Role of Monoacylglycerol Acyltransferase-1 and Monoacylglycerol Acyltransferase-2 in Adipocyte Lipid Metabolism

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ABSTRACT

Adipose tissue is the main storage site of triacylglycerol which provides energy to other tissues by producing fatty acids during fasting. Any dysregulation in triacylglycerol synthesis and breakdown in adipocytes can be responsible for obesity, diabetes, cardiovascular diseases and so on. The sequential acylation of glycerol-3-phosphate to produce triacylglycerol is well studied in adipocytes, whereas little is known about the role of the monoacylglycerol pathway in adipocyte triacylglycerol metabolism. Two monoacylglycerol acyltransferase (MGAT) isoforms, MGAT1 and MGAT2, have been found in serving the substrate-diacylglycerol for diacylglycerol acyltransferase (DGAT) to produce triacylglycerol in liver and intestine, respectively, but their roles in adipocyte lipid metabolism are not clear. We hypothesize that one or both of these MGAT enzymes contribute to adipose tissue triacylglycerol metabolism.

We used 3T3-L1 pre-adipocytes which can differentiate to adipocytes within 10 days by adding proper differentiation media. To explore the role of MGAT1 and MGAT2 in adipocytes, we used shRNA to knockdown MGAT1 or MGAT2 separately in pre-adipocytes. We have found increased *in vitro* MGAT activity during adipocyte differentiation, but most of this MGAT activity come from DGAT1. Also, DGAT1 may try to compensate for the absence of MGAT1 in adipocytes by increasing both MGAT and DGAT activity. This result suggests a post-translational modification of DGAT1 in adipocytes since the mRNA expression level of DGAT1 did not change during adipocyte differentiation. MGAT1 may have a role in triacylglycerol synthesis and storage in lipid droplets since the size of lipid droplets and the synthesis of triacylglycerol was decreased in the absence of MGAT1. The function of MGAT2 in adipocytes is not clear but may have a role in upregulating DGAT1 activity in the absence of MGAT1. However, the differentiation of adipocytes does not depend on either MGAT enzyme. We also tried to explore the role of MGAT1 and MGAT2 in lipolysis in adipocytes but due to a technical problem, we could not conclude anything from this lipolytic experiment.

In summary, this study suggests the role of monoacylglycerol pathway in regulating triacylglycerol metabolism in adipocytes.

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LIST OF ABBREVIATIONS

ACP	Acyl carrier protein
AGPT	1-acyl-*sn*-glycerol-3-phosphate O-acyltransferases
BGS	Bovine growth serum
BMI	Body mass index
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer binding protein
CGI-58	Comparative gene identification-58
CoA	Coenzyme A
CPT-cAMP	8-(4-Chlorophenylthio) adenosine 3′,5′-cyclic monophosphate sodium salt
DGAT	1,2-diacyl-*sn*-glycerol O-acyltransferase
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
GPAT	Acyl-CoA: *sn*-1-glycerol-3-phosphate O-acyltransferase
IBMX	3-Isobutyl-1-methylxanthine
MGAT	Monoacylglycerol acyltransferase
NBD	N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)-methyl] amino
PPAR	Peroxisome proliferator activated receptor
RT	Reverse transcriptase
Sh	Short hairpin
TLC	Thin layer chromatography

CHAPTER 1: Introduction

1.1 Triacylglycerol is a form of stored energy

Lipids are hydrophobic biomolecules that are insoluble in water but are instead soluble in organic solvents, such as diethyl ether, hexane, benzene. Lipids are a structurally diverse group of molecules that belong to different categories, such as fatty acids, glycerolipids, sphingolipids, saccharolipids, sterol lipids, and prenol lipids (Fahy et al., 2009). In living organisms, these lipids have diverse roles in many vital biological functions. For example, fatty acids store energy in the form of triacylglycerol in adipose tissue. Phospholipids serve as a primary component of the lipid bilayer of cells and also are involved in eukaryotic cell signaling. Sterol lipids, such as cholesterol are also found in biological membranes and are used as a precursor for some hormones. However, this thesis will focus on triacylglycerol metabolism in adipocytes as triacylglycerols store most of the energy in a lipid form in eukaryotic organisms.

Triacylglycerol consists of three fatty acids linked to a glycerol backbone via an ester bond. In eukaryotes, under certain metabolic conditions, triacylglycerols are broken down to release fatty acids. These fatty acids produce ATP via β-oxidation in mitochondria, which is used as energy by eukaryotic cells (Bartlett and Eaton, 2004; Houten and Wanders, 2010).

Fatty acids consist of a nonpolar hydrocarbon chain that is 4-36 carbons in length and terminates with a carboxylic acid group (Fig. 1.1). As a result, the longer the carbon chain, the higher the hydrophobicity and melting temperature of fatty acids. Moreover, these two physical properties depend on the number of double bonds. Saturated fatty acids have a high melting temperature and are solid at room temperature. Unsaturated fatty acids have a lower melting point and tend to be liquid at room temperature.

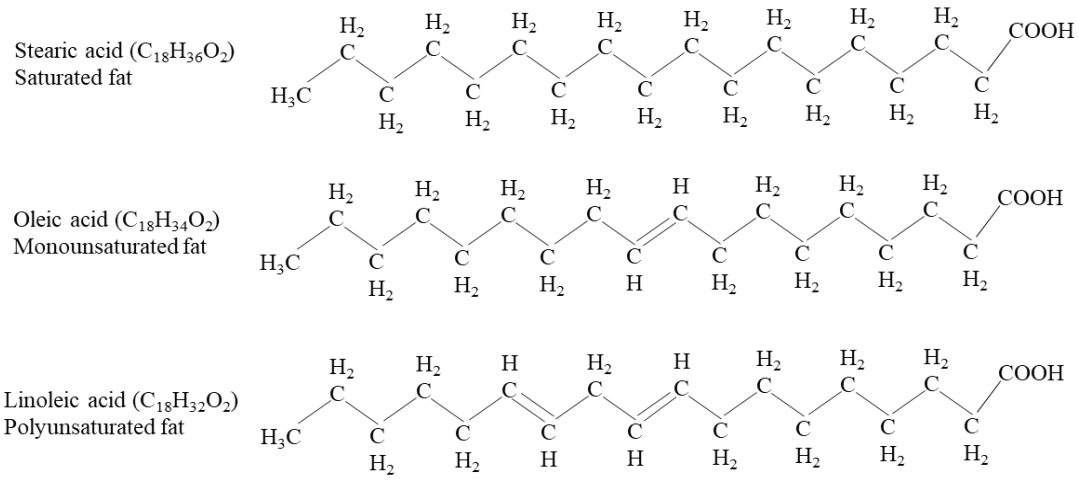


Figure 1.1: Fatty acids structure. Stearic acid (18:0) is a saturated fatty acid and has no double bonds. Oleic acid (18:1) and linoleic acid (18:2) have one and two double bonds, respectively.

Fatty acids synthesis occurs in the cytosol (Fig. 1.2). The production of malonyl coenzyme A (CoA) from acetyl CoA by acetyl CoA carboxylase is the rate limiting step in fatty acid synthesis (Wolfgang and Lane, 2006). The acetyl CoA is derived from glucose via the glycolytic pathway (Wolfgang and Lane, 2006). The malonyl CoA serves as a precursor for the synthesis of fatty acids and has no other metabolic role. Fatty acid synthesis is catalyzed by fatty acid synthase that contains all of the enzymatic activities required to synthesize a fatty acid (Wakil, 1989, Smith, 1994). Fatty acid synthase is an enzyme complex that is located on cytoplasm and intracellular membranes in mammalian cells. This enzyme complex is a product of one gene in mammals, whereas in yeast and fungi, two genes produce polypeptide products, which combine to form a multifunctional fatty acid synthase complex. The acetyl-CoA and malonyl CoA are attached to the acyl carrier protein (ACP) to tether covalently to the fatty acid synthase. By the action of β-ketoacyl-ACP synthetase, 3-oxobutanoate is produced from acetyl-CoA and malonyl CoA. The intermediate, 3-oxobutanoate is then reduced to 3-hydroxy-butanoate by β-ketoacyl-ACP reductase, which is in turn dehydrated to trans-2-butenoate by β-hydroxyacyl-ACP hydratase, followed by reduction to butanoate by enoyl-ACP reductase. These reactions are repeated six times until the end product of fatty acid synthesis, 16-carbon palmitic acid produced (Berg et al., 2002).

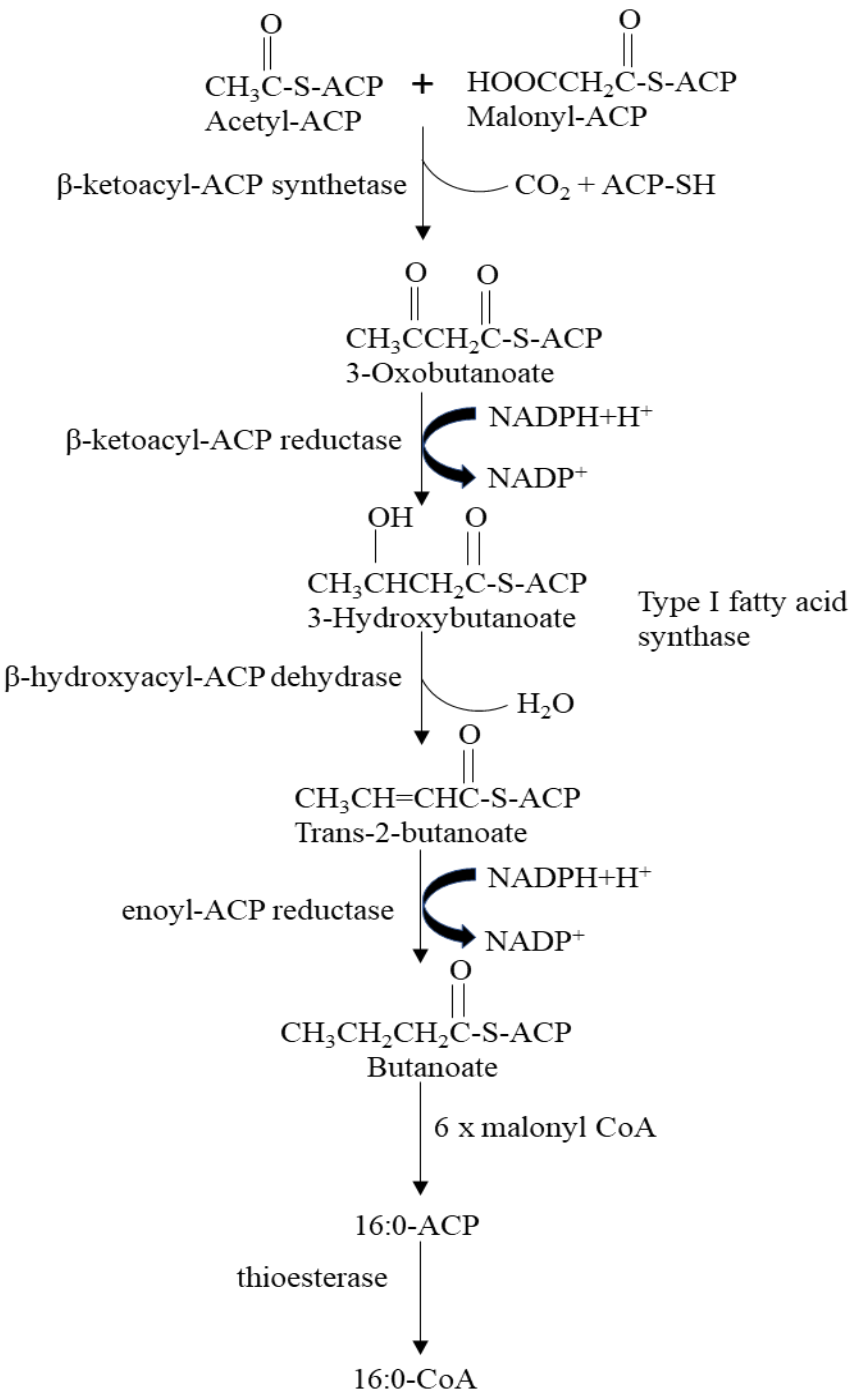


Figure 1.2: Fatty acid synthesis. In the presence of fatty acid synthase, acetyl CoA groups are added into malonyl CoA to make the acyl chain of a fatty acid through the sequential condensation reaction to generate, for example, the 16-carbon saturated fatty acid palmitate.

1.2 Triacylglycerol biosynthesis

Triacylglycerol is a neutral lipid and consists of a glycerol backbone to which three fatty acids are attached by ester bonds (Fig. 1.3). Triacylglycerol is non-polar and very hydrophobic in nature. In animals, triacylglycerol is the major source of energy, stored in white adipose tissue, whereas plants store triacylglycerol in their seeds. In addition to storing energy in adipocytes, triacylglycerols have different biological functions in hepatocytes, enterocytes, myocytes and mammary epithelial cells in animals. For example, triacylglycerol synthesis in mammalian enterocytes and hepatocytes plays an important role in lipoprotein assembly and secretion, that transport fatty acids between tissues (Shelness and Sellers, 2001). Also, triacylglycerol, synthesized in the mammary epithelial cells, is a vital component of mammalian milk lipids (Rudolph et al., 2007).

Oxidation of fatty acids, derived from breakdown of triacylglycerol, produces more than twice the energy (approximately 9 kcal/g) compared to an equal mass of other energy storage molecules, such as glycogen and protein (approximately 4 kcal/g) (Drummond and Brefere, 2001). Besides energy storage, triacylglycerol prevents the cellular toxicity associated with the detergent-like properties of free fatty acids (lipotoxicity) by storing fatty acids as a non-toxic compound in mammals (Listenberger et al., 2003).

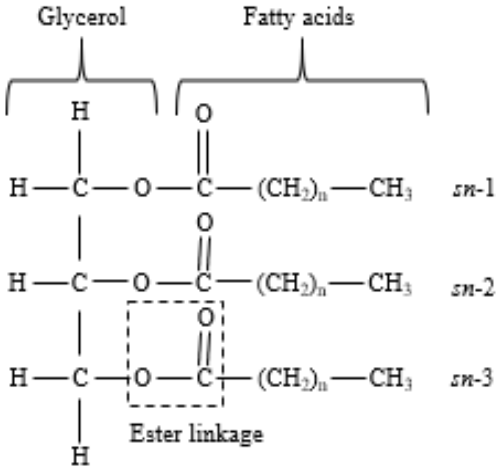


Figure 1.3: Triacylglycerol molecule. Triacylglycerol consists of three fatty acids bound to a glycerol backbone by ester bonds.

Although triacylglycerol stores energy in living organisms, excessive accumulation of triacylglycerol in human adipose tissue is responsible for different pathological conditions like obesity, type 2 diabetes, cardiovascular diseases and non-alcoholic fatty liver diseases (Millar and Billheimer, 2005, Mokdad et al., 2000, Kopelman, 2000). Moreover, deposition of triacylglycerol in non-adipose tissues, such as pancreatic β cells, skeletal muscle, and liver, is associated with insulin resistance and apoptosis (Seip and Trygstad, 1996, Zhou et al., 2000, Unger and Orci, 2002). Obesity is defined as a chronic condition in which excessive intake of food energy coupled with decreased energy expenditure, increases the adipose tissue mass and impairs health. Clinically, obesity is estimated by body mass index (BMI) which is defined as the subject's weight divided by the square of their height and is expressed as kg/m^2. According to the World Health Organization, when BMI exceeds 30 kg/m^2, it can be referred as obesity, and 25-29.9 kg/m^2 is referred as overweight. The Public Health Agency of Canada stated that the obesity rate in adults has increased from 49% to 64% between 1978 and 2017, whereas child obesity also increased from 23% to 30%. Obesity is also a global concern as 39% of the global adult population are overweight and 13% are obese. In many instances, individuals who become obese are predisposed to other metabolic diseases, such as diabetes, hypertension, dyslipidemia, and thus reduces the life expectancy and increase the socio-economic burden. Therefore, understanding the mechanisms responsible for increased adipose tissue content in obese individuals is critical for the development of effective therapies to reverse obesity and minimize the risk of other diseases.

1.2.1 The Kennedy pathway

Triacylglycerol synthesis occurs via the Kennedy pathway (also known as glycerol-3-phosphate pathway) which is present on the endoplasmic reticulum (ER) membrane in most of the cell types. It mainly involves the sequential esterification of three fatty acyl groups to a glycerol backbone to form triacylglycerol (Kennedy, 1957) (Fig. 1.4).

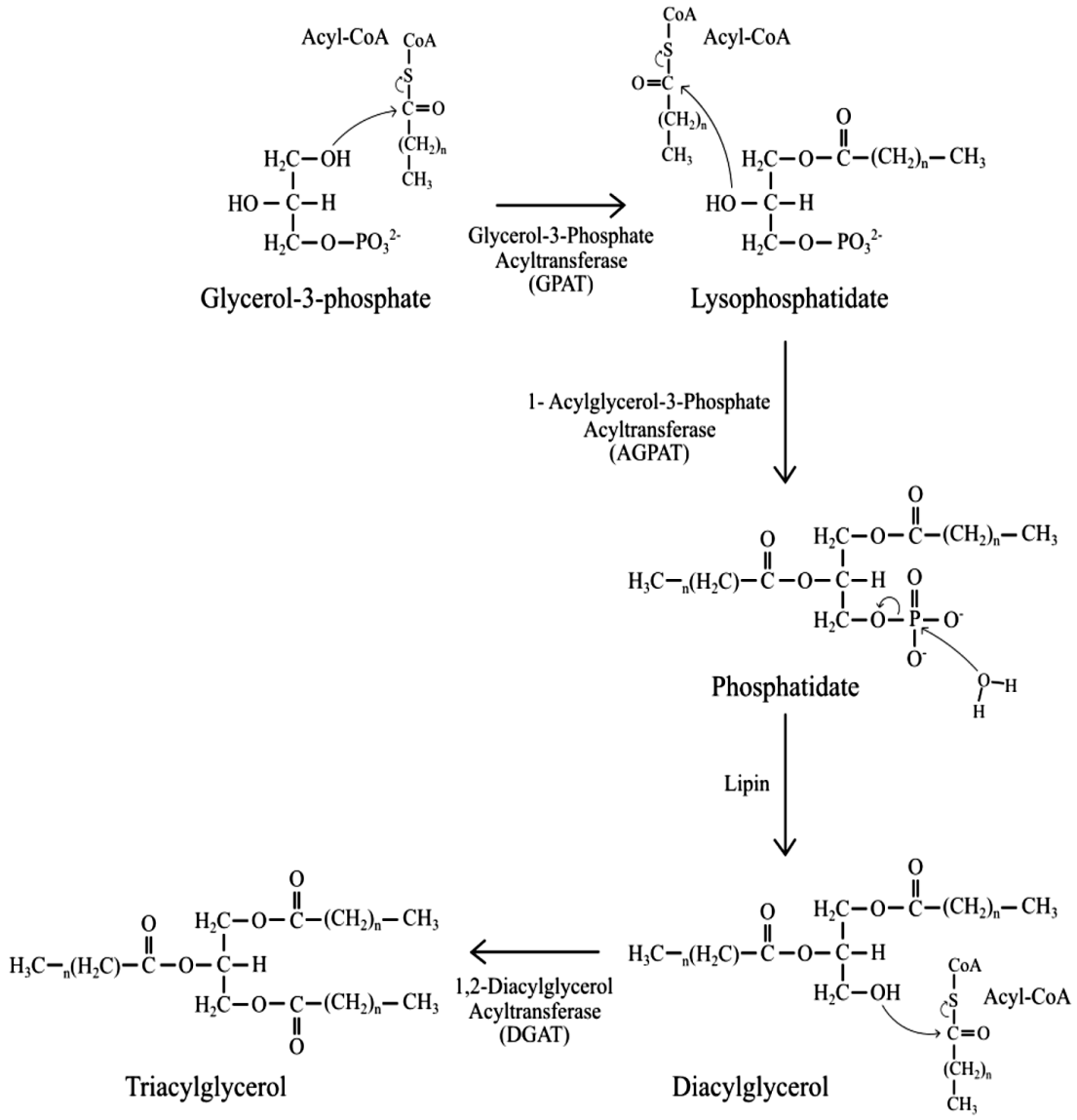


Figure 1.4: The Kennedy pathway. Glycerol-3-phosphate acyltransferase catalyzes the rate limiting step where glycerol-3-phosphate is esterified into lysophosphatidate. Next esterification is mediated by 1-Acyl-sn-glycerol-3-phosphate O-acyltransferase to produce phosphatidate. Diacylglycerol is then derived from the dephosphorylation of phosphatidic acid by lipin. In the last step, 1,2-diacylglycerol acyltransferase produces energy storage lipid-triacylglycerol from diacylglycerol.

