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Monoacylglycerol O-acyltransferase 1 is regulated by peroxisome proliferator-activated receptor γ in human hepatocytes and increases lipid accumulation

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ABSTRACT

Monoacylglycerol *O*-acyltransferase (MGAT) is an enzyme that is involved in triglyceride synthesis by catalyzing the formation of diacylglycerol from monoacylglycerol and fatty acyl CoAs. Recently, we reported that MGAT1 has a critical role in hepatic TG accumulation and that its suppression ameliorates hepatic steatosis in a mouse model. However, the function of MGAT enzymes in hepatic lipid accumulation has not been investigated in humans. Unlike in rodents, MGAT3 as well as MGAT1 and MGAT2 are present in humans. In this study, we evaluated the differences between MGAT subtypes and their association with peroxisome proliferator-activated receptor γ (PPARγ), a regulator of mouse MGAT1 expression. In human primary hepatocytes, basal expression of MGAT1 was lower than that of MGAT2 or MGAT3, but was strongly induced by PPARγ overexpression. A luciferase assay as well as an electromobility shift assay revealed that human MGAT1 promoter activity is driven by PPARγ by direct binding to at least two regions of the promoter in 293T and HepG2 cells. Moreover, siRNA-mediated suppression of MGAT1 expression significantly attenuated lipid accumulation by PPARγ overexpression in HepG2 cells, as evidenced by oil-red-O staining. These results suggest that human MGAT1 has an important role in fatty liver formation as a target gene of PPARγ, and blocking MGAT1 activity could be an efficient therapeutic way to reduce nonalcoholic fatty liver diseases in humans.

1. Introduction

With the rise in obesity, the incidence of fatty liver disease has gradually increased and is now a significant healthcare issue. Hepatic steatosis, characterized by an increase in intrahepatic triacylglycerol (TG), is an important marker of metabolic dysfunction and is associated closely with insulin resistance and dyslipidemia [1,2]. Because hepatic steatosis appears in the early stage of metabolic disease, the early detection and management of the disease, as well as understanding the mechanism of hepatic steatosis, are extremely important. To date, the exact etiology of hepatic steatosis has not been defined, but transcription factors such as sterol regulatory element-binding protein 1c (SREBP1c) and carbohydrate response element-binding protein (ChREBP) that control the expression of lipogenic genes have been found to be important in hepatic lipid accumulation [3,4].

In a past decade, peroxisome proliferator-activated receptor γ (PPARγ) has been described as a protein that is involved in hepatic steatosis [5–7]. PPARγ is a ligand-activated transcription factor that mainly controls adipogenesis [8]. Although PPARγ expression in hepatic tissue is low (about 10–30% of that in adipose tissue [9]), it

Abbreviations: PPAR, peroxisome proliferator-activated receptor; MGAT, monoacylglycerol *O*-acyltransferase; DGAT, diacylglycerol acyltransferase; TG, triacylglycerol; MAG, monoacylglycerol; PPRE, PPAR regulatory element; EMSA, electrophoretic mobility shift assay.

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is significantly increased in a rodent model of obesity and has an important role in fatty liver formation [6,10,11]. Under a high-fat diet, PPARγ stimulates the expression of genes involved in TG synthesis, such as adipose differentiation-related protein (ARDP) and fat-specific protein 27 (FSP27), and increases fatty acid uptake by the induction of fatty acid binding protein (FABP) and CD36 in the liver [12].

