-palmitic acid incorporation into TG in muscle and adipose tissue, and an 2-fold increase in labeled diacylglycerols in isolated adipocytes. However, there was no change in the activity of the enzyme responsible for the conversion of diacylglycerols to TG, diacylglycerolacyltransferase (DGAT). There was no change in the long chain acyl-CoA synthetase enzymes in muscle and adipose tissues. The authors postulate that a decreased rate of TG production in these tissues may be due to the lower affinity of DGAT for fatty acyl-CoAs (Coburn et al., 2000).
In the intestine, CD36 deficiency was not associated with decreased uptake of either \([^{3}H]\)-palmitic acid or BMIPP (Coburn et al., 2000), despite its similar localization to other proteins implicated in FA uptake (Poirier et al., 1996). However, Drover, et al. demonstrated that CD36 is involved in the uptake of FA through its involvement in chylomicron formation in CD36\(^{-/-}\) mice enterocyte (Drover et al., 2005). In this study, CD36\(^{-/-}\) or wild type mice were given a bolus of olive oil of FAs by intragastric gavage and the secretion of lipids into the lymph was measured. There was an increase in BMIPP accumulation in the proximal section of the small intestine in CD36\(^{-/-}\) mice compared to wild type mice, which was not due to an impairment of FA uptake (Drover et al., 2005). There was a decrease in microsomal TG in CD36\(^{-/-}\) enterocytes compared to wild type animals, but unlike adipocytes and muscle, DAG did not accumulate in the microsome fraction of isolated enterocytes from CD36\(^{-/-}\) mouse intestine (Drover et al., 2005). These data suggest that CD36 is responsible for directing FAs towards the microsomal secretion TG pool (Drover et al., 2005). How CD36 accomplishes the intracellular trafficking of FAs is completely unknown, but Drover, et al. hypothesized that CD36-rich membrane vesicles shuttle FA with the help of trafficking proteins that cycle between the ER and plasma membrane (Drover et al., 2005).

Examination of lipid output from the intestine of these animals revealed that TG secretion into the lymph was significantly decreased in CD36\(^{-/-}\) mice compared to wild type mice (Drover et al., 2005). Again, these data emphasize a role for CD36 in intestinal lipid absorption and CM secretion.

1.6.2. The Role of Fatty Acid Binding Proteins in FA Uptake

Once the FAs are transported across the plasma membrane, they encounter a family of lipid chaperones called fatty acid binding proteins (FABP). In the intestine, there are a two main FABPs, intestinal-FABP (FABP2) and liver-FABP (FABP1) (Iseki et al., 1990). L-FABP
is a small (14 kDa) cytoplasmic protein that can bind long chain FAs, long chain fatty-acyl CoA and an array of other molecules including carcinogens, lysophospholipids, acyl-coenzyme A, eicosanoids and heme (Coe and Bernlohr, 1998). The promoter of L-FABP contains a peroxisome proliferators response element, but its mRNA is also regulated by FAs, dicarboxylic acid and retinoic acid (Rolf et al., 1995). It has been suggested that L-FABP can act as a co-activator of PPARα-mediated gene activation, since L-FABP and PPARα physically interact (Furuhashi and Hotamisligil, 2008). Unlike other members of the FABP family, L-FABP can bind two ligands simultaneously (Furuhashi and Hotamisligil, 2008). This property of L-FABP is suggested to serve as a feature enabling ligand delivery through interactions with target receptors (Furuhashi and Hotamisligil, 2008).

L-FABP deficient mice have no change in appearance, gross morphology or viability. They were of normal weight and had normal serum TG and FA levels (Cianflone et al., 2008). However, the metabolic parameters in these mice upon exposure to high-fat/cholesterol diet differed between studies (Cianflone et al., 2008). Some evidence suggests that L-FABP is necessary for the formation of chylomicrons (also discussed in section 1.10. The Process of Chylomicron Assembly and Secretion). Charles Mansbach’s group demonstrated the involvement of L-FABP in the formation of chylomicrons. L-FABP was shown to select cargo and its presence in the COPII protein-deficient cytosol was sufficient to bud prechylomicron transport vesicles (PCTV) from the ER in an in vitro budding assay (Neeli et al., 2007).

I–FABP is only expressed in the small intestine and thought to be involved in the transport of long-chain FAs (Kim et al., 2001). Studies in Caco-2 cells suggest that I-FABP may actually be involved in the reduction of FA uptake (Darimont et al., 2000). Through an unknown mechanism, overexpression of I-FABP inhibited FA incorporation into triglyceride in differentiated Caco-2 cells supplemented with FA (Darimont et al., 2000). Characterization of the I-FABP-null mouse has lead to a new hypothesis for the role of I-FABP; its deletion did not
affect development or dietary fat absorption. The mice were hyperinsulinemic, but showed genetic differences in body weight and plasma parameters (Darimont et al., 2000). Male I-FABP-null mice had elevated plasma TG and weighed more than wild type animals on both low and high fat diets (Darimont et al., 2000), while female I-FABP-null mice gained less weight than wild-type mice. The authors suggest that I-FABP functions as a lipid-sensing factor in the maintenance of energy homeostasis and may not be directly involved in the uptake of FAs (Darimont et al., 2000).

In a recent study, protein levels of both intestinal I-FABP and L-FABP were measured in two hereditary lipid malabsorption syndromes, Abetalipoproteinemia (ABL) and Anderson's disease (AD) (Guilmeau et al., 2007). Both of these disorders are characterized by absent or abnormal lipid and lipoprotein metabolism. ABL results from a mutation in microsomal triglyceride transfer protein (MTP), a chaperone necessary for the lipidation of apoB (Wetterau et al., 1992). In patients with this disorder, apoB100 and apoB48 are absent from the plasma and accumulation of lipid droplets in the cytoplasm of enterocytes (Wetterau et al., 1992; Shoulders et al., 1993). A mutation in the SARA 2 gene encoding the Sar1 GTPase causes Anderson’s disease (Jones et al., 2003). This disorder is characterized by a retention of chylomicrons in the enterocyte, presumably because of a lack of the appropriate intracellular trafficking machinery (Jones et al., 2003). Protein levels, but not mRNA levels, of I-FABP and L-FABP were decreased in patients with either ABL or AD compared to normal subjects (Guilmeau et al., 2007). The authors suggest that a reduction of I-FABP or L-FABP may be beneficial in these pathological conditions associated with intracellular lipid accumulation (Guilmeau et al., 2007). Their postulation is that a decrease in I-FABP or L-FABP would prevent conversion of FA to TG, preventing additional intestinal injury (Guilmeau et al., 2007).
1.6.3. The Role of Fatty Acid Transport Proteins in Fatty Acid Uptake

CD36 is thought to be involved in the uptake of FAs by binding them at the plasma membrane (Ibrahimi and Abumrad, 2002; Abumrad et al., 1999), and FABPs are chaperones that are involved in lipid signaling, trafficking, esterification, and chylomicron formation. Another family of lipid binding proteins, FATPs, play a role in the translocation of FAs across the plasma membrane (Stahl et al., 2001). There are six mammalian FA transport protein members that are expressed in fat utilizing tissues (Ehehalt et al., 2006). Of the six, only FATP4 is expressed in the intestine (Ehehalt et al., 2006; Stahl et al., 1999). FATP4 deletion causes embryonic lethality or perinatal lethality with a phenotype that similar to lethal restrictive dermopathy (Herrmann et al., 2003). Herrmann, et al. showed that FATP4 is implicated in ceramide biosynthesis, perhaps by acylating very long chain FAs. There was an increase in ceramide and cholesterol content in the skin of these animals, however, very long chain FA substitutes were significantly reduced in the ceramide fraction (Herrmann et al., 2003). In another study, FATP4 null mice died shortly after birth and it appeared that FATP4 plays an essential role in the formation of the epidermal barrier. FATP4 heterozygosity in the same study lead to reduced LCFA uptake by the enterocyte, but did not affect lipid absorption or body composition in vivo (Hall et al., 2005).

Recently, a group rescued the defective skin phenotype in FATP4 null mice by using an FATP4 transgene driven by a keratinocyte-specific promoter (Fatp4−/−;Ivl-Fatp4+g/+ in order to study intestinal lipid absorption (Shim et al., 2009). In short, there were no differences in food consumption, weight gain, plasma lipid parameters, or intestinal lipid absorption in WT vs. Fatp4−/−;Ivl-Fatp4+g/+ on normal chow diets, although Western diet fed Fatp4−/−;Ivl-Fatp4+g/+ mice showed a significant increase in enterocyte TG and FA content. There was no compensation observed by any other FATP family member, or by any FA or cholesterol
transporter in Fatp4−/−;Ivl-Fatp4+ mice (Shim et al., 2009). The current proposed mechanism by which FATP4 facilitates uptake of FAs is by providing acyl CoA synthetase activity (Herrmann et al., 2003). Moreover, FA uptake is indirectly driven by FATP4 activity; activation of FAs to fatty acyl-CoA moieties shifts the equilibrium towards uptake of FAs at the plasma membrane (Herrmann et al., 2003). The products of FATP4 activity, fatty acyl-CoAs, are substrates for TG synthesis and are directed towards TG synthesis enzymes that are located on the ER membrane.

1.7. **Intestinal Cholesterol Absorption**

In the lumen of the intestine, cholesterol is taken up by enterocytes and secreted into the intestinal lymph mostly as cholesteryl ester. Cholesterol is esterified through the action of acyl-CoA cholesterol acyl transferase (ACAT) (Gallo et al., 1984). Our understanding of the process cholesterol absorption increased dramatically from the discovery of ezetimibe, an inhibitor of cholesterol absorption (Clader, 2004). The target of ezetimibe is now known to be the Niemann-Pick C1-like protein 1 (NPC1L1) (Garcia-Calvo et al., 2005). Scavenger Receptor Class B Type I (SR-BI) is another key player in the absorption of cholesterol. SR-BI binds lipoprotein particles on the surface of cells to facilitate cholesterol uptake (Connelly and Williams, 2004). CD36 has also been shown to mediate cholesterol uptake in enterocytes (Nauli et al., 2006). Important to this study, is the regulation of intestinal chylomicron assembly and secretion by cholesterol. Pal, et al. examined the regulatory role of sterols, cholesterols, and cholesteryl esters on the secretion apoB48 containing lipoproteins in Caco-2 cells (Pal et al., 2002). Their results indicate that cholesteryl ester formation regulates apoB48 secretion in these cells (Pal et al., 2002).
1.8. Chylomicron Assembly: A Characteristic Property of Enterocytes

CMs are very large buoyant lipoprotein particles produced by the intestine upon ingestion of fat. Their physiological role is to transport dietary fat throughout the circulation, thereby delivering energy to peripheral tissues. Chylomicrons consist of a neutral lipid core of unordered TG and cholesteryl ester (CE) and are surrounded by a phospholipid monolayer embedded with free cholesterol (Hussain, 2000). The key structural component of chylomicrons is a large monomeric protein apolipoprotein B48 (apoB48). The liver synthesizes apoB100 for the assembly of VLDL particles. In the intestine, apoB48 is translated from the full length message, or apoB100, but is posttranscriptionally modified by deamination of a cytosine to uracil, ending protein synthesis at codon 2153 (reviewed in (Hussain, 2000)). Since the liver does not have the capacity to assembly CM or synthesize apoB48, it was thought that CM synthesis dependent on apoB48 synthesis. This hypothesis was investigated by Dr. Hussain’s group, who created Caco-2 cells that stably express apoB48 independent of mRNA editing activity (Luchoomun et al., 1997). Their observation that undifferentiated Caco-2 cells secrete apoB-containing lipoprotein particles of different sizes, but not CM particles, suggested that expression of apoB48 could not drive the assembly of larger CM particles (Luchoomun et al., 1997). Since apoB48 expression was not sufficient for CM assembly, this group postulated that formation of CM particles may be a unique property of differentiated enterocytes. To this end, Caco-2 cells were allowed to differentiate and challenged with fatty acid (FA) (Luchoomun and Hussain, 1999). However, both apoB100 and apoB48 had the capacity to assemble CM particles (Luchoomun and Hussain, 1999). Studies in mice that produce only apoB100 from the liver and intestine showed that apoB100 could replace apoB48 in the intestine (Farese, Jr. et al., 1996). Numerous studies in rodent hepatocytes that can synthesize apoB48 in addition to
apoB100 showed that this cell type cannot be induced to assemble CM particles (Boren et al., 1994; Hussain et al., 1989; Hussain et al., 1995). Therefore, CM assembly is a characteristic property of differentiated enterocytes (reviewed in (Hussain, 2000)).

Figure 1. Absorption of dietary lipid, and the assembly and secretion of chylomicrons. CD36 facilitates FA uptake. FAs are bound by cytosolic FA binding proteins that deliver substrates to TG synthesis enzymes (MGAT and DGAT) on the ER membrane. Chylomicron assembly is thought to proceed in three distinct steps: the co-translational lipidation of apoB with phospholipids and some TG by MTP, to form a primordial particle (1); formation of TG-rich lipid droplets (2); and ‘core expansion’, or fusion of the primordial particle and TG-rich lipid droplets (3). The budding of the prechylomicron transport vesicle may be the site of ‘core expansion’ and involves the selection of cargo by FABP1. PCTVs fuse with the Golgi in a COPII dependent manner. Mature chylomicrons are secreted via the basolateral membrane into the lymphatic circulation.
1.9. **TG Synthesis**

The intestinal enterocyte can assimilate TG via two separate pathways: glycerol phosphate (G3P) or Kennedy pathway (CLARK and HUBSCHER, 1961) and the monoacylglycerol (MG) pathway (HUBSCHER and CLARK, 1960) (Fig. 2.). The enzymes involved in the two pathways of TG synthesis are found in different subcellular regions, keeping the products of acylation compartmentalized (Higgins and Barnett, 1971). Higgins, et al. showed that acyltransferases from the MG pathway were localized to the smooth ER while those from the G3P pathway were localized to the rough ER (Higgins and Barnett, 1971). The first step in the MG pathway is catalyzed by monoacylglycerol acyltransferase (MGAT), which acylates monoacylglycerol with fatty acyl-CoA (FA-CoA) to form diacylglycerol (DG) (reviewed in (Mansbach and Gorelick, 2007)). Subsequent acylation of DG by DGAT yields TG (Mansbach and Gorelick, 2007).