1.2.1.1 Glycerol-3-phosphate acyltransferase

In the Kennedy pathway, acyl-CoA: *sn*-1-glycerol-3-phosphate O-acyltransferase (GPAT) serves as a rate limiting enzyme in triacylglycerol synthesis (Wendel et al., 2009). GPAT catalyzes

the first step of the Kennedy pathway where lysophosphatidate is produced by the formation of an ester bond between glycerol-3-phosphate and fatty acyl-CoA. However, four GPAT isoforms (GPAT1-4) have been identified, each the product of an independent gene, which mainly differ from each other in tissue expression, localization and substrate specificity (Lewin et al., 2008). For example, GPAT1 and GPAT2 are found in the outer mitochondrial membrane, whereas GPAT3 and GPAT4 are present at the ER (Pellon-Maison et al., 2007, Lewin et al., 2004, Cao et al., 2006, Nagle et al., 2008). All GPAT isoforms are integral membrane proteins that have a cytosol facing active site and two predicted transmembrane domains (Gonzalez-Baro et al., 2001). The analysis of GPAT in *E. coli* showed four active site motifs (Lewin et al., 1999), and further these four active sites are found in all GPAT isoforms (Coleman and Mashek, 2011).

In rat tissues, the level of GPAT1 protein is high in the heart, but the highest activity is found in liver and adipocytes (Lewin et al., 2001). Post-translational modification may have a role in regulating the GPAT activity. In the lipogenic state, activated GPAT1 regulates triacylglycerol production in hepatocytes and contributes 30–50% of total hepatic GPAT activity (Hammond et al., 2005). Overexpression of murine mitochondrial GPAT1 in rat hepatocytes supports the view that increased hepatic GPAT1 activity is associated with obesity related lipid disorders by increasing lipid synthesis and inhibiting fatty acid oxidation (Lindén et al., 2004). Knockout of hepatic mitochondrial GPAT1 results in the use of fatty acids in oxidation and ketogenesis instead of triacylglycerol synthesis (Hammond et al., 2005).

During adipocyte differentiation in mouse, insulin activates the transcription factor sterol regulatory element-binding protein-1c which upregulates GPAT1 expression and activity by binding with the *Gpat1* promoter (Cha and Repa, 2007, Takeuchi and Reue, 2009).

GPAT2 was cloned from mouse testis and kidney (Wang et al., 2007, Harada et al., 2007), though less is known about GPAT2. Unlike GPAT1, GPAT2 mRNA abundance is not altered by fasting or refeeding in liver (Wang et al., 2007). GPAT2 may have a specialized role in testis that has yet to be elucidated.

GPAT3 is highly expressed in mouse tissues that have high rates of triacylglycerol synthesis, such as small intestine, heart and adipose tissue. Knockdown of GPAT3 reduces triacylglycerol synthesis by 60% in adipocytes (Shan et al., 2010). Increased triacylglycerol formation is found in HEK293 cells with overexpressed GPAT3 (Cao et al., 2006).

The expression level of GPAT4 is high in brown adipose tissue and testis in mice, while in white adipose tissue, its expression is low (Beigneux et al., 2006). *Gpat4* knockout mice have a low level of diacylglycerol and triacylglycerol in milk, suggesting the importance of GPAT4 in triacylglycerol production during lactation (Beigneux et al., 2006).

1.2.1.2 1-Acyl-glycerol-3-phosphate acyltransferases

The second step of the Kennedy pathway is catalyzed by 1-acyl-*sn*-glycerol-3-phosphate O-acyltransferases (AGPATs), where a second fatty acid is esterified to the second carbon of lysophosphatidate to form phosphatidate. AGPATs belong to a homologous family of enzymes, and they are also known as lysophosphatidic acid acyltransferases (Bradley and Duncan, 2018). Eleven isoforms of AGPATs have been reported in humans which are products of different genes and differ in their tissue expression patterns (Agarwal, 2012). In an earlier study, all eleven isoforms of AGPATs were thought to exhibit minor *in vitro* AGPAT activity in mice and humans (Kitson et al., 2012). Recent studies show that five of these enzymes, named AGPAT1-5, catalyze the esterification of lysophosphatidate to produce phosphatidate (Bradley et al., 2017a, Bradley and Duncan, 2018). Like GPATs, AGPATs are integral membrane proteins, found in ER and mitochondrial membranes (Agarwal et al., 2011, Prasad et al., 2011, Pagliuso et al., 2016).

In animals, relatively high levels of AGPAT1 mRNA was found in the brain, however, the expression of AGPAT1 is present in most tissues (Kume and Shimizu, 1997, Kitson et al., 2012). Overexpression of AGPAT1 in 3T3-L1 adipocytes caused increased fatty acid uptake and lipogenesis (Ruan and Pownall, 2001). A study on *Agpat1* knockout mice has shown impaired phospholipid homeostasis, which caused early death of the mice (Agarwal et al., 2017). This study suggests that AGPAT1 may have roles in maintaining metabolic, reproductive and neurologic health in mice by regulating phospholipid homeostasis (Agarwal et al., 2017).

A high level of sequence homology is found between mouse *Agpat1* and *Agpat2* (Lu et al., 2005). However, knockout mouse models revealed different metabolic abnormalities based on the absence of these genes. For example, *Agpat2* knockout mice are hyperglycemic (Cortés et al., 2009), whereas *Agpat1* knockout mice are hypoglycemic (Agarwal et al., 2017). In addition,

genetic loss of *Agpat2* in adipocytes results in congenital generalized lipodystrophy, a syndrome of severe fat loss which is not compensated by AGPAT1 (Agarwal et al., 2002).

AGPAT2 may have a role in adipocyte differentiation, as the *Agpat2* promoter has binding sites for peroxisome proliferator activated receptor γ (PPARγ) and CCAAT/enhancer binding protein β (C/EBPβ), key transcriptional activators of adipogenesis (Gale et al., 2006). During differentiation of 3T3-L1 cells into adipocytes, the mRNA expression level of AGPAT2 is increased 30-fold, while knockdown of *Agpat2* slows down the adipogenesis by preventing the induction of C/EBPβ and PPARγ (Gale et al., 2006).

The mRNA of AGPAT3 is found in both brown and white adipose tissue, liver and testis (Yuki et al., 2009). AGPAT3 is a transmembrane protein, localized to the ER and Golgi (Schmidt et al., 2010). A study on AGPAT3 knockdown in rat hepatocytes showed fragmented Golgi and accelerated protein trafficking as the level of AGPAT3 catalyzed product, phosphatidate was decreased (Schmidt and Brown, 2009). In that study, overexpression of AGPAT3 increased phosphatidate in Golgi that inhibited membrane tubule formation and trafficking (Schmidt and Brown, 2009). Taken together, these results suggest the role of AGPAT3 in the structure and trafficking functions of the Golgi by regulating the level of phosphatidate.

AGPAT4 is highly expressed in brain. A study on *Agpat4* null mice showed impaired learning and memory as the level of different phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol was reduced (Bradley et al., 2017b).

AGPAT4 also has different roles in different types of mice adipose tissue depots. For example, the mass of epididymal white adipose tissue is increased in male *Agpat4* knockout mice, while there was no change in perirenal white adipose tissue mass (Mardian et al., 2017).

The expression of AGPAT5 in testis suggest its role in sperm maturation though the mechanism is unclear (Prasad et al., 2011). Also, AGPAT5 may play a role in maintaining mitochondrial membrane structure and function by providing phospholipids (Prasad et al., 2011).

1.2.1.3 Lipin

Following the AGPAT acylation reaction, Lipins, also known as phosphatidate phosphatases, hydrolyze phosphatidate to form 1,2-diacylglycerol. Lipins remove a phosphate group from the 3-carbon of phosphatidate in the presence of magnesium. Cytoplasmic Lipins

translocate to the ER membrane, where their substrate, insoluble phosphatidic acids are present. There are three mammalian Lipin isoforms (Lipin1-3) which share some common structural elements and differ in their tissue expression pattern (Péterfy et al., 2001, Coleman and Mashek, 2011). All three isoforms have a nuclear localization sequence, a DIDGT motif (required for enzyme activity) and an LXXIL motif that suggests Lipin as a transcriptional coactivator (Finck et al., 2006, Donkor et al., 2009).

Lipin1 mRNA is highly expressed in mouse adipose tissue, skeletal muscle and testis (Péterfy et al., 2001). Transcription of *Lipin1* is positively regulated by glucocorticoids, cAMP, sterol regulatory element-binding protein-1 and nuclear factor Y, whereas insulin acts as a negative regulator (Manmontri et al., 2008, Ishimoto et al., 2009). Overexpression of Lipin1 in mouse adipose tissue leads to a marked increase in triacylglycerol storage (Phan and Reue, 2005), while siRNA mediated knockdown of *Lipin1* in 3T3-L1 preadipocytes inhibits adipocyte differentiation by downregulating PPARγ (Koh et al., 2008). Since Lipin1 acts as a transcriptional coactivator, it can also induce PPARα expression, a regulator of ATP production in oxidative tissues (Finck et al., 2006). Therefore, Lipin1 helps in energy metabolism in mitochondria through fatty acid oxidation.

Deficiency of Lipin1 has different effects in mice and humans. For example, mice with a mutated *Lipin1* gene are characterized by lipodystrophy, hypertriglyceridemia, insulin resistance, neonatal fatty liver, and peripheral neuropathy (Péterfy et al., 2001). While in humans, *LIPIN1* mutation causes muscle pain, weakness, and myoglobinuria in childhood rather than lipodystrophy (Michot et al., 2012, Zeharia et al., 2008).

There are three isoforms of human Lipin1 (Lipin1α, Lipin1β, Lipin1γ) that have been derived from alternative mRNA splicing (Han and Carman, 2010). The expression of Lipin1γ in human brain is very high where it is localized to lipid droplets (Wang et al., 2011). Lipin1γ is assumed to direct diacylglycerol for phospholipid synthesis rather than triacylglycerol, and thus it can alter the morphology of lipid droplets in brain without affecting the triacylglycerol level (Wang et al., 2011). By this process, Lipin1γ may provide phospholipids to regulate the brain functions.

Little is known about Lipin2 and Lipin3. The expression of Lipin2 is high in the liver, and it is also expressed in the kidney, brain, and lungs (Donkor et al., 2009). Besides phosphatidate

phosphatases activity, Lipin2 also acts as a transcriptional coactivator that combines with PPARα (Donkor et al., 2009).

Lipin3 is the least studied of the Lipin family members. Lipin3 is highly expressed in mammalian liver and intestine. Study on *Lipin* knockout mouse models showed that Lipin3 and Lipin1 cooperate *in vivo* to influence adipose tissue phosphatidate phosphatase activity and adiposity, which suggest that Lipin3 may protect lipin-1-deficient humans from overt lipodystrophy (Csaki et al., 2014).

1.2.1.4 1,2-Diacyl-*sn*-glycerol O-acyltransferase

The last step of the Kennedy pathway is catalyzed by 1,2-diacyl-*sn*-glycerol O-acyltransferase (DGAT) to produce triacylglycerol. DGAT forms an ester bond between the free hydroxyl group of diacylglycerol and a long chain fatty acid to produce triacylglycerol. DGAT activity is found in almost all tissues, but the highest activity is present in adipose tissue, liver, lactating mammary gland, small intestinal mucosa, and adrenal gland in animals, where high levels of triacylglycerols are synthesized (Cases et al., 1998, Cases et al., 2001). Two DGAT genes have been identified in eukaryotes, *DGAT1* and *DGAT2,* which belong to two distinct gene families, and have no sequence similarity in their protein products. The murine and human DGAT1 genes were first identified by their similarity to the sequences of acyl- CoA:cholesterol acyltransferase enzymes and belong to the membrane-bound O-acyltransferases family (Cases et al., 1998). DGAT1 is homotetramer and contains a FYxDWWN motif for binding of acyl-CoA (Buhman et al., 2001, McFie et al., 2010). DGAT1 has additional acyltransferase activities such as, acyl-CoA:retinol acyltransferase, lecithin:retinol acyltransferase, monoacylglycerol acyltransferase (MGAT) beyond that of esterifying diacylglycerol *in vitro* (Yen et al., 2005, Batten et al., 2004).

In contrast, DGAT2 appears to have a more prominent role in triacylglycerol synthesis in eukaryotes than DGAT1 (Stone et al., 2004). DGAT2 was first purified from fungus which led to the cloning of mammalian DGAT2 (Yen et al., 2008). DGAT2 is a member of the DGAT2/ MGAT family that includes MGAT1, MGAT2, MGAT3 and wax synthase (Cases et al., 2001, Yen et al., 2002, Buhman et al., 2001).

Both murine DGAT1 and DGAT2 are integral membrane proteins of the ER. DGAT1 has three transmembrane domains (McFie et al., 2010) and DGAT2 has two transmembrane domains (Stone et al., 2006). The C-terminus of DGAT1 is found in the ER lumen and the N-terminus is exposed to the cytosol (McFie et al., 2010). Studies showed that this N-terminal domain may be required for tetramer formation instead of catalytic activity, whereas a highly conserved histidine residue (H426) in the C-terminus is important for enzymatic activity (McFie et al., 2010). In contrast, DGAT2 has a highly conserved histidine-proline-histidine-glycine sequence in the cytosol which is required for the full catalytic activity (Stone et al., 2006). The N- and C-terminus of DGAT2 are exposed to the cytosol. DGAT2 also interacts with lipid droplets to promote the expansion of lipid droplets by catalyzing triacylglycerol synthesis in there (Jin et al., 2014, McFie et al., 2014).

In 3T3-L1 pre-adipocytes, the mRNA expression level and the DGAT activity of both DGAT1 and DGAT2 are upregulated during adipocyte differentiation (Harris et al., 2011). PPARγ and C/EBPα/β regulate the transcription of DGAT1 and DGAT2, respectively, during adipocyte differentiation by binding to the promoter (Ludwig et al., 2002, Payne et al., 2007).

Moreover, both DGAT1 and DGAT2 mRNA are positively regulated by glucose (Hirata et al., 2006), while insulin increases the mRNA expression of DGAT2 in adipocytes (Meegalla et al., 2002). Different studies also suggest that DGAT activity is regulated by post-transcriptional modification. For example, in differentiating 3T3-L1 adipocytes, DGAT activity increases 60-fold, whereas mRNA expression of DGAT1 and DGAT2 increase only 8- and 30-fold, respectively (Reed, 1978, Cases et al., 1998, 2001).

Dgat1 knockout mice have reduced triacylglycerol levels in liver and skeletal muscle with improved glucose metabolism (Chen et al., 2003). Also, these *Dgat1* knockout mice are resistant to diet-induced obesity by increasing the energy expenditure (Smith et al., 2000). In contrast, DGAT2 knockout mice become lipopenic and die within hours of birth due to rapid dehydration caused by skin abnormalities and decreased triacylglycerol storage used for energy metabolism (Stone et al., 2004). In *Dgat2* knockout mice, DGAT1 expression could not compensate for the loss of DGAT2 which suggested that mammalian DGAT1 and DGAT2 may have some different functions in triacylglycerol metabolism (Stone et al., 2004). Studies on overexpressed DGAT1

and DGAT2 in mouse liver suggest that DGAT2 plays a role in bulk triacylglycerol synthesis while DGAT1 regulates whole-body energy homeostasis (Monetti et al., 2007).

DGAT enzymes may be potential therapeutic candidates to treat metabolic diseases, such as obesity, type 2 diabetes and hyperlipidemia. Studies in homozygous and heterozygous *Dgat1* knockout mice have improved health, such as, insulin sensitization and weight reduction (Chen et al., 2002, Smith et al., 2000). Clinical trials of DGAT1 inhibitors showed reduced postprandial triacylglycerol levels, but also found significant gastrointestinal adverse effects, such as nausea, vomiting and diarrhoea (Denison et al., 2013, Meyers et al., 2015, Maciejewski et al., 2013). On the other hand, inhibition of DGAT2 by compound PF-06424439 did not have any severe side effects and decreased plasma triacylglycerol levels more than 50% in rats (Lee et al., 2013, Kim et al., 2014, Naik et al., 2014, Futatsugi et al., 2015).

1.2.2 The monoacylglycerol pathway

Although the Kennedy pathway plays a prominent role in triacylglycerol synthesis in most of the cell types, 70% of triacylglycerol is synthesized via the monoacylglycerol pathway in the small intestine (Kayden et al., 1967) (Fig. 1.5). About 95% dietary fat consists of triacylglycerol and this triacylglycerol must be hydrolyzed to 2-monoacylglycerol and free fatty acids by the pancreatic lipase in the intestinal lumen (Mattson and Volpenhein, 1968). Pancreatic lipase prefers ester bonds at the *sn*-1 and *sn*-3 positions, which leads to production of *sn*-2 monoacylglycerol and fatty acids (Mattson and Volpenhein, 1968). These *sn*-2 monoacylglycerols and fatty acids are transported out of the intestinal lumen and across the apical membrane of intestinal epithelial cells. MGAT then catalyzes the synthesis of diacylglycerol in enterocytes (Yen and Farese, 2003). DGAT uses this diacylglycerol to join with fatty acyl-CoAs, resynthesizing triacylglycerol in enterocytes. This triacylglycerol is then packaged into chylomicrons and released into the circulation. The highest MGAT activity is found in the small intestine, however, other tissues such as stomach, kidney, brown and white adipose tissues, liver, testis, brain, skeletal muscle, and heart also have much lower MGAT activity (Yen et al., 2002). The possible reason for less MGAT activity in the liver may be due to the presence of very high lipase activity in adult liver that

decreases the availability of monoacylglycerol required for diacylglycerol synthesis by MGAT (Hall et al., 2012).