Previously, we investigated the role of PPARγ in hepatic steatosis, identifying monoacylglycerol *O*-acyltransferase 1 (MGAT1) as a novel therapeutic target gene for hepatic steatosis in mice [10]. Subsequently, other groups also reported the beneficial metabolic effects of hepatic MGAT1 suppression in mouse models [13,14]. MGAT is an enzyme that catalyzes monoacylglycerol (MAG) and fatty acyl CoAs to form diacylglycerol (DAG), which is then acylated to form TG by diacylglycerol acyltransferase (DGAT) [15]. In mice, there are two subtypes of MGATs (MGAT1 and MGAT2), but only MGAT1 is associated with hepatic steatosis [10]. However, in humans, there is one more subtype, designated MGAT3, which is mainly expressed in gastrointestinal tracts along with MGAT2 [16]. Interestingly, the mouse gene corresponding to human MGAT3 has not been found; it appears to be a pseudogene [17]. It was reported that the human liver exhibits MGAT activity; the expression levels of MGAT2 and MGAT3 are higher than that of MGAT1, and the hepatic expression of MGAT3 is highly correlated with total MGAT activity [18]. However, the regulation of the expression of each subtype of MGAT, and its association with diet-induced non-alcoholic fatty liver diseases, remain uncertain. For these reasons, although the inhibition of MGAT1 expression resulted in a dramatic reduction of hepatic steatosis in mouse models, it is important to investigate whether human MGATs are regulated under conditions of pathological lipid accumulation, in order to develop a therapeutic agent.

Therefore, we investigated the differences among MGATs gene and their roles in human hepatocyte models. In particular, we focused on the responsiveness of the MGAT gene by PPARγ that is known as a regulator of murine MGAT1 gene. In this study, we report that among the MGAT family, MGAT1 is the most sensitive target gene of PPARγ, suggesting its role in human fatty liver formation as well as its potential as a target for therapeutics.

2. Materials and methods

2.1. Analysis of gene expression by quantitative reverse transcription polymerase chain reaction (RT-PCR)

Human primary hepatocytes were purchased from BD Biosciences (Metabolism-Qualified Cat. No.454543), and RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. For quantitative RT-PCR, cDNA was synthesized from 5 μg of total RNA using a random hexamer and SuperScript reverse transcriptase III (Invitrogen). RT-PCR was performed using the following primers: MGAT1, 5′-CTCGG GCCGA TGTCC ATTGG A-3′, 5′-GGGTA TGCCA GTCAA AGTAA AGC-3′; MGAT2, 5′-CCTTC GGGGA GAATG ACCTAT-3′, 5′-GAGGG AGATG CCCAT GATCT T-3′; MGAT3, 5′-ATGGG AGTTG CCACA ACCC-3′, 5′-CAGAG TGACG TGAAG AGGAG G-3′; CD36, 5′-CGCTG AGGAC AACAC AGTCT-3′, 5′-CTGCC ACAGC CAGAT TGAGA-3′; aP2/422, 5′-GCTTT GCCAC CAGGA AAGTG-3′, 5′-TCCTG GCCCA GTATG AAGGA-3′. The level of 18s mRNA was also measured as an invariant control.

2.2. Human primary hepatocytes culture

Human primary hepatocytes were purchased from BD Biosciences, and plated using BD Gentest™ High Viability Recovery Kit (BD Biosciences). Cells were plated on six-well dishes (collagen-coated six-well plate, BD Gentest™) at 1.0×10^6 cells per well and incubated for 12 h in Hepato-STIM with 100 mM L-glutamine, 500 μg epidermal growth factor to allow cells to attach. Cell counts and viability were confirmed before use (Adam cell counter; Digital Bio); viability was routinely >80%. After attachment, cells were infected with adenoviruses, as described below.

2.3. Preparation of recombinant adenovirus

The construction of a recombinant adenovirus expressing PPARγ2 has been described previously [10]. All viruses were propagated in 293 cells and purified by CsCl density purification, dissolved in 1x HBSS (Invitrogen) and stored at −70 °C. The multiplicity of infection (MOI) was calculated from viral particle numbers. Recombinant adenovirus containing the GFP gene was used as a control.

2.4. Transfection and luciferase assay

Cells (HepG2 cells or 293T cells) were transfected with 0.8 μg pGL3-MGATs promoter plasmid, 0.1 μg expression vector plasmid, and 10 ng pRL-CMV (Promega), using Lipofectamine (Invitrogen), according to the manufacturer's instructions. After 24 h, luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions. Firefly luciferase activity was standardized to *Renilla* luciferase activity.