Two mammalian DGAT enzymes have been identified. DGAT1 was first identified in 1998 as having a similar sequence to acyl-CoA:cholesterol transferase (ACAT) and possessing diacylglycerol transferase activity (Cases et al., 1998). DGAT1 is a member of a family of membrane-bound O-acyltransferases (MBOAT) that catalyze the addition of acyl-CoA moieties to hydroxyl or thiol groups of lipids or protein molecules (reviewed in (Yen et al., 2008)). Other MBOAT family members include acyl-CoA: cholesterol acyltransferase (ACAT) which covalently joins cholesterol and fatty acyl-CoA to form cholesteryl ester. DGAT2 was sought after following the development and characterization of the DGAT1−/− mouse, since lack of DGAT1 did not cause a defect in TG assimilation (reviewed in (Yen et al., 2008)).

Mammalian DGAT2 was cloned in 2001, followed by identification of other members of DGAT2 family, including MGAT1, MGAT2 and MGAT3 (reviewed in (Yen et al., 2008)). Studies in DGAT2−/− mice suggest that DGAT2 is the major DGAT involved in TG synthesis.
and that it may be involved in FA biosynthesis (Stone et al., 2004). Evidence for this comes from studies by Man, et al. who have shown that DGAT2 and stearoyl-CoA desaturase1 (SCD1) colocalize and interact in HeLa cells (Man et al., 2006). This suggests that DGAT2 may be responsible for incorporating the monounsaturated FA, oleoyl-CoA, into TG (Man et al., 2006).

TG can also be synthesized by the G3P pathway, which begins with the consecutive acylation of glycerol-3-phosphate by GPAT and AGPAT to form lysophosphatidate and phosphatidate, respectively. Phosphatidate is then dephosphorylated by PAP to form DG, which undergoes acylation to yield TG. The DG intermediate in the G3P pathway may also go on to form phospholipids, however, DG formed by the MG pathway can only form TG (Mansbach and Gorelick, 2007).

The MG pathway is used in tissues that re-esterify TG from its dietary lipolytic products, such as the liver and the intestine. The MG pathway contributes to approximately 80% of CM TG, while the G3P pathway contributes only 20% of CM TG (Breckenridge and Kuksis, 1975).

**Figure 2.** TG synthesis pathways. The predominant pathway for synthesis of TG in normal primary enterocytes is the monoglyceride (MG) pathway, while the predominant pathway used by Caco-2 cell is the glycerol phosphate (G3P) pathway, involving the enzymes glycerol-3-phosphate acyltransferase (GPAT), 1-acylglycerol-3-P-acyltransferase (AGPAT), phosphatidic acid phosphatase (PAP), and diacylglycerol acyltransferase (DGAT). DAG produced via the G3P pathway can be used for the biosynthesis of phospholipids, while DAG produced by the MG pathway cannot.
1.10. The Process of Chylomicron Assembly and Secretion

Overall, the assembly of chylomicrons is thought to proceed in a similar manner to that of VLDL assembly in the liver, since lack of MTP has a similar detrimental effect on the assembly of CM and VLDL particles. The co-translational lipidation of apoB by MTP is necessary for the release of apoB from the ER and for the formation of primordial lipoprotein particles (reviewed in (Hussain, 2000)). However, the subsequent steps involved in lipidation of the primordial particle to form either VLDL or CM are still unclear. Tso, et al. proposed an independent model of chylomicron assembly, suggesting that VLDL and CM assembly proceed by different mechanisms (Tso et al., 1984). Moreover, that VLDL assembly is constitutive, while CM assembly occurs in the postprandial state. A key piece of evidence supporting this model is that Pluronic L81 (PL81) affects the secretion of CM but not VLDL particles (Tso and Gollamudi, 1984). PL81 is a hydrophobic surfactant that has been shown to increase cytosolic TG (Fatma et al., 2006). Mammoud Hussain’s group proposes that PL81 disrupts the transport of cytosolic TG to the ER for secretion (Fatma et al., 2006), which may explain why PL81 inhibits assembly of more buoyant CM particles but not of smaller particles.

On the other hand, Dr. Hussain proposed a sequential model of assembly, involving the same initial lipidation of apoB by MTP (Hussain et al., 2005), followed by ‘core expansion’ of the primordial particle by the addition of TG to form a nascent chylomicron particle (Hussain, 2000). This model involves three distinct steps, the first being the co-translational lipidation of apoB, where synthesis of apoB is associated with interaction with phospholipids and requires the activity of MTP. Second, is the formation of lipid droplets in the lumen of the smooth ER. This stage is independent of apoB synthesis, but would differ in the postprandial state as more substrate is available for TG synthesis. Presumably, the increase in substrate would lead to the formation of larger TG droplets and hence, yield larger lipoprotein particles during the third step.
of this model, ‘core expansion’ (Hussain, 2000). This step involves the fusion of the primordial particles with the preformed lipid droplets residing in the smooth ER.

Charles Mansbach’s group proposed that a vesicle transporting the developing chylomicron from the ER to the Golgi, is the rate limiting step in TG transport across the enterocyte (Kumar and Mansbach, 1997; Kumar and Mansbach, 1999). Evidence to support this came from studies that showed the rapid esterification of oleic acid into TG, despite a long (4 hour) lag time to obtain steady state TG secretion (Mansbach and Nevin, 1998). They identified a vesicular subcellular fraction, named the prechylomicron transport vesicle (PCTV) that functions to transport the nascent chylomicron particles from the ER to the Golgi (Kumar and Mansbach, 1999). The formation of this vesicle is hypothesized to be the site of the ‘core expansion’ step (Hussain, 2000). The developing CM particle traverses the Golgi, undergoing further maturation, and is secreted via the basolateral membrane into the lymphatic circulation.

1.11. Specific Aims of the Study

1.11.1. Experimental Models

Caco-2 cells are an intestinal cell line derived from a human colorectal carcinoma and have been extensively used to study intestinal lipid absorption. Caco-2 cells are the only enterocyte cell culture model available for the study of lipid and lipoprotein processing (reviewed in (Levy et al., 1995)). The differentiation of Caco-2 cells proceeds through three states, the first being a homogenous undifferentiated state. Upon plating on polycarbonate microporous membrane plates (Transwell® filter plates), the cells begin to heterogeneously differentiate, displaying differences in morphology and brush border organization. Finally, after approximately 21-30 days of culture, Caco-2 cells become homogenously polarized and differentiated (reviewed in (Levy et al., 1995)). The potential for genetic manipulation and their
enterocyte-like secretory profile makes Caco-2 cells a suitable model for the study of intestinal chylomicron assembly and secretion.

Differentiated Caco-2 cells secrete mainly LDL-sized lipoprotein particles when they are not stimulated with lipid (Hughes et al., 1987a). Several investigators have explored the effect of different fatty acids on the formation of larger lipoproteins (Dashti et al., 1990; Hughes et al., 1987b; Luchoomun et al., 1997; Luchoomun and Hussain, 1999; Traber et al., 1987). Specifically, Luchoomun, et al. performed a detailed analysis of chylomicron secretion from Caco-2 cells using different concentrations of oleate and the bile salt derivative, taurocholate (Luchoomun and Hussain, 1999). The optimal concentration of oleate and taurocholate for the induction of CM assembly and secretion was found to be 1.6 mM and 1.0 mM, respectively (Luchoomun and Hussain, 1999).

Importantly, there is a difference in the TG synthesis pathway that predominates in Caco-2 cells and normal absorptive enterocytes. As mentioned above, the MG pathway is characteristic of absorptive enterocytes, and is especially important in the postprandial state. In mature Caco-2 cells, however, MGAT activity was less than 1/10th of that in rat jejunal cells (Levin et al., 1992). The G3P pathway, therefore, is the main pathway for TG synthesis in Caco-2 cells under both basal and lipid stimulated conditions.

In order to compare the in vitro results obtained in this study to an in vivo model, we made use of the CD36−/− mouse model. To study the effect of CD36 knockdown on intestine lipid metabolism, and more specifically, FA and TG synthesis, we followed a protocol that has been optimized in our lab for the isolation of primary enterocytes (Haidari et al., 2002).

1.11.2. Rationale

The process of lipid absorption, defined as the uptake, assembly into lipoproteins, and secretion of lipid into the lymphatic circulation, may be subject to regulation at several steps.
Under pathological conditions, such as obesity and insulin resistance, dysregulation of this process leads to dyslipidemia. Changes in levels of FA transporters such as CD36 and FABPs may lead to aberrant uptake or mistargetting of FAs (Coburn et al., 2000; Drover et al., 2005; Kim et al., 2001; Masuda et al., 2008; Nassir et al., 2007; Nauli et al., 2006; Neeli et al., 2007) and changes in proteins involved in the assembly and trafficking of lipoprotein particles causes lipid malabsorption disorders (Avramoglu et al., 2003; Jones et al., 2003; Lewis et al., 2004; Masuda et al., 2008; Wetterau et al., 1992). It remains unclear however, how deficiency of these intestinal lipid transporters contributes to aberrant intestinal lipoprotein production. Furthermore, whether the expression of these lipid transporters reflects the rate of lipid absorption, or if it determines the incorporation of lipid into lipoprotein particles, is also of significant interest. Elucidating the role of proteins potentially involved in the assembly of chylomicrons in an enterocyte cell culture model may provide insight into the mechanisms responsible for the dyslipidemia observed under pathological conditions.

1.11.3. Hypothesis

The hypothesis of the proposed research is that depletion of either intestinal FA transporter, CD36 or FABP1, will impair intestinal lipoprotein assembly and secretion. Both proteins are highly expressed in the proximal intestine and their involvement in lipid absorption has been well documented. However, in studies of whole body CD36 or FABP1 knock out animals, their influence on lipid and lipoprotein metabolism may be masked by peripheral factors. To delineate a role for these proteins in intestinal chylomicron assembly and secretion, we will make use of an enterocyte cell culture model. In this isolated model, external factors such as hormonal changes and peripheral clearance of lipoproteins should not impede the interpretation of the role of these proteins in the intestine.
1.11.4. Objectives

GENERAL OBJECTIVE: The overall objective of this project was to examine potential molecular factors involved in intestinal chylomicron assembly.

SPECIFIC AIM 1: To knock down putative factors involved in chylomicron assembly, CD36 and FABP1, in Caco-2 cells, an enterocyte cell culture model.

SPECIFIC AIM 2: To examine assembly of CM particles, apoB synthesis and secretion, and TG synthesis and secretion under knockdown conditions.
CHAPTER 2

2. MATERIALS and METHODS

2.1. Animals

Male CD36⁻/⁻ mice bred on the C57BL/6 background, and wild type C57BL/6 mice from 12 to 15 weeks of age were obtained from Dr. Maria Febbraio, Cleveland Clinic, via Dr. Kevin Kain, University of Toronto. Following a one week acclimatization period, animals underwent surgery as described below. Anaesthetics used for surgery (isofluorane, nitrous oxide, and oxygen) were from Baxter (Toronto, ON).

2.2. Chemicals and Reagents

Cell Recovery Solution was from BD Biosciences (Bedford, MA). Complete-Mini, EDTA-free protease inhibitor cocktail (PI) and Colourimetric TG assay was purchased from Roche (Mississauga, ON). [³H]-oleic acid and [³H]-acetate were obtained from PerkinElmer (Boston, MA). [³⁵S]-methionine was from PerkinElmer Life Science, Inc. (Woodbridge, ON). Bovine Serum Albumin (BSA), β-mercaptoethanol, glycerol, polyoxyethylenesorbitam monolaurate (Tween 20), calcium chloride, cholesterol, cholesteryl oleate, L-α-phosphatidylcholine, and oleic acid etc etc etc, were purchased from Sigma Aldrich (St. Louis, MO). Acetic acid (glacial) and petroleum ether were from Fisher Scientific (Nepean, ON). Methanol, ethanol, and 2-propanol were from Caledon Laboratories Ltd. (Georgetown, ON).

SureSilencing™ shRNA plasmids and CD36 and FABP1 RT-PCR primers were purchased from SA Biosciences Corp. (Frederick, MD). Lipofectamine™ 2000 was purchased from Invitrogen (Carlsbad, CA). Power SYBR Green master mix was purchased from Applied Biosystems (CA, USA). Antibiotics G418 and Puromycin were purchased from Sigma Aldrich (St. Louis, MO). Eagle's Minimal Essential Medium (EMEM), Dulbecco’s Minimal Essential
Medium (DMEM), methionine, cysteine, and glutamine-free DMEM, L-glutamine, fetal bovine serum, and Trypsin-EDTA, were purchased from Wisent, Inc. (Montreal, QC).

Agarose was purchased from Invitrogen Life Technologies (Grand Island, NY). Molecular weight marker was from Fermentas (Burlington, ON). All Real-time polymerase chain reaction primers were purchased from Sigma Aldrich (St. Louis, MO). All SDS-PAGE including 40% acrylamide/bis solution (29:1 with 3.3% C), tris(hydroxymethyl)-aminomethane (TRIS), glycine, sodium dodecyl sulfate (SDS), ammonium persulfate (APS), and N,N,N,N’,N’-tetramethyl-ethylenediamine (TEMED), blocking-grade non-fat dry milk and the Bradford-based protein assay kit were purchased from Bio-Rad. Horse radish peroxidase (HRP)-conjugated secondary antibodies and Western Lightning chemiluminescence detection reagents (ECL) were purchased from Amersham Biosciences. PVDF membranes were purchased from PerkinElmer (Boston, MA).