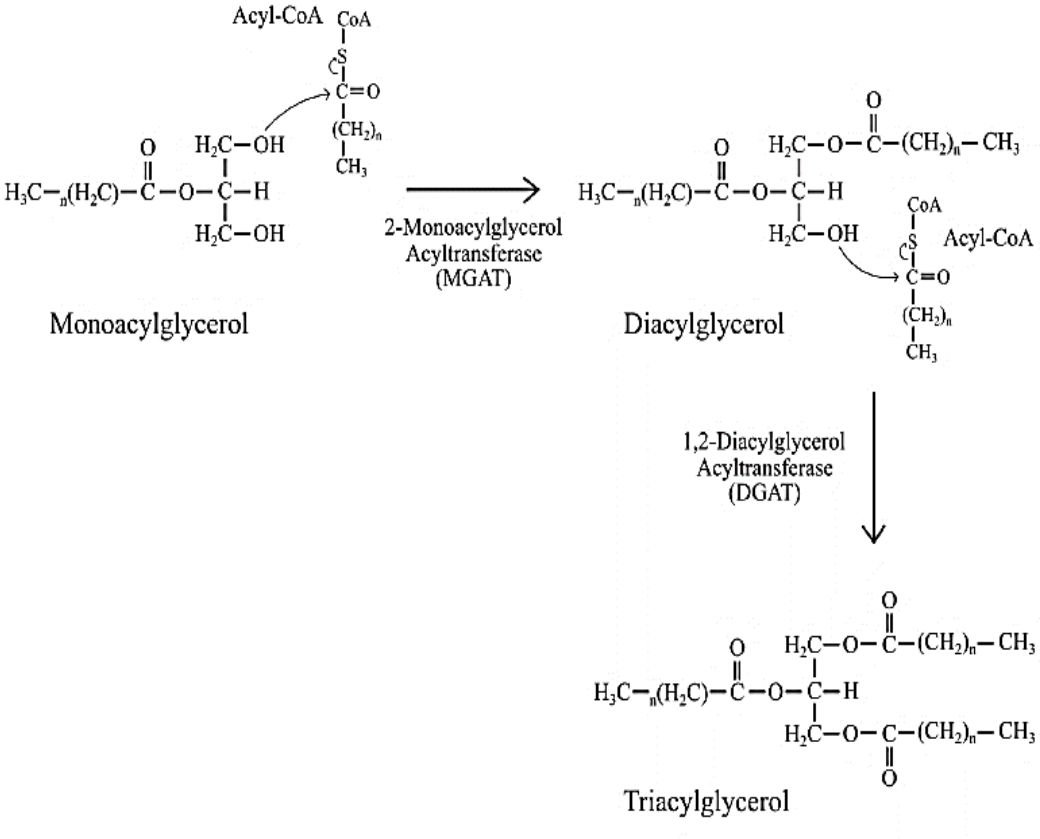


Figure 1.5: Monoacylglycerol pathway. Monoacylglycerol acyltransferase catalyzes the acylation of monoacylglycerol to produce diacylglycerol. Diacylglycerol acyltransferase then converts diacylglycerol to triacylglycerol.

Three MGAT isoforms have been identified, encoded by genes *MOGAT1-3* (Cases et al., 2001). All three *MOGAT* genes were identified based on their high sequence homology with the *DGAT2* gene (Cao et al., 2007, Yen and Farese, 2003, Yen et al., 2002, Cases et al., 2001). Like DGAT2, the MGAT enzymes have a highly conserved four amino acid sequence histidine-proline-histidine-glycine which are important for their catalytic activity (Lee and Kim, 2017).

1.2.2.1 Monoacylglycerol acyltransferase 1

The *Mogat1* gene, located on chromosome 1 (GenBank accession no. AC079223), was first cloned from mouse liver based on sequence homology to DGAT2 (Yen et al., 2002, Cases et al., 2001), which is 40% identical to mouse DGAT2. However, the human *MOGAT1* gene is located on chromosome 2 (Gene ID: 116255) with 6 exons. MGAT1 can acylate at the position of either *sn*-1 or *sn*-2 of all three stereoisomers of monoacylglycerol- *sn*-1-monooleoylglycerol, *sn*-2-monooleoylglycerol, or *sn*-3-monostearoylglycerol (Yen et al., 2002). Moreover, MGAT1 has both MGAT and DGAT activities.

MGAT1 is an integral ER membrane protein with two transmembrane domains (Lee and Kim, 2017). The transmembrane domain contains two hydrophobic regions in the N-terminus that help localize MGAT1 to the ER membrane (Lee and Kim, 2017). Both the N and C termini of MGAT1 are localized in the cytoplasm (Lee and Kim, 2017). This study also suggests that the N-terminus of MGAT1 is important for the formation of a homodimer as well as a heterodimer with DGAT2 (Lee and Kim, 2017). The interaction of MGAT1 with DGAT2 in lipid droplets is important for triacylglycerol synthesis and lipid droplet expansion in the liver (Lee and Kim, 2017).

MGAT1 is not expressed in intestine, but is detected in the stomach, kidney, uterus, liver and adipocytes (Yen et al., 2002). MGAT1 is thought to preserve polyunsaturated fatty acids in tissues, where fast turnover of triacylglycerol degradation and re-synthesis occurs (Xia et al., 1993). Though the expression level of MGAT1 is lower than that of MGAT2 and MGAT3 in both mice and human liver, PPARγ regulated MGAT1 is found to cause hepatic steatosis by accumulating excessive lipids in the liver (Lee et al., 2012, Yu et al., 2015). As a transcription factor, PPARγ regulates many lipogenic genes by directly binding to a promoter site, called the PPAR response element (Yu et al., 2015b). Studies on human hepatoma-derived cells, HepG2 revealed that unlike *MOGAT2* or *MOGAT3*, the human *MOGAT1* promoter is directly regulated by PPARγ, which suggests a possible role for MGAT1 in lipid accumulation in human hepatocytes (Yu et al., 2015b). Different studies have shown that liver steatosis as well as obesity can be treated by inhibiting MGAT1 (Hayashi et al., 2014, Soufi et al., 2014, Yu et al., 2015).

MGAT1 is also expressed in mammalian adipocytes, but the role of MGAT1 in adipocyte lipid metabolism have not been studied carefully.

1.2.2.2 Monoacylglycerol acyltransferase 2

The mouse *Mogat2* gene was first cloned based on *Mogat1* and *Dgat2* sequence homology and located on chromosome 7 (GenBank accession number NW_000328) (Yen and Farese, 2003, Cao et al., 2003). Mouse MGAT2 shares 52.5% amino acid identity to mouse MGAT1 and 47.5% identity with mouse DGAT2 (Cao et al., 2003). The hydrophobicity profile of mouse MGAT2 closely matches with mouse MGAT1 rather than DGAT2 which suggested that MGAT2 is a member of the MGAT family (Cao et al., 2003). Mouse MGAT2 and human MGAT2 share 81% amino acid identity (Yen and Farese, 2003). Like *MOGAT1*, *MOGAT2* has 6 exons that are located on chromosome 11 (GenBank accession number NT_033927) (Yen and Farese, 2003). Like MGAT1 and DGAT2, MGAT2 has two transmembrane domains that direct MGAT2 to the ER (McFie et al., 2016). Both C- and N-termini of MGAT2 face the cytosol like MGAT1 and DGAT2. However, the N-terminus is partially buried in the ER membrane (McFie et al., 2016).

MGAT2 is expressed in human liver, small intestine, stomach, kidney, colon and white adipose tissue (Yen and Farese, 2003). However, the low MGAT activity in liver compared to the small intestine suggests post-transcriptional regulation of MGAT2 (Yen and Farese, 2003).

Although MGAT2 prefers the *sn*-2-isomer of monoacylglycerol, it can acylate other stereoisomers of monoacylglycerol (Cao et al., 2003). Like mMGAT1, hMGAT2 has the lowest activity in the presence of long chain saturated fatty acids (Yen and Farese, 2003). Studies have shown that mammalian MGAT2 can interact with both DGAT1 (Zhang et al., 2014) and DGAT2 (Jin et al., 2014). Thus, MGAT2 may help in channeling diacylglycerol directly to DGAT enzymes for efficient triacylglycerol synthesis.

As mammalian MGAT2 plays a role in triacylglycerol synthesis in intestine, MGAT2 may be a useful drug target for treating obesity. Mice lacking MGAT2 are protected from metabolic disorders, such as high fat diet-induced obesity and insulin resistance (Yen et al., 2009, Tsuchida et al., 2012). In addition, although dietary fat can be absorbed fully by the small intestine of MGAT2-deficient mice, fat absorption was delayed (Yen et al., 2009, Tsuchida et al., 2012). Recently, JTP-103237 (7-(4,6-Di-tert-butyl-pyrimidin-2-yl)-3-(4-tri-fluoromethoxy-phenyl)-5,6,7,8-tetrahydro-[1,2,4] triazolo [4,3-a] pyrazine derivative) has been found to enhance energy expenditure and decrease fat absorption by the inhibition of intestinal MGAT2 in humans, rats and mice (Okuma et al., 2015).

1.2.2.3 Monoacylglycerol acyltransferase 3

Human *MOGAT3* is located on chromosome 7 and has 8 exons (Gene ID: 346606). Although MGAT3 is an isoform of the MGAT enzymes, it has more amino acids sequence similarity to DGAT2 (60%) than MGAT1 (51%) or MGAT2 (55%) (Cheng et al., 2003). However, MGAT3 does not have the lysine residues that are responsible for DGAT2 degradation, and thus MGAT3 becomes more stable than DGAT2 (Brandt et al., 2016).

Mogat3 is a pseudogene in mice, while in rats, it encodes an active enzyme (Yue et al., 2011). The mRNA level of human MGAT3 is highly expressed in gastrointestinal tract and liver (Cheng et al., 2003, Hall et al., 2012). In the intestine, human MGAT3 is mostly expressed in the ileum, while MGAT2 is expressed in the proximal intestine (Yue et al., 2011). However, MGAT3 is responsible for the most of the MGAT activity in obese human liver, and weight loss can downregulate the expression of MGAT3 in the liver (Hall et al., 2012).

MGAT3 has both MGAT as well as DGAT activity. Its DGAT activity is robust when the concentration of monoacylglycerol is low (Cheng et al., 2003, Cao et al., 2007). MGAT3 has a high affinity for *sn-2*- monoacylglycerol compared to MGAT1 and MGAT2. MGAT3 has a strong DGAT activity compared to that of MGAT1, and its DGAT activity is closely similar to that of DGAT2 (Brandt et al., 2016). However, unlike DGAT2, MGAT3 is not present around the lipid droplets after oleate-loading, suggesting unique biochemical properties of these two enzymes to produce triacylglycerol (Brandt et al., 2016).

1.3 Storage of triacylglycerol in lipid droplets

Lipid droplets are an organelle that are found in almost all organisms. They are dynamic organelles able to move via the cellular cytoskeleton. The diameters of lipid droplets varies from 0.1-100 µm depending on cell type (Tauchi-Sato et al., 2002). In adipocytes, lipid droplets occupy almost the entire cytoplasm by having a diameter of more than 100 µm. Lipid droplets consist of a neutral lipid core enveloped by a phospholipid monolayer, such as, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and specific surface proteins (Tauchi-Sato et al., 2002, Walther and Farese, 2012). Phosphatidylcholine is found as the primary phospholipid in the monolayer as its cylindrical shape reduces the surface tension, and thus stabilizes the lipid droplets

(Tauchi-Sato et al., 2002, Thiam et al., 2013). Lipid droplets store mainly triacylglycerols and sterol esters in their hydrophobic core and thus separate the nonpolar triacylglycerols from the aqueous intracellular environment. Lipid droplets in adipocyte mainly store triacylglycerols, while in macrophage, lipid droplets store sterol esters (Buhman et al., 2001). These different neutral lipids in lipid droplets suggests that lipid droplets have specialized functions in different cell types.

In eukaryotes, three models of lipid droplet formation from the ER have been proposed. The first one is ER budding, in which lipid droplets are formed from the ER bilayer. After formation, the lipid droplets either stay connected with or bud off from the ER membrane (Walther and Farese, 2009). The next model is bicelle formation where both membranes of the ER form lipid droplets by entire excision (Ploegh, 2007). Finally, in the vesicular budding system, triacylglycerols accumulate in the intramembranous space of a bilayer vesicle (Walther and Farese, 2009). Association of the ER with lipid droplets is thought to be mediated by small GTPase Rab18 which is found after lipid droplets formation (Ozeki et al., 2005).

Lipid droplets have several other functions besides energy storage. For example, they can store histones during *Drosophila* embryogenesis until needed for nuclear division in embryo segmentation (Cermelli et al., 2006). In addition, before proteasomal degradation, lipid droplets may also store unfolded membrane proteins (Welte, 2007). Hepatitis C virus assembly also take place in lipid droplets (Herker and Ott, 2011).

1.4 Digestion, mobilization and transport of lipids

Eukaryotic cells have three sources of fatty acids that can be used as energy: dietary fat, lipid droplet as stored fat, and *de novo* synthesis of fat that is transported from one organ to other. After consuming a fatty meal, bile salts solubilize dietary fat, cholesterol and lipid-soluble vitamins in the small intestine (Zhou and Hylemon, 2014). Bile salts act as biological detergents which are synthesized from cholesterol in the liver, followed by storage in the gallbladder (Zhou and Hylemon, 2014). During fat digestion, pancreatic lipase is released into the small intestine and hydrolyzes triacylglycerols at *sn*-1 and *sn*-3 positions to produce 2-monoacylglycerol and fatty acids (Mattson and Volpenhein, 1968) (Fig. 1.6). These products are taken up by the apical membrane of enterocytes through passive diffusion and protein transport, such as fatty acid binding proteins, fatty acid transport protein 4 and fatty acid translocase (Patton and Carey, 1979, Abumrad

and Davidson, 2012). The absorbed fatty acids and monoacylglycerol are directed to the ER to resynthesize triacylglycerol via the MGAT pathway. Triacylglycerol and cholesterol then combine with specific apolipoproteins to make chylomicrons, which are released into the lymphatic system.

In the capillaries of peripheral tissues, lipoprotein lipase hydrolyses triacylglycerol into fatty acids and glycerol which are taken up by cells (Young and Zechner, 2013). In muscle, the fatty acids are oxidized to produce energy, whereas in adipocytes, fatty acids are re-esterified to store triacylglycerol as a lipid droplet.

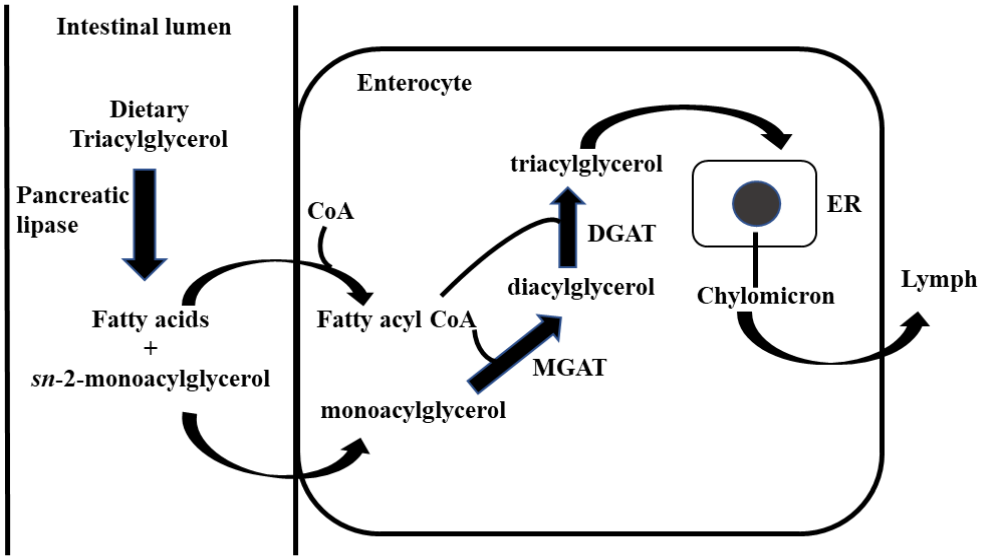


Figure 1.6: Digestion and absorption of triacylglycerol in the small intestine. Dietary triacylglycerol is hydrolyzed into sn-2-monoacylglycerol and fatty acids by pancreatic lipase in intestinal lumen, and then absorbed by the enterocyte, where triacylglycerol is re-synthesized by the monoacylglycerol pathway. Triacylglycerol is then incorporated into chylomicron and released into the lymph.

1.5 Triacylglycerol catabolism: Lipolysis

Triacylglycerols in lipid droplets are broken down into glycerol and fatty acids through lipolysis (Fig. 1.7). Lipolysis derived fatty acids bind with albumin to circulate to the tissues such as, skeletal muscle, heart, and renal cortex through the blood, and serve as energy by β-oxidation (van der Vusse, 2009). During fasting, lipolysis is stimulated by catecholamines and stress

hormones, such as epinephrine in adipocytes, while inhibited by insulin after feeding (Large et al., 2004). When blood glucose level is low, catecholamines stimulate β-adrenergic signaling on the cell-surface membrane of adipocytes, so which increases cAMP and activate protein kinase A (Carmen and Víctor, 2006). This protein kinase A phosphorylates perilipin-1, that is found along with fat specific protein-27 in large lipid droplets in adipocytes, which then dissociates from comparative gene identification-58 (CGI-58) (Grahn et al., 2013, Tansey et al., 2001, Yamaguchi et al., 2006). Released CGI-58 activates adipose triglyceride lipase which hydrolyses triacylglycerol at *sn*-1 and *sn*-2 position to produce *sn*-1,3-diacylglycerol or *sn*-2,3-diacylglycerol, respectively (Zimmermann et al., 2009). CGI-58 thus regulates lipolysis by interacting with either perilipin-1 or adipose triglyceride lipase.

Protein kinase A also phosphorylates cytosolic hormone-sensitive lipase at Ser650. After phosphorylation, hormone-sensitive lipase forms a complex with fatty acid-binding protein-4 (Smith et al., 2007) and moves to the lipid droplets surface to bind with phosphorylated perilipin-1. Cytosolic fatty acid-binding protein-4 is highly expressed in adipocytes and facilitates fatty acids intracellular trafficking and lipolysis. Hormone-sensitive lipase hydrolyses diacylglycerol at *sn*-3 position (Rodriguez et al., 2010, Wang et al., 2009) to produce monoacylglycerol. The monoacylglycerol is then hydrolyzed by monoacylglycerol lipase to produce glycerol and fatty acids which are released into the circulation and delivered to tissue.