2.5. Electrophoretic mobility shift assay (EMSA)

Briefly, pcDNA3.0-PPARγ and pcDNA3.0-RXRα were used for an *in vitro* translation reaction using TNT T7 quick master mix (Promega). EMSA was performed by the following method. Reaction mixture containing 50,000 cpm of the appropriate [^{32}P]-labeled oligonucleotide probe, 1 μg poly (dI-dC), and 1 μg protein in 30 μl binding buffer (50 mM HEPES, 5 mM EDTA, 10 mM DTT, and 35% glycerol) were incubated on ice for 20 min. Proteins and labeled oligonucleotide probes were separated electrophoretically on 4% polyacrylamide gels with 0.25x TBE. The probes were double-stranded oligonucleotides corresponding to the potential PPAR regulatory elements (PPREs) on the MGAT1 promoter.

2.6. Gene silencing with small inhibitory RNA (siRNA)

The siRNA sequence 5′-GAAACAUCUCUGUCAUUGUUU-3′ (si-MGAT1) was designed to target human MGAT1 mRNA. The scrambled siRNA was purchased from Genolution Inc. They were introduced into HepG2 cells using the Lipofectamine RNAiMAX reagent (Invitrogen).

2.7. Statistical analysis

All results are expressed as mean ± SD. Statistical comparisons of groups were made using unpaired Student's *t* test.

3. Results

3.1. Human MGAT1 is upregulated by PPARγ in human primary hepatocytes

MGAT genes show a variety of subtypes in different species. In human, unlike in mouse or rat, there are three subtypes of MGAT enzymes, MGAT1, MGAT2, and MGAT3 (Fig. 1A). In a phylogenetic tree with inferred evolutionary relationships, MGAT3 shares higher sequence homology with the diacylglycerol *O*-acyltransferase 2

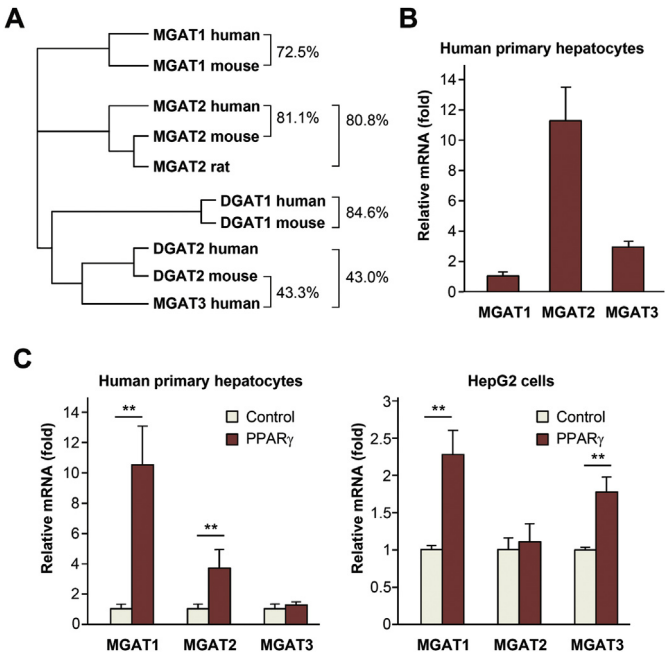

Fig. 1. Expression patterns of MGATs in human primary hepatocytes and their responsiveness to PPARγ. (A) Phylogenetic tree of mouse, rat, and human MGAT or DGAT genes. Similarities in amino acids sequences are shown. (B) Human primary hepatocytes (5×10^5 per well) were cultured in six-well plates for 24 h and harvested for real-time qPCR analysis. (C) Human primary hepatocytes (1×10^6 per well) or HepG2 cells in six-well plates were infected with either Ad-GFP or Ad-PPARγ at an MOI of 100. After 24 h, media change was done with 10% FBS-DMEM. After 48 h, total RNA was prepared and analyzed by real-time qPCR. Data represent the mean ± SD. $^{**}P < 0.01$.

(DGAT2) enzyme than with MGAT1 or MGAT2 [19]. It is considered that MGAT3 has a significant DGAT activity [18,19], suggesting that it plays a different role in lipid-accumulating organs in higher organisms. Nevertheless, since several studies in rodents have focused on hepatic PPARγ-regulated MGAT1 expression and its role in hepatic steatosis [7,10,13,14], we investigated whether human MGAT subtypes in the liver are associated with increased lipid accumulation in response to PPARγ expression.