The anti-MTP, anti-FATP4, and anti-β-actin antibodies were purchased from Sigma Aldrich (St. Louis, MO). The anti-Sar1 antibody was from Stressgen Bioreagents (Ann Arbor, MI). The anti-FAS antibody was from Novus Biologicals (Littleton, CO). The anti-CD36 antibody was purchased from Abcam Inc. (Cambridge, MA).

2.3. Laboratory Supplies and Apparata

Micropipette tips (10 μl- 5 ml), screw-cap tubes (15 ml & 50 ml) and microfuge tubes (1.5 ml & 2 ml) were from Sarstedt Inc. (Montreal, QC). Syringes (1 ml, 3 ml, 5 ml, and 10 ml) and needles (18- 25 gauge) were from Becton Dickinson & Co. (Franklin Lakes, NJ). Petri dishes were from Fisher Scientific (Nepean, ON). Beckman Optima LE-80 ultracentrifuge, rotor (SW55Ti), ultracentrifuge tubes (5 ml), and table top refrigerated centrifuge were from Beckman (Palao Alto, CA). Transwell filter plates (1.12cm² insert diameter) and 6-well cell culture plates were purchased from Corning Inc. (Corning, NY).
Mini-gel electrophoresis/transfer systems and power supplies were from Bio-Rad. The orbital shaker was from VWR (Mississauga, ON). The recording film was from Kodak (Cedex, France). The gel dryer was from Savant Instruments (Holbrook, N.Y.).

2.4. Animal Surgery

The animal surgery was performed essentially as described by Haidari et al. 2002 (Haidari et al., 2002). Briefly, male CD36<sup>−/−</sup> or wild type mice were housed separately with free access to food and water. The mice were fasted for 8 hours prior to surgery. The animals were anesthetized with a continuous flow mixture of isofluorane, nitrous oxide, and oxygen. An incision was made in the animal’s abdomen and the jejunum (10 cm of the proximal end of the small intestine) was excised and placed in a Petri dish of ice cold 1xPBS to be used for primary enterocyte collection. The intestine was rinsed several times with ice cold 1xPBS using an 18-gauge needle and 5 ml syringe to clear intestinal contents. The intestine was cut longitudinally and subsequently into 2 cm fragments.

2.5. Primary Enterocyte Isolation

The intestine fragments were immersed in 5 ml Matrisperse Cell Recovery Solution for 1 h at 4°C. The fragments were then washed with 1xPBS with agitation on an orbital shaker for 5 min at 4°C. On the shaker, 5 ml of 1xPBS was added to the fragments, which were then gently taped with a sterile dissection instrument. At this time, the villi begin dissociating from the intestinal fragments. The PBS with suspended villi was collected and the villi collection was repeated 5 more times or until no more villi dissociated from the fragments. The villi were pelleted with a 3 min centrifugation at 1000 RPM at 4°C. The supernatant was removed and the pelleted villi were weighed to estimate the number of cells. The pelleted villi were then washed with 1x PBS followed by another centrifugation at 1000 RPM at 4°C.
2.6. **Primary Enterocyte Cell Culture**

Primary enterocytes isolated from CD36−/− or wild type mice were resuspended in a volume of media in order to obtain a culture with 1 x 10^6 cells/ ml. To achieve this cell density, 2.5 ml of media was added per 0.1g of cells. One ml of cells plated per well of a 6-well culture dish. Primary cells underwent lipid micelle stimulation and lipid labeling as described below.

2.7. **Caco-2 Cell Culture**

Caco-2 cells (American Type Culture Collection, Manassas, VA) were grown in 75 cm² tissue culture flasks at 37°C in air and 5% CO₂ in Eagle’s Minimal Essential Medium (EMEM, GIBCO, USA), supplemented with 20% fetal bovine serum (FBS, GIBCO, USA), 100IU/ml penicillin. The culture medium was changed every other day. For the subculture, the medium was removed and the cells were detached from the culture dish with 0.25% trypsin diluted in phosphate-buffer saline (PBS) containing 0.2g/L EDTA. Culture medium with FBS was added to stop trypsinization. For all experiments, Caco-2 cells were plated at a density of 5x10⁴ cells in 400μl onto the apical compartment of Transwell filter plates. The basolateral compartment was supplemented with 1 ml of culture media. The media was changed every two days and transepithelial resistance (TER) was monitored using an Ohmmeter (homemade by the Grinstein lab). After 21 days, the Caco-2 cells had formed a confluent monolayer (TER was >600Ω) and were fully differentiated.

2.8. **LB broth and agar plate preparation**

LB broth was made by dissolving 25g of LB broth MILLER in 1L Millipore-purified water and autoclaving. LB agar plates were made by dissolving 37g of LB agar MILLER in 1L of purified water and autoclaved. When the agar had cooled down to approximately 50 °C, ampicillin was added to a final concentration of 50μg/ml, and poured into Petri dishes. Once the agar had polymerized, the plates were stored at 4 °C.
2.9. **Transformation and plasmid DNA amplification**

shRNA plasmids obtained were amplified by transforming into DH5α competent *E. coli* cells by heat-shock method. Approximately 2 ng of plasmid DNA was combined with 50µL of freshly thawed DH5α competent cells. The mixture was incubated on ice for 1 hr, placed in a 37°C water bath for 1 min, and returned on ice for another 5 min. The transformed culture was plated onto LB-ampicillin agar plates (50µg/ml ampicillin) and incubated overnight at 37°C. Several colonies were picked off the plates using sterile pipettes and were used to inoculate separate tubes of LB broth containing 50µg/ml ampicillin. The tubes were incubated overnight at 37°C with shaking at 250rpm.

Plasmid DNA was isolated using a QIAprep Miniprep kit according to the manufacturer’s protocol. Plasmids were eluted using purified, autoclaved water. Their concentrations were determined by Nanodrop. The purity of the plasmid DNA was assessed by the ratio of optical density units at A$_{260}$/A$_{280}$, which was between 1.8 and 2.

2.10. **shRNA Transfection in Caco-2 Cells**

Transfection of CD36- or FABP1-specific shRNA plasmid, or their respective transfection control shRNA plasmid was performed essentially as described by the manufacturer. Briefly, one day before transfection, Caco-2 cells were plated at a density of 2x10$^4$ cells in 12-well culture plates. For each well, 4.0 µl Lipofectamine™ 2000 was diluted in 100 µl EMEM without serum and incubated at room temperature for 5 min. Two µg of either control shRNA plasmid or CD36- or FABP1-targetting shRNA plasmid DNA was diluted in 100 µl EMEM without serum and mixed gently. The diluted Lipofectamine™ 2000 and DNA mixtures were mixed together and incubated for 45 min at room temperature. The complexed DNA and Lipofectamine™ 2000 solutions were added to each well and mixed by rocking the plate back and forth. The cells were returned to the 37°C incubator and after 6 hours, the media
was replaced with EMEM supplemented with 20% FBS. Three replicate transfections for each of the four gene-specific and the scrambled control shRNA plasmids were performed.

2.11. Selection of Stably Transfected Caco-2 Cell Clones

CD36-specific shRNA plasmid and its scrambled control shRNA plasmid conferred puromycin resistance, while the FABP1-specific shRNA plasmid and its scrambled control shRNA plasmid conferred G418 resistance. Dose response curve for G418 and puromycin selection were generated and concentrations of 800 μg/ml G418 and 4 μg/ml puromycin were found to be the effective concentrations for selection in Caco-2 cells. Forty-eight hours post-transfection, Caco-2 cells were subjected to selection using the appropriate antibiotic. The antibiotic containing media was changed every two days for up to 21 days. When a stably-transfected colony was observed, it was aspirated from the plate and re-plated into a 24-well plate to generate separate populations of stably-transfected cells, or a monoclonal population. These clones were grown for one week and then re-plated onto 6 well plates and grown until enough cells were available for generating a frozen stock and for isolating total RNA.

2.12. Total RNA Extraction

Total RNA was extracted from Caco-2 cells. Caco-2 cells grown on Transwell filters were washed with 1xPBS and the cells were disrupted directly in the Transwell filter plates by adding Buffer RLT. RNA was extracted using RNeasy Plus reagent (Qiagen, ON, Canada). All subsequent steps were performed as recommended by the manufacturer. RNA was quantified using a NanoDrop Spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, USA). RNA integrity was examined by running a 2% agarose gel and by measuring the ratio of the optical density at 260 nm to that at 280 nm. RNA was considered to be of good quality if the 28S:18S RNA was 2:1, and if the ratio of optical density at 260 nm to that at 280 nm exceeded 1.8.
2.13. Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was used to quantify the expression of specific genes (Table 1). cDNA was synthesized using Taq Man Reverse Transcription Reagent cDNA Synthesis Kit (Applied Biosystems, CA, USA). Reverse transcription was performed using 200 ng of total RNA and random hexamer primers according to the manufacturer’s instructions. The qRT-PCR conditions were 95°C for 10 min, 40 cycles at 95°C for 15s, 60-62°C (primer specific, optimal annealing temperature) for 1 min. Melting curve analysis was performed by increasing the temperature (1°C/s) from 65°C to 95°C, with continuous fluorescence acquisition. Primers for the target genes, ACC1, DGAT2, ApoAIV, SCD1, and FABP2 were designed with Primer 3.0 (Rozen and Skaletsky, 2000), using publicly available sequences. PCR conditions for all primers were optimized and specificities of all reactions were verified by melting curves and electrophoresis on 1% agarose gels. Primers for target genes and 3 reference genes are shown in Table 1. Threshold cycle (Ct) values were obtained in triplicate for each sample using the Power SYBR Green master mix according to the manufacturer’s protocol. The mean of three experiments in triplicate was used for the statistical analyses. PCR efficiencies for each set of primers were calculated for each sample using serial dilution curve method. The differences in expression between control and treated group means were assessed for statistical significance by pair-wise fixed reallocation randomization test, using the relative expression software tool (REST)(Pfaffl et al., 2002). An index (part of the REST program) was generated with three reference genes selected: actin and 18S and GAPDH. These genes were not differentially expressed between samples (assessed by qRT-PCR) and the index generated with their values was used to normalize the data. Probability less than 0.05 was considered statistically significant.
Table 1. RT-PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse primer</th>
<th>Amplicon size (bp)</th>
</tr>
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<td>5’-TGTAGACCATGATGGAGGTC-3’</td>
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<tr>
<td>18S</td>
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<td>5’- GATCGAGGGCCCCTCCTAAAC-3’</td>
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</tr>
<tr>
<td>Actin</td>
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<td>5’- TACTCCTGCTTGATGCAC-3’</td>
<td>300</td>
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<tr>
<td>Acetyl CoA Carboxylase 1 (ACC1)</td>
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<td>5’- CAATGGCCCACGCATTG-3’</td>
<td>61</td>
</tr>
<tr>
<td>Apolipoprotein AIV (ApoAIV)</td>
<td>5’- CAGCAAGCAGCTCAGATGTT-3’</td>
<td>5’- TCCACGGCCTCTTGCCATT-3’</td>
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</tr>
<tr>
<td>FA binding protein 2 (I-FABP)</td>
<td>5’- CTGATGCAGCTCCTGCCACTA-3’</td>
<td>5’- TTTTGAAGCCTTGAAAAATTG-3’</td>
<td>143</td>
</tr>
<tr>
<td>Diacylglycerol acyltransferase 2 (DGAT2)</td>
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</table>

2.14. SDS-PAGE and Western Blotting

For western blotting analysis of Caco-2 cells lysate, Transwell filters were washed with 1xPBS and cut out of the plastic insert. The filter was immersed in 500 μl solubilizing buffer (150 mM NaCl, 1.0 mM Tris, 1.0 mM EDTA, 1.0 mM EGTA, 0.01% Triton X-100, 0.01% NP-40, 100 mM NaF, 10 mM Na₂P₂O₇, 2 mM sodium orthovanadate, 2 mM PMSF, pH 7.4) and vortexed for 2 min. Lysates were centrifuged at 13,000 g for 10 min to remove debris and the protein concentrations in the resulting supernatants were determined as described by Bradford (Bradford, 1976). Aliquots of lysate containing equal amounts of protein were used for SDS-PAGE. 6-12% SDS-PAGE resolving gels were cast and allowed to polymerize for 30 min. The resolving gels were overlaid with 3-5% stacking gels and either 10 or 15-well combs were inserted. The cell lysates from Caco-2 cells were prepared for PAGE by diluting in 4X sample buffer (8% SDS, 0.25 M Tris, 40% glycerol) containing β-mercaptoethanol. The sample mixture was boiled for 5 min at 100°C to fully denature the proteins in the sample. The
denatured samples were spun at 13,000 RPM in a tabletop centrifuge to pellet any remaining cell membranes and loaded into the wells cast in the stacking gel, along with a protein molecular weight marker. The Bio-Rad mini-gel electrophoresis system was filled with 1x running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.5) and the gels were subjected to electrophoresis at 100V for a period of approximately 2 hours. For apoB and FAS SDS-PAGE, gels were subjected to electrophoresis overnight at 35V. The gels were then transferred to methanol activated PVDF membranes in the Bio-Rad mini-gel protein transfer apparatus filled with 1x transfer buffer (pH 7.5) and run at 225 mA for 2 hours at 4°C. When the transfer was complete, the PVDF membranes were soaked in methanol for 15 seconds and left to air-dry. The dried membranes were soaked in 5% blocking-grade non-fat milk in 1x PBST (pH 7.5) for at least 1 hour on an orbital shaker at RT. The membranes were then incubated in a primary antibody dilution (1:1000 in 1% BSA, 1xPBST) overnight at 4°C. The membranes were then washed with 1xPBS 3 times for 5 minutes each, followed by incubation with a secondary antibody dilution (for anti-mouse and ant-rabbit secondary antibodies the dilution was 1:10,000 in 1% BSA, 1xPBST, and for anti-goat secondary antibody the dilution was 1:80,000 in 1% BSA, 1xPBST) for 1 hour at RT. The membranes were then washed with 1xPBS 3 times for 10 minutes each, followed by incubation in ECL reagent containing HRP substrate to yield chemiluminescence. The membranes were exposed to recording film and then developed. ImageJ software (available at rsb.info.nih.gov/ij/) was used for quantification of intensity of each band. The graphs show the densitometry (mean ± SE). In the case of western blotting, a typical blot was shown. Data comparisons were analyzed for statistical significance using a student’s t-test. Differences with probability values lower than 0.05 were considered significant.
2.15. **Preparation of lipid micelles**