However, the release of glycerol and fatty acids from adipocytes does not occur in the ratio of 1:3 because up to 70% of fatty acids are re-esterified to produce new triacylglycerols in adipocytes (Forest et al., 2003, Vaughae, 1962, Jensen et al., 2001) (Fig. 1.7). Re-esterification of fatty acids is an energy-consuming recycling process, but the exact pathway for re-esterification is unknown (Chitraju et al., 2017). During lipolysis, high rates of fatty acid re-esterification have been found in mammalian adipocytes (Reshef et al., 2003). This esterification needs glycerol-3-phosphate to incorporate fatty acids into triacylglycerol, but the supply of glycerol-3-phosphate from glycolysis is reduced during lipolysis. The glycerol, derived from lipolysis in adipocytes, cannot be re-phosphorylated to glycerol-3-phosphate since the activity of glycerol kinase is very low in adipocytes (Reshef et al., 2003). Moreover, most of the glycerol that is released into the circulation is taken up by the liver and used for gluconeogenesis. So, the question is-'from where does the glycerol-3-phosphate arise to re-esterify fatty acids during lipolysis'. Different studies

proposed a pathway, named glyceroneogenesis, where glycerol-3-phospahte is synthesized from non-carbohydrate precursors, such as pyruvate, lactate, and amino acids (Ballard et al., 1967, Reshef et al., 1970).

A recent study has found that DGAT1, not DGAT2, plays an important role in fatty acid re-esterification in adipocytes, and thus inhibits the lipotoxic stress, caused by increased fatty acid levels during lipolysis (Chitraju et al., 2017). This study also shows that in the absence of DGAT1, the level of glycerol and fatty acids become increased in stimulated lipolysis that suggests the complete hydrolysis of triacylglycerol to produce glycerol and fatty acids (Chitraju et al., 2017). Though the level of the intermediate products, such as monoacylglycerol, diacylglycerol was not measured, the possibility of fatty acid re-esterification by the MGAT pathway cannot be excluded.

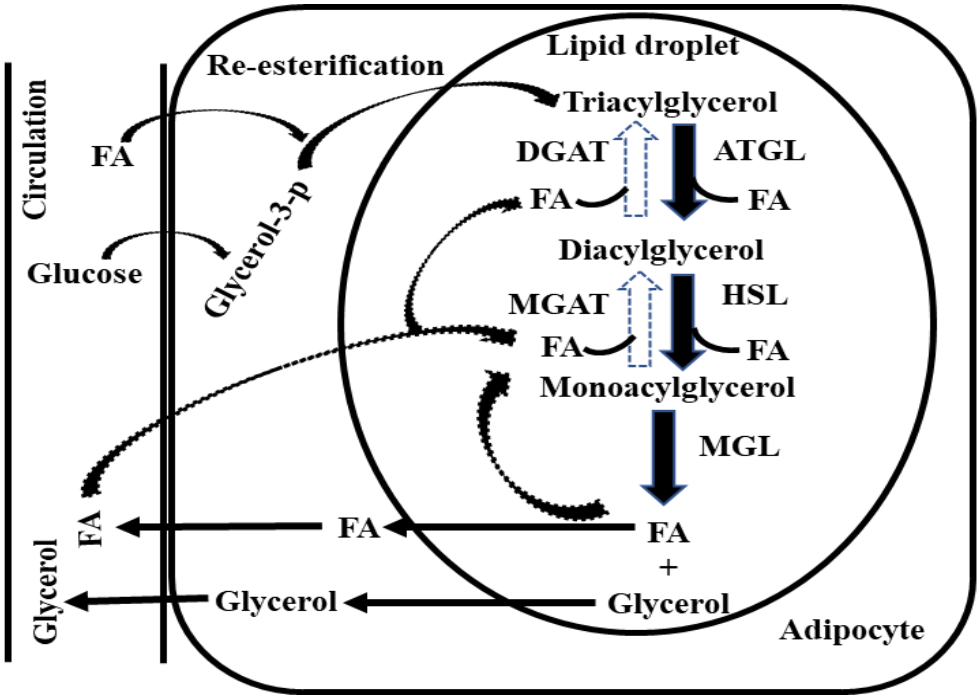


Figure 1.7: Lipolysis and re-esterification of triacylglycerol in adipocyte. Complete hydrolysis of triacylglycerol produces fatty acids and glycerol. Glycerol is released into the circulation, whereas fatty acids can be re-esterified with glycerol-3-phosohate to produce triacylglycerol again. Partial hydrolysis of triacylglycerol produces monoacylglycerol and diacylglycerol which are assumed to re-cycle to produce triacylglycerol. ATGL: adipose triglyceride lipase; HSL: hormone-sensitive lipase; MGL: monoacylglycerol lipase; FA: fatty acids

1.6 Differentiation and lipid metabolism in adipocytes

Adipose tissue is a loose connective tissue that is derived from preadipocytes in the embryonic mesoderm and composed of mostly adipocytes as well as fibroblast, vascular endothelial cells and a variety of immune cells (Poulos et al., 2016). Adipocytes are classified into three main types based on the color of the adipose tissue: white, brown and beige. White adipocytes are composed of triacylglycerol and cholesterol esters that store energy, while brown adipocytes regulate thermogenesis (Cannon and Nedergaard, 2008). However, beige cells are derived from white adipocytes, though they have a similar role as brown adipocytes (Cinti, 2012).

Progenitors of adipocytes are generated from pluripotent and multipotent mesenchymal stem cells (Otto and Lane, 2005). Terminal differentiation of these progenitors into mature functional adipocytes are required for adipogenesis. Different studies showed that proliferation and differentiation of adipocyte progenitors is regulated by fibroblast growth factor 1 and activin A. Fibroblast growth factor 1 stimulates both proliferation and differentiation, while activation of activin A promotes only proliferation and impairs differentiation (Widberg et al., 2009, Zaragosi et al., 2010). Cell cycle regulator cyclin dependent kinase 4 also plays an important role in adipocyte differentiation by activating transcription of PPARγ targeted genes (Abella et al., 2005). Nuclear hormone receptor PPARγ is known as the 'master regulator' of adipogenesis as it is dramatically upregulated during adipogenesis (Rosen et al., 2002, Shao et al., 2016). In adipose precursors, expression of PPARγ is very low, but once activated, it creates a positive feedback loop to activate its own expression that drives adipocyte differentiation. PPARγ also activates the expression of its upstream regulator, C/EBPα which further induces PPARγ expression in adipogenesis by binding to the PPARγ promoter (Rosen et al., 2002, Lee and Ge, 2014). However, PPARγ knockout mice can not form differentiated adipose tissues and thus develop fatty liver and lipodystrophy (Rosen et al., 1999). In addition, mutations of the PPARγ gene play role in lipodystrophy as well as other metabolic diseases such as hypertension and insulin resistance in humans (Monajemi et al., 2007, Agarwal and Garg, 2002). Several inhibitors of PPARγ have been identified. For example, tumor necrosis factor α and interleukin 1 inhibit PPARγ which in turn suppress adipose conversion (Suzawa et al., 2003). Moreover, the Wnt signalling pathway inhibits adipocyte differentiation by suppressing PPARγ (Ross et al., 2000). However, C/EBPβ, an important early transcription factor of adipogenesis, induces the expression of PPARγ and C/EBPα by binding to their promoters (Guo et al., 2014). Knockdown of C/EBPβ in 3T3-L1 preadipocytes

inhibits adipocyte differentiation (Zhang et al., 2011), and a lack of C/EBPβ in mice also impaired the development of adipose tissue (Tanaka et al., 1997). Taken together, these results suggested that C/EBPβ plays an important role in adipocyte differentiation (Guo et al., 2014). Therefore, adipogenesis is a multistep process with a cascade of transcription factors and cell cycle proteins that regulate gene expression and lead to adipocyte development.

The main role of the adipose tissue is to store energy. During a time of excess energy, fatty acids are stored as triacylglycerol in adipocytes. When energy is needed, lipolysis of triacylglycerol release fatty acids back into the circulation. Thus, these two opposite processes in adipocytes, lipolysis and re-esterification regulate the concentration of fatty acids in the circulation (Zimmermann et al., 2003). High level of circulating fatty acids due to dysregulated lipolysis can lead to insulin resistance and diabetes mellitus (Jiao et al., 2011). In contrast, increased mass of adipose tissue in animals is responsible for obesity, and this condition also plays a crucial role in obesity related complications such as type 2 diabetes, hypertension, hyperlipidemia, and arteriosclerosis (Haslam and James, 2005).

However, compared to the Kennedy pathway, the role of MGAT pathway in adipocyte lipid metabolism has not been studied. Both MGAT1 and MGAT2 are found in mouse adipocytes, but their function in this tissue is not clear yet. A recent study showed the involvement of MGAT1 in triacylglycerol synthesis and suppression of lipolysis in adipocytes (Liss et al., 2018).

CHAPTER 2: Hypothesis and Objectives

Hypothesis

MGAT1 and/or MGAT2 synthesize diacylglycerol that contributes to triacylglycerol formation in adipocytes and are important for adipocyte differentiation.

Objectives

1. Assess MGAT activity during adipocyte differentiation.
2. Determine levels of MGAT1 and MGAT2 mRNA expression during adipocyte differentiation.
3. Determine if MGAT1 or MGAT2 are required for adipocyte differentiation.
4. Determine the roles of MGAT1 and MGAT2 in adipocyte lipid metabolism.

CHAPTER 3: Materials and Methods

3.1 Reagents

Table 3.1 shows the names of reagents and their corresponding supplier.

Table 3.1 Reagents and suppliers

General reagents and consumables	**Supplier**
BODIPY 493/503	Invitrogen
Bovine serum albumin – fatty acid free	Sigma- Aldrich
CPT-cAMP	Sigma- Aldrich
1,2 Dioleoyl-*sn*-glycerol	Sigma- Aldrich
DGAT1 inhibitor (PF-04620110)	Sigma- Aldrich
DGAT2 inhibitor (PF-06424439)	Sigma- Aldrich
1 kb DNA ladder	FroggaBio
ProLong Diamond Antifade Mountant with DAPI	Fisher
Fugene 6 transfection reagent	Roche
[^{3}H] glycerol	American Radiolabeled Chemicals
Insulin	Sigma- Aldrich
Iodine	Sigma- Aldrich
2- oleoylglycerol	Sigma
NBD – palmitoyl CoA	Avanti
Oleic acid	Sigma- Aldrich
Purelink RNA mini kit	Ambion
Superscript III one-step RT-PCR system with platinum *Taq* DNA polymerase	Invitrogen
Scintillation liquid	Beckman coulter
Trypsin	Sigma- Aldrich
Free glycerol reagent	Sigma
Non-esterified fatty acids detection 500-point kit	Zenbio
Bacterial cell culture reagents	**Supplier**
Ampicillin	Fisher
Bacto agar	Difco
Chloramphenicol	Fisher
DH5α Competent *E. coli*	Invitrogen
Tryptone	EMD
Yeast extract	EMD
Mammalian cell culture reagents	**Supplier**
Antibiotic-Antimycotic 100x	Gibco
Dulbecco's modified eagle's medium	Lonza
Dexamethasone	Sigma
3-Isobutyl-1-methylxanthine (IBMX)	Sigma
Puromycin	Invivogen
Troglitazone	Sigma

Table 3.2 Reagent supplier addresses

Supplier	Location
Ambion	Waltham, Massachusetts, USA
American Radiolabeled Chemicals	St. Louis, Missouri, USA
Beckman coulter	Fullerton, California, USA
Difco	Franklin Lakes, New Jersey, USA
EMD	Chicago, Illinois, USA
Fisher	Waltham, Massachusetts, USA
FroggaBio	Toronto, Ontario, Canada
Gibco	Waltham, Massachusetts, USA
Invitrogen	Waltham, Massachusetts, USA
Invivogen	San Diego, California, USA
Lonza	Basel, Switzerland
Roche	Indianapolis, Indiana, USA
Sigma-Aldrich	St. Louis, Missouri, USA
Zenbio	North Carolina, USA

3.2 Adipocyte differentiation

3T3-L1 mouse preadipocytes (American Type Tissue Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine growth serum (BGS) and 1x antibiotic-antimycotic to confluency at 37 °C, 5% CO_2, and 95% humidity. We used 3T3-L1 preadipocyte cells because of their potential to differentiate from fibroblasts to adipocytes (Poulos et al., 2010). After 48 hours of cell seeding, the cells became 90% confluent and plates were washed with PBS and refed with DMEM/10% BGS containing 1 µM dexamethasone, 10 µM troglitazone, 5 µg/mL insulin and 0.5 mM IBMX to induce adipocyte differentiation. At day 2 and 4 after initiation of differentiation, the medium was replaced with the fresh differentiating cocktail. At day 7, cells were incubated with DMEM containing 10% BGS and 5 µg/mL insulin. Fresh maintenance medium was replaced every 2 days thereafter.

3.3 *In vitro* MGAT activity assay

In vitro MGAT activity was determined by measuring the formation of N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)-methyl] amino (NBD)-diacylglycerol from NBD-palmitoyl-CoA in total cell extracts of preadipocytes and adipocytes samples. NBD-palmitoyl-CoA is a fluorescent fatty acyl-CoA (McFie and Stone, 2011). The reaction mixture consisted of: 100 mM Tris-HCl (pH 7.6), 20

mM $MgCl_2$, 0.625 mg/mL of bovine serum albumin (BSA) (fatty acid free), 200 µM 2-monooleoylglycerol, 25 µM NBD-palmitoyl-CoA, 100 mM sucrose, brought to a final volume of 150 µL with double-distilled water. Total cell extracts, at a volume of 47.5 µL (diluted to 1 µg/µL in 50 mM Tris-HCl pH 7.6, 250 mM sucrose) were incubated for 30 minutes on ice with 2.5 µL of 40 mM DGAT1 inhibitor (pyrimidooxazepinone, PF-04620110) (Dow et al., 2011) to get the final volume of 50 µL. In the absence of DGAT1 inhibitor, 2.5 µL dimethyl sulfoxide (DMSO) was added to the final volume of 50 µL, and kept on ice for 30 minutes. Samples were then incubated at 37 °C for 10 minutes with the reaction mixture. Reactions were terminated by the addition of 4 mL $CHCl_3$:MeOH (2:1, v/v), followed by addition of 800 µL water which was mixed by vortexing. Samples were centrifuged at room temperature for 5 minutes at 775 x g. The upper aqueous layer was aspirated followed by air-drying of the organic phase. Lipids were dissolved in 65 µL of $CHCl_3$:MeOH (2:1) and applied to a channeled 20 x 20 cm thin layer chromatography (TLC) plate. The plate was developed in diethyl ether/hexane/methanol/acetic acid (55:45:5:1, v/v/v/v). The plate was then allowed to air dry for 1 hour. NBD-diacylglycerol was detected with a VersaDoc 4000 molecular imaging system (Bio-Rad Laboratories, Inc.) and Quantity One software (Bio-Rad Laboratories, Inc.).

3.4 *In vitro* DGAT activity assay

DGAT assays were performed the same as MGAT assays except the substrate 1,2-dioleoyl-sn-glycerol was used instead of 2-monooleoylglycerol. After the reaction, fluorescently active NBD-triacylglycerol was detected with a VersaDoc 4000 molecular imaging system (Bio-Rad Laboratories, Inc.) and Quantity One software (Bio-Rad Laboratories, Inc.).

3.5 Reverse transcriptase (RT)-PCR to determine mRNA expression levels

Total RNA was isolated from cells using Purelink RNA Mini Kit (Ambion, life technologies) according to the manufacturer's instructions. Primer pairs are detailed in Table 3.3. Superscript III one-step RT-PCR system with platinum *Taq* DNA polymerase (Invitrogen) was used for RT-PCR. PCR reactions were conducted in a final volume of 25 µL containing the following reagents: 12.5 µL 2 x reaction mix, 1 µL Superscript III RT/Platinum *Taq* mix, 50 ng of template RNA, 10 µM of each primer (forward and reverse) and 9.5 µL cross-linked ultrapure

water. The thermal cycler was programed as described in Table 3.4. Reaction products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide and detected with a VersaDoc 4000 molecular imaging system (Bio-Rad Laboratories,Inc.) as well as Quantity One software (Bio-Rad Laboratories, Inc.).

Table 3.3 Primers

Primer name	**Primer sequence**
MGAT1 F	5'-GCAGCCTAATTGGGGCGAA-3'
MGAT1 R	5'-ATTTCCAAAGGCTCCAGGCA-3'
MGAT2 F	5'-GAGCGCAGGTTACAGACCTT-3'
MGAT2 R	5'-AGCTGTCTTGACCAAAGAGACA-3'
DGAT1 F	5'-GTGCACAAGTGGTGCATCAG-3'
DGAT1 R	5'-CAGTGGGACCTGAGCCATC-3'
β-actin F	5'-GACATGGAGAAAATCTGGCA-3'
β-actin R	5'-AATGTCACGCACGATTTCCC-3'
PPARγ F	5'-GAGCTGACCCAATGGTTGCTG-3'
PPARγ R	5'-GCTTCAATCGGATGGTTCTTC-3'
CEBPα F	5'-GAACAGCAACGAGTACCGGGTA-3'
CEBPα R	5'-GCCATGGCCTTGACCAAGGAG-3'

Table 3.4 Thermal cycle

cDNA synthesis and pre-denaturation		Denature	Anneal	Extend	Final extension
1 CYCLE		40 CYCLES			1 CYCLE
55°C	94°C	94°C	57°C	68°C	68°C
30 minutes	2 minutes	15 seconds	30 seconds	26 seconds	5 minutes

3.6 Bacterial strain and media preparation

DH5α competent *E. coli* were transformed with plasmids for bulk amplification. Transformation was conducted according to manufacturer specifications. A mix of 20 µL DH5α competent cells with 1 µL of 1 ng/µL plasmid was incubated on ice for 30 minutes, followed by 25 seconds heat shock at 42ºC. After that, the mixture was kept on ice for 2 minutes and then 280 µL prewarmed Luria-Bertani media was added to incubate at 37ºC for 1 hour with shaking. The bacterial transformation mix was plated onto Luria-Bertani broth agar plates. Luria-Bertani broth,

consisting of 10 g tryptone, 5 g yeast extract and 10 g of NaCl in 1 L of double-distilled water, was supplemented with 15 g of agar and autoclaved for 20 minutes at 15 psi. Once cooled, appropriate antibiotics were added (34 µg/mL chloramphenicol for MGAT1/2 shRNA plasmids, and 100 µg/mL ampicillin for psPAX2 and pMD2.G) and the Luria-Bertani agar solution was transferred into petri dishes. Colonies were cultured overnight in Terrific broth, composed of 2.4 g tryptone, 4.8 g yeast extract and 0.8 mL glycerol – brought to a final volume of 200 mL with double-distilled water. The solution was autoclaved for 20 minutes at 15 psi, before the addition of 20 mL solution containing 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 and appropriate antibiotic (ampicillin or chloramphenicol).