In human primary hepatocytes, MGAT2 expression was highest, compared to MGAT1 and MGAT3 (Fig. 1B). Interestingly, however, MGAT1 was highly induced by PPARγ overexpression, up to 10-fold, in human primary hepatocytes, as shown by real-time qPCR (Fig. 1C, *left panel*). This pattern was also seen in the human hepatoma cell line HepG2 (Fig. 1C, *right panel*). This result suggests that human MGAT1 could be responsible for increased hepatic TG synthesis in response to PPARγ expression, as in mice. In the previous study, we revealed that PPARγ is a strong regulator of the mouse MGAT1 gene and modulates hepatic steatosis in an obese mouse model. Although MGAT2 or MGAT3 could play a role in hepatic steatosis, the dramatic elevation of MGAT1 gene expression by PPARγ might be an excellent target for the treatment of hepatic steatosis in humans.

3.2. PPARγ directly binds to the human MGAT1 promoter

PPARγ is a transcription factor that regulates many lipogenic genes via direct binding to a promoter site, called the PPAR response element (PPRE). In order to investigate whether human MGAT1 promoter activity is regulated by PPARγ, we constructed human MGAT promoter constructs (MGAT1, MGAT2, and MGAT3, ~2 kb of each promoter) and performed the luciferase assay. Among the three isoforms, we observed that MGAT1 promoter activity was the highest compared to that of MGAT2 or MGAT3, upon PPARγ overexpression (Fig. 2A) in human hepatoma-derived cells, HepG2. A similar pattern is also observed in 293T cells (Fig. 2B). This result was concordant with the regulation of endogenous MGAT gene expression levels by PPARγ in primary human hepatocytes (Fig. 1C).

Next, in order to explore functional PPRE sites on the human MGAT1 promoter, we generated several mutant promoters based on predictions from computer analysis (Dragon PPRE Spotter v.2.0, http://www.cbrc.kaust.edu.sa/ppre/). We selected three sites (-1384, -380, and -300 from the transcription initiation site) among several candidates based on their possibility scores and sequences. The mutant and wild-type sequences are shown in Fig. 3A. The luciferase assay revealed that a mutation in potential PPRE at position -1384 or -300 abrogated MGAT1 luciferase activity, but the mutation in -380 did not (Fig. 3B).

To confirm this result, we carried out an electrophoretic mobility shift assay using ^{32}P-labeled oligonucleotide probes. As a result, we identified that the locations -1384 and -300 are strong binding element for PPARγ in the MGAT1 promoter (particularly -1384 site) and mutations in these sites efficiently interfered with the binding of PPARγ (Fig. 3C). Consistent with the results of the luciferase assay, the -380 site did not show any significant binding of PPARγ. Taken together, our results revealed that the human MGAT1 promoter is directly regulated by PPARγ by its binding to both the -1384 and -300 sites, suggesting a possible role for MGAT1 in lipid accumulation in human hepatocytes.

3.3. The inhibition of MGAT1 ameliorates hepatic steatosis in a human liver cell line

Although PPARγ is not significantly expressed in normal liver cells, an increasing number of observations report that the aberrant expression of PPARγ is observed in hepatic steatosis and plays an important role in lipid accumulation in rodent models of obesity [5–7,20–22]. In humans, it was reported that the PPARγ mRNA was increased in the livers of obese patients with nonalcoholic fatty liver diseases. Using HepG2 cells, we tested whether PPARγ expression causes lipid accumulation. In the presence of oleic acid (40 μM) as a lipid substrate, PPARγ overexpression facilitated lipid accumulation (Fig. 4A). Moreover, inhibition of MGAT1 by siRNA treatment efficiently ameliorated the lipid accumulation (Fig. 4A and B). Western blot revealed that PPARγ overexpression was not changed by si-MGAT1 treatment (Fig. 4C), and MGAT1 knockdown did not affect other lipogenic PPARγ target genes (Fig. 4D). Interestingly, the expression level of aP2 gene was significantly