Stock solutions of cholesterol (0.5 mg/ml) and oleic acid (100 mM) were prepared in chloroform and ethanol, respectively. L-α-phosphatidylcholine, oleic acid, and cholesterol were added to a large Pyrex tube to obtain the final concentrations of 0.5 mg/ml, 1.6 mM, and 0.2 mg/ml, respectively. With constant rotation, the tube was held nearly horizontal and lipids were evaporated with a mild stream of Argon gas until a film was deposited on the Pyrex tube. The Pyrex tube was returned upright and maintained under a constant stream of Argon gas for 5 min to continue drying the lipids. During this time, 5.37g taurocholate was dissolved in 0.5 ml media to obtain a final concentration of 1.0 mM. The dried lipids were resuspended in 0.5 ml taurocholate-containing media and left to hydrate for 1 min. The solution was dissolved by sonication for 1 min and dissolved in 9.5 ml of EMEM supplemented with 20% FBS to obtain a total of 10 ml of 1.6 mM oleate-containing lipid micellar media. The micellar solution was filtered through a 0.45 μm cellulose acetate filter before adding to cells. For lipid labeling experiments, 125 μCi [3H]-oleate (5 μCi of [3H]-oleate per well) was added to the Pyrex tube with the other lipids, followed by drying and resuspension as described above.

2.16. **Lipid Micelle Stimulation of Caco-2 Cells**

Differentiated Caco-2 cells were grown for 21 days and the basolateral media was replaced with serum-free EMEM immediately before lipid micelle stimulation. The apical compartment of the cells was washed with sterile 1xPBS that had been warmed to 37°C in a water bath. The apical compartment was supplemented with 400 μl of 1.6 mM oleate-containing lipid micellar media or with 400 μl EMEM supplemented with 20% FBS. The cells were incubated for 12 hours at 37°C in air and 5% CO2.
2.17. Lipid Extraction and Thin Layer Chromatography

Following the 12 hour lipid micelle stimulation, the basolateral media was transferred to 2 ml tubes. A volume of 600 μl of organic solvent hexane:isopropanol (3:2, v/v) was added to 1 ml of basolateral media and vortexed for 1 min. The media sat on ice for 30 min to allow separation of organic and aqueous phases. During this time, the apical and basolateral membrane of cells were washed with cold 1X PBS and 600 μl of hexane:isopropanol was added directly to the apical membrane of washed cells. After 30 min, the upper layer of organic solvent was carefully removed from the basolateral media by Pasteur pipette and transferred to a 1.5 ml tube and the lipid extraction was repeated. The organic solvent was removed from the apical membrane of the cells and transferred to a 1.5 ml tube and the lipid extraction was repeated. The extracted lipids were dried in a dessicator with vacuum connection (NALGENE® Labware, Thermo Fisher Scientific Inc., NY) for 12 hours and resuspended by adding 30 μl ice cold hexane and vortexing for 1 min. Solubilized lipids were spotted onto Silica plates in 2 μl volumes for a total of 10 μl for each sample. Each sample spot was overlayed with 2 μl of standards cholesteryl oleate, cholesterol (50 mg/ml), TG (1:20 dilution of olive oil), and oleate (10mM). Development was with petroleum ether: diethyl ether: glacial acetic acid (80:20:1) in a glass chamber for 90 minutes. The plates were air-dried at room temperature in and the lipid standards were visualized by iodine vapour. The lipid spots were cut out from the plates and placed into scintillation vials with 4 ml Ready Safe® liquid scintillation cocktail (Beckman Coulter, USA). Scintillation vials were vortexed for 1 min and allowed to sit overnight before counting in scintillation spectrometer.

2.18. Metabolic Pulse Labeling and Immunoprecipitation of ApoB

To assess newly synthesized apoB, differentiated Caco-2 cells were incubated in 20%-FBS containing methionine and cysteine-free DMEM for 1 hr (pre-pulse) before they were pulse
labeled with 50 μCi [35S]-methionine. The pulse media was removed from the apical compartment and replaced with chase media (20% FBS containing EMEM). Basolateral media was collected after a 3 hour chase period and subjected to immunoprecipitation. Anti-apoB-antibody (5 μl/ per ml of media) was added to the samples and incubated for 18 hours at 4°C on an orbital shaker. The following day, 50 μl of zysorbin was added to each sample and incubated at room temperature for 1 hour on an orbital shaker. The samples were subjected to centrifugation at 13,000 RPM for 10 min to pellet antibody-bound proteins. The supernatant was removed and saved for a subsequent immunoprecipitation of a control secretory protein used for normalization. The pellet was washed 3x10 min each with 1xPBS supplemented with PI, and finally resuspended in 50 μl 1x sample buffer containing β-mercaptoethanol. The sample was then centrifuged to pellet zysorbin. The supernatant containing labeled apoB was resolved by SDS-PAGE as described above, and the SDS-PAGE gels were dried onto filter paper using a gel dryer for approximately 2.5 hours. Protein bands were visualized by exposing the dried gels to a phosphoimager screen and quantified using ImageJ, as described above.

2.19. Chylomicron Density Ultracentrifugation

Isolation of secreted lipoproteins of different sizes was accomplished by using a method to isolate large chylomicrons, small chylomicrons, and VLDL from cell culture media by sequential ultracentrifugation (Haidari et al., 2002). The basolateral media from experiments where Caco-2 cells were stimulated with lipid micelles was adjusted to a density of 1.1 g/ml using KBr. The adjusted media was then overlaid with 3 ml of 1.006 g/ml KBr solution containing PI in a 4 ml ultracentrifuge tube. To obtain large CM (Sf > 400), samples were ultracentrifuged for 33 min at 40,000 rpm 10°C, and the top 333 μl was collected. The samples in the ultracentrifuge were then overlaid with 333 μl of 1.006 g/ml KBr solution. The gradient was spun again at 40,000 rpm at 10°C for 2 hours, and the top 333 μl containing small CM (Sf =
60–400) was collected. The gradient was then spun at 40,000 RPM for 17 hours at 10°C, and then top 333 μl, containing VLDL (Sr = 20–60) was collected.

2.20. Triglyceride, cholesterol, and liver enzyme assays

To measure triglyceride mass in different lipoprotein fractions, 10 μl of each fraction was used in a colourimetric assay from Roche according to the manufacturer’s protocol. For mice studies, plasma TG, cholesterol, and liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by colourimetric assay using the VITROS 250/350/950 and 5,1 FS Chemistry Systems and the VITROS 5600 Integrated System in the Clinical Biochemistry lab at The Hospital for Sick Children.

2.21. Cerulenin treatment

Cerulenin, a fatty acid analog, was used to inhibit fatty acid synthesis in Caco-2 cells. A dose response curves was generated to determine the optimal concentration of cerulenin to inhibit fatty acid synthesis. Cerulenin was added to the apical compartment of differentiated Caco-2 cells to obtain a final concentration of 0, 5, 10, or 15 μg/mL. The apical media was EMEM supplemented with 20% FBS, while the basolateral compartment media was replaced with serum-free EMEM. Five μCi of 3H-acetate was added to label newly synthesized fatty acids. After 12 hours, cellular lipids were resolved by TLC and counted, as described above. The optimal concentration was found to be 10 μg/mL and further experiments were conducted as above using 10 μg/mL cerulenin.

2.22. Statistical Analysis

Data were entered into, and all graphs were created using GraphPad Prism Software (Version 4, April 2003). Data were analyzed by two-way ANOVA or by student’s T-test where appropriate. A p-value less than 0.05 was considered significant.
CHAPTER 3

3. RESULTS

3.1. shRNA Mediated Knockdown of CD36 and FABP1 in Caco-2 Cells

After selection of Caco-2 cell clones that stably express control, or CD36- or FABP1-specific shRNA plasmids, the knockdown of CD36 or FABP1 gene products were verified by real-time quantitative polymerase chain reaction. Messenger RNA expression in each clone was normalized to 3 housekeeping (internal control) genes and expressed as a percentage of CD36 mRNA in the control puromycin transfected clones. Clones CD36-1 and CD36-3 had 3.66 fold (p=0.001) and 3.75 fold (p=0.04) level of CD36 knockdown, respectively (Fig. 3A). To assess protein knockdown in these clones, cell lysates were resolved by SDS-PAGE and immunoblotting for CD36 was performed (Fig. 3B). The trend in RNA knockdown correlated with the level of protein knockdown (except for shRNA-CD36-4) (Fig. 3C). Clones CD36-1 and CD36-3 express CD36 protein at 40% (p=0.02) and 60% (p=0.03), respectively, of control cell protein levels (Fig. 3C). One clone expressing FABP1-specific shRNA was quantified (Fig. 3D). This clone had an 18.8 fold decrease in expression of FABP1 at the mRNA level (Fig. 3D). Immunoblotting for FABP1 protein knockdown was not performed because we could not obtain an antibody for FABP1 that could detect FABP1 by immunoblot.
3.2. Depletion of CD36 or FABP1 does not impair FA uptake in Caco-2 cells

To examine whether CD36- or FABP1-deficiency results in impaired lipid uptake, Caco-2 cells were treated with $^3$H-oleate containing micelles, and uptake was measured after 1, 2, 4, and 12 hours (Fig. 4). There were no differences in $^3$H cellular counts between CD36- or FABP1-deficient Caco-2 cells and their respective controls at any time point (Fig. 4). Therefore, differences in fatty acid uptake do not contribute to the increase in TG secretion from CD36- or FABP1-deficient Caco-2 cells (Fig. 4).
3.3. CD36 or FABP1-Deficiency is Associated with Increased TG Secretion in Caco-2 Cells

CD36- or FABP1-deficient Caco-2 cells were stimulated with [3H]-oleate-containing lipid micelles to determine whether CD36- or FABP1-deficiency affects TG assimilation or secretion in Caco-2 cells. Assimilation of neutral lipids, TG and CE, were not affected by knockdown of CD36 or FABP1 in Caco-2 cells (Fig. 5A & B). Levels of FA and cholesterol were not different between CD36- or FABP1-deficient Caco-2 cells and their respective controls (Fig. 5A & B). Although intracellular [3H]-TG content was not different between control and knockdown cell lines, TG secretion from CD36 knockdown Caco-2 cells was significantly increased (Fig. 5C). A trend towards increased TG secretion was also observed in FABP1 knockdown cells compared to the control cells, although this was not significant (Fig. 5D). Secretion of CE, FA, or cholesterol was not significantly altered by knockdown of CD36 (Fig. 5C). Although secretion of CE and FA were not altered, secretion of cholesterol from FABP1
knockdown Caco-2 cells was significantly increased compared to control cells (Fig. 5D).

**Figure 5.** Lipid assimilation and secretion in CD36 & FABP1 knockdown Caco-2 cells. Cellular ³H-lipids in CD36- (A) and FABP1- (B) deficient Caco-2 cells. ³H-lipids secreted from CD36- (C) and FABP1- (D) deficient Caco-2 cells. The apical compartment of control or CD36, or FABP1 knockdown Caco-2 cells were treated with 1.6 mM oleate-containing lipid micelles also containing 5 μCi ³H-oleate for 12 hours. Lipids were extracted from the cells (A & B) or the basolateral media (C & D) using hexane:isopropanol (3:2), dried, and resuspended in hexane. Lipids were resolved by thin layer chromatography using petroleum ether:diethyl ether:acetic acid (80:20:2) as solvent. The ³H-oleate incorporation into each lipid was measured by scintillation counting. n=9, *p=<0.05.

### 3.4. TG Content of Lipoproteins Secreted by CD36- or FABP1-Deficient Caco-2 Cells

To examine which lipoprotein fraction contained the observed increase in TG and apoB, density gradient ultracentrifugation of the basolateral media was performed. Without lipid stimulation, the amount of TG present in the large CM and small CM lipoproteins secreted from control and CD36-deficient cells was not significantly different (Fig. 6). Interestingly, the amount of TG present in VLDL secreted from CD36 knockdown Caco-2 cells was significantly
greater than in control cells (Fig. 6). When control cells were treated with lipid, most of the TG was present in the CM fraction (Fig. 6). The same trend was observed in CD36 knockdown Caco-2 cells treated with lipid micelles, however, the small CM fraction contained more TG than in control cells (Fig. 6).

![Lipoprotein Triacylglycerol](image)

**Figure 6.** TG content of lipoproteins secreted from normal and CD36-deficient Caco-2 cells. The basolateral media from cells treated with (+) or without (-) 1.6 mM oleate-containing lipid micelles was subjected to density gradient ultracentrifugation to isolate large chylomicron (LG CM), small chylomicron (SM CM), and VLDL. To determine the mass of TG in each fraction, TG was detected in 10 µL from each fraction by colorimetric assay. n=3. *p<0.05 compared to the control VLDL fraction without lipid treatment.