3.7 Knockdown of MGAT1 and MGAT2 by using short hairpin RNA (shRNA)

For lentivirus production, HEK-293T cells (American Type Tissue Culture Collection) were used as a packaging cell line, and transfected with packaging plasmid-psPAX2, envelope plasmid-pMD2.G and specific shRNAs by using Fugene transfection reagent. Four unique mouse 29mer shRNAs were constructed in lentivirus GFP vector for each gene (MGAT1 and MGAT2). A scrambled non-targeting shRNA was used as a negative control. After 18 hours of transfection, media was replaced with high-BSA growth media, and after 24 hours, media containing lentiviruses were harvested. 3T3-L1 pre-adipocytes were transduced with lentivirus for 24 hours and then selected with puromycin for 48 hours. After 48 hours, stable infected cells were harvested for further experiments.

3.8 Fluorescence microscopy

Cells on glass coverslips were fixed with 4% paraformaldehyde in PBS for 10 minutes followed by permeabilization of cellular membranes with 0.2% Triton X-100 in PBS for 5 minutes at room temperature. Cells were washed with PBS and incubated with BODIPY 493/503 (1:50) for 1 hour to visualize lipid droplets. The cells were then washed three times with PBS and the coverslips were mounted on glass slides with a drop of DAPI containing mounting media. To stimulate triacylglycerol synthesis, preadipocytes were incubated in the presence or absence of 0.5 mM oleic acid for 12 hours. Images were taken by Zeiss LSM700 laser scanning confocal

microscope. Image processing was conducted using ImageJ (National Institutes of Health, rsb.info.nih.gov/ij/).

3.9 Lipid extraction

Total cell extracts, prepared in 0.5% Triton X-100 in PBS (pH 7.4) were diluted to a final volume of 1 mL with double-distilled water. Four mL of $CHCl_3$:MeOH was added and samples were vortexed for 30 seconds, followed by centrifugation at 775 x g for 5 minutes. The upper aqueous layer was removed followed by air-drying of the organic phase. Lipids were resuspended in 65 µL of $CHCl_3$:MeOH (2:1) and applied to a channeled 20 x 20 cm TLC plate. The plate was developed in hexane:ethyl ether:acetic acid (80:20:1, v/v/v). Plates were air-dried and then briefly submerged in a solution of 10% cupric sulfate (w/v) and 8% phosphoric acid. Lipids were observed by charring at 180ºC until bands appeared. Images were taken by using a VersaDoc 4000 molecular imaging system (Bio-Rad Laboratories,Inc.) and Quantity One software (Bio-Rad Laboratories, Inc.).

3.10 Quantification of radiolabeled triacylglycerol synthesis

Differentiated adipocytes were incubated at 37 ºC with 10 µCi of [^{3}H] glycerol in DMEM+10 % BGS for 4 hours. The cells were then lysed with 1 % Triton X-100 in PBS and the protein concentration was determined. Total cell extracts, at a volume of 100 µL were diluted to a final volume of 1 mL double-distilled water. Four mL $CHCl_3$:MeOH was added and vortexed for 30 seconds, followed by centrifugation at 775 x g for 5 minutes. The upper aqueous layer was removed followed by air-drying of the organic phase. Lipids were dissolved in 65 µL of $CHCl_3$:MeOH (2:1) and applied to a channeled 20 x 20 cm TLC plate. The plate was developed in a hexane:ethyl ether:acetic acid (80:20:1, v/v/v) solvent system. After completely air-dried, iodine vapor was used to visualize lipids. The triacylglycerol band was scraped into a scintillation vial with 5 mL scintillation liquid, followed by vortexing. Radioactivity was quantified using a Beckman Coulter LS 6000 IC liquid scintillation counter.

3.11 Lipolysis assay

To determine the role of MGAT1 and MGAT2 in free fatty acid re-esterification, differentiated adipocytes were incubated in DMEM containing 2% BSA (fatty acids free). To stimulate lipolysis, 0.5 mM 8-(4-Chlorophenylthio) adenosine 3′,5′-cyclic monophosphate sodium salt (CPT-cAMP) (Sigma-Aldrich) was added in the media for 4 hours, whereas in basal state, no CPT-cAMP was added. The media was saved for glycerol and free fatty acids measurement and the cells were lysed to determine the intracellular protein concentration. Glycerol and free fatty acids concentrations were measured by free glycerol reagent (Sigma-Aldrich) and non-esterified fatty acids detection 500-point kit (Zenbio), respectively, according to the manufacturer's instructions.

CHAPTER 4: Results

4.1 MGAT activity increased during adipocyte differentiation

The role of MGAT1 and MGAT2 in triacylglycerol synthesis is well studied in mammalian liver and intestine, respectively. Both enzymes are also found in adipose tissue, but their roles in lipid metabolism in this tissue are not clear. Since adipocytes accumulate triacylglycerols during adipocyte differentiation, we were interested in determining whether MGAT1 or MGAT2 plays any role in triacylglycerol synthesis in adipocytes. 3T3-L1 pre-adipocytes were induced to differentiate to adipocytes. Total cell extracts were isolated at day 0, 4 and 10 of adipocyte differentiation. MGAT activity of cell lysates was assessed by measuring the incorporation of fluorescent NBD-palmitoyl-CoA into NBD-diacylglycerol. Since DGAT1 has MGAT activity, we used the DGAT1 inhibitor, PF-04620110 (Dow et al., 2011) to measure only MGAT-derived MGAT activity. In the absence of DGAT1 inhibitor, MGAT activity increased by approximately 4-fold by day 10 (Fig. 4.1). In the presence of DGAT1 inhibitor, MGAT activity was reduced compared to the MGAT activity without DGAT1 inhibitor. However, in the presence of the inhibitor, the MGAT activity still increased during adipocyte differentiation. This suggests that DGAT1 accounts for a large amount of the MGAT activity in adipocytes in an *in vitro* assay.

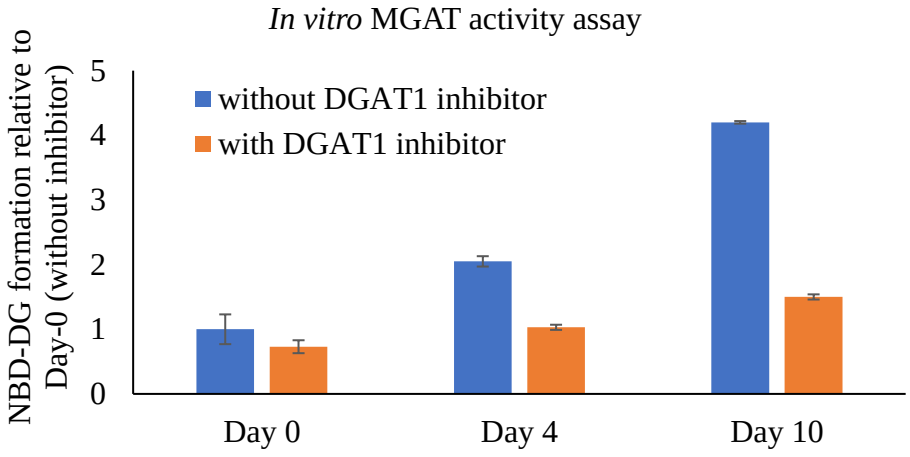


Figure 4.1: Increased *in vitro* MGAT activity during adipocyte differentiation. *In vitro* MGAT activity of total cell extracts, isolated from day 0, 4 and 10 of differentiating 3T3-L1 adipocyte was determined. MGAT activity was measured in both the presence and absence of a DGAT1 inhibitor. Results shown represent the mean ± standard deviation obtained from duplicate samples in one experiment; the experiment was repeated, yielding similar results.

4.2 No change in MGAT1 and MGAT2 mRNA expression during adipocyte differentiation

Having identified that *in vitro* MGAT activity increased during adipocyte differentiation, we were interested in examining if the mRNA expression levels of MGAT1 and MGAT2 also increased. 3T3-L1 cells were treated with the differentiation cocktail and RNA was isolated at day 0, 4 and 10. The mRNA expression levels of PPARγ, C/EBPα, β-actin, MGAT1 and MGAT2 were determined by RT-PCR (Fig. 4.2). Both PPARγ and C/EBPα are transcription factors which activate genes that stimulate lipid uptake and adipogenesis in fat cells (Cornelius et al., 1994), and are routinely used as markers of adipocyte differentiation. Since the mRNA level of PPARγ and C/EBPα was increased in this experiment, it indicates the differentiation of pre-adipocytes to adipocytes. β-actin was used to show that the quality of the RNA was satisfactory. During adipocyte differentiation, there was no change in the mRNA expression of both MGAT1 and MGAT2, while the MGAT-derived MGAT activity increased (Fig. 4.1). These results suggest the post-translational modification of MGAT1 and MGAT2 during adipocyte differentiation.

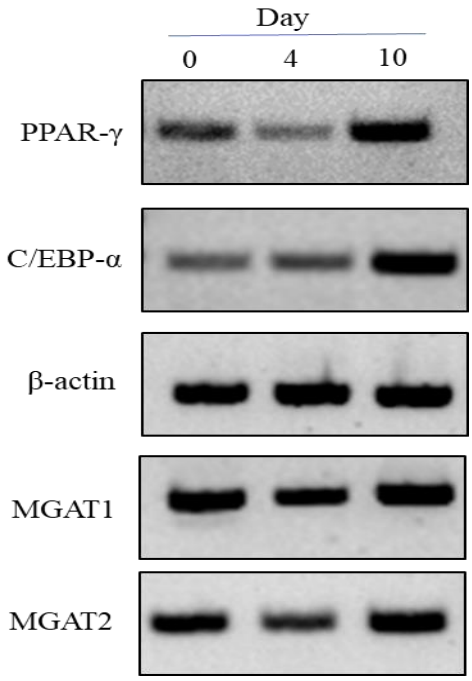


Figure 4.2: MGAT1 and MGAT2 mRNA expression levels during adipocyte differentiation. RNA was isolated from day 0, 4 and 10 of differentiating 3T3-L1 adipocyte. PPARγ, C/EBPα, β-actin, MGAT1 and MGAT2 mRNA expression levels were determined by reverse transcriptase (RT)-PCR.

4.3 Knockdown of MGAT1 and MGAT2 gene expression in 3T3-L1 pre-adipocyte

To determine the roles of MGAT1 and MGAT2 in adipocyte lipid metabolism, we used shRNA to knockdown MGAT1 and MGAT2 gene expression in 3T3-L1 preadipocytes. Knockdown was confirmed by RT-PCR (Fig 4.3). Scrambled shRNA was used as a negative control. MGAT1 mRNA was not detectable in MGAT1 knockdown cells, while MGAT2 mRNA expression was unaffected by shMGAT1 treatment. On the other hand, in MGAT2 knockdown cells, MGAT2 mRNA expression was decreased. Unexpectedly, MGAT1 mRNA expression was also decreased. β-actin mRNA was detected in all three samples to confirm the RNA quality.

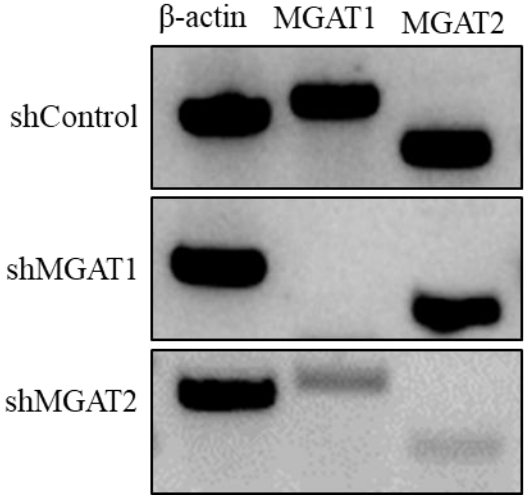


Figure 4.3: Knockdown of MGAT1 and MGAT2 gene expression in 3T3-L1 pre-adipocytes by using shRNA. After 24 hours of lentivirus transduction and 48 hours of puromycin selection, stable shControl or shMGAT1 or shMGAT2 containing 3T3-L1 preadipocytes were harvested for RNA isolation. Knockdown was confirmed by reverse transcriptase (RT)-PCR. β-actin was used to confirm RNA quality. Scrambled shRNA was used for a negative control.

4.4 Adipocyte differentiation does not require MGAT1 or MGAT2

One of our objectives was to determine if adipocyte differentiation required MGAT1 or MGAT2. To detect this, MGAT1 and MGAT2 knockdown preadipocytes were induced to differentiate. RNA was then isolated at day 0 and day 10 of differentiation from both MGAT1 and MGAT2 knockdown cells. The expression levels of PPARγ mRNA as well as C/EBPα mRNA were compared between day 0 and 10 samples by RT-PCR (Fig. 4.4). Since the mRNA levels of both adipocyte differentiation markers, PPARγ and C/EBPα, increased from day 0 to day 10 in both MGAT1 and MGAT2 knockdown cells, this indicate that adipocyte differentiation is not dependent on MGAT1 or MGAT2.

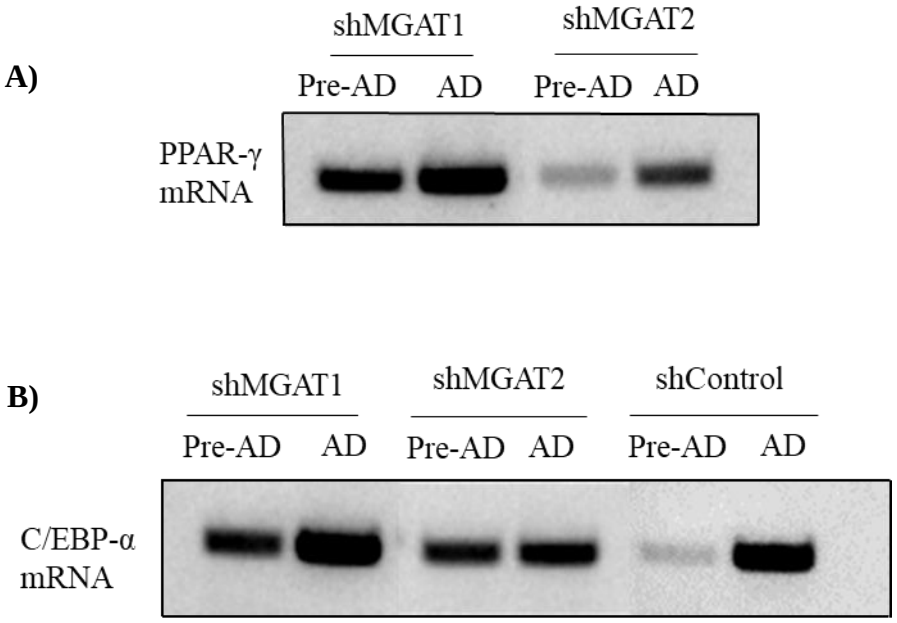


Figure 4.4: Adipocyte differentiation is not dependent on MGAT1 or MGAT2. shControl, shMGAT1 or shMGAT2 containing 3T3-L1 preadipocytes were treated with differentiation media and RNA was isolated from pre-adipocytes and adipocytes. Reverse transcriptase (RT)-PCR of adipocyte differentiation markers, A) PPARγ mRNA and B) C/EBPα mRNA was done to see any change in knockdown pre-adipocyte differentiation. Scrambled shRNA cell was used as a control. The RNA quality was confirmed by using β-actin, shown in Fig. 4.3. AD: adipocyte.

In addition, we examined the size of lipid droplets in control and knock down adipocytes. During adipogenesis, the size of lipid droplets is increased as triacylglycerol biosynthesis is

increased. Since the mRNA expression level of PPARγ and C/EBPα was increased during differentiation of knockdown cells, we expected an increased size of lipid droplets in knockdown adipocytes. We found that the size of the lipid droplets was increased in shControl as well as MGAT1 and MGAT2 knock down adipocytes compared to preadipocytes (Fig 4.5). These results also suggested that adipocyte can differentiate without MGAT1 or MGAT2. The lipid droplet analysis was conducted on confocal images of shControl, shMGAT1 and shMGAT2 pre-adipocytes and adipocytes (Fig 4.6). Larger lipid droplets were seen in shMGAT2 adipocyte compared to shControl. However, the number of lipid droplets was increased in shControl during adipocyte differentiation, while decreased in shMGAT1 and shMGAT2 adipocytes. One possible way is the fusion of small lipid droplets to grow as a large lipid droplet in shMGAT1 and shMGAT2 adipocyte. Fat specific protein-27, induced by PPARγ, is known to catalyze the fusion of lipid droplets in adipocyte by binding with perilipin1 in the lipid droplet contact site, and thus it can form pore-like channels to allow the flow of triacylglycerol from the smaller to the larger lipid droplets (Puri et al., 2007). As the number of lipid droplets decreased in shMGAT1 and shMGAT2 adipocytes and they formed larger lipid droplets, MGAT1 and MGAT2 may have a role in fat specific protein-27 regulation, though no study has been done on this.