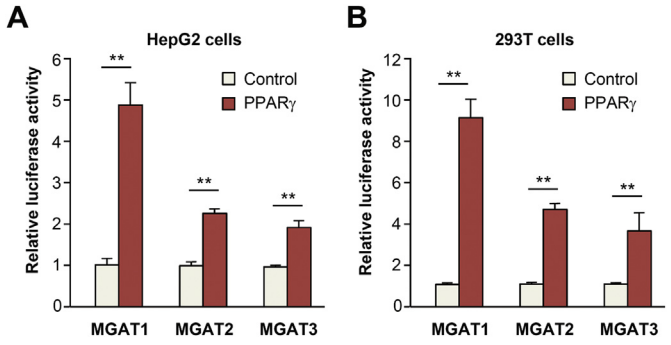

Fig. 2. The MGAT1 promoter is highly regulated by PPARγ. Luciferase assay was performed using human MGAT promoters (~2 kb of each promoter). Promoter activity was shown by relative luciferase activity in either (A) HepG2 cells or (B) 293T cells. MGAT promoter constructs were co-transfected with or without PPARγ/RXRα overexpression vectors. The data are presented as mean ± SD from measurements collected from three independent experiments and two measurements for each experiment. $^{**}P < 0.01$.

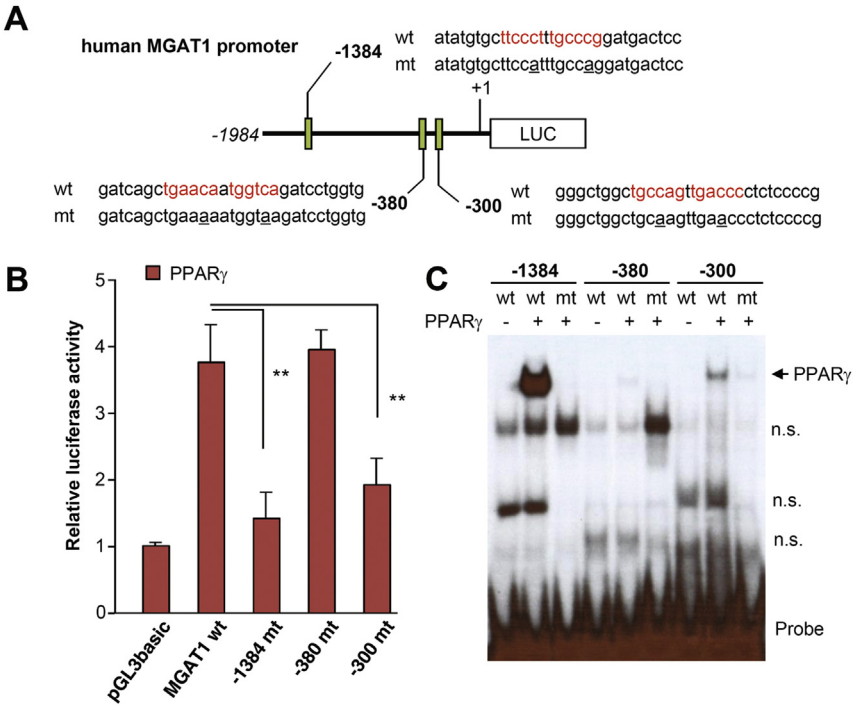

Fig. 3. Analysis of PPARγ binding sites on the MGAT1 promoter. (A) The MGAT1 promoter-luciferase construct (~2 kb) is shown with PPRE sites predicted by computer analysis. Wild type (wt) and mutant (mt) sequences of the -1384, -380, and -300 region are shown. Predicted PPRE (DR1) sequences are marked in red, and mutated sequences are underlined. (B) Luciferase assay using the human MGAT1 promoter and mutant promoters. Promoter activity was revealed by the relative luciferase activity, with overexpression of RXRα and PPARγ in HepG2 cells. The data are presented as mean ± SD from measurements collected from three independent experiments, with two measurements for each experiment. $^{**}P < 0.01$. (C) EMSA of three putative PPREs on the human MGAT1 promoter. The PPARγ protein was prepared using TNT *in vitro* translation, and the position of the PPARγ-DNA complex is marked by an arrow. n.s., non-specific. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

increased by PPARγ overexpression in HepG2 cells, but CD36 was not, which is unlikely in murine cell models [10]. This suggests that the regulation of human CD36 expression is different from that of rodent models. Nevertheless, our data indicate that the MGAT1 induced by PPARγ has a role in hepatic lipid accumulation in humans.