### 3.5. **CD36 or FABP1-Deficiency in Caco-2 cells is Associated With an Increase in ApoB Secretion**

The basolateral media from experiments in which Caco-2 cells were treated with oleate-containing lipid micelles was used for immunoblot analysis of apoB. As expected, lipid stimulation increased apoB secretion in control cells and in CD36 knockdown Caco-2 cells (Fig. 7A & B). An increase in apoB secretion upon lipid stimulation was not observed in FABP1-
deficient Caco-2 cells (Fig. 7B). Under basal conditions, depletion of CD36 was associated with an increase in apoB100 secretion (Fig. 7A & C).

Metabolic labeling of proteins was used to examine apoB secretion under basal conditions. Caco-2 cells were pulse-labeled with $[^{35}S]$-methionine for one hour, followed by a 3 hour chase period (Fig. 7E & F). Radiolabeled apoB100 and apoB48 could be detected in these samples by immunoprecipitation of apoB using an anti-apoB antibody followed by resolution of bound proteins by SDS-PAGE. Under basal conditions, CD36-deficient Caco-2 cells secreted significantly more apoB100 and apoB48 compared to control cells (Fig. 7G). Compared to control cells, FABP1-knockdown Caco-2 cells secreted significantly more apoB100, while levels of secreted apoB48 remained the same (Fig. 7H).
Figure 7. Apolipoprotein B secretion from CD36 or FABP1 knockdown Caco-2 cells. Immunoblot of apoB100 secreted from CD36- (A) or FABP1- (B) deficient Caco-2 cells. Cells were treated with 1.6 mM oleate-containing lipid micelles for 12 hours, the basolateral media was resolved by SDS-PAGE. Quantification of immunoblots in A (C) & B (D). Metabolic labeling of apoB100 and apoB48 in CD36- (E) and FABP1- (F) deficient Caco-2 cells. Cells were labeled for 1 hr with [35S]-methionine and chased for 3 hours. ApoB was immunoprecipitated from the basolateral media and was resolved by SDS-PAGE. Quantification of phosphoimages in E (G) & F (H). n=6, *p<0.05, **p<0.01
3.6. **Expression of Genes Involved in FA Synthesis**

We hypothesized that depletion of either CD36 or FABP1 would impair lipoprotein assembly and secretion in Caco-2 cells. Upregulation of FA synthesis could lead to increased formation of neutral lipid and, in turn, may provide more substrate for apoB lipidation. We thus sought to determine the mechanisms responsible these paradoxical results. The expression of genes involved in FA synthesis, fatty acid synthase (FAS) and acetyl coA carboxylase1 (ACC1), was examined.

Cells were treated for 12 hours with 1.6 mM oleate-containing lipid micelles, lysates were resolved, and immunoblotting for FAS was performed. FAS levels were normalized to the amount of E-Cadherin, a loading control protein (Beaslas et al., 2009). In CD36 control cells (Fig. 8A), FAS expression increased with lipid micelle stimulation, while in FABP1 control cells (Fig. 8B), levels of FAS remained the same with or without lipid stimulation. The differences in response to lipid stimulation in the control cell lines and this may be due to variation in mRNA levels in different control cell preparations (Fig. 8C and 8D). Under basal conditions, an increase in FAS expression in both CD36 and FABP1 knockdown cells was observed, compared to their respective controls (Fig. 8A & B). Levels of FAS did not change significantly in either CD36 or FABP1 knockdown cells when treated with lipid micelles (Fig. 8A & B).
Levels of ACC1 mRNA were measured in control and knockdown cells by real-time PCR. Control cells that had been treated with lipid for 12 hours had a significantly reduced level of ACC1 mRNA, compared to control cells that were not treated with lipid (Fig. 9A & B). Surprisingly though, significantly less ACC1 mRNA was detected in both CD36 and FABP1 knockdown cells compared to their respective controls under basal conditions (Fig. 9A & B). A trend of decreased ACC1 mRNA was observed in both knockdown cell lines compared to their controls when treated with lipid, but this was statistically significant only in CD36 knockdown cells (Fig. 9A & B).
Since FAS levels were increased under basal conditions in both CD36- and FABP1-deficient Caco-2 cells, we decided to investigate whether de novo lipogenesis (endogenous FA synthesis) was increased, and if cerulenin (a FAS inhibitor) would inhibit FA synthesis in these cells.

### 3.7. Cerulenin Decreases FA Synthesis in Caco-2 Cells

Differentiated Caco-2 cells were treated overnight with acetate and varying concentrations of cerulenin. In both control and knockdown Caco-2 cells, doses of 5 or 10 μg/ml cerulenin reduced [³H]-acetate incorporation into FAs (Fig. 10). In CD36 control cells (Fig. 10A), inhibition of [³H]-acetate incorporation into FAs was greatest at 5 μg/ml cerulenin, and cerulenin decreased the [³H]-acetate incorporation into TG. Levels of CE and cholesterol decreased as the concentration of cerulenin was increased (Fig. 10A). FA synthesis was decreased in a dose-dependent manner in CD36 knockdown cells (Fig. 10B), however, it was only decreased by approximately 30% at 5 μg/ml cerulenin as compared to a 60% decrease in FA synthesis observed in control cells (Fig 10A). Similar to control cells, [³H]-acetate incorporation into CE, cholesterol, and TG were decreased with cerulenin treatment in CD36

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**Figure 9.** Real-time PCR measurement of ACC1 mRNA in control and CD36 or FABP1 knockdown Caco-2 cells. Differentiated Caco-2 cells were treated with or without 1.6 mM oleate-containing lipid micelles for 12 hours. Ct values for ACC1 were normalized to an internal control gene, 18S, and expressed as fold-change compared to their respective controls that were not treated with lipid. n=9, **p<0.01, compared the control without lipid treatment.
knockdown cells (Fig 10B). We observed a similar level of inhibition of FA synthesis in control and CD36 knockdown Caco-2 cells, therefore a concentration of 10 μg/ml was used in further experiments. Also, our laboratory had previously used a concentration of 10 μg/ml cerulenin in primary enterocytes to successfully inhibit FA synthesis (Haidari et al., 2002).

**Figure 10.** Dose-dependent effect of different concentrations (0, 5, 10, 15 mg/ml) of cerulenin, a FA synthase inhibitor, on incorporation of [3H]-acetate into CE, TG, FA, and CH in control Caco-2 cells (A & C) and CD36 knockdown (B) or FABP1 knockdown (C) Caco-2 cells. Lipids were extracted from the cells using hexane: isopropanol (3:2), dried, and resuspended in hexane. Lipids were resolved by thin layer chromatography using petroleum ether: diethyl ether: acetic acid (80:20:1) as solvent. The [3H]-acetate incorporation into each lipid species was assessed by scintillation counting. [3H]-acetate incorporation into lipid species at different concentrations of cerulenin are expressed as a percent of the untreated condition. n=3.

Cerulenin treatment also inhibited FA synthesis in FABP1 control cells, as measured by [3H]-acetate incorporation into FA (Fig. 10C). However, the dose-dependent decrease in [3H]-acetate incorporation into CE, TG or FA, was not observed in FABP1 control cells (Fig. 10C). FA synthesis was also decreased by approximately (80%) in FABP1 knockdown Caco-2 cells by 5 μg/ml cerulenin treatment (Fig. 10D). At 10 μg/ml cerulenin treatment, [3H]-acetate
incorporation into FA increased to approximately 60% of untreated cell levels (Fig. 10D). In FABP1 knockdown Caco-2 cells, [³H]-acetate incorporation into cholesterol, TG and CE were decreased in a dose-dependent manner (Fig. 10D). We next determined if an increase in de novo lipogenesis may be responsible for the increase in TG and apoB secretion observed in CD36 or FABP1 knockdown Caco-2 cells.

3.8. De novo Lipogenesis May Contribute to the Increase in TG or ApoB

Secretion Observed in CD36, but not FABP1-Deficient Caco-2 Cells

The incorporation of [³H]-acetate into CE, TG, and FA was examined in differentiated control and knockdown Caco-2 cells after a 12 hour treatment with or without 1.6 mM oleate-containing lipid micelles and with or without 10 μg/ml cerulenin. The incorporation of [³H]-acetate into CE was increased following treatment with oleate-containing lipid micelles in CD36- or FABP1-deficient Caco-2 cells and in their respective control cell lines (Fig. 11A & B). This is expected, as the availability of FA substrate for esterification of cholesterol would increase with oleate supplementation. Overall, cerulenin had no effect on the incorporation of [³H]-acetate into CE except in FABP1 knockdown cells, where cerulenin treatment decreased the incorporation of [³H]-acetate into CE when the cells were also treated with lipid micelles (Fig. 11B).

The incorporation of [³H]-acetate into TG followed a similar trend in CD36 and FABP1 knockdown, and the FABP1 control cell lines. That is, with lipid micelle supplementation, [³H]-acetate incorporation into TG increased significantly, although this was not seen in the CD36 knockdown control cells in the absence of cerulenin treatment (Fig. 11C & D). Cerulenin treatment had no major effect on [³H]-acetate incorporation into TG in CD36 knockdown cells, however, in their respective control cell line, cerulenin treatment decreased [³H]-acetate incorporation into TG in the absence of lipid stimulation (Fig. 11C).
The objective of this experiment was to determine whether CD36 or FABP1 knockdown cells have an increase in *de novo* lipogenesis compared to their respective controls. With no lipid stimulation or cerulenin treatment, the level of *de novo* lipogenesis was not different between control and CD36 knockdown cell lines (Fig. 11E, first and fifth bars). When the cells were treated with lipid, there was a trend towards an increased level of FA synthesis in CD36 knockdown cells compared to control cells (Fig. 11E, second and sixth bars). When CD36 knockdown cells were treated with cerulenin, FA synthesis was significantly greater than in control cells (Fig. 11E).

Surprisingly, the level of *de novo* lipogenesis was decreased in FABP1 knockdown cells compared to their control (Fig. 11D, first and fifth bars). Cerulenin treatment decreased the incorporation of [³H]-acetate into FA, an indication that FAS was inhibited (Fig. 10E & F), the exception being in FABP1 knockdown cells, where cerulenin had no effect on the already blunted incorporation of [³H]-acetate into FA (Fig. 11F).

There were no significant differences between the level of secreted [³H]-CE from CD36 or FABP1 knockdown cells and their respective control cell lines (Fig. 12A & B). Without cerulenin treatment, secreted [³H]-TG levels from CD36 knockdown cells were increased compared to controls, while cerulenin treatment reduced the level of secreted [³H]-TG to control levels (Fig. 12C). There were no significant differences between secreted [³H]-TG from FABP1 knockdown cells compared to the control with or without cerulenin treatment (Fig. 12D). There were no significant differences between secreted [³H]-FA from CD36 knockdown cells compared to the control with or without cerulenin treatment (Fig. 12E). FABP1 knockdown cells secreted significantly less [³H]-FA with cerulenin treatment (Fig. 12F).
Figure 11. Cellular $[^3\text{H}]$-lipids extracted from CD36 or FABP1 knockdown Caco-2 cells. A & B. Cellular $[^3\text{H}]$-esterified CH. C & D. Cellular $[^3\text{H}]$-TG. E & F. Cellular $[^3\text{H}]$-FA. The apical compartment of control or CD36 or FABP1 knockdown Caco-2 cells were treated with (+) or without (-) cerulenin (10μg/ml), and with (+) or without (-) 1.6 mM oleate-containing lipid micelles and 10μCi $[^3\text{H}]$-acetate for 12 hours. Lipids were extracted from the cells using hexane: isopropanol (3:2), dried, and resuspended in hexane. Lipids were resolved by thin layer chromatography using petroleum ether: diethyl ether: acetic acid (80:20:2) as solvent. The $[^3\text{H}]$-acetate incorporation into each lipid species was measured by scintillation counting. Data is plotted as mean DPM per mg protein ± SEM. Comparisons are indicated by lines and statistical significance was determined by two-way ANOVA. n=3, *p<0.05, **p=<0.001.

Overall, these results suggest that de novo lipogenesis may be contributing to the increase in TG or apoB secretion in CD36 knockdown Caco-2 cells (Fig 10E & F). Cerulenin treatment showed a trend towards decreased cellular FA in CD36 knockdown cells treated with lipid (Fig. 11, bars six and eight). Finally, cerulenin did not have an effect on the secretion of $[^3\text{H}]$-acetate labeled lipids from Caco-2 cells (Fig. 12).
Figure 12. Secreted $^3$H-lipids extracted from the medium CD36 or FABP1 knockdown Caco-2 cells. A & B. Cellular $^3$H-esterified CH. C & D. Cellular $^3$H-TG. E & F. Cellular $^3$H-FA. The apical compartment of control of CD36 or FABP1 knockdown Caco-2 cells were treated with (+) or without (-) Cerulenin (10$\mu$g/ml) to inhibit FA synthesis, and with (+) or without (-) 1.0mM oleate-containing lipid micelles and 10$\mu$Ci $^3$H-acetate for 12 hours. Lipids were extracted from the cells using hexane:isopropanol (3:2), dried, and resuspended in hexane. Lipids were resolved by thin layer chromatography using petroleum ether:diethyl ether:acetic acid (80:20:2) as solvent. The $^3$H-acetate incorporation into

3.9. **CD36$^+/-$ Mice Have Impaired cholesterol and TG Production**

We observed that de novo lipogenesis may be contributing to the increase in TG and apoB secretion in CD36-deficient Caco-2 cells so we wanted to test the hypothesis that an impairment
in de novo lipogenesis contributes to the dyslipidemia observed in the CD36<sup>−/−</sup> mouse. CD36<sup>−/−</sup> mice were anesthetized, the intestine was removed and primary enterocytes were isolated using an established method (Haidari et al., 2002). The cells were radiolabeled with either [³H]-acetate, or [³H]-oleate. The cellular and secreted lipids were then analyzed. Overall, we observed that cholesterol synthesis (Fig. 13A) and TG synthesis (Fig. 13B) were significantly decreased in CD36<sup>−/−</sup> mouse enterocytes, compared to their WT littermates.