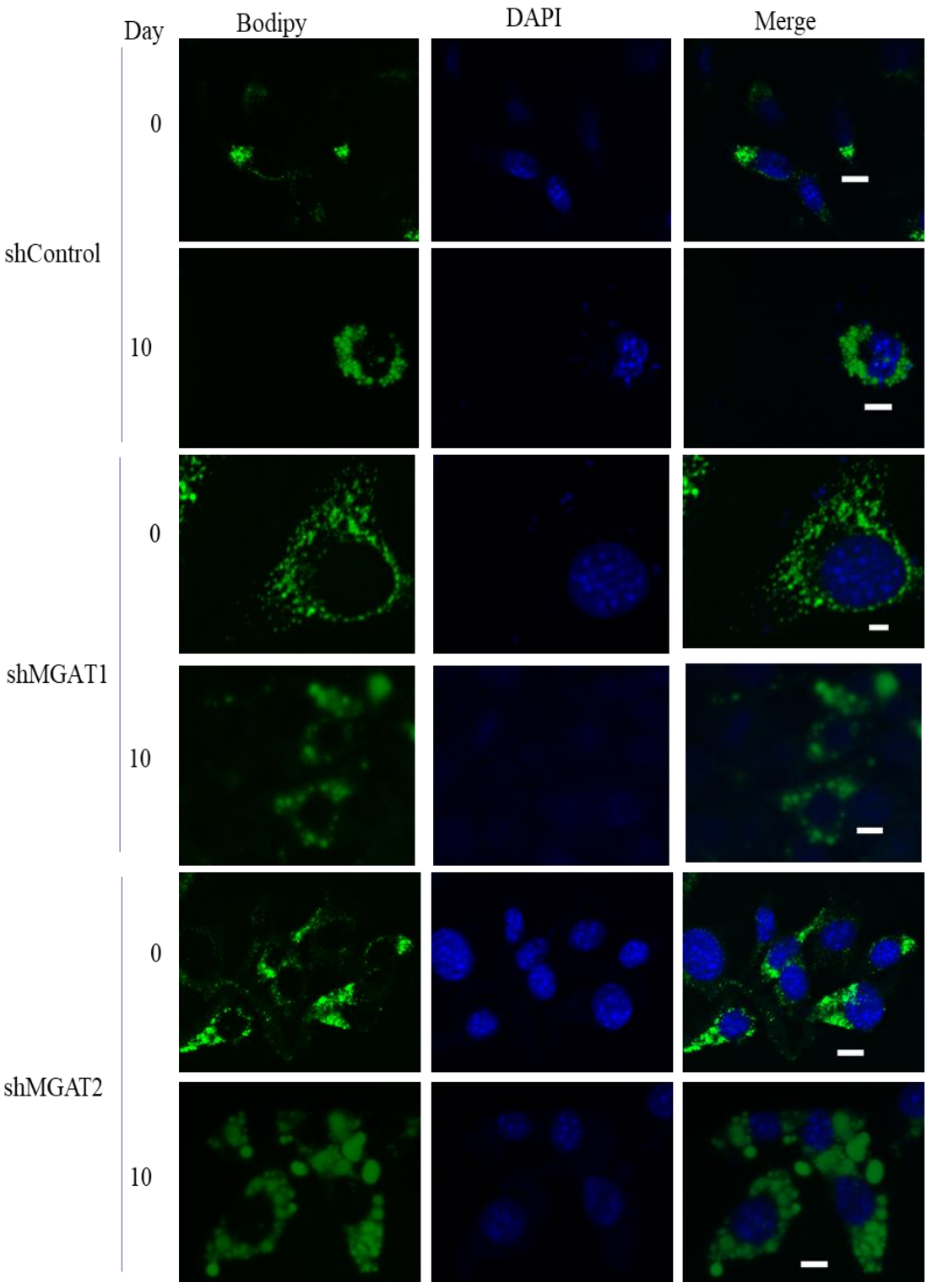
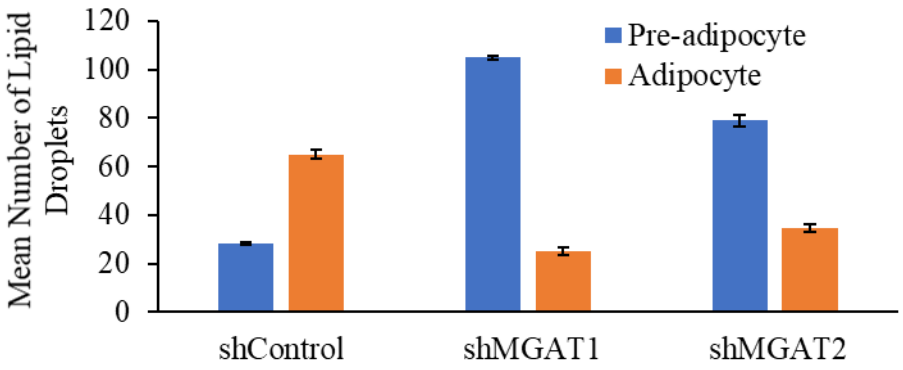


Figure 4.5: Adipocyte differentiation does not require MGAT1 or MGAT2. At day 0 and 10 of adding differentiating media, shControl, shMGAT1 and shMGAT2 cells were stained with BODIPY 493/503 to visualize lipid droplets. Cover slips were mounted with DAPI containing medium. Scale bar, 10 µm.

A)



B)

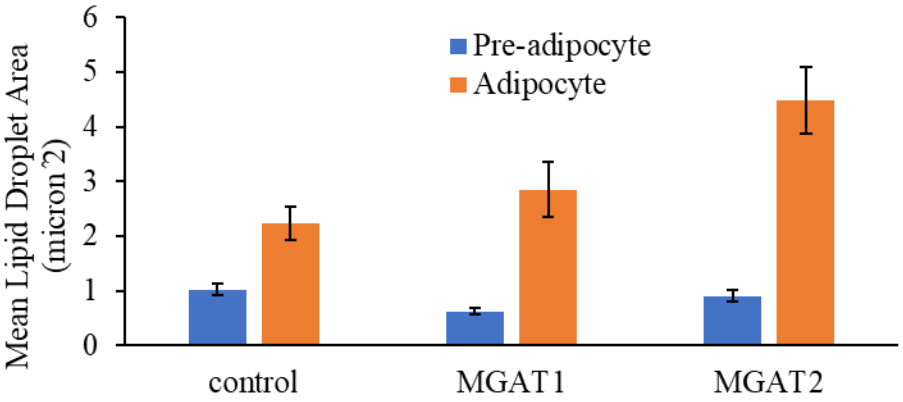


Figure 4.6: Quantification of lipid droplet area and number in MGAT1 and MGAT2 knockdown cells. Confocal images were analyzed using ImageJ analysis software. All analysis was conducted on seven cells per construct. (A) Lipid droplet number was calculated as mean ± standard error. (B) Lipid droplet area was counted from ~150–770 lipid droplets as mean ± standard error.

We also examined lipid droplets in knockdown pre-adipocytes that were loaded with oleate to stimulate triacylglycerol synthesis (Fig 4.7). Treatment with oleic acid, an MGAT substrate, drives the acylation of monoacylglycerol to form diacylglycerol. DGAT then adds oleic acid to diacylglycerol and thus produce triacylglycerol. We found larger lipid droplets in shMGAT2 preadipocyte compared to shControl, while the number of lipid droplets was lower in shMGAT2 than that of the shControl (Fig 4.8). On the other hand, the size of lipid droplets was smaller in shMGAT1 compared to shControl. Since large lipid droplets were formed in shMGAT2 cells, MGAT1, and not MGAT2, may have a role in the synthesis of triacylglycerol.

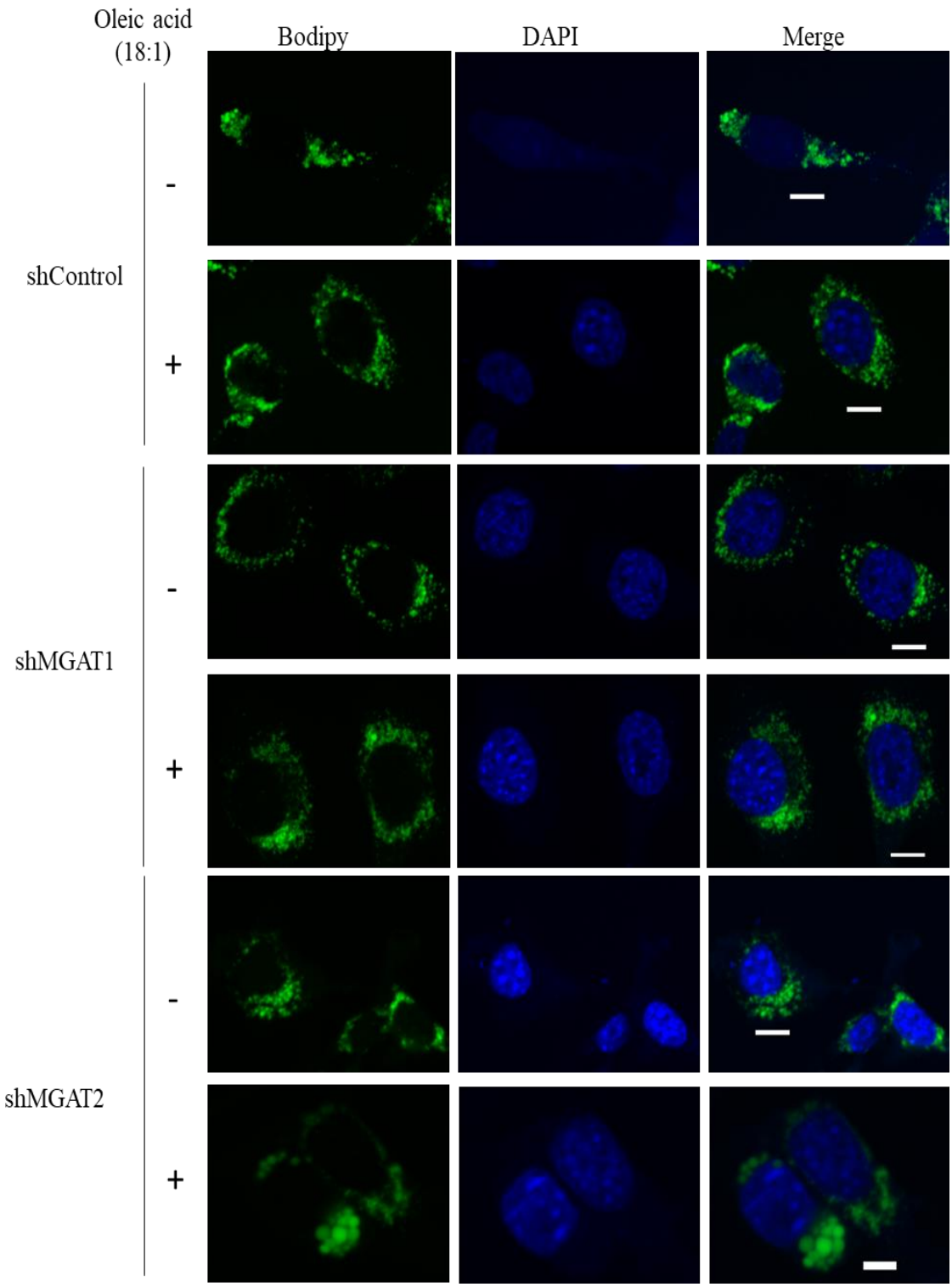


Figure 4.7: Larger lipid droplets in shMGAT2 cells. shControl, shMGAT1 and shMGAT2 preadipocyte were treated with 0.5 mM oleate for 12 hour and then stained with BODIPY 493/503 to visualize lipid droplets. Cover slips were mounted with DAPI containing medium. Scale bar, 10 μm.

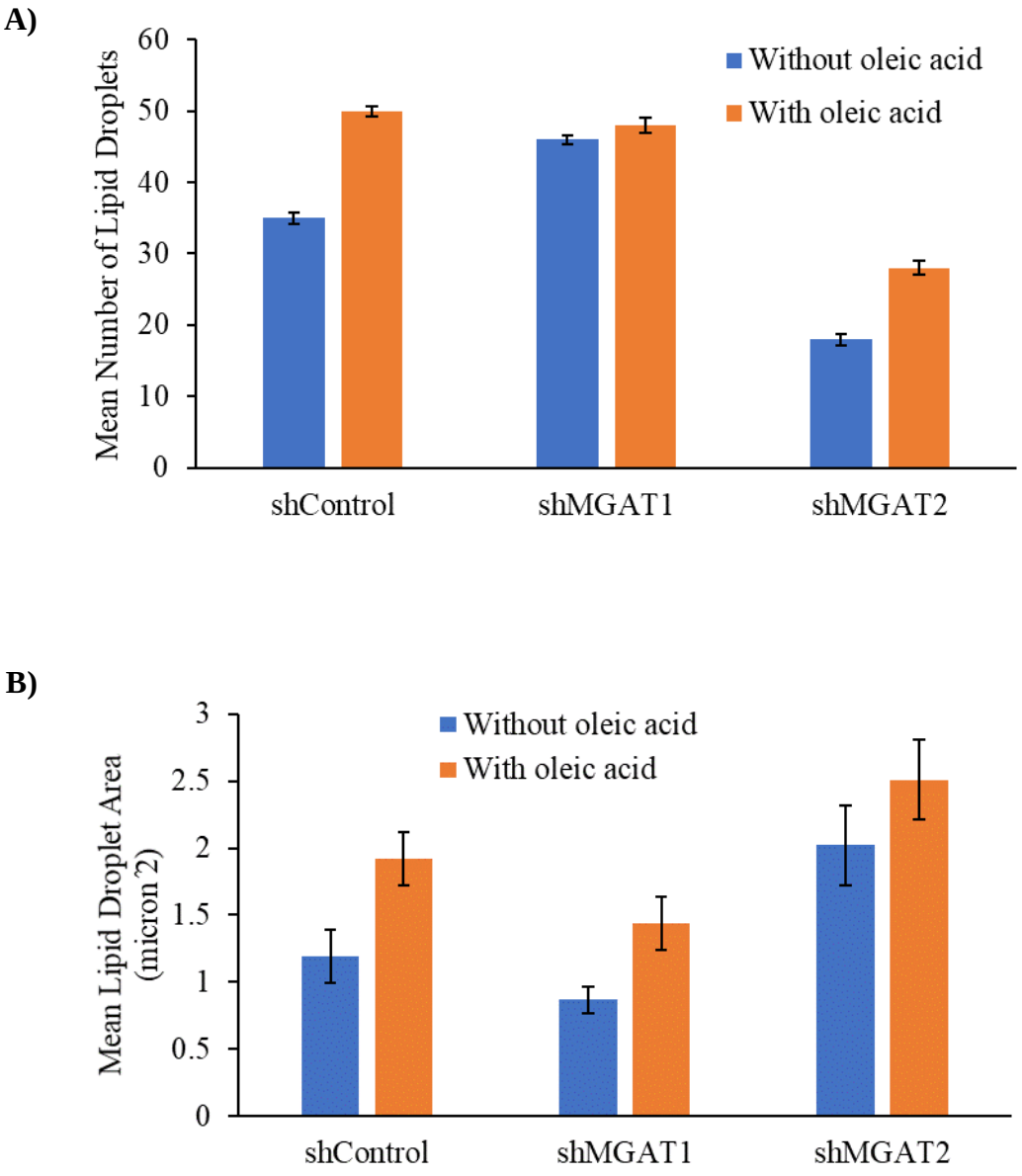


Figure 4.8: MGAT1 is important in triacylglycerol synthesis compared to MGAT2 in preadipocyte. shControl, shMGAT1 and shMGAT2 cells were treated with 0.5 mM oleate for 12 hours and stained with BODIPY 493/503 and DAPI to visualize lipid droplets and nucleus, respectively. Images were taken by confocal system and analyzed using ImageJ analysis software. All analysis was conducted on seven cells per construct. (A) Lipid droplet number was counted as mean ± standard error. (B) Lipid droplet area was calculated from ~110–380 lipid droplets as mean ± standard error.

4.5 Altered triacylglycerol mass in knockdown adipocytes

Next, we were interested in determining if knockdown of MGAT1 or MGAT2 had an effect on triacylglycerol levels in adipocytes. Lipids were extracted from knockdown adipocytes and separated by thin layer chromatography, followed by charring to visualize the lipid bands (Fig 4.9A). Triacylglycerol was not increased so much in shMGAT2 adipocytes compared to shcontrol, whereas triacylglycerol was decreased in shMGAT1 adipocytes compared to shcontrol (Fig 4.9B). These results suggest that in the absence of MGAT1, MGAT2 is not able to compensate the triacylglycerol mass in adipocyte.

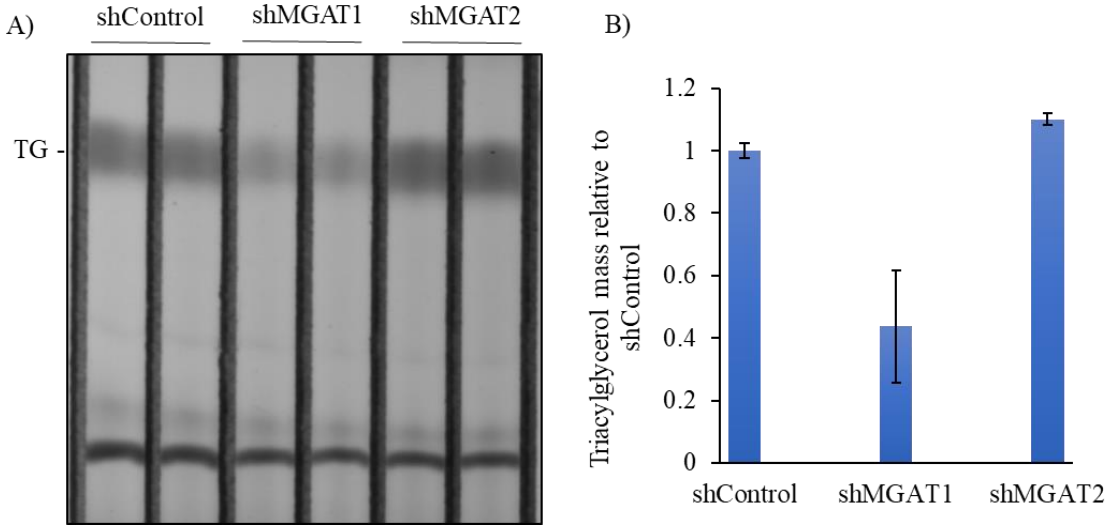


Figure 4.9: Low triacylglycerol mass in shMGAT1 adipocytes. (A**)** Lipids were extracted from equal amounts of cellular protein from control and knockdown adipocytes and separated by TLC. Lipids were visualized by charring. (B) Quantification of triacylglycerol levels. Data are shown as mean ± standard deviation.

4.6 Altered triacylglycerol synthesis in shMGAT1 and shMGAT2 adipocyte

Since triacylglycerol levels were altered in both shMGAT1 and shMGAT2 adipocytes, we wanted to look at triacylglycerol synthesis in intact adipocytes. Adipocytes were incubated with [^{3}H] glycerol for 4 hours which was incorporated into triacylglycerol. There was in decrease in incorporation of [^{3}H] glycerol into triacylglycerol in shMGAT1 adipocytes compared to shControl, suggesting triacylglycerol synthesis was reduced (Fig 4.10). However, the increased incorporation of [^{3}H] glycerol into triacylglycerol in shMGAT2 adipocyte compared to shControl indicates increased triacylglycerol synthesis. Taken together, these results suggest a major role of MGAT1, and not MGAT2, in triacylglycerol synthesis.