4. Discussion

Metabolic syndrome is characterized by abdominal obesity, elevated blood pressure, elevated fasting plasma glucose, high serum triglycerides, and low high-density cholesterol levels, and is closely associated with diabetes mellitus and cardiovascular disease. Hepatic steatosis is defined as intrahepatic TG content with 5% or more of liver volume or liver weight, and appears in the early phase of metabolic syndrome [23]. Because hepatic lipid accumulation affects systemic insulin sensitivity and cardiometabolic risk and can lead to fibrosis and cirrhosis [1,24,25], it is important to identify liver-specific therapeutic targets for the treatment of hepatic steatosis.

PPARγ is mainly considered as a regulator of adipogenesis and lipid accumulation in fat tissues. Over the past decade, the thiazolidinedione (TZD) class of insulin-sensitizing drugs, known as PPARγ agonists, has been used for diabetic patients [26]. This drug is believed to act predominantly on adipose tissue, where PPARγ expression is considerably high compared to other tissues. The mechanism by which TZDs lower blood glucose is not clear; however, it is accepted that this drug enhances insulin sensitivity by sequestrating free fatty acid in fat depots, thereby reducing a metabolically harmful conditions such as chronic inflammation [9]. Despite their beneficial effect, the clinical use of TZDs is now limited due to the risk of heart failure [27]. The effect of TZDs on the human liver in diabetic patients has not been clearly investigated, partly due to the lack of proper evaluation methods in humans. Furthermore, the role of PPARγ has been underestimated because of its low expression in the normal liver. However, recent studies have revealed that PPARγ plays a critical role in hepatic steatosis by regulating the expression of lipogenic genes. Hepatic PPARγ expression is associated with fatty acid transport and the TG synthesis pathway [11,12]. Thus, it is possible that the effect of PPARγ agonists in the liver is directed towards increased lipid accumulation, aggravating hepatic steatosis. It is obvious that PPARγ agonists improve insulin sensitivity; therefore, it is important to identify new therapeutic target candidates from among the PPARγ target genes in liver.

In a previous study, we reported the role of MGAT1, a new target gene of PPARγ, in a mouse model of obesity [10]. The suitability of MGAT1 as a therapeutic target for hepatic steatosis was confirmed in subsequent studies [13,14]. However, before developing effective chemicals or drugs to modulate MGAT1 activity, it is essential to investigate the role of MGAT1 in the human liver. Several studies revealed that the human liver has MGAT enzyme activity [17,18], and has three isotypes of the MGAT enzyme. Although MGAT3 has a role in the human liver, MGAT3 is classified as a different group from that of MGAT1 and MGAT2, one that is closer to DGAT2 in the phylogenetic tree. We investigated whether PPARγ regulates the promoter activities of these three MGAT subtypes. Our results indicate that neither MGAT3 expression nor its promoter activity are regulated by PPARγ (Fig. 1C and Fig. 2A), suggesting that the role of MGAT3 in hepatic steatosis accompanied by increased PPARγ expression is not significant. On the other hand, MGAT2 expression is high in human primary hepatocytes, but its response to PPARγ expression is not as obvious as with MGAT1 (Fig. 1B and Fig. 2A). Moreover, siRNA-mediated suppression of MGAT1 alone led to a