**Figure 13.** Lipogenesis in enterocytes isolated from WT and CD36<sup>−/−</sup> mice. Enterocytes were isolated from WT and CD36<sup>−/−</sup> animal and resuspended in serum-free DMEM. Cells were pulse labeled with A. 5μCi [³H]-acetate or B. 1.0mM oleate-BSA + 5μCi [³H]-oleate. After 3 hour labeling period, lipids were extracted from the cells (A & B) or the medium (C) using hexane: isopropanol (3:2), dried, and resuspended in hexane. Lipids were resolved by thin layer chromatography using petroleum ether: acetoc acid (80:20:2) as solvent. The ³H-acetate or ³H-oleate incorporation into each lipid was assessed by measuring radioactivity by scintillation counting. Data is expressed as a percent of WT ³H-acetate or ³H-oleate incorporation. (D) Plasma ALT (U/l) (E) Plasma ALT (U/l) (F) Plasma CH (mmol/l) (G) Plasma triglyceride (mmol/l). n=3, *p<0.05, **p<0.01, ***p<0.001 are statistically significant compared to WT based on a student’s t-test.
plasma levels of liver enzymes AST and ALT. There were no significant differences in plasma levels of liver enzymes AST and ALT (Fig. 13D & E) or in levels of TG or cholesterol (Fig. 13F & G) between CD36^-/- and WT mice. We also wanted to confirm our findings from Caco-2 cells that CD36 deficiency is associated with an increase in TG secretion. In contrast to the increase secretion observed from CD36-deficient Caco-2 cells, we found that TG secretion from CD36^-/- enterocytes was decreased compared to WT enterocytes (Fig. 13C).

It is possible that in CD36-deficient Caco-2 cells, an increase in FA substrate would feed into TG synthesis pathways. In FABP1-deficient Caco-2 cells, there was no observed increase in FA synthesis, as measured by [3H]-acetate incorporation into FA, although FAS levels were increased. Thus, protein levels of enzyme involved in TG synthesis were measured next.

3.10. Cellular levels of proteins involved in TG synthesis in CD36- and FABP1-deficient Caco-2 cells

To determine if there was an upregulation of proteins involved in TG synthesis, we measured levels of fatty acid transport protein 4 (FATP4) and diacylglycerol acyltransferase (DGAT). FATP4 is a membrane protein found in the plasma membranes and ER membranes of the small intestine and has both FA transport and acyl-coA synthetase activities (Hall et al., 2005), allowing it to convert long-chain FAs to fatty acyl-CoA. Lipid treatment increased levels of FATP4 in control cells and in FABP1 knockdown cells (Fig. 14A & B). Under basal conditions, FATP4 was increased in both CD36 and FABP1 knockdown cells compared to control cells (Fig. 14A & B). Lipids did not have a stimulatory effect on FATP4 protein levels in CD36-deficient Caco-2 cells (Fig. 14A). The increase in FATP4 under basal conditions might provide more activated substrate (fatty acyl-CoA) for the enzymes in either the MG or G3P pathways of TG synthesis (Fig. 2).
The synthesis of TG is mediated by two diacylglycerol transferases, DGAT1 and DGAT2. DGAT1 knockout mice can synthesize TG and have normal serum TG levels, even when fed a high fat diet (Buhman et al., 2002). DGAT2, on the other hand, is important for TG synthesis and is essential for survival (Stone et al., 2004). In control cells, DGAT2 expression was decreased by lipid supplementation (Fig. 15), and in both knockdown cell lines, DGAT2 mRNA levels were decreased under basal and lipid stimulated conditions compared to their respective controls (Fig. 15).

Figure 14. FATP4 protein expression levels in CD36 and FABP1 knockdown cells. Cells were treated with or without 1.6 mM oleate containing micelles and harvested after 12 hours. Representative immunoblot of cell lysates from CD36- (A) or FABP1- (B) deficient Caco-2 cells using an anti-FATP4 antibody. C & D. FATP4 protein expression was normalized to the loading control, E-Cadherin. n=9, *p<0.05.
To further investigate mechanisms responsible for the increase in TG and apoB secretion observed from CD36 and FABP1 knockdown Caco-2 cells, we examined the expression of genes or proteins involved in chylomicron assembly.

3.11. Factors involved in chylomicron assembly in CD36- and FABP1-deficient Caco-2 cells

Differentiated CD36 and FABP1 knockdown Caco-2 cells were treated with or without 1.6 mM oleate-containing micelles for 12 hours, after which protein or RNA were isolated. The mRNA expression or protein level of proteins involved in chylomicron assembly, such as microsomal triglyceride transfer protein (MTP), Sar1 GTPase and ApoAIV was examined.

MTP possesses lipid transfer activity and plays a role not only in the initial lipidation of newly translated apoB with ER-membrane bound TG (Black, 2007), but also in the core expansion of the chylomicron particle. CD36 and FABP1 knockdown Caco-2 cells were probed for MTP, however, MTP protein levels were variable among the samples, and the expected

Figure 15. Real-time PCR measurement of DGAT2 mRNA in control and CD36 or FABP1 knockdown Caco-2 cells. Differentiated Caco-2 cells were treated with or without 1.0mM oleate-containing lipid micelles for 12 hours. Ct values for DGAT2 were normalized to an internal control gene, 18S, and expressed as fold-change compared to their respective untreated controls. n=9, *p<0.05.
increase in MTP after lipid stimulation was not observed, except in CD36 knockdown cells (Fig. 16A).

Figure 16. Microsomal triglyceride transfer protein levels in CD36 and FABP1 knockdown cells. Cells were treated with or without 1.6 mM oleate-containing micelles and harvested after 12 hours. Representative immunoblot of cell lysates from CD36- (A) or FABP1- (B) deficient Caco-2 cells using an anti-MTP antibody. C & D. MTP protein expression was normalized to the loading control, beta actin. n=6, *p<0.05, **p<0.01.

Nascent chylomicrons exit the ER in a specialized transport vesicle, the prechylomicron transport vesicle or PCTV. Work by Charles Mansbach’s group has shown that PCTVs can bud from the ER in a COPII independent manner, but that the trafficking and fusion with the Golgi are dependent on Sar1, Sec23/24, and its unique SNARE complex (Siddiqi et al., 2006). Sar1 expression was examined in CD36 and FABP1 knockdown cells, and in all cells, Sar1 levels
increased slightly after lipid micelle stimulation (Fig. 17). However, there were no differences in levels of Sar1 between knockdown cells and their respective controls (Fig. 17).

ApoAIV is a 46 kDa glycoprotein that associates with nascent chylomicrons in the ER and is secreted on the surface of chylomicrons at the basolateral membrane (Black, 2007). It has been proposed to play a role in the enlargement and stabilization of the maturing chylomicron (Black, 2007). The control cells for each knockdown cell lines responded differently to lipid stimulation, that is, apoAIV mRNA levels decreased in the CD36 KD control cell line (Fig. 18A) and increased significantly in the FABP1 KD control cell line (Fig. 18B). There were no statistically significant differences in the amount of apoAIV mRNA between the knockdown

Figure 17. Sar1 GTPase protein expression levels in CD36 and FABP1 knockdown cells. Cells were treated with or without 1.6 mM oleate-containing micelles and harvested after 12 hours. Representative immunoblot of cell lysates from CD36- (A) or FABP1- (B) deficient Caco-2 cells using an anti-Sar1 antibody. C & D. Sar1 protein expression was normalized to the loading control, E-Cadherin. n=9, *p<0.05, **p<0.01.

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cells and their respective control, untreated cells (Fig. 18). Changes in the expression of proteins involved in chylomicron assembly may not explain the increase in TG and apoB secretion that we observe in the CD36 and FABP1 knockdown cell lines.

**Figure 18.** Real-time PCR measurement of ApoAIV mRNA in control and CD36 or FABP1 knockdown Caco-2 cells. Differentiated Caco-2 cells were treated with or without 1.0mM oleate-containing lipid micelles for 12 hours. Ct values for ApoAIV were normalized to an internal control gene, 18S, and expressed as fold-change compared to their respective controls that were not treated with lipid. n=9, **p<0.01.
CHAPTER 4

4. DISCUSSION

4.1. shRNA Mediated Knockdown of CD36 and FABP1 in Caco-2 Cells

The discovery of RNA interference (RNAi) in 1998 (Fire et al., 1998) brought about exciting opportunities for elucidating the role of your gene of interest. RNAi is a conserved process of double-stranded RNA post-transcriptional gene expression modulation. Since its discovery, RNAi technology has been harnessed for uses in cell biology and therapeutics. With the improved algorithms for the design of short interfering and short hairpin RNA sequences, investigators can attribute the observed phenotype to the loss of gene of interest.

We employed the use of shRNA plasmids, containing sequences complementary to CD36 and FABP1 to study the effect of knockdown of these proteins on intestinal lipid absorption and lipoprotein assembly. CD36 and FABP1 mRNA levels were reduced by more than 70% in Caco-2 cells stably expressing the shRNA plasmids compared to control cells stably expressing the scrambled shRNA plasmid (Fig. 3A &D). CD36 protein was found to be decreased by less than 60% of control cell levels (Fig 3C). As mentioned in Section 4.1., there is no commercially available anti-FABP1 antibody that is suitable for detection of FABP1 by immunoblot.

Researchers have struggled to delineate the role of CD36 in chylomicron production because its intestinal function is complicated by its functions in peripheral tissues. The intestinal specific contribution of FABP1 to lipoprotein metabolism is not clear in studies of FABP1−/− mouse, however, Mansbach and colleagues, showed that FABP1 is important for chylomicron secretion, since FABP1-depleted enterocyte cytosol had a decreased capacity to bud nascent chylomicrons from the ER (Neeli et al., 2007). Therefore, the development of these
cell lines should aid in the resolution of the role of CD36 and FABP1 in intestinal chylomicron assembly.

4.2. **CD36- or FABP1-deficiency in Caco-2 cells did not impair fatty acid uptake**

Fatty acid uptake was measured by treating cells with [³H]-oleate-containing lipid micelles and measuring the amount of radioactivity in the cells after 1, 2, 4, or 12 hours (Fig. 4). At every time point, we did not observe a difference in oleic acid uptake by CD36-deficient Caco-2 cells compared to their control cells (Fig. 4A). Several studies have implicated CD36 in the absorption of very-long chain fatty acids (Drover et al., 2008d; Nassir et al., 2007). Drover, et al. observed that FA absorption in CD36 expressing COS-7 cells was directly related to the length of the acyl chain when the FA backbone was greater than 18 carbon atoms in length (Drover et al., 2008c). However, there were no differences in absorption properties of oleic acid (18:0), and palmitic acid (16:0). We did not measure the uptake of very long chain fatty acids in the current study. In contrast, a study by Nassir and colleagues suggests that CD36 is important for oleic acid uptake. Isolated enterocytes from WT and CD36⁻/⁻ mice were incubated with different ratios of FA/BSA for 0 to 30 min to examine FA uptake (Nassir, et al., 2007). CD36-deficient enterocytes had significantly reduced levels of oleic acid uptake between 5 and 30 minutes, compared to WT enterocytes (Nassir et al., 2007). We conclude that CD36-deficiency does not impair oleic acid uptake in Caco-2 cells, and these results are in line with previous studies in cell culture (Drover et al., 2008b).

Fatty acid uptake was not different, at any measured time point, between FABP1-deficient Caco-2 cells compared to its control cell line (Fig. 4B). Previous studies have shown that FABP1 enhances fatty acid uptake (McArthur et al., 1999; Prows et al., 1995; Wolfrum et
al., 1999). Deficiency of FABP1 has not been shown to affect the absorption of oleic acid. Our studies indicate that in Caco-2 cells, FABP1-deficiency does not impair FA uptake.

4.3. CD36- or FABP1-deficiency in Caco-2 cells is associated with an increase in TG and ApoB secretion

The intracellular lipid content, as measured by [3H]-oleate labelling experiments, was not significantly different in either CD36- or FABP1-deficient Caco-2 cells (Fig. 5A & B). However, there was a significant increase in TG secretion from CD36-deficient cells with lipid stimulation (Fig. 5A). A number of studies have documented a postprandial increase in plasma TG in both CD36−/− mice and humans (Masuda et al., 2008; Miyaoka et al., 2001), however, some investigators attribute this to impaired clearance of lipoprotein TG (Masuda et al., 2008).

Masuda, et al. investigated the mechanisms of postprandial hypertriglyceridemia observed in CD36-states (Masuda et al., 2008). Patients with CD36-deficiency displayed increased FA, TG and apoB48 levels in both fasting and postprandial states (Miyaoka et al., 2001). After an oral fat load, there was a delayed peak in plasma TG and apoB48 in CD36-deficient patients (Masuda et al., 2008; Miyaoka et al., 2001), suggesting that there is either an overproduction of CM or impaired clearance of CM remnants in CD36-deficient states (Masuda et al., 2008). These results support those observed in a study by Drover, et al. who showed that TG secretion into the lymph was delayed in CD36−/− mice compared to wild type (Drover et al., 2005). It seemed that there may be a defect in lipoprotein lipase activity, since the size of CM particles (measured by electron microscopy) were much larger (from 110nm to 200-700nm in diameter) in the plasma of CD36−/− mice (Drover et al., 2005). However, Masuda, et al. measured lipase activity in the plasma of CD36−/− and wild type animals and found no difference in either lipoprotein lipase or hepatic lipase activity (Masuda et al., 2008). This supports the
premise that synthesis of lipoproteins is increased in the intestines of CD36-deficient animals and humans.