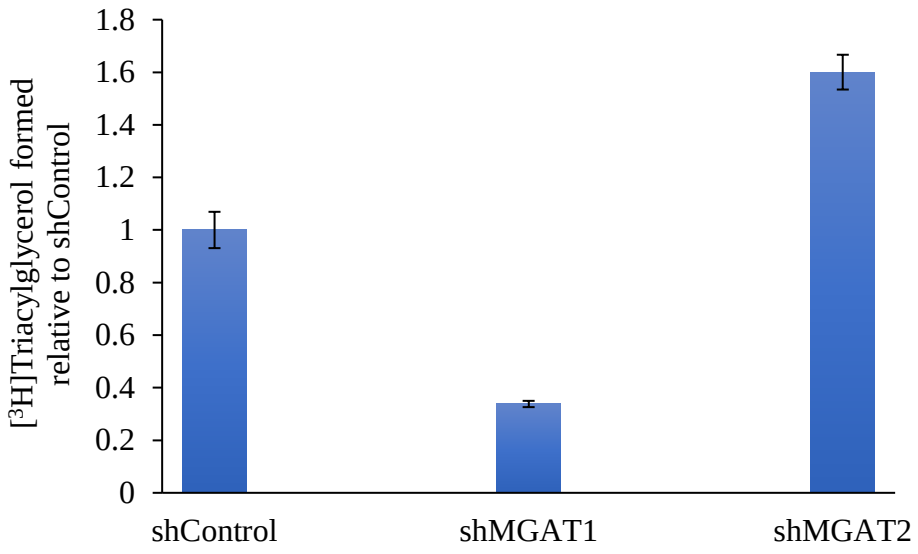


Figure 4.10: MGAT1 is important for triacylglycerol synthesis in adipocytes. Knockdown adipocytes were plated in 60-mm dishes and incubated for 4 hours with 10 µCi of [^{3}H] glycerol. Lipids were then extracted from cells and separated by TLC. The incorporation of [^{3}H] glycerol into triacylglycerol was determined by liquid scintillation counting and normalized to cellular protein. Average values from duplicate samples in one experiment are shown. The experiment was repeated once with similar results. Data are represented as mean ± standard deviation.

4.7 *In vitro* MGAT activity in MGAT1 and MGAT2 knockdown adipocytes

We next measured the *in vitro* MGAT activity of shMGAT1 and shMGAT2 adipocytes (Fig 4.11). Unexpectedly, in shMGAT1 cell lysates, the MGAT activity was higher than shControl adipocytes, and not decreased, which was expected. To determine if the increased MGAT activity was from DGAT1, a DGAT1 inhibitor was included in the assay. In the presence of DGAT1 inhibitor in shMGAT1 adipocyte, MGAT activity was reduced, but still greater than shControl. On the other hand, compared to shControl adipocytes, MGAT activity was lower in shMGAT2 adipocytes in the presence or absence of DGAT1 inhibitor. Taken together, these results suggest that DGAT1 is at least partly responsible for the increased MGAT activity in the absence of MGAT1, since the DGAT1 inhibitor decreases it.

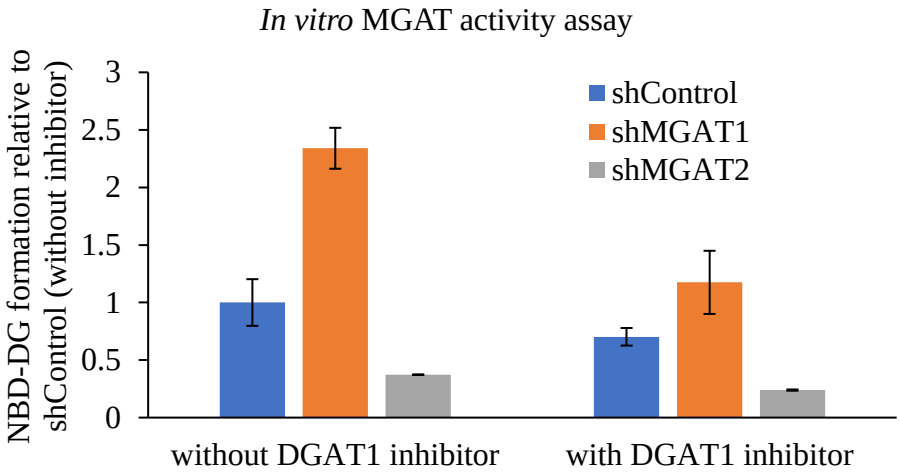


Figure 4.11: Increased *in vitro* MGAT activity in shMGAT1 adipocytes. *In vitro* MGAT activity of total cell extracts, isolated from shControl, shMGAT1 and shMGAT2 adipocyte were determined by measuring the formation of (NBD)-diacylglycerol from fluorescent NBD-palmitoyl-CoA. A DGAT1 inhibitor was used to measure only MGAT-derived MGAT activity. Results shown represent the mean ± standard deviation obtained from duplicate samples in one experiment; the experiment was repeated, yielding similar results.

4.8 Similar DGAT1 mRNA levels between pre-adipocyte and adipocyte of shMGAT1 or shMGAT2

Since MGAT activity was found in shMGAT1 in the presence of DGAT1 inhibitor, we examined the DGAT1 mRNA expression level in MGAT1 and MGAT2 knock down cells (Fig 4.12). We did not find any difference in the level of DGAT1 mRNA expression between pre-adipocytes and adipocytes. However, DGAT1 expression has previously been shown to increase during adipocyte differentiation (Cases et al., 1998). Therefore, the increased MGAT activity of the DGAT1 enzyme by post-translational modification cannot be disregarded.

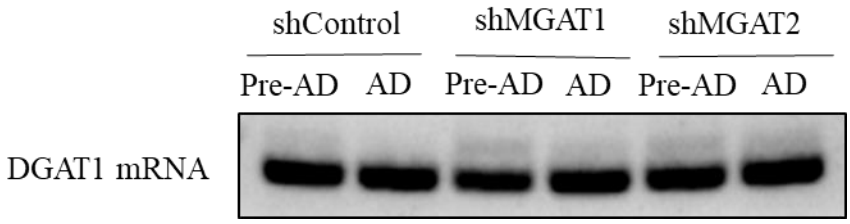


Figure 4.12: Similar DGAT1 mRNA expression levels between knockdown pre-adipocytes and adipocytes. RNA was isolated from knockdown pre-adipocytes and adipocytes. DGAT1 mRNA expression levels were determined by reverse transcriptase (RT)-PCR. The quality of RNA was confirmed by using β-actin, shown in Fig. 4.3. AD: adipocyte.

4.9 *In vitro* DGAT activity in MGAT1 and MGAT2 knockdown adipocytes

Next, we measured the *in vitro* DGAT activity in shMGAT1 and shMGAT2 adipocytes. DGAT activity was increased approximately 70-fold in shMGAT1 adipocytes compared to shControl adipocytes (Fig 4.13A). Moreover, in the presence of DGAT1 inhibitor, DGAT activity was still detectable in MGAT1 knock down adipocytes which may indicate that either inhibition of DGAT1 was not 100%, or the remaining activity was due to DGAT2.

To identify *in vitro* DGAT activity of DGAT2, we used a DGAT2 inhibitor in shControl adipocytes. However, the DGAT activity in shControl adipocytes was not reduced when the DGAT2 inhibitor was included in the assay. We used DGAT2 inhibitor at different concentrations (data not shown), however, it had no effect on the DGAT activity (Fig 4.13B). This indicated that the DGAT2 inhibitor was not effective and was not used in further experiments.

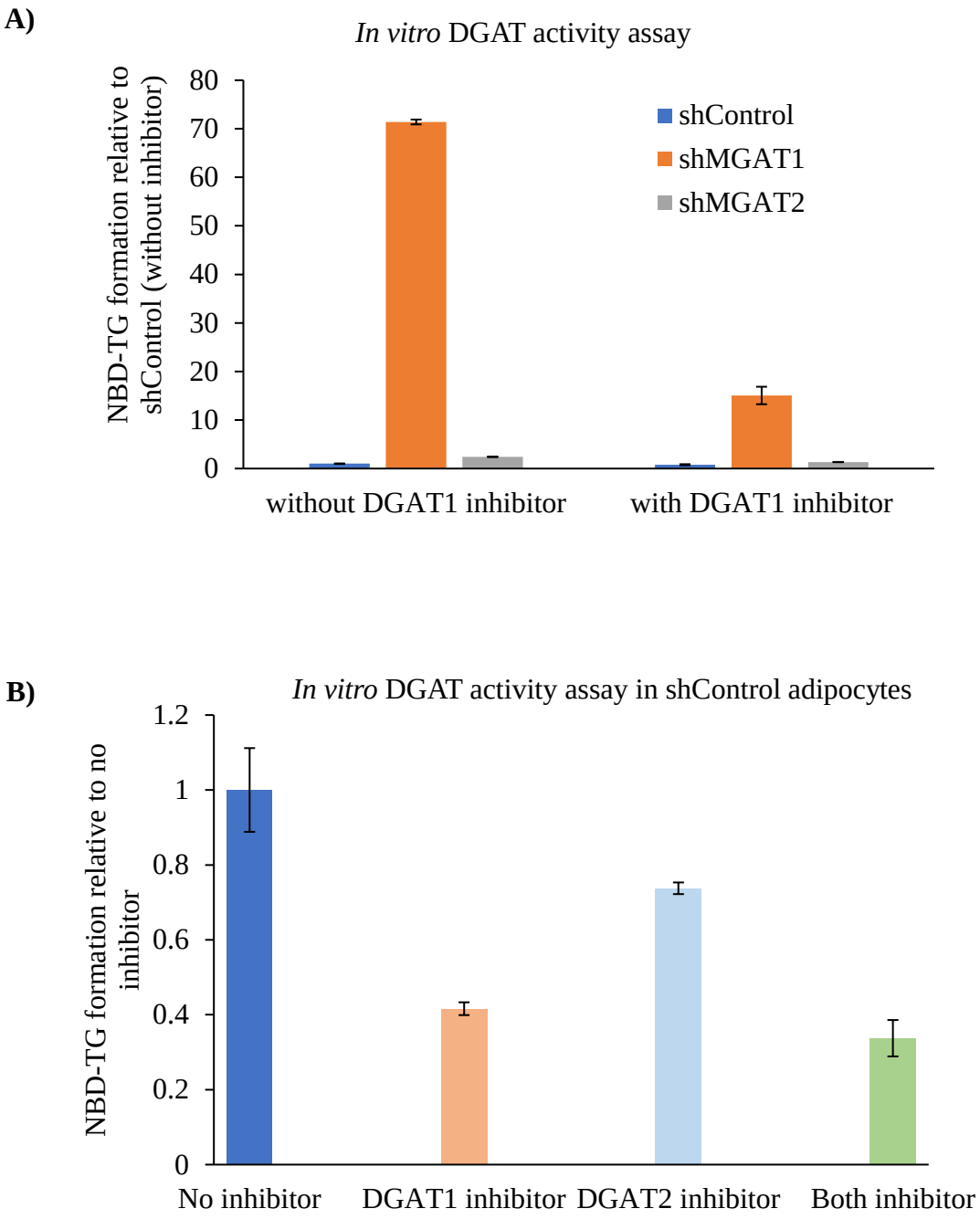


Figure 4.13: High level of *in vitro* DGAT activity in shMGAT1 adipocytes. Total cell extracts were isolated from shControl, shMGAT1 and shMGAT2 adipocyte. *In vitro* DGAT activity was determined by measuring the formation of NBD-triacylglycerol from fluorescent NBD-palmitoyl-CoA. (A) *In vitro* DGAT activity was measured in the absence or presence of DGAT1 inhibitor. (B) DGAT2 inhibitor did not reduce the DGAT activity in shControl adipocytes. The final concentration of DGAT1 and DGAT2 inhibitor was 2 mM. Results shown represent the mean ± standard deviation obtained from duplicate samples in one experiment; the experiment was repeated, yielding similar results.

4.10 Inconclusive lipolysis experiment in knockdown adipocyte

Through lipolysis, triacylglycerols are sequentially broken down into diacylglycerols, monoacylglycerols, glycerol and fatty acids. Glycerol cannot be re-used to produce triacylglycerol due to the lack of glycerol kinase in adipocytes. However, approximately 70% of free fatty acids released during lipolysis can be activated and re-esterified into newly synthesized triacylglycerol in adipocytes (Forest et al., 2003, Vaughae, 1962, Jensen et al., 2001). However, the specific pathway for fatty acids re-esterification in adipocytes has not been well characterized. One possible way is that glycerol-3-phosphate can be produced from non-carbohydrate precursors like pyruvate, lactate or certain amino acids in adipocyte though glyceroneogenesis. This glycerol-3-phosphate is then used by free fatty acids to re-esterify triacylglycerol.

Since monoacylglycerol is also produced during lipolysis, we hypothesized that either MGAT1 or MGAT2 may have a role in fatty acid re-esterification with monoacylglycerol in adipocytes. We attempted to determine if MGAT1 or MGAT2 had a role in fatty acid re-esterification by measuring free fatty acid and glycerol release from adipocytes during lipolysis. If MGAT1 or MGAT2 has a role in this process, fatty acid release would be higher in the knockdown adipocytes. However, the levels of fatty acids and glycerol did not increase when lipolysis was stimulated in shControl adipocytes. Therefore, we did not get any significant data (shown in supplementary data) to assess the role of MGAT1 and MGAT2 in re-esterification of fatty acids in adipocyte.

CHAPTER 5: Discussion

In eukaryotes, adipocytes are the major site for triacylglycerol storage. The main role of triacylglycerol is to store energy. During excess energy after food consumption, the adipocytes store energy in the form of triacylglycerol, which is then hydrolyzed to produce fatty acids for use of other organs in periods of low energy. Therefore, the synthesis and breakdown of triacylglycerol is important for maintaining energy homeostasis in the body. However, excessive accumulation of triacylglycerol in adipocytes causes obesity (Kopelman, 2000), whereas loss of adipose tissue, which is known as lipodystrophy, is responsible for insulin resistance and diabetes (Simha and Garg, 2006). Therefore, targeting enzymes to regulate triacylglycerol metabolism in adipocytes is important to treat metabolic diseases.

The synthesis of triacylglycerol through the Kennedy pathway in adipocytes is well studied (Ahmadian et al., 2007). The Kennedy pathway involves incorporation of fatty acids into glycerol-3-phosphate through sequential acylation reactions, and thus mediates triacylglycerol formation (Kennedy, 1957). The other synthesis pathway of triacylglycerol involves the acylation of monoacylglycerol by MGAT enzymes to form diacylglycerol (Kayden et al., 1967). This MGAT pathway is predominant in the small intestine. After feeding, a large amount of 2-monoacylglycerol and fatty acids are released from the dietary lipids in the intestine, which are then absorbed by enterocytes to produce triacylglycerol through the monoacylglycerol pathway. Three MGAT isoforms, MGAT1-3 have been identified in eukaryotes of which tissue expression is different between species (Cases et al., 2001, Yen and Farese, 2003). The metabolic effects of MGAT enzymes have been studied broadly in the intestine and liver (Yen and Farese, 2003, Cao et al., 2003, Cheng et al., 2003, Lee et al., 2012, Soufi et al., 2014). However, both MGAT1 and MGAT2 are expressed in adipocytes, but the role of the MGAT pathway in adipocyte lipid metabolism is less well understood relative to the Kennedy pathway. In this study, we focused on determining the role of MGAT1 and MGAT2 in triacylglycerol metabolism in adipocytes.

First, we examined whether the MGAT activity is altered during adipocyte differentiation. From our *in vitro* MGAT activity assay, MGAT activity increased during adipocyte differentiation. However, MGAT activity was reduced when we added DGAT1 inhibitor into the reaction mixture. In addition to DGAT activity, DGAT1 also has MGAT activity. Therefore, DGAT1 can convert monoacylglycerol to diacylglycerol and then to triacylglycerol by sequential esterification of fatty

acids (Yen et al., 2005). Though the MGAT-derived MGAT activity was increased during adipocyte differentiation, the total *in vitro* MGAT activity was reduced after adding DGAT1 inhibitor. This suggests that most of the MGAT activity in adipocytes is mediated by DGAT1. A recent study on MGAT activity in adipocytes also found increased MGAT activity during adipocyte differentiation, but that study did not mention any DGAT1-derived MGAT activity in adipocytes (Liss et al., 2018). In fact, the role of DGAT1 in adipocyte MGAT activity has not been studied yet.

In our study, the mRNA expression level of MGAT1 and MGAT2 did not increase considerably during adipocyte differentiation. Though the MGAT-derived MGAT activity increased during adipocyte differentiation in this study, the unchanged mRNA expression of MGAT1 and MGAT2 during differentiation suggests post-translational modification of these enzymes. However, our findings on MGAT1 are contradictory to a recent study on the metabolic role of MGAT1 in adipocytes that shows a marked increase in MGAT1 expression and activity, induced by PPARγ during adipocyte differentiation in both mice and human cells, (Liss et al., 2018). That study indicates MGAT1 protein is a major contributor in the overall adipocyte MGAT activity. In contrast, they found a very small increase in MGAT2 mRNA expression during adipogenesis, which is consistent with our findings.

To study the role of MGAT1 and MGAT2 in adipocyte lipid metabolism, we separately knocked down the mRNA expression of MGAT1 and MGAT2 in pre-adipocytes by using shRNA. After confirming that MGAT1 and MGAT2 mRNA expression was reduced in shMGAT1 and shMGAT2 pre-adipocytes, respectively, we collected stable cells to continue our study. Since PPARγ, the key regulator of adipogenesis, is robustly upregulated during adipocyte differentiation (Shao et al., 2016), and also directly regulates the MGAT1 expression (Lee et al., 2012), we were interested in identifying any changes in adipogenesis without MGAT1. Unlike MGAT1, the transcriptional regulation of MGAT2 by any adipogenic transcription factor has not been determined. We were, therefore, also interested to find out the role of MGAT2 in adipocyte differentiation. From our results, we have found increased mRNA expression of both PPARγ and C/EBPα in both the MGAT1 and MGAT2 knockdown adipocytes, which clearly revealed that differentiation of adipocyte does not depend on MGAT1 or MGAT2. This conclusion is bolstered

by the presence of large lipid droplets in the knockdown adipocytes. The formation of large lipid droplets has been used as an indirect marker for adipocyte differentiation.

Unexpectedly, the size of lipid droplets was larger in shMGAT2 adipocytes compared to shControl adipocytes. Why shMGAT2 adipocytes form larger lipid droplets is unclear. It is possible that knockdown of MGAT2 may upregulate other enzymes that are involved in the synthesis of triacylglycerol, which in turn accumulates in lipid droplets. However, the number of lipid droplets was decreased in shMGAT2 adipocytes compared to shControl adipocytes which may be due to the fusion of lipid droplets to produce larger lipid droplets in shMGAT2 adipocytes. Fat specific protein-27, regulated by PPARγ, promotes fusion of lipid droplets by inhibiting lipolysis in adipocytes (Puri et al., 2007). Since the lipid droplets became larger and the number of lipid droplets was decreased in shMGAT2 adipocytes, fat specific protein-27 function may be upregulated in the absence of MGAT2. In contrast, the number of lipid droplets was decreased in shMGAT1 adipocytes, although the size of the lipid droplets were similar to those observed in shControl adipocytes.

Moreover, stimulation of triacylglycerol synthesis through substrate loading showed larger lipid droplets in shMGAT2 pre-adipocytes than shControl pre-adipocytes. This result suggests that MGAT1 contributes to triacylglycerol synthesis by the MGAT pathway in adipocytes.