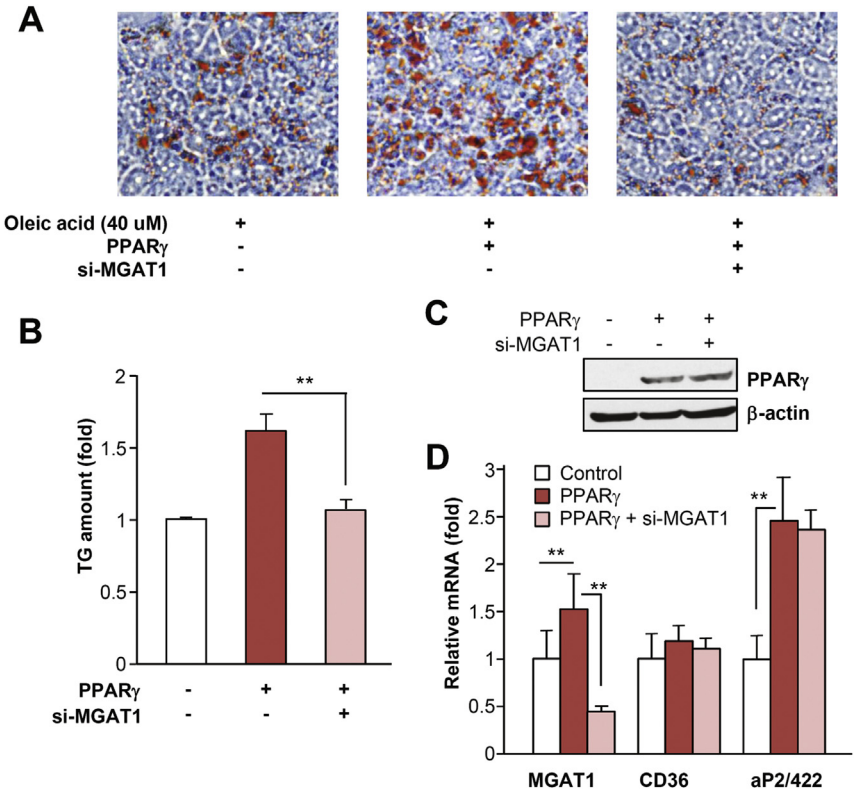

Fig. 4. MGAT1 knockdown by siRNA attenuated the lipid accumulation upon PPARγ overexpression in HepG2 cell. (A) HepG2 cells (1×10^6 per well) in six-well plates were infected with either Ad-GFP or Ad-PPARγ at an MOI of 100. After 24 h, either si-control or si-MGAT1 RNA was added. After 48 h, the media were changed to include 40 μM oleic acid and 10% FBS-DMEM. After 4 d, oil red-O staining to detect hepatic lipid accumulation was performed. (B) Triglyceride content was measured by spectrometric analysis. Spectrophotometric quantification of staining from three independent experiments. (C) Western blot reveals that PPARγ overexpression was successful and was not changed by si-MGAT1 treatment. (D) MGAT1 knockdown efficiency was monitored by quantitative RT-PCR. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reduction in lipid accumulation to the basal level in HepG2 cells overexpressing PPARγ, suggesting that increased lipid accumulation in human hepatocytes is largely dependent on increased MGAT1 activity, as seen in mice.

There are two convergent pathways for diacylglycerol (DAG) and TG biosynthesis. One is the glycerol phosphate pathway and the other is the monoacylglycerol pathway [11]. In the normal liver, most TG is thought to be synthesized via the glycerol phosphate pathway through sequential acylation of glycerol-3-phosphate. On the other hand, the monoacylglycerol pathway is active in neonatal and diabetic rodent livers [28] and is important for TG synthesis in intestinal epithelial cells. However, the relevance of these two pathways in hepatic steatosis is not clear. Because the MGAT enzyme is associated only with the monoacylglycerol pathway, inhibition of MGAT activity does not completely block TG synthesis and hepatic lipid accumulation. In murine models of obesity, the expression of enzymes involved in the glycerol phosphate pathway is not as highly induced as that of MGAT1, and inhibition of MGAT1 expression efficiently decreased the development of hepatic steatosis [10], indicating that excessive lipid accumulation beyond the normal level in the liver is considerably dependent on the monoacylglycerol pathway.

To date, many therapies have been investigated for the treatment of nonalcoholic fatty liver disease. However, weight loss is the only therapy with reasonable evidence to suggest that it is beneficial and safe. Conservative management such as moderate exercise, abstinence from alcohol, and hepatitis vaccination is useful to control fatty liver disease [29,30]. Considering the worldwide increase in the incidence of human nonalcoholic fatty liver disease, the development of new therapeutic agents based on the mechanism underlying hepatic steatosis, such as MGAT1 inhibitors, is important.

Conflict of interest

None.

Acknowledgments

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