In our study, FABP1-deficient Caco-2 cells treated with lipid showed a trend towards increased TG secretion (Fig. 5B). These are consistent with another study examining intestinal lipid output in FABP1−/− mice (Newberry et al., 2003). When TG secretion rates were examined using tyloxapol, there were no apparent differences in intestinal TG output between FABP1−/− and WT mice (reported as unpublished observations in (Newberry et al., 2003)).

Using two different approaches; quantifying apoB mass by immunoblotting or radiolabelling newly synthesized apoB, we have shown that under basal conditions, knockdown of FABP1 results in increased secreted apoB100, and that knockdown of CD36 results in increased apoB100 and apoB48 secretion in Caco-2 cells. It is well established that apoB secretion is enhanced by lipid stimulation (reviewed in (Ginsberg and Fisher, 2009)). TG and phospholipids act to stabilize the nascent apoB polypeptide, thereby rescuing it from degradation pathways (reviewed in (Fisher and Ginsberg, 2002)). Although Liao and Chan have shown that apoB does not undergo intracellular degradation in Caco-2 cells (Liao and Chan, 2000), our results are consistent with the former observations. Scrambled shRNA control Caco-2 cells secrete apoB under basal conditions, and the secretion of apoB is enhanced upon lipid micelle stimulation (Fig. 6A and B). Interestingly, CD36-deficient Caco-2 cells secrete significantly more apoB than control cells under basal conditions (Fig. 6A). When stimulated with lipid, there is an increase in the amount of secreted apoB, however, this is comparable to lipid-stimulated control cell amounts (Fig. 6A).

A potentially novel finding from these studies is the absence of lipid-induced enhancement of apoB secretion from FABP1-deficient Caco-2 cells (Fig. 6B). Newberry, et al., reported no differences in plasma apoB100 or apoB48 from FABP1−/− mice compared to WT mice (Newberry et al., 2003). The link between FABP1 and apoB secretion has been
investigated by Mansbach and colleagues, who found that FABP1 was necessary for the exit of nascent chylomicrons (or prechylomicrons) from the ER (Neeli et al., 2007). In these studies, WT mouse enterocytes were pre-incubated with $[^3$H]-oleate for 30 minutes, followed by isolation of the ER. Nascent chylomicron particles were budded from the ER by incubation with WT or FABP1$^{-/-}$ cytosol. FABP$^{-/-}$ cytosol provided only 60% of the budding activity of WT cytosol, indicating that FABP1 plays a role in the budding of the prechylomicron particle (Neeli et al., 2007). The decrease in TG output from the ER treated with FABP1$^{-/-}$ cytosol, and the authors are currently investigating whether this is a result of a decrease in number of particles, or a decrease in the size of the budded prechylomicron particles (Neeli et al., 2007).

In our study, depletion of FABP1 resulted in a greater amount of secreted TG, but the same amount of apoB. A possible explanation for these observations is that FABP1 restricts the size of nascent chylomicron particles exiting the ER. This scenario would allow for the formation of bigger, but not more, prechylomicron particles, and explain the absence of the lipid-induced enhancement in apoB secretion.

4.4. Depletion of CD36 in Caco-2 cells results in a shift in secreted lipoprotein profile

The TG in large and small CM, and in VLDL particles secreted from Caco-2 cells was measured (Fig. 6). Under basal conditions, Caco-2 cells secrete small, dense lipoproteins of HDL size (Levy et al., 1995). We also made this observation in our control Caco-2 cell line. The three fractions of lipoproteins analyzed contained very little TG in control cells that were not stimulated with lipid (Fig. 6). However, Fatma, et al. showed that when stimulated with an appropriate concentration of oleate and taurocholate-containing lipid micelles, Caco-2 cells secrete large CM particles (Fatma et al., 2006). The TG content in the CM fraction of control cells that were with stimulated with lipid micelles was significantly greater than in control cells
in the basal condition. This indicates that the control Caco-2 cells can assemble TG-rich large CM-sized particles (Fig. 6).

In CD36-deficient Caco-2 cells, there is a significant increase in the TG content of the VLDL fraction compared to control cells under basal conditions (Fig. 6). This was an unexpected observation, and would require a significant upregulation of de novo TG synthesis. The culture conditions used expose Caco-2 cells to a high concentration of FA (20% FBS), therefore, there may have been an increase in esterification of FA to neutral lipids.

In mice lacking CD36, there is a shift in particle size from LDL to VLDL in the fasting state (Febbraio et al., 1999). The authors attributed this increase in particle size to a defect in peripheral fatty acid utilization. As mentioned above, a deficiency in lipoprotein lipase activity was suggested to account for the defect in uptake by peripheral tissues, although no change in activity was noted (Masuda et al., 2008). In a more recent study, fasting and postprandial lipoprotein profiles were examined in CD36−/− mice (Masuda et al., 2008). The fasting TG content of the VLDL-sized fraction was significantly higher in CD36−/− mice (Masuda et al., 2008). In fact, when lipoprotein lipase activity was blocked by an inhibitor of Lpl, triton WR-1339, fasting plasma TG levels were not different between wild type and CD36−/− animals (Masuda et al., 2008). This supports the notion that hepatic lipoprotein synthesis is not different between wild type and CD36−/− animals, hence, the difference in fasting levels of VLDL-associated TG may be attributed to intestinal output.

In our CD36-deficient Caco-2 cell model, we observed an increase in VLDL-associated TG secreted under basal conditions, and an increase in small CM-associated TG secreted under lipid stimulated conditions. In the postprandial state, significant increases in CM, VLDL, and LDL TG was observed in CD36−/− mice (Masuda et al., 2008). Furthermore, inhibition of Lpl activity after an oral fat load resulted in a significant accumulation of TG in the plasma of CD36−/− mice compared to wild type (Masuda et al., 2008). Perhaps, upon lipid stimulation,
there is an even further induction of the assembly of buoyant particles (>VLDL in size) in CD36-deficient states. We examined this possibility by measuring levels of de novo lipogenesis and proteins involved in the assembly of chylomicrons.

4.5. Evidence for an increase in de novo lipogenesis in CD36-deficient Caco-2 cells

FAS protein levels were elevated under basal conditions in both CD36- and FABP1-deficient Caco-2 cells compared to control Caco-2 cell levels (Fig. 8). When stimulated with lipid, FAS levels increased in CD36 knockdown control cells and in FABP1-deficient cells (Fig. 8C & D). No change was observed in CD36-deficient cells or in FABP1 knockdown control cells (Fig. 8C &D). This suggests that, under basal conditions, de novo lipogenesis is increased in CD36- and FABP1-deficient Caco-2 cells. In animals, FAS is transcriptionally regulated by feeding and insulin. FAS expression is stimulated by insulin in the liver (Paulauskis and Sul, 1989) and by leptin in adipose tissue, through the action of and sterol regulatory element binding protein-1c (SREBP-1c) and Upstream Stimulatory Factor (USF) (Latasa et al., 2003). FAS expression is also indirectly regulated by fatty acids. Studies in HepG2 cells showed that SREBP-1-activated gene transcription was decreased by supplementation with oleic acid and other polyunsaturated fatty acids, while saturated fatty acids did not regulate SREBP-1-activated genes (Worgall et al., 1998). Thus, we would expect control Caco-2 cells treated with oleate-containing lipid micelles to have decreased levels of FAS compared to basal conditions. The presence of taurocholate in the culture media may also provide an explanation for this observation, since transcription of FAS is also regulated by bile acids through an FXR dependent mechanism (Matsukuma et al., 2006). It is unclear why FAS expression in the control cell lines did not respond to lipid micelle stimulation in the same way.
The reaction catalyzed by ACC is the first committed step in FA synthesis, converting malonyl CoA to acetyl CoA. ACC1 expression is controlled by at least three promoters, PI-PIII, and key transcription factors include sterol-regulatory-element-binding protein 1C (SREBP1C) as well as liver X receptor, retinoid X receptor, PPARs, FOXO, and PPARγ co-activator (Brownsey et al., 2006). Levels of ACC mRNA were significantly reduced in control and CD36- and FABP1-deficient Caco-2 cells treated with oleate-containing lipid micelles (Fig. 9A and B). Like FAS, ACC expression is controlled by SREBP1C (Brownsey et al., 2006). Therefore, the above results are consistent with the regulation of ACC1 by SREBP1C. On the other hand, we observed a decreased in ACC1 expression in CD36 and FABP1 knockdown Caco-2 cells under basal conditions (Fig. 9A and B). Reduced activity of ACC would limit the supply of malonyl-CoA, the essential substrate for fatty acid synthesis.

Our observations that an increase in FAS protein levels under basal conditions led us to hypothesize that there may be an increase in basal levels of FA synthesis. However, the expression of ACC does not support this hypothesis. To directly measure the level of FA synthesis in Caco-2 cells, we labeled cells with [3H]-acetate and measured its incorporation into FA, CE, and TG (Fig. 11). Oleate treatment significantly reduced FA synthesis in CD36 control cells. Interestingly, FA synthesis in CD36-deficient Caco-2 cells seemed resistant to the inhibitory effects of either FA or cerulenin, however, FA and cerulenin together modestly reduced FA synthesis (Fig. 11E). The observation that CD36-deficient cells are more resistant to cerulenin treatment than control Caco-2 cells points to a role for CD36 in the targeting of FA to the appropriate subcellular locations. Moreover, CD36 may act in FA targeting or signalling, and its ablation could result in a loss of FAS feedback inhibition.

As mentioned above, evidence from our lab suggests that upon induction of an insulin resistant state, there is an increase in FA synthesis in the intestine as measured by [3H]-oleate incorporation into FA (Haidari et al., 2002). The increase in FA synthesis was associated with
the observed fasting and postprandial increase in TG and apoB48 secretion observed in insulin resistant hamsters (Haidari et al., 2002). Interestingly, Masuda, et al. showed that mRNA levels of FAS were increased in the intestines of CD36<sup>−/−</sup> mice in both the fasted and postprandial conditions, although they did not measure protein levels or FA synthesis directly (Masuda et al., 2008).

These studies both support the idea that FA biosynthesis is susceptible to both dietary and genetic influences and may perturb normal FA metabolism. How CD36 and FABP1 are involved in the regulation of FA metabolism in the intestine has yet to be investigated. It is tempting to speculate that these lipid binding proteins are involved in the targeting of fatty acids to their appropriate metabolic fate. CD36 and FABP1 may interact with other proteins to accomplish their role in targeting of FA. For example, FABP1 has been shown to directly interact with PPARα, which induces a number of genes involved in lipid metabolism (Hostetler et al., 2009). Furthermore, the observed interaction between FABP1 and PPARα suggest that FABP1-bound ligands may be transferred to PPARα (Hostetler et al., 2009). PPARα induces a number of genes involved in the oxidation of fatty acids. If FABP1 is responsible for the delivery of FA to induce PPARα-mediated activation of genes, and ultimately the oxidation of FA, its absence could reduce the level of FA oxidation in the cell.

We propose that CD36 is acting as a lipid sensor in Caco-2 cells, such that its absence perturbs endogenous FA metabolism. SREBP1 induces transcription of genes involved in de novo lipogenesis, including FAS, ACC1 and SCD1. FA and cholesterol are suppressors of de novo FA synthesis pathway through inhibition of cleavage of SREBP1. Moreover, we suggest that CD36 normally functions to sense FA and cholesterol. In support of this, another member of scavenger receptors, SR-B1, plays a role in lipid sensing in the intestine (Beaslas et al., 2009). Perhaps CD36 and FABP1 function together, and the absence of one of these key regulatory molecules would perturb normal FA metabolism.
4.6. Triacylglycerol synthesis in CD36- or FABP1-deficient Caco-2 cells

Intracellular \(^{3}H\)-oleate incorporation into TG was not different, while secreted \(^{3}H\)-TG was increased from CD36- or FABP1-deficient Caco-2 cells compared to their respective control cell lines (Fig. 4). In this study, differentiated Caco-2 cells were incubated with \(^{3}H\)-oleate-containing lipid micelles for 12 hours, according to a previously published protocol (Luchoomun and Hussain, 1999). Drover, et al. showed that FA transport to microsomes was defective in CD36\(^{-/-}\) enterocytes treated with \(^{3}H\)-oleate-containing micelles (Drover et al., 2005). The \(^{3}H\) incorporation into CE, FA, and DAG, was not significantly different in microsomes isolated from CD36\(^{-/-}\) enterocytes compared to wild type (Drover et al., 2005), although there was a significant increase in \(^{3}H\) incorporation into TG. In their study, the primary enterocytes were incubated with lipid micelles for 1 hour (Drover et al., 2005). Therefore, the defect in FA delivery to microsomes can explain the initial lag in TG output to the lymphatic circulation observed in this model (Drover et al., 2005). In the present study, there may be an initial defect in targeting of FA in CD36- or FABP1-deficient Caco-2 cells, however, at the end of the 12 hour incubation with lipid micelles, a mistargetting of FA or lag in TG secretion was not observed. As mentioned above, CD36 was proposed to play a role in the targeting of exogenous fatty acids to the microsomal TG secretion pool (Drover et al., 2005). On the contrary, we conclude that CD36 is not necessary for the formation and secretion of TG, as its deficiency was actually associated with an increase in TG secretion.