We also measured the level of triacylglycerol mass in MGAT1 and MGAT2 knockdown adipocytes. Thin layer chromatographic analysis of cellular lipids showed that in the absence of MGAT1, the level of triacylglycerol was low compared to the control. Study on MGAT1 activity in obese mouse liver has found that knockdown of MGAT1 improved glucose tolerance and hepatic insulin signaling with increased diacylglycerol content in the liver, though the mechanism is unknown (Hall et al., 2014). Another study showed that MGAT1 knockdown decreased triacylglycerol content in liver and adipocytes, while the content of diacylglycerol in liver was not decreased (Soufi et al., 2014). We have also found decreased triacylglycerol level in the absence of MGAT1, although we did not measure the level of diacylglycerol.

The incorporation of [^{3}H]-glycerol into triacylglycerol also revealed that triacylglycerol synthesis was reduced in the absence of MGAT1. In contrast, the rate of triacylglycerol synthesis in shMGAT2 adipocytes was much higher than the shControl adipocytes. This result indicates that MGAT1 can compensate for the absence of MGAT2 in the triacylglycerol synthetic pathway,

whereas MGAT2 is not able to compensate the absence of MGAT1 in adipocytes. Adipocytes are supposed to lack glycerol kinase (Reshef et al., 2003). How is [^{3}H]-glycerol then incorporated into triacylglycerol? One possible way is the direct acylation of glycerol by glycerol:acyl-CoA acyltransferase to produce monoacylglycerol, and then sequential acylation will produce triacylglycerol at the end. For example, a study on hepatocytes and myoblasts had found that direct acylation of glycerol was prominent either with a high level of exogenous glycerol or attenuation of the glycerol-3-phosphate pathway (Lee et al., 2001). Although different studies did not find significant glycerol uptake by adipose tissue (Coppack et al., 1999, Coppack et al., 2005), we have found glycerol-isotope uptake by adipocytes that was also reported in Kurpad et al. (Kurpad et al., 1994). The pathway for glycerol uptake in adipocytes has yet to be elucidated.

Since the synthesis of triacylglycerol was high and the lipid droplets were large in shMGAT2 adipocytes, we thought that both *in vitro* MGAT and DGAT activity were high in shMGAT2 adipocytes. However, the MGAT activity was lower in shMGAT2 adipocytes compared to that of shControl adipocytes. Why shMGAT2 adipocytes showed low level of MGAT activity, while having larger lipid droplets compared to shControl is not clear. Different studies found the interaction of MGAT2 with DGAT1 (Zhang et al., 2014) and DGAT2 (Jin et al., 2014) to enhance triacylglycerol synthesis. The absence of MGAT2 may disrupt this interaction, and as a result it may down regulate the MGAT activity of DGAT1.

In contrast, the MGAT activity was higher in shMGAT1 adipocytes rather than shControl adipocytes. Since the addition of a DGAT1 inhibitor reduced MGAT activity in shMGAT1 adipocytes, it indicates that much of the increased MGAT activity likely comes from DGAT1. This result suggests the role of DGAT1 in MGAT activity which may be regulated by MGAT2.

Since we found increased *in vitro* MGAT activity in shMGAT1 adipocytes, we were interested in measuring *in vitro* DGAT activity in the knock down adipocytes. Interestingly, in shMGAT1 adipocytes, *in vitro* DGAT activity was higher than shControl adipocytes, even after using a DGAT1 inhibitor. Since both DGAT1 and DGAT2 enzymes are present in adipocytes, we used a DGAT2 inhibitor along with a DGAT1 inhibitor to examine the role of the DGAT2 enzyme in DGAT activity in adipocytes. However, the DGAT2 inhibitor was not effective in inhibiting DGAT activity in shControl adipocytes. Therefore, it is not possible to conclude anything about the contribution of DGAT2 in *in vitro* DGAT activity.

A study on the role of DGAT enzymes in triacylglycerol synthesis and lipid droplet formation in adipocytes showed that adipocytes without both DGAT1 and DGAT2 did not have triacylglycerol or lipid droplets (Harris et al., 2011). Single deletion of either DGAT enzyme did not affect the formation of triacylglycerol as well as lipid droplets in adipocytes. In addition, DGAT enzymes did not play any role in lipid droplet formation in other cell types, such as macrophages. Taken together, that study indicates that DGAT1 and DGAT2 account for nearly all triacylglycerol synthesis in adipocytes and are required for lipid droplet formation in adipocytes (Harris et al., 2011). MGAT2 may have a role in regulating this DGAT activity in adipocytes as DGAT activity was decreased in the absence of MGAT2.

It is possible that the mRNA expression of DGAT1 is upregulated in shMGAT1 adipocytes, and thus it increases both the *in vitro* MGAT and DGAT activity. We did not find any change in the mRNA expression level of DGAT1 during adipocyte differentiation, whereas a study showed increased DGAT1 mRNA expression during adipocyte differentiation (Cases et al., 1998). Since the DGAT1 promoter has a binding site for PPARγ, it is possible that PPARγ upregulates the DGAT1 mRNA expression during adipogenesis (Ludwig et al., 2002). However, DGAT1 is found to be regulated by post-transcriptional modification during adipocyte differentiation (Yu et al., 2002). A study had shown that DGAT1 protein level increased by 90-fold in differentiated 3T3-L1 adipocytes, while DGAT1 mRNA expression increased 7-fold (Yu et al., 2002). Another study had found that phosphorylation of DGAT1 increased DGAT activity in skeletal muscle, which indicated post-translational modification of DGAT1 (Yu et al., 2015a). Since DGAT1 inhibitor reduced both *in vitro* MGAT and DGAT activity in shMGAT1 adipocytes, and also since no change in the mRNA expression of DGAT1, it follows that DGAT1 activity was increased due to post-translational modification in shMGAT1 adipocytes.

From this study, we have found increased MGAT and DGAT activity in shMGAT1 adipocytes while the level of triacylglycerol mass and synthesis are reduced. This indicates that in the absence of MGAT1, DGAT1 tries to normalize the triacylglycerol mass by increasing its activity in adipocytes (Fig. 5.1B). The recent finding of DGAT1 in fatty acid re-esterification in adipocytes may indicate that in the monoacylglycerol pathway, DGAT1 plays a more important role in triacylglycerol synthesis than DGAT2 (Chitraju et al., 2017).

Since the size of the lipid droplets was decreased in shMGAT1 adipocytes after oleate loading, it seems that MGAT1 may have a role in lipid droplet formation. Different studies on hepatocytes showed that MGAT1 localizes to the lipid droplets and interacts with DGAT2 to promote triacylglycerol synthesis (Lee and Kim, 2017). Although such an experiment was not done in adipocytes, the smaller lipid droplets in shMGAT1 adipocytes may be the result of loss of MGAT1 localization in lipid droplets.

However, the monoacylglycerol, substrate for the MGAT enzymes, may be derived from the partial hydrolysis of triacylglycerol during lipolysis (Fig. 5.1A). It is possible that in the absence of MGAT1, the rate of lipolysis becomes increased, and thus produces more substrate for MGAT and DGAT enzymes, which in turn stimulates MGAT and DGAT activity (Fig. 5.1B). A recent study showed an inverse correlation of *Mogat1* expression with lipolysis in adipocytes, but also showed decreased MGAT activity in the absence of *Mogat1* (Liss et al., 2018).

In contrast, in the presence of MGAT1 in shMGAT2 adipocytes, the breakdown of triacylglycerol may become decreased, so that the size of lipid droplets, the synthesis and mass of triacylglycerol increased.

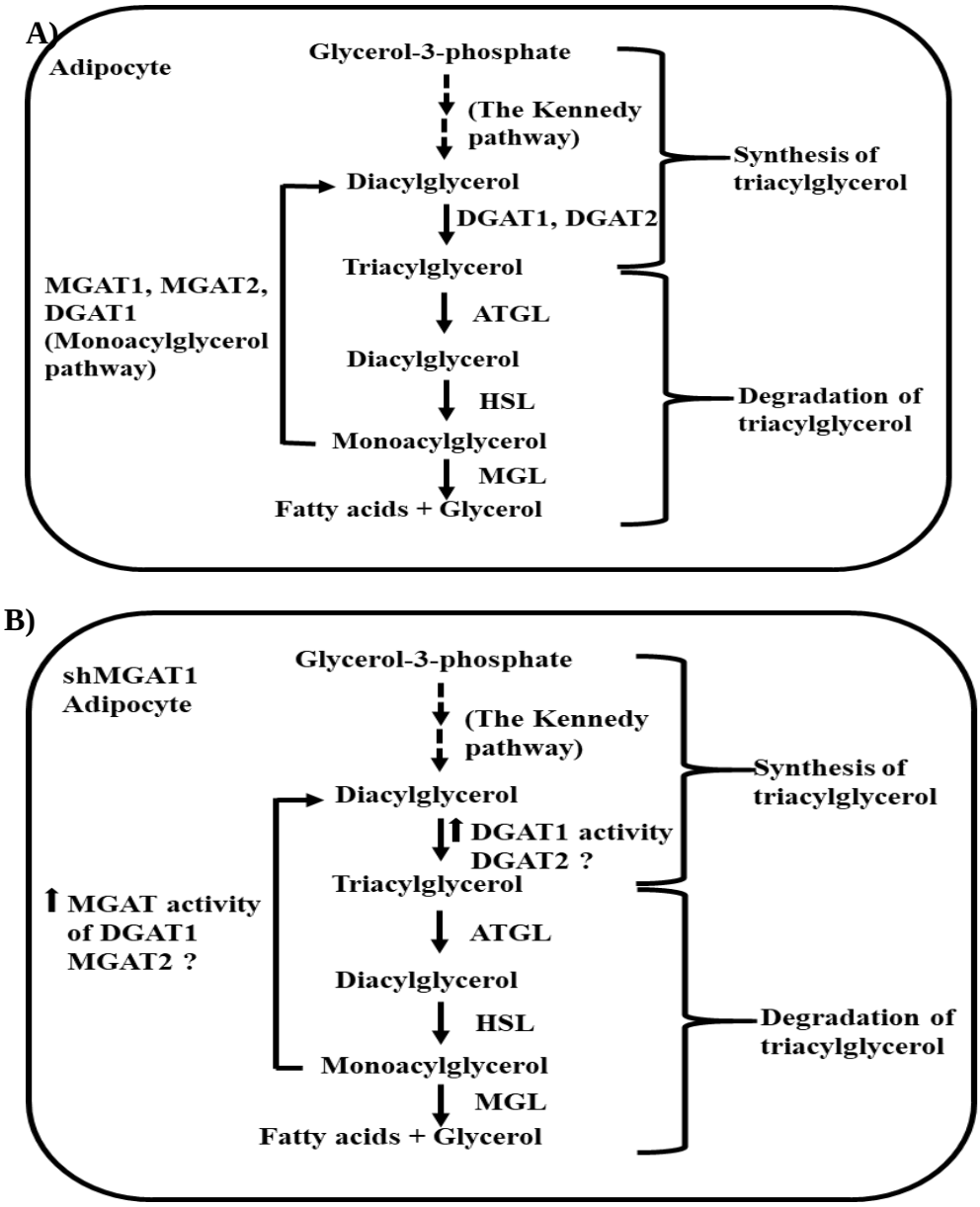


Figure 5.1: Hypothetical model for the roles of MGAT1 in adipocytes. A) In normal adipocytes, the lipolysis and the synthesis of triacylglycerol is balanced properly, whereas, B) in shMGAT1 adipocytes, it seems that DGAT1 is trying to compensate the absence of MGAT1 by increasing the MGAT and DGAT activity. Also, the lipolysis of triacylglycerol may become increased in the absence of MGAT1. ATGL: adipose triglyceride lipase; HSL: hormone-sensitive lipase; MGL: monoacylglycerol lipase.

CHAPTER 6: Conclusion

Triacylglycerol is the main storage form of energy in human. Excessive storage of triacylglycerol in adipose tissue leads to obesity that promotes detrimental effects on health. In many instances, individuals who become obese are predisposed to hyperlipidemia, atherosclerotic coronary heart disease, and diabetes (Kopelman, 2000). Therefore, understanding the mechanisms responsible for increased adipose tissue content in obese individuals is critical for the development of effective therapies to reverse obesity and minimize the risk of other metabolic diseases.

This thesis focused on understanding the role of MGAT enzymes in triacylglycerol metabolism in adipose tissue. MGAT synthesizes diacylglycerol, a substrate for enzymes responsible for triacylglycerol synthesis. There are two MGAT isoforms, MGAT1 and MGAT2, in adipose tissue. We hypothesize that one or both of these enzymes contribute to adipose tissue triacylglycerol synthesis.

In summary, we have found that differentiation of adipocyte does not depend on MGAT1 or MGAT2. However, the MGAT pathway plays an important role in regulating the level of triacylglycerol in adipocytes. DGAT1 derived MGAT and DGAT activity may be upregulated in the absence of MGAT1. Also, MGAT1 may regulate the lipolytic pathway in adipocytes. On the other hand, the role of MGAT2 in adipocytes is not clear. It may have a prominent role in triacylglycerol re-synthesis in intestine rather than other tissues.

CHAPTER 7: Future directions

Further study on fatty acid re-esterification during lipolysis in adipocytes may help clarify the role of MGAT1 and MGAT2 in more detail. Although our attempt was unsuccessful, a recent study found MGAT1 as a negative regulator of lipolysis in adipocyte (Liss et al., 2018). Re-esterification of free fatty acids inhibits lipotoxic stress as increased fatty acids causes insulin resistance and obesity and diabetes (Jiao et al., 2011). Lipolysis derived 70% fatty acids are re-used to produce triacylglycerol in adipocyte, but different studies indicate different pathways for this re-esterification process (Forest et al., 2003, Jensen et al., 2001). Recently, a study found DGAT1 as an important enzyme to re-esterify fatty acids (Chitraju et al., 2017). Although in which pathway DGAT1 uses these free fatty acids is not clear, since DGAT1 is found in both the Kennedy pathway and the monoacylglycerol pathway. In our study, we showed rapid turnover of triacylglycerol in shMGAT1 adipocytes, but the molecular mechanism has not been found yet.

Also, direct acylation of glycerol in adipocyte has been assumed in different studies, so it could be a substrate for the MGAT enzymes in adipocytes. More studies on finding out the substrate source for the MGAT enzymes in adipocytes may help regulate lipid metabolism.

In our study, the mRNA levels of MGAT1, MGAT2 and DGAT1 were not consistent with previous studies. It will be a good idea to see the protein levels of these enzymes by doing western blots. Due to lack of appropriate antibody, we did not do western blot in our study. However, finding out the protein levels along with the mRNA levels will give a hint about post-transcriptional modification of MGAT and DGAT enzymes.

We did do radioactive assays to see the level of triacylglycerol synthesis at certain times. However, the triacylglycerol synthesis rate in shMGAT1 and shMGAT2 adipocytes will give an idea about the turnover of triacylglycerol as well.

CHAPTER 8: Supplementary data

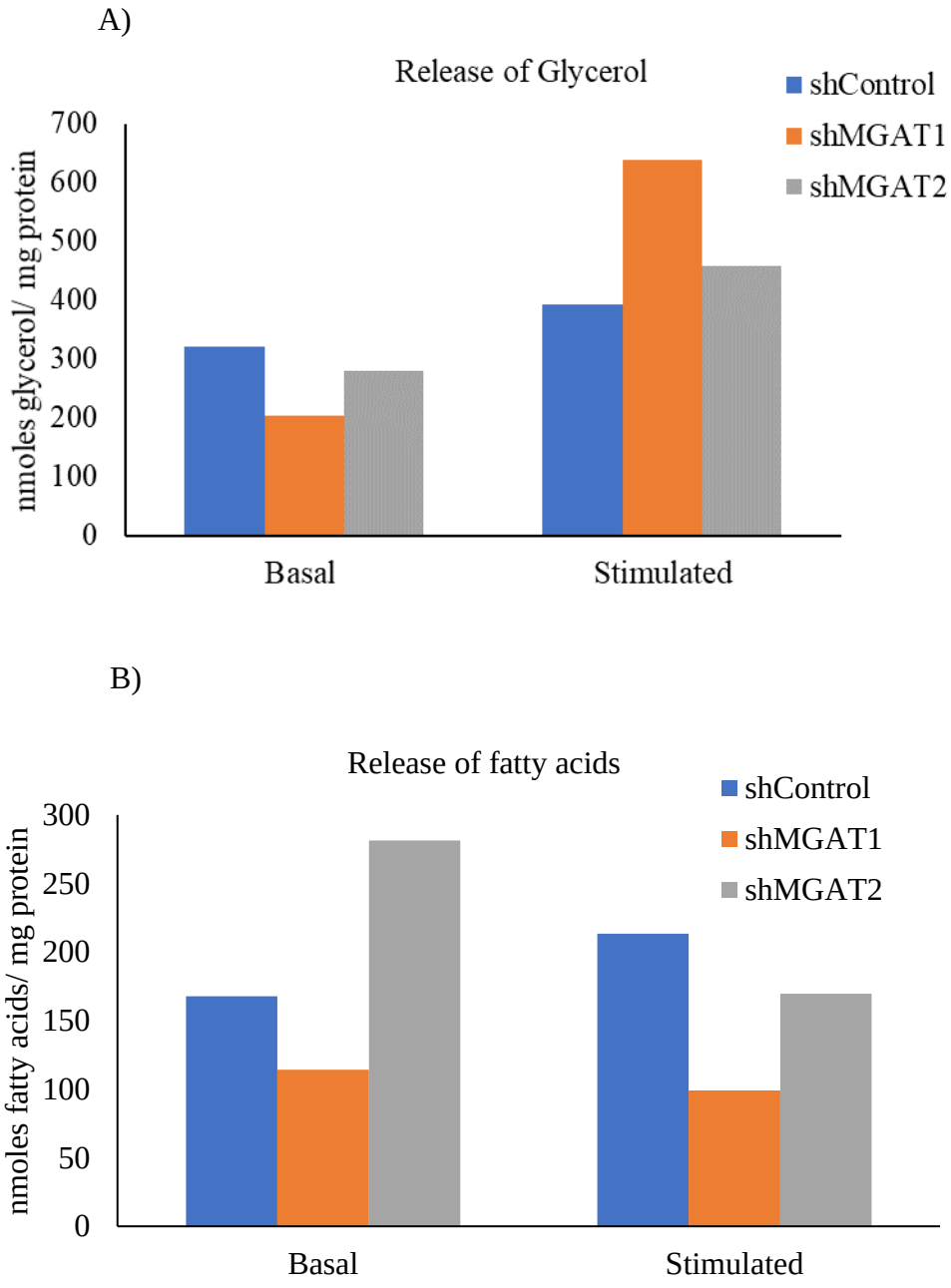


Figure 8.1: Effect of MGAT1 and MGAT2 knockdown on lipolysis. shControl, shMGAT1, shMGAT2 adipocytes were incubated with or without CPT-cAMP for 4 hours. Media was collected to perform the lipolytic assay and normalized with cellular protein. The experiment was repeated with similar results. (A) Released glycerol was measured by free glycerol reagent. (B) Free fatty acids were measured by non-esterified fatty acids detection kit.

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