We have previously shown that DGAT activity in the intestine is associated with an increase in TG accumulation and secretion in a dietary model of insulin resistance, the fructose-fed Syrian golden hamster (Casaschi et al., 2005). DGAT activity was increased 2.5- and 5-fold in the proximal intestine upon feeding of fructose and fat-enriched diets, respectively (Casaschi et al., 2005), but was not associated with an increase in mRNA levels of either DGAT1 or
DGAT2. In Caco-2 cells deficient in CD36, DGAT2 mRNA levels were not changed between control and knockdown cell lines, despite an increase in TG secretion (Fig. 15A). In addition, fatty acid supplementation did not increase DGAT2 mRNA levels in either control or CD36-deficient Caco-2 cells (Fig. 15A). Under basal conditions, DGAT2 expression was not changed in FABP1 knockdown Caco-2 cells compared to their control, however, there was a significant decrease in DGAT2 mRNA levels when these cells were supplemented with lipid (Fig. 15B). The regulation of DGAT enzymes under some conditions is at the level of mRNA, however, transcription factors responsible for its regulation are not well characterized (reviewed in (Yen et al., 2008)).

Recently, the contribution of DGAT1 activity to the synthesis of TG in Caco-2 cells was investigated (Cheng et al., 2008). They showed that, in vitro, approximately 76% TG synthesis from MG is mediated by DGAT1 in Caco-2 cell (Cheng et al., 2008). Recall that the majority of TG synthesis in Caco-2 cells is formed via the G3P pathway (reviewed in (Levy et al., 1995)), therefore, the relative amount of TG produced by DGAT1 may not account for 76% of total TG in normal Caco-2 cells. DGAT2 may catalyze the formation of TG from intermediates produced by the G3P pathway. It is also possible that DGAT1 catalyzes the assimilation of TG from G3P pathway-derived DAG, however, this was not tested.

Incorporation of oleate into TG was reduced in adipocytes from CD36-/- mice, while incorporation into DAG was increased (Febbraio et al., 1999). It followed then, that there was an impairment of DAG conversion to TG in CD36-deficient conditions (Febbraio et al., 1999). In the intestine, however, Drover, et al. showed that DAG species do not accumulate. They also did not detect changes in either DGAT1 or DGAT2 mRNA levels (Drover et al., 2005).

Fatty acids that are taken up by cells either diffuse or are transported to the appropriate subcellular location, for example, the mitochondria, peroxisomes, or the ER and are activated by their conversion to CoA thioesters. FATP4 has been implicated in fatty acid uptake via this
process, which is termed vectorial acylation. In the current study, we observed an increase in FATP4 protein levels under basal conditions in both CD36- and FABP1-deficient Caco-2 cells. Since FATP4 is present on the ER membrane (Ehehalt et al., 2006; Stahl et al., 1999), it is possible that FATP4 is curbing normal cellular metabolism towards secretion.

4.7. Factors involved in chylomicron assembly and secretion

We have previously shown that MTP gene expression is increased by oleate in an SREBP1-independent manner in Hep-G2 cells (Qiu et al., 2005), while cholesterol increases MTP expression in a SREBP1-dependent manner (reference- chapter 14). MTP expression is highly regulated in the liver, but little is known about the regulation of MTP in the intestine. If MTP expression is regulated in a similar manner in liver and enterocytes, we may expect to see an increase in MTP levels in our Caco-2 cells treated with oleate-containing lipid micelles. However, we do not see an increase in MTP upon lipid stimulation except for in CD36-deficient Caco-2 cells (Fig. 16). Although the lack of FABP1 in FABP-deficient Caco-2 cells could perturb normal FA signaling mechanism, this is probably not the case in this experiment, since control Caco-2 cells did not respond to oleate either.

The intestine is the first organ to process dietary lipid, and the absorption of lipid is clearly very complex and highly regulated, although it is not well understood. Many of the findings from hepatocyte lipoprotein assembly are assumed to be true for enterocyte lipoprotein assembly as well. However, there is evidence that regulation of protein involved in chylomicron assembly do not respond in a similar fashion in liver and the intestine. For example, MTP expression in the liver of Sprague-Dawley rats is downregulated in response to a 6- and even more so in a 12-hour fast, while its expression in the intestine does not change (Lally et al., 2007).
There may also be differences in the regulation of proteins involved in chylomicron assembly during different developmental stages. Lu, et al. have shown that in enterocytes of newborn swine, both MTP and ApoAIV mRNAs are upregulated by duodenal lipid infusion (Leng et al., 2007b; Lu et al., 2002). HNF4α regulates transcription of ApoAIV in enterocytes from newborn swine (Leng et al., 2007a). The MTP gene also contains an HNF4α binding elements in its promoter region (Hussain et al., 2003). In our hands, the expression of ApoAIV did not change in response to oleate-containing lipid micelle stimulation (Fig. 18).

The involvement of Sar1 to the secretion of lipoprotein is unique to the intestine, as mutations in the SARA2 gene lead to chylomicron retention disease (Dannoura et al., 1999; Jones et al., 2003). Patients with this rare-lipid malabsorption disorder secrete VLDL particles from the liver but retain chylomicrons in enterocytes. Previous work in our lab has shown that the protein level of Sar1 are increased 2.13-fold in chow fed hamsters after receiving a bolus of olive oil (Wong, et al. Manuscript under revision). Furthermore, we have shown that Sar1 protein levels are increased 2.33-fold in fructose-fed hamsters, a well-established model of a mild-insulin resistant state (Wong, et al. Manuscript under revision; Avramoglu et al., 2003; Avramoglu et al., 2006; Taghibiglou et al., 2000). On this dietary background, Sar1 protein expression is not induced further by a lipid bolus (Wong, et al. Manuscript under revision). In this study, Sar1 levels were not induced by oleate-containing micelles stimulation in Caco-2 cells, except when CD36 was deficient.

4.8. Fatty acid synthesis in enterocytes isolated from CD36−/− mice

In order to determine whether our observations in Caco-2 cells accurately represent what is happening in vivo, we examined secretion of lipids from isolated enterocytes from CD36−/− mice. We observed a significant reduction in secreted TG from CD36−/− enterocytes and these results are consistent with several studies examining intestinal lipid absorption and secretion in
CD36<sup>−/−</sup> mice (Drover <i>et al.</i>, 2005; Drover <i>et al.</i>, 2008a). We next wanted to examine FA synthesis in CD36<sup>−/−</sup> enterocytes, since a more recent study has suggested that FA synthesis may be upregulated in the intestine of CD36<sup>−/−</sup> mice (Masuda <i>et al.</i>, 2008), and we have similar evidence of this in CD36-deficient Caco-2 cells. To assess FA synthesis in isolated enterocytes, we measured the incorporation of [<sup>3</sup>H]-acetate into CE, TG, FA, and cholesterol (Fig. 13A). There were no significant differences in the incorporation of [<sup>3</sup>H]-acetate into CE, TG, or FA, but we observed a significant reduction in the incorporation of [<sup>3</sup>H]-acetate into cholesterol. CD36 has been implicated in intestinal cholesterol metabolism (Nassir <i>et al.</i>, 2007; Nauli <i>et al.</i>, 2006); our observations also support a relationship between CD36 and cholesterol metabolism.

To assess enterocyte de novo lipogenesis, we measured the incorporation of [<sup>3</sup>H]-oleate into CE, TG, FA, and cholesterol (Fig. 13B). There were no significant differences in the incorporation of [<sup>3</sup>H]-oleate into CE, FA, or cholesterol, but we observed a significant reduction in the incorporation of [<sup>3</sup>H]-acetate into TG. The reduction in TG synthesis in CD36<sup>−/−</sup> enterocytes is consistent with the decrease in microsomal TG observed in CD36<sup>−/−</sup> enterocytes (Drover <i>et al.</i>, 2005).

Fatty acid and triacylglycerol metabolism in Caco-2 cells is different from mature villus enterocytes (discussed above). The impairment of TG synthesis associated with CD36-deficiency implicates CD36 in the MAG (dominant) pathway of TG synthesis. Differences in TG synthesis pathway predominance in Caco-2 cells and normal enterocytes may explain the differences in TG synthesis and secretion observed in these two models. It is also possible that the level of CD36 expression is important to its function, and that a model in which CD36 is completely absent would react differently than a model in which CD36 is expressed at 50% of control cell levels.
4.9. **Concluding remarks**

The wealth of information concerning CD36 and FABP1 and their role in FA metabolism is complicated by their tissue-specific roles and the differences in phenotypes under different dietary conditions. It is clear that both CD36 and FABP1 are important players in FA and lipoprotein metabolism in the intestine, as polymorphisms in both genes are associated with the metabolic syndrome (Ma et al., 2004). We have investigated the role of these two proteins in a cell culture model of mature villus enterocytes, Caco-2 cells. We have used a Caco-2 cell culture model in order to avoid interference of various circulating factors that can metabolize intestinally secreted lipoproteins. From these studies, we report a number of observations: (i) shRNA-mediated knockdown of CD36 or FABP1 in Caco-2 cells was associated with increased TG secretion under lipid stimulated conditions, (ii) shRNA-mediated knockdown of CD36 or FABP1 in Caco-2 cells was associated with increased apoB secretion under basal conditions, (iii) deficiency of CD36 results in a shift in lipoprotein associated TG, from large CM only, to both large and small CM, (iv) deficiency of CD36 or FABP1 results in increased FAS expression under basal conditions, (v) cerulenin reduces FA synthesis in Caco-2 cells, but that CD36-deficient cells are more resistant to cerulenin-mediated inhibition of FA synthesis, (vi) FATP4 levels are increase under basal conditions in CD36- or FABP1-deficient Caco-2 cells, (vii) levels of MTP, Sar1, and ApoAIV were not dramatically different in the knockdown cells versus control cells, under basal or lipid-stimulated conditions.

We have also performed several experiments in CD36−/− mice in order to compare our *in vitro* findings to an *in vivo* model. From these studies, we report that CD36−/− mice (i) have impaired cholesterol and TG synthesis and (ii) a significant reduction in secreted TG compared to WT animals.
Patients with CD36-deficiency and the CD36\textsuperscript{\textminus} mouse display an increase in fasting and postprandial apoB48 and TG (Miyaoka \textit{et al.}, 2001), which is not associated with a defect in Lpl activity (Masuda \textit{et al.}, 2008). This suggests that overproduction of intestinal lipoproteins is responsible for this postprandial increase in apoB48 and TG (Masuda \textit{et al.}, 2008). In our study, CD36 or FABP1-deficiency is associated with an increase in FAS expression. Although FAS protein levels did not correlate with fatty acid synthesis in these cells, there may be an increase in FA synthesis in CD36 knockdown Caco-2 cells.

As mentioned above, FABP1 has been shown to directly interact with PPAR\(\alpha\), a transcription factor responsible for the induction of genes involved in FA oxidation (Hostetler \textit{et al.}, 2009). Rivabene, \textit{et al.} have shown that the pre-existing redox state of Caco-2 cells affects cellular lipid metabolism (Rivabene \textit{et al.}, 2001a). Modulation of the redox status in Caco-2 cells using antioxidant N-acetylcysteine (NAC) or the pro-oxidizing agent CuSO\(_4\), altered the metabolism of oleate supplemented to Caco-2 cells (Rivabene \textit{et al.}, 2001b). The synthesis of TG was significantly lowered under more reducing conditions using NAC. In the absence of FABP1, the normal oxidative and reductive potential of Caco-2 cells may be altered. Upregulation of a number of genes would be necessary to accommodate diminished FA oxidation in order to relieve the cells of the potential lipotoxic effects of FFA. For example, upregulation of FATP4 could be an adaptive response, activating FA and shuttling them towards secretion or storage as neutral TG.

We postulate that CD36 and FABP1 are intimately involved in lipid sensing and downstream regulation of lipid metabolism in the cell, and our results indicate that modulation of their expression leads to aberrant intestinal lipid and lipoprotein production.
CHAPTER 5

5. FUTURE DIRECTIONS

The absence of CD36 may result in the overcompensation by a molecule that was not measured in our studies, SR-B1. SR-B1 is a scavenger receptor that is present on the apical membrane of Caco-2 cells and acts as a lipid sensor (Beaslas et al., 2009). Supplementation of Caco-2 cells with postprandial lipid micelles results in downstream signaling events, dependent on SR-B1, and a re-localization of apoB to apical compartments (Beaslas et al., 2009). The relationship between SR-B1 and CD36 in Caco-2 cells should be examined; SR-B1 levels in Caco-2 cells deficient in CD36 could be measured by immunoblotting. Co-immunoprecipitation studies should be performed to determine whether CD36 and SR-B1 interact. Confocal immunofluorescence based assays could be used to determine the subcellular localization of CD36 in relation to SR-B1 and in response different stimuli (basal and postprandial conditions).

Another experimental approach could have yielded some important information with respect to the importance of CD36 or FABP1 to fatty acid uptake in Caco-2 cells. A study should be designed in which CD36- or FABP1-knockdown Caco-2 cells are challenged with various concentrations of fatty acid. This experiment could answer the question of whether CD36 or FABP1 are critical for fatty acid uptake when fatty acid concentrations are extremely low.

A novel observation in the current study was the lack of lipid-induced enhancement of apoB secretion from FABP1-deficient Caco-2 cells. As discussed above, this observation is supported by the findings of Neeli, et al., which have shown that FABP1 participates in the selection of cargo for, and budding of, the prechylomicron transport vesicle (Neeli et al., 2007). When FABP1 is deficient, we propose that there is less regulation of cargo budding, which may
allow for the formation of larger particles. Future experiments could focus on characterizing pre-chylomicron particles formed from FABP1-deficient Caco-2 cells or from isolated enterocytes from FABP1⁻/⁻ mice. Our lab has performed previous experiments on the proteome of prechylomicron transport vesicles isolated from enterocytes from the Syrian golden hamster (Wong et al., 2009).